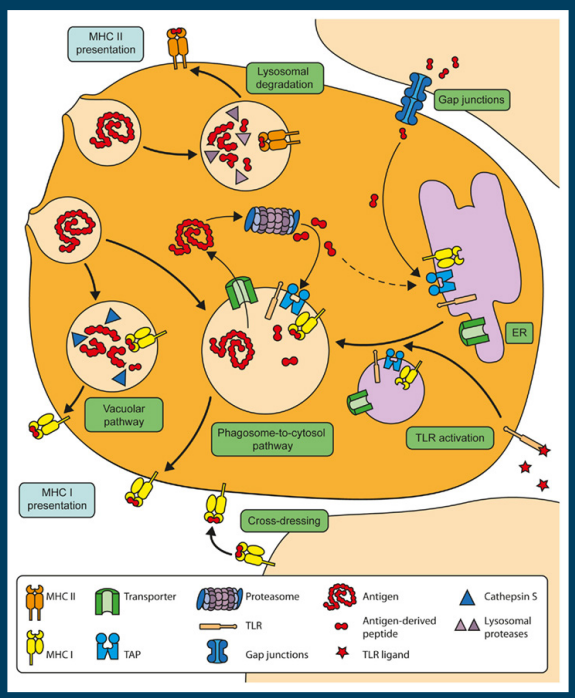
frontiers

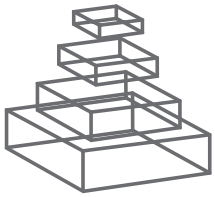
RESEARCH TOPICS



MOLECULAR AND CELL-BIOLOGICAL MECHANISMS OF ANTIGEN CROSS-PRESENTATION

Topic Editor
Christian Kurts

frontiers in IMMUNOLOGY



frontiers

ISSN 1664-8714
ISBN 978-2-88919-231-1
DOI 10.3389/978-2-88919-231-1

ABOUT FRONTIERS

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

FRONTIERS JOURNAL SERIES

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing.
All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

DEDICATION TO QUALITY

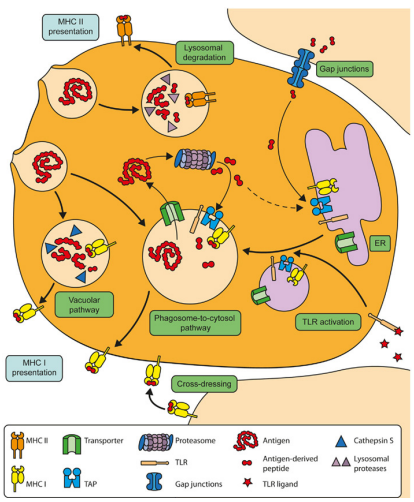
Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view.
By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

WHAT ARE FRONTIERS RESEARCH TOPICS?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area!
Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: researchtopics@frontiersin.org

MOLECULAR AND CELL-BIOLOGICAL MECHANISMS OF ANTIGEN CROSS-PRESENTATION

Topic Editor:
Christian Kurts, Institute of Experimental Immunology, Friedrich Wilhelms-Universität Bonn, Germany



Overview of the molecular mechanisms of cross-presentation. In the vacuolar cross-presentation pathway, extracellular antigens are internalized and degraded in endosomal compartments by Cathepsin S. The resulting peptides are subsequently loaded onto MHC I molecules within the endosomal compartment. In the phagosome-to-cytosol pathway, internalized antigens are transported out of the endosomes into the cytosol for proteasomal degradation. The resulting peptides can be re-imported into the same endosomal compartment by endosomal TAP to be loaded onto MHC I molecules there. The transport of the cross-presentation machinery toward antigen-containing endosomes is induced after stimulation of TLRs. Alternatively, DCs can obtain peptides from neighboring cells via gap junctions. These peptides are thought to subsequently enter the endogenous MHC I-restricted presentation pathway in the ER.

Taken from: Kreer C, Rauen J, Zehner M and Burgdorf S (2012) Cross-presentation: how to get there – or how to get the ER. *Front. Immun.* 2:87. doi: 10.3389/fimmu.2011.00087

Cross-priming serves to activate cytotoxic T lymphocytes for immune defense against viruses and tumors and plays an important role in vaccinations. Only certain DC subsets can cross-present and these are characterized by expression of cell surface markers like CD8a, CD24 CD103, BDCA-3 or XCR1. Classifying DC subsets by such markers is convenient for flow-cytometric analysis, but does not mechanistically explain why a cell can cross-present. Recent studies have proposed two mechanistic explanations, which are not mutually exclusive: 1. only cross-presenting DCs possess the antigen processing machinery that loads endocytosed antigen onto MHC class I molecules (e.g. by proteases like IRAP). 2. cross-presentation depends on distinct endocytosis mechanisms (Mannose receptor, DNGR1, DC-SIGN, DEC205) (CD205) that can introduce antigen directly into distinct organelle(s) in which cross-presentation occurs. This Research Topic is focused on articles that can help understanding how cross-presentation occurs mechanistically, with a special emphasis on further endocytosis receptors, intracellular organelles and molecular antigen processing or membrane translocation mechanisms that can facilitate or are associated with cross-presentation and that can be exploited for vaccine optimization.

Table of Contents

frontiers in
IMMUNOLOGY

EDITORIAL
published: 28 February 2013
doi: 10.3389/fimmu.2013.00051

Molecular and cell-biological mechanisms of antigen cross-presentation

Christian Kurts*

Institute of Molecular Medicine and Experimental Immunology, Friedrich Wilhelms-Universität Bonn, Bonn, Germany
**Correspondence: ckurts@uni-bonn.de*

Edited by:
Hermann Wagner, Technical University Munich, Germany

Reviewed by:
Hermann Wagner, Technical University Munich, Germany

Cross-priming serves to activate cytotoxic T lymphocytes for immune defense against viruses and tumors and plays an important role in vaccinations. Only certain dendritic cell (DC) subsets can cross-present. Several cell surface markers have been described that more or less specifically and sensitively characterize these subsets. The cell-biological mechanism(s) why a DC subset can cross-present are less clear. Theoretically, the task of cross-presentation can be divided into several mechanistic steps: (1) Antigen uptake by various endocytosis mechanisms, (2) Intracellular antigen routing into distinct organelles including the crossing of organelle membranes, (3) Antigen processing into peptides, (4) Peptide loading onto MHC molecules, and (5) Transport of these complexes to the cell surfaces for presentation to T cells. Each of these steps is dependent on numerous parameters, not only the DC subtype, but also the nature of the antigen or the presence of further signals that impact DC function or signify the presence of danger or infection.

This research topic contains 10 articles by leading experts in the field of antigen presentation that cover our current knowledge on the molecular mechanisms underlying cross-presentation (Chopin et al., 2012; Compeer et al., 2012; Harriff et al., 2012; Kreer et al., 2012; Kroczek and Henn, 2012; Murshid et al., 2012; Neefjes and Sadaka, 2012; Saveanu and van Endert, 2012; Thacker and Janssen, 2012; Wagner et al., 2012). The authors describe the influence of endocytosis receptors or heat shock proteins for antigen uptake, the intracellular logistics of antigen routing, membrane translocation mechanisms and proteases, the transcriptional DC regulation, the chemokine-mediated crosstalk between cross-presenting DCs and the cytotoxic T cells to be cross-primed and immune-escape mechanisms of pathogens.

REFERENCES

Chopin, M., Allan, R. S., and Belz, G. T. (2012). Transcriptional regulation of dendritic cell diversity. *Front. Immunol.* 3:26. doi: 10.3389/fimmu.2012.00026

Compeer, E. B., Flinsenberg, T. W. H., van der Grein, S. G., and Boes, M. (2012). Antigen processing and remodeling of the endosomal pathway: requirements for antigen cross-presentation. *Front. Immunol.* 3:37. doi: 10.3389/fimmu.2012.00037

Harriff, M. J., Purdy, G. E., and Lewinsohn, D. M. (2012). Escape from the phagosome: the explanation for MHC-I processing of mycobacterial antigens? *Front. Immunol.* 3:40. doi: 10.3389/fimmu.2012.00040

Kreer, C., Rauen, J., Zehner, M., and Burgdorf, S. (2012). Cross-presentation: how to get there – or how to get the ER. *Front. Immunol.* 2:87. doi: 10.3389/fimmu.2011.00087

Kroczek, R. A., and Henn, V. (2012). The role of XCR1 and its ligand XCL1 in antigen cross-presentation by murine and human dendritic cells. *Front. Immunol.* 3:14. doi: 10.3389/fimmu.2012.00014

Murshid, A., Gong, J., and Calderwood, S. K. (2012). The role of heat shock proteins in antigen cross presentation. *Front. Immunol.* 3:63. doi: 10.3389/fimmu.2012.00063

Neefjes, J., and Sadaka, C. (2012). Into the intracellular logistics of cross-presentation. *Front. Immunol.* 3:31. doi: 10.3389/fimmu.2012.00031

Saveanu, L., and van Endert, P. (2012). The role of insulin-regulated aminopeptidase in MHC class I antigen presentation. *Front. Immunol.* 3:57. doi: 10.3389/fimmu.2012.00057

Thacker, R. I., and Janssen, E. M. (2012). Cross-presentation of cell-associated antigens by mouse splenic dendritic cell populations. *Front. Immunol.* 3:41. doi: 10.3389/fimmu.2012.00041

Wagner, C. S., Grotzke, J. E., and Cresswell, P. (2012). Intracellular events regulating cross-presentation. *Front. Immunol.* 3:138. doi: 10.3389/fimmu.2012.00138

Received: 01 February 2013; accepted: 10 February 2013; published online: 28 February 2013.
*Citation: Kurts C (2013) Molecular and cell-biological mechanisms of antigen cross-presentation. Front. Immunol. **4**:51. doi: 10.3389/fimmu.2013.00051*
This article was submitted to Frontiers in Antigen Presenting Cell Biology, a specialty of Frontiers in Immunology.

frontiers in
IMMUNOLOGY

REVIEW ARTICLE
published: 04 June 2012
doi: 10.3389/fimmu.2012.00138

Intracellular events regulating cross-presentation

Claudia S. Wagner†, Jeffrey E. Grotzke† and Peter Cresswell*

Department of Immunobiology, Yale University Medical Center, New Haven, CT, USA

Edited by:
Christian Kurts, Friedrich Wilhelms-Universität Bonn, Germany

Reviewed by:
William Ross Heath, The University of Melbourne, Australia
Sammy Bedoui, The University of Melbourne, Australia
Sven Burgdorf, Rheinische Friedrich-Wilhelms-University, Germany

***Correspondence:**
Peter Cresswell, Department of Immunobiology, Yale University Medical Center, 300 Cedar Street, TAC S669, PO Box 208011, New Haven, CT, USA. e-mail: peter.cresswell@yale.edu

† Claudia S. Wagner and Jeffrey E. Grotzke have contributed equally to this work.

Cross-presentation plays a fundamental role in the induction of CD8-T cell immunity. However, although more than three decades have passed since its discovery, surprisingly little is known about the exact mechanisms involved. Here we give an overview of the components involved at different stages of this process. First, antigens must be internalized into the cross-presenting cell. The involvement of different receptors, method of antigen uptake, and nature of the antigen can influence intracellular trafficking and access to the cross-presentation pathway. Once antigens access the endocytic system, different requirements for endosomal/phagosomal processing arise, such as proteolysis and reduction of disulfide bonds. The majority of cross-presented peptides are generated by proteasomal degradation. Therefore, antigens must cross a membrane barrier in a manner analogous to the fate of misfolded proteins in the endoplasmic reticulum (ER) that are retrotranslocated into the cytosol for degradation. Indeed, some components of the ER-associated degradation machinery have been implicated in cross-presentation. Further complicating the matter, endosomal and phagosomal compartments have been suggested as alternative sites to the ER for loading of peptides on major histocompatibility complex class I molecules. Finally, the antigen presenting cells involved, particularly dendritic cell subsets and their state of maturation, influence the efficiency of cross-presentation.

Keywords: dendritic cell, MHC class I, endocytosis, phagocytosis

CROSS-PRESENTATION: AN OVERVIEW

In the broadest sense, cells contain two different antigen processing pathways that serve to present peptides to T lymphocytes. These pathways and the machinery required for them have distinct roles in the immune system and function to sample different environments for antigenic peptides. Major histocompatibility complex class I (MHC-I) molecules are loaded in the endoplasmic reticulum (ER) with peptides derived from degradation of cytosolic proteins by the proteasome, and these MHC-I/peptide complexes are then surface expressed and presented to $CD8^+$ T cells. MHC-II molecules exit the ER in association with the invariant chain, which occupies their peptide binding groove. In the endocytic pathway proteolysis results in the degradation of the invariant chain leaving the residual CLIP fragment in the binding groove. In a process catalyzed by HLA-DM (in humans) CLIP is replaced by peptides derived by proteolysis from proteins resident or internalized into the endocytic pathway. After surface expression, these are presented to $CD4^+$ T cells. Hence, MHC-I generally serves as a reporter of intracellular infection, while MHC-II senses the antigens present in the extracellular milieu.

We now know that in professional antigen presenting cells (APC), peptides derived from exogenously acquired antigens can be presented on MHC-I. This process is known as cross-presentation. Furthermore, $CD8^+$ T cells can be primed to such antigens by dendritic cells (DCs), a process termed cross-priming. The term was originally introduced to describe $CD8^+$ T cell sensitization to minor histocompatibility antigens in transplantation situations (Bevan, 1976). The importance of cross-priming for the generation of $CD8^+$ T cell-mediated immunity in general is a topic of debate, but cross-priming certainly plays an important role during priming of anti-tumor $CD8^+$ T cells as well as priming the $CD8^+$ response to pathogens which do not directly infect DCs.

Although various cell types, even including non-professional antigen-presenting cells like endothelial cells or modified 293T cells (Bagai et al., 2005; Giodini et al., 2009), are able to cross-present under certain conditions, DCs are the most important cross-presenting cells *in vivo* (Jung et al., 2002). In mice, several DC subsets are competent for cross-presentation, with the lymphoid-organ resident $CD8\alpha^+$ DC (Heath et al., 2004; Hildner et al., 2008) and dermal migratory $CD103^+$ DCs (Bedoui et al., 2009; Henri et al., 2010) defined as the main cross-presenting DCs. The search for human counterparts specialized in cross-presentation is ongoing, and considerable progress in characterization of human DC subsets has been made lately, including the discovery of a likely equivalent to the mouse $CD8\alpha^+$ DC subset (Villadangos and Shortman, 2010).

Cross-presentation of exogenous antigens raises interesting and important biochemical and cell biological questions. How do internalized proteins that are localized in the endocytic system gain access to the MHC-I processing and presentation machinery normally present in the ER? Over three decades have been spent defining how this process occurs. In a seminal study, Rock and colleagues demonstrated that presentation of exogenously derived antigens required proteasomal degradation, and therefore access to the cytosol (Kovacsovics-Bankowski and Rock, 1995). Consistent with this the ribosomal inhibitor protein gelonin was found to inhibit protein synthesis when added to cells, demonstrating that an intact protein can access the cytosol. An alternative mechanism, described by Harding and colleagues, involves endosomal

processing of exogenous antigens and peptide binding to MHC-I in the endocytic system (Pfeifer et al., 1993). This is known as the vacuolar pathway. We now know that cross-presentation can occur through multiple pathways, including antigenic processing in the endocytic system and/or in the cytosol after translocation from endosomes or phagosomes. MHC-I binding after cytosolic processing may occur through several different mechanisms. After antigens reach the cytosol and are degraded by the proteasome, the resulting peptides can be transported into the ER or phagosomes via the transporter associated with antigen processing (TAP), where loading onto MHC-I can occur (Ackerman et al., 2003; Guermonprez et al., 2003; Houde et al., 2003; Grotzke et al., 2009). To complicate matters further, recent evidence suggests that peptide transport into phagosomes may involve an as yet unidentified, novel, transporter (Merzougui et al., 2011).

Although the broad pathways by which cross-presentation occurs have been elucidated, only now are more definitive molecular studies emerging. Moreover, many questions are still unanswered. Which cytosolic pathway is most widely used by cells? What role, if any, do endocytic receptors play in cross-presentation? How do antigens reach the cytosol? What factors help achieve the exquisite efficiency of this process that must occur *in vivo*? Are pathogens able to specifically inhibit cross-presentation? How is cross-presentation regulated in DCs? This review will focus on the known molecular mechanisms of cytosolic cross-presentation, while the other mechanisms will be covered by other authors. The second half of the review concentrates on the role of DC maturation in the context of cross-presentation.

ANTIGEN UPTAKE BY PROFESSIONAL APC

Early studies of cross-presentation demonstrated that the process is not simply a matter of extracellular processing and cell surface peptide loading (Pfeifer et al., 1993; Kovacsovics-Bankowski and Rock, 1995). For particulate antigens, phagocytosis is required for subsequent cross-presentation, and considerable evidence suggests that endocytosis is required for soluble antigens. The endocytic pathway ultimately leads to lysosomes and the phagocytic pathway progresses to phagolysosomes, but extensive proteolysis is incompatible with successful cross-presentation and a number of papers have implicated early endosomes and phagosomes in cross-presentation. A key question is whether different receptors deliver cargo preferentially to compartments that are capable of mediating cross-presentation. Several groups have shown that during and/or subsequent to phagocytosis, ER components are delivered to the phagosome (Ackerman et al., 2003, 2006; Guermonprez et al., 2003; Houde et al., 2003). A major focus has been on the role of this process in delivering the components necessary for translocation of antigenic proteins into the cytosol (see below), but in fact most or all of the components necessary for cross-presentation, including MHC-I and the peptide loading complex (PLC), as well as ER-derived retrotranslocation machinery, are recruited to the phagosome, creating a cross-presentation competent organelle. Whether delivery of ER components to the phagosome is required for cytosolic cross-presentation has been difficult to determine, but at least one study suggests that MyD88 signaling enhances delivery of ER components to endosomes and that this is required for cross-presentation of soluble ovalbumin (OVA; Burgdorf et al., 2008).

If phagocytosis of antigens results in the formation of a compartment that is competent for cross-presentation, is the ability to cross-present determined solely by the ability to phagocytose the antigen? It should be noted that an ER contribution to phagosomes occurs not only in DC, but also in cells that are much less efficient at cross-presentation, such as macrophages (Gagnon et al., 2002; Houde et al., 2003) and even a normally non-phagocytic cell line (Giodini et al., 2009). Furthermore, in *Dictyostelium*, not only are the ER proteins calnexin and calreticulin recruited to phagosomes, they appear to be required for phagocytosis (Muller-Taubenberger et al., 2001). This suggests that ER-recruitment is an evolutionarily conserved process and not restricted to cells that can cross-present. Consistent with this, introduction of a phagocytic receptor (FcγRIIa) into the non-cross-presenting cell line HEK293T leads to ER-recruitment to the phagosome and cross-presentation (Giodini et al., 2009). However, it is difficult to tease apart the role of receptor-specific effects and phagocytosis itself. Moreover, there is experimental evidence demonstrating that phagocytosis is not always sufficient to mediate cross-presentation, even in DC (Schnorrer et al., 2006).

Macrophages and DCs express a multitude of cell surface receptors that can mediate endocytosis and phagocytosis, but there is a dearth of data regarding the role that individual cell surface receptors and their associated intracellular trafficking and signaling pathways play in the process. Under certain conditions, specific receptors such as DEC-205 may induce cross-presentation more efficiently than other receptors such as DC-SIGN (Bozzacco et al., 2007), but the mechanisms that lead to this superiority have not been addressed. How could the internalizing receptor influence cross-presentation? First, certain receptors could traffic to a specific compartment that is highly competent for cross-presentation. Such a compartment may avoid the decreased pH and increased proteolysis detrimental for the process (Savina et al., 2006). Second, receptors could directly recruit additional components necessary for cross-presentation. Third, receptors and associated signaling adaptors could initiate a specialized signaling response that initiates cross-presentation. These receptor-specific responses could also serve to determine whether the cross-presented antigen promotes tolerance or an active CD8 response. The ability of a receptor to mediate cross-presentation and cross-priming *in vivo* is likely dependent on the antigen and its immunogenic potential, the delivery mechanism, co-stimulation, and the immune microenvironment.

The following receptors have been implicated in cross-presentation: Fc receptors (FcR), scavenger receptors (SR), DC-SIGN (Dendritic Cell-Specific ICAM-3 Grabbing NonIntegrin), MR (Mannose Receptor), DEC-205 (Dendritic and Epithelial Cells, 205 kDa), CLEC9A (C-type Lectin domain family 9A), DCIR (DC ImmunoReceptor), and LOX-1 (Lectin-like Oxidized Low-density Lipoprotein Receptor 1; Regnault et al., 1999; Bonifaz et al., 2002; Delneste et al., 2002; Berwin et al., 2003; Shakushiro et al., 2004; Tacken et al., 2005; Burgdorf et al., 2006; Bozzacco et al., 2007; Sancho et al., 2008, 2009; Klechevsky et al., 2010). In the case of MR, which internalizes OVA due to mannosylation, cross-presentation is dependent on co-ligation of

TLR4 from contaminating endotoxin and subsequent MyD88 signaling (Burgdorf et al., 2008). Thus, internalization alone is not necessarily sufficient for cross-presentation, even by professional APC. Consistent with this, cross-presentation by CLEC9A, a receptor specifically expressed on the cross-presenting DC subset (Sancho et al., 2008), requires a hemITAM motif (containing only a single YxxL motif) in the cytoplasmic tail and subsequent syk signaling for efficient cross-presentation (Sancho et al., 2009). Notably, CLEC9A is required for recognition, but not uptake of its physiological ligand (necrotic cells). This suggests that cross-presentation may use one receptor for antigen uptake and a different receptor/adaptor protein to trigger the appropriate pathway in DCs.

The precise mechanisms by which receptor-induced signaling pathways initiate cross-presentation are poorly understood. In the case of CLEC9A-mediated cross-presentation, syk was found to associate with the hemITAM motif and be required for cross-presentation (Sancho et al., 2009). Similarly, cells deficient in FcRγ or DAP12 adaptors, which signal through ITAM motifs, are deficient in cross-presentation of particulate antigens (Graham et al., 2007). Although the ITAM present in FcR has not been reported to be required for cross-presentation, FcR signaling is initiated by ITAM phosphorylation and syk recruitment (Swanson and Hoppe, 2004). These cases suggest that ITAM or hemITAM phosphorylation and subsequent syk signaling may be an important pathway. However, an antigen bound to MR can be cross-presented even though MR lacks an ITAM motif or known association with an ITAM-containing adaptor (Burgdorf et al., 2006). Similarly, the scavenger receptor SR-A1 can mediate cross-presentation while lacking an ITAM or known association of an ITAM-containing adaptor (C. Wagner and P. Cresswell, unpublished data), and mutation of the hemITAM motif in DC-SIGN does not affect cross-presentation of OVA bound to latex beads (C. Wagner and P. Cresswell, unpublished data). Finally, DCIR is able to cross-present targeted antigen even though it has an ITIM inhibitory motif (Klechevsky et al., 2010). These data suggest that there may be a general, unidentified signaling requirement for cross-presentation, that receptor-specific pathways can influence cross-presentation, and/or that incorporation of signals from multiple pathways may determine the fate of internalized antigens. Identification of receptors and associated signaling pathways that can activate cross-presentation should yield promising candidates to target for more efficient anti-tumor therapies and anti-pathogen vaccines.

To date, there is little to no evidence to show that individual phagocytic receptors impact the recruitment to the phagosome of additional proteins required for cross-presentation. In the case of MR-mediated cross-presentation, contaminating endotoxin is required for TLR4 ligation, MyD88 signaling, and recruitment of PLC members to endosomes (Burgdorf et al., 2008). Also, an ITAM signaling pathway not involved in uptake of bead- or bacteria-associated antigens was shown to be required for recruitment of NOX2 to phagosomes and efficient cross-presentation (Graham et al., 2007). These data suggest that recruitment of factors that enhance cross-presentation is determined not by the phagocytic receptor, but more likely by co-ligation of a second receptor.

ANTIGEN UNFOLDING AND CYTOSOLIC TRANSLOCATION

One of the more intriguing and perplexing questions about cross-presentation is how antigens access the cytosol. After initial studies showed that the proteasome is required for most examples of cross-presentation, with the underlying assumption that this reflects cytosolic proteolysis, how antigens cross the phagosomal membrane has been a major topic of study. A major leap forward was the finding that at least some of the machinery that functions in ER-associated degradation (ERAD), which translocates unfolded proteins from the ER lumen to the cytosol for degradation (Vembar and Brodsky, 2008), is localized to phagosomes in APCs and facilitates antigen translocation to the cytosol (Guermonprez et al., 2003; Houde et al., 2003; Imai et al., 2005; Ackerman et al., 2006). Recently it has been found that the ER–Golgi intermediate compartment (ERGIC) SNARE protein Sec22b is required for the process (Cebrian et al., 2011). When Sec22b function was inhibited using RNAi, DCs were much less efficient in translocating antigens to the cytosol. However, the components involved in antigen translocation to the cytosol and whether the nature of the antigen influences the process remain unclear. It is also unclear if there are specialized components of the retrotranslocation machinery in the phagosome and cytosol that function in cross-presentation but not in ERAD. Other open questions are whether there is a specific time window in which the retrotranslocation machinery is localized to phagosomes/endosomes, and whether the process is regulated by factors such as the maturation state of the cell or identity of the internalizing receptor. Also, as is the case with ERAD, the nature of the channel through which antigens are translocated remains a subject of debate.

Cells devote considerable energy to the production of secretory proteins. Not all of these proteins fold correctly and therefore need to be degraded before they are allowed to accumulate in the ER. Cells contain a structured complex of proteins that functions constitutively in the ER to facilitate degradation of misfolded proteins, i.e., ERAD. ERAD requires chaperones that recognize terminally misfolded proteins, a translocation channel, ubiquitination machinery, deglycosylating enzymes, and other accessory proteins (Vembar and Brodsky, 2008). In mammalian and yeast cells, the ERAD complex forms around a central E3 ubiquitin ligase, whose identity differs depending on the aberrant protein (Kostova et al., 2007). In mammalian cells, the most widely studied E3 ligases involved in ERAD are hrd1 and gp78, which function in the translocation of a number of ERAD substrates. Once a protein is identified as misfolded and targeted to the retrotranslocation channel it can be ubiquitinated during translocation, targeting it for degradation. After the substrate has partially entered the cytosol, the AAA ATPase VCP/p97 (cdc48 in yeast) generally functions to extract it into the cytosol (Ye et al., 2001; Braun et al., 2002; Jarosch et al., 2002; Rabinovich et al., 2002). The cytosolic chaperone hsp90 (Taylor et al., 2010), or even proteasomes themselves (Mayer et al., 1998; Lee et al., 2004; Lipson et al., 2008), have been proposed to mediate the dislocation event for substrates which are not dependent on p97. Overall, the function of ERAD is translocating misfolded proteins across a membrane and targeting them for proteasomal degradation before their accumulation results in induction of the unfolded protein response.

The process of ERAD is very similar to antigen translocation into the cytosol for cross-presentation. Initial studies demonstrated that one potential retrotranslocation channel, sec61, is localized to phagosomes and that ubiquitinated proteins and proteasomes are associated with phagosomes (Guermonprez et al., 2003; Houde et al., 2003). Furthermore, ERAD components such as sec61, p97, and Bip can be co-immunoprecipitated with exogenously added OVA and vice versa (Imai et al., 2005). Together, these data suggest that the factors necessary for retrotranslocation are accessible to internalized antigens, even if the nature of the retrotranslocation channel(s) is unclear. The presence of functional retrotranslocation machinery has been confirmed by our laboratory and others. After internalization of latex beads and luciferase by a DC-like cell line and subsequent phagosome purification, translocation of phagosomal luciferase across the membrane requires addition of cytosol or recombinant p97 (Ackerman et al., 2006), demonstrating that p97 can function in phagosomal translocation as well as ERAD. Moreover, knockdown of p97 function by either expression of a dominant negative mutant or by siRNA inhibits cross-presentation (Imai et al., 2005; Ackerman et al., 2006). In addition to p97, hsp90 has also been implicated in both retrotranslocation and cross-presentation. Cells deficient in Hsp90α by knockout, siRNA knockdown, or pharmacological inhibition show decreased cross-presentation of soluble or cell-associated OVA (Ichiyanagi et al., 2010). Hsp90 can contribute to antigen translocation to the cytosol (Imai et al., 2011) or to cytosolic refolding of proteins after translocation (Giodini and Cresswell, 2008).

Although ERAD components are recruited to phagosomes from the ER as described above, other mechanisms may contribute to recruitment of ERAD components in the case of endosomes. Mannose receptor ligation by OVA results in polyubiquitination of the receptor leading to increased recruitment of p97, OVA translocation to the cytosol, and cross-presentation (Zehner et al., 2011). Poly- but not mono-ubiquitination was required for the recruitment of p97 and OVA translocation to the cytosol, suggesting that receptor ubiquitination can serve to recruit cytosolic components required for antigen dislocation. As p97 can directly interact with members of the retrotranslocation complex (Zhong et al., 2004; Li et al., 2005; Schulze et al., 2005; Ye et al., 2005; Morreale et al., 2009), which must already be localized to endosomes for translocation to occur, it is unclear why receptor polyubiquitination is required for this recruitment. However, the retrotranslocation complexes present in phagosomes and endosomes are still ill-defined and this may be a mechanism governing the ability of certain receptors to mediate cross-presentation.

The retrotranslocation of exogenously added antigens has been difficult to study due to the lack of a good readout for their access to the cytosol. Studies have relied on the use of toxins such as gelonin or exotoxin A (exoA) from *Pseudomonas aeruginosa* (Kovacsovics-Bankowski and Rock, 1995; Ackerman et al., 2006; Giodini and Cresswell, 2008; Giodini et al., 2009), enzymes such as HRP (Gil-Torregrosa et al., 2004), cytochrome *c* (Lin et al., 2008) or luciferase (Giodini and Cresswell, 2008), or pulsing large amounts of soluble OVA onto cells and examination of OVA in the cytosolic fraction (Burgdorf et al., 2008; Imai et al., 2011). No assay system for tracing particulate antigens has been devised yet, and all of these methods using soluble antigens have their drawbacks. In the case of exoA, there is evidence demonstrating that exoA can inhibit retrotranslocation of radioactive-labeled peptides from microsomes (Koopmann et al., 2000). ExoA has also been shown to inhibit cross-presentation of soluble proteins and immune complexes as well as inhibit presentation of bacterially and parasite-derived antigens (Ackerman et al., 2006; Giodini et al., 2009; Goldszmid et al., 2009; Grotzke et al., 2009). However, the target of inhibition remains unknown. When pulsing enzymes or large amounts of protein antigen onto cells, a common problem is that protein or enzymatic activity found in the cytosol could be due to contamination during processing or lysosomal "bursting" and not true retrotranslocation. Until an assay is developed that directly measures retrotranslocation, results need to be interpreted with caution.

During ERAD, many substrates are unfolded before translocation to the cytosol. If cross-presentation utilizes the same retrotranslocation machinery as ERAD, then cross-presented antigen should also be unfolded before translocation. Indeed, several reports have shown a requirement for acidification or partial lysosomal proteolysis of antigens that are cross-presented in a proteasome-dependent manner (Fonteneau et al., 2003; Giodini et al., 2009), suggesting that processing of the internalized protein or at least breakdown of immune complexes or apoptotic cells are required prior to translocation. Chaperones such as calnexin, calreticulin, Bip, and the ER enzyme protein disulfide isomerase (PDI), have all been shown to localize to phagosomes and may play a role in protein unfolding (Ackerman et al., 2003; Guermonprez et al., 2003; Houde et al., 2003; Imai et al., 2005). For the case of ERAD substrates containing disulfide bonds, several studies demonstrate that reduction is required for, or enhances, retrotranslocation (Molinari et al., 2002; Dong et al., 2008; Ushioda et al., 2008). Similarly, for cross-presented antigens with disulfide bonds, it is likely that reduction and unfolding is required for cytosolic access. The only known thiol reductase present in phagosomes and lysosomes prior to ER acquisition is γ-interferon inducible lysosomal thiol reductase (GILT). GILT-deficient DC are deficient in their ability to cross-present gB, a HSV-1 glycoprotein that contains five disulfide bonds, but showed no defect in cross-presentation of a protein that did not contain disulfide bonds (Singh and Cresswell, 2010). Furthermore, GILT-deficient mice showed a decrease in cross-priming anti-gB and anti-influenza-specific $CD8^+$ T cells. These results underscore the importance of protein unfolding for cross-presentation, and demonstrate that further characterization of the mechanisms that aid protein unfolding while at the same time limiting lysosomal proteolysis are needed.

CROSS-PRESENTATION IN THE CONTEXT OF DC MATURATION

The ability to cross-present not only differs between various cell types or DC subsets, but the maturation state of the cross-presenting DCs also plays an important role. Microbial products, inflammatory cytokines or mediators of tissue damage induce a process of maturation in DCs that, besides changes in phenotype and motility, also involves changes in handling and presentation

of antigens. These modifications significantly affect MHC-II-restricted presentation, but also influence cross-presentation. Maturation is a potentially attractive approach to dissecting the molecular mechanisms regulating cross-presentation. However, care needs to be taken to separate effects on T cells resulting from enhanced co-stimulation from a true effect on the formation of MHC-I/peptide complexes.

Depending on the timing there are different scenarios for how DC maturation may affect cross-presentation. One can ask how DC activation affects cross-presentation of antigens that are acquired together with or shortly prior to the maturation event, such as antigens acquired from virally infected cells. Experimentally this may be represented by the administration of a defined antigen followed by or combined with specific ligand for innate immune receptors, such as Toll-like receptors (TLRs). Alternatively one can ask if DCs are still able to cross-present antigens that they encounter in an already matured state. This would be important in a situation in which an individual encounters a pathogen while already undergoing a response to prior infection. We will discuss the latter situation first.

CROSS-PRESENTATION IN MATURE DCs

A rationale for cross-presentation being controlled through maturation comes from analogy to presentation on MHC-II. Both cross-presentation and MHC-II presentation serve to prime T cell responses, with antigens presented by either MHC-I to $CD8^+$ T cells or to $CD4^+$ T cells via MHC-II. MHC-II-restricted antigen presentation is tightly regulated during maturation at several levels, including alterations in endocytic proteolysis, re-distribution of peptide loaded MHC-II molecules to the cell surface, enhanced stability of surface complexes and reduction of MHC-II biosynthesis (Wilson and Villadangos, 2005). With few exceptions (Drutman and Trombetta, 2010; Platt et al., 2010), these maturation-induced changes generally prevent MHC-II presentation of antigens by mature DCs. The proposed benefit is preservation of the MHC-II/peptide complexes derived from antigens acquired at the onset of maturation (Villadangos et al., 2005). A pathogen-derived antigen that is acquired in the periphery by immature DCs will still be presented by mature DCs that have migrated to lymph nodes to prime $CD4^+$ T cells.

For cross-presentation, the situation is more complex. There is a considerable overlap with pathways used simultaneously for endogenous MHC-I presentation, a process that is still operational in mature DCs (Gil-Torregrosa et al., 2004; Wilson et al., 2006). Supporting this notion, MHC-I synthesis and trafficking is not subjected to the same control during maturation as MHC-II. MHC-I synthesis is increased during maturation (Cella et al., 1997; Rescigno et al., 1998) and stability is not affected (Cella et al., 1997; Delamarre et al., 2003) or is only moderately affected (Ackerman and Cresswell, 2003). One could argue that cross-presentation should still be operational in mature DCs to allow initiation of responses toward secondary pathogens, although the relatively short lifespan of matured DCs and the constant renewal by fresh immature DCs could take care of that problem (Kamath et al., 2002). If cross-presentation is indeed regulated by maturation in order to focus or preserve certain antigens, what could be the mechanism? Exogenous material taken up at the onset of maturation may be retained intracellularly until the mature DC reaches the lymph node, creating a form of antigenic memory. In mouse DC-like cell lines and to some extent in bone marrow DCs, storage of soluble (Lutz et al., 1997) or immune complexed antigens (van Montfoort et al., 2009) in distinct compartments with reduced proteolytic activity has been observed, either in immature DCs (Lutz et al., 1997) or in mature DCs (van Montfoort et al., 2009). Whether specialized storage compartments for exogenous antigens exist in all cross-presenting DCs is unknown. Also unknown is how antigen deposition and release are regulated. In a human DC-like cell line trafficking of MHC-I molecules to the cell surface was delayed in an immature state, suggesting that intracellular retention of preformed MHC-I/peptide complexes may occur (Ackerman and Cresswell, 2003).

Regardless of theoretical considerations, published evidence does not clearly argue for or against regulation of cross-presentation during maturation. As often, the truth may lie somewhere in between: cross-presentation in mature DCs may be compromised under certain circumstances, depending on the maturation stimulus and the nature or the form of the antigen that is encountered. With a few exceptions, studies have employed the OVA antigen to study the effect of maturation on cross-presentation. It should be noted that bone marrow-derived DCs require an additional maturation stimulus during or shortly after antigen uptake to cross-present soluble OVA (Delamarre et al., 2003; Gil-Torregrosa et al., 2004). Depending on the study, pre-treatment of DCs with certain TLR ligands such as CpG, LPS, or poly (I:C), either did not alter or enhanced cross-presentation of subsequently acquired antigens (Regnault et al., 1999; Machy et al., 2000; Datta et al., 2003; Henri et al., 2007; Weck et al., 2007; Drutman and Trombetta, 2010; Platt et al., 2010). On the other hand, the ability to cross-present can also be lost after contact with TLR ligands, both after administration *in vivo* (Wilson et al., 2006) as well as after prolonged treatment of DCs *in vitro* (Gil-Torregrosa et al., 2004; Weck et al., 2007). We found that peptidoglycan, a common impurity in LPS preparations, can inhibit cross-presentation of viral antigens via signaling through cytoplasmic NOD receptors (Wagner and Cresswell, 2012).

Decreased antigen uptake (Gil-Torregrosa et al., 2004; Wilson et al., 2006; Weck et al., 2007) and a lack of transfer of antigen to the cytosol (Gil-Torregrosa et al., 2004) have been proposed to explain reduced cross-presentation by mature DCs. Decreased uptake is likely a contributing factor but cannot be the sole explanation, as mature DCs still take up considerable amounts of antigens, even if reduced compare to immature DCs (Datta et al., 2003; Drutman and Trombetta, 2010; Platt et al., 2010; Wagner and Cresswell, 2012; C. Wagner and P. Cresswell, unpublished data). *In vivo*, antigen availability is certainly also a regulatory factor. For example, immature DCs residing in the periphery may have access to pathogen-derived antigens at a local site of infection, unlike mature DCs that have already migrated to the lymph node and never come in contact with the new antigen. Regarding inhibition of antigen transfer to the cytosol, it would be very interesting to understand exactly how this process is impaired in mature DCs. Are the antigens routed to different compartments that lack components of the still unidentified transport machinery? Are the

antigens degraded within endosomal/lysosomal compartments before they can be retrotranslocated? These are potential levels of regulation that are specific for cross-presentation and would not interfere with endogenous MHC-I peptide loading and presentation.

PHAGOSOME MATURATION AND ANTIGEN DEGRADATION

Major contributions to our understanding of cross-presentation came from a series of studies on the regulation of proteolysis and pH in DCs. DCs have a lower content of lysosomal proteases compared to macrophages (Delamarre et al., 2005) and the kinetics of acquisition of distinct proteases during phagosome maturation are slower in DCs than macrophages (Lennon-Dumenil et al., 2002). Amigorena and co-workers demonstrated that, unlike macrophages, DCs limit acidification in phagosomes and inhibit proteolysis, thus promoting cross-presentation (Savina et al., 2006). DCs maintain pH levels above pH 7 for several hours post-phagocytosis (Savina et al., 2006), in contrast to macrophages where the pH drops to pH 5 within 15 min (Yates et al., 2005). The mechanism involves recruitment of the NADPH oxidase NOX2 to the phagosome, which drives alkalinization of the phagosomal lumen and is recruited in a Rab27-dependent fashion (Savina et al., 2006; Jancic et al., 2007). In CD8α$^+$ DC, the GTPase Rac2 is responsible for assembly of the NOX2 complex on phagosomes, while Rac1 directs NOX2 to the plasma membrane in CD8α$^-$ DC (Savina et al., 2009). A high pH would serve to limit proteolysis and thus favor cross-presentation. An elevated pH might also contribute in other ways to successful cross-presentation, such as influencing the conformation of proteins associated with translocation of antigens or proper assembly of the PLC. Although limited acidification may facilitate cross-presentation, a certain degree of proteolysis is necessary for pre-processing particulate antigens before translocation into the cytosol or for complete processing of antigens in case of the vacuolar pathway of cross-presentation. DCs may selectively use proteases active at a higher pH than most lysosomal proteases. One example is cathepsin S (Kirschke et al., 1989) which is enriched in DCs (Lennon-Dumenil et al., 2002) and has been shown to be involved in processing of antigens for the vacuolar pathway of cross-presentation (Shen et al., 2004).

Dendritic cell activation has been demonstrated to modify phagosome maturation and proteolysis. The assembly of the vacuolar proton pump in lysosomes is enhanced after activation with LPS, resulting in enhanced acidification and higher protein degradation (Trombetta et al., 2003), while phagocytosis induction combined with LPS treatment of DCs was found to delay acquisition of active proteases by phagosomes (Lennon-Dumenil et al., 2002). This could mean that despite an overall enhancement of lysosomal activity after TLR4 triggering, proteolytic activity in phagosomes is actually reduced, potentially preserving antigens for cross-presentation. However, the latter study was based on a method using internalized beads coated with probes specific for cysteine proteases, and LPS-treated DC tend to be "sticky" and immobilize beads at the cell surface. Surface bound beads could potentially lead to an overestimation of initial uptake compared to untreated DC and also result in a mixed population of phagosomes, due to slow internalization of the surface bound beads over time (C. Wagner and P. Cresswell, unpublished data). Two further reports link NOX2 activity to TLR signaling and efficient intracellular bacterial killing: in human DC, NOX2 activity was increased in TLR ligand matured DCs (Vulcano et al., 2004), and in macrophages, NOX2 assembly was regulated by MyD88, a central adaptor protein for TLR signaling (Laroux et al., 2005). Thus, TLR signaling appears to regulate cross-presentation by modulating NOX2 activity and phagosomal pH.

CROSS-PRESENTATION OF ANTIGENS COMBINED WITH MATURATION STIMULI

How does maturation that occurs simultaneously with or after antigen uptake relate to the ability to cross-present? CD8α$^-$ cells, which are inferior to CD8α$^+$ DCs in terms of cross-presentation, can be activated through FcγR triggering and become competent for cross-presentation of immune complexes (den Haan and Bevan, 2002). Besides immune complexes (Regnault et al., 1999; den Haan and Bevan, 2002), cross-presentation can be induced by certain stimuli such as LPS (Gil-Torregrosa et al., 2004; West et al., 2004), disruption of cell contacts, or CD40L stimulation (Delamarre et al., 2003), but not by CpG (Datta et al., 2003; Delamarre et al., 2003), low-dose LPS, Poly (I:C), or TNFα (Delamarre et al., 2003). This means that OVA cross-presentation is induced by only a subset of maturation conditions that stimulate MHC-II presentation. Cross-presentation of physiological relevant antigens, such as viral proteins from infected cells, may have different requirements for maturation stimuli. These antigens are already delivered in a complex mix of activating signals, such as pathogen-derived TLR ligands and signals from dying cells. Under experimental settings, no additional external maturation stimuli are needed to induce cross-presentation.

What mechanisms are responsible for maturation-induced changes in cross-presentation? Early after activation, DCs transiently sequester endogenous ubiquitinated proteins in cytosolic aggregates, termed DALIS by Pierre and colleagues, a phenomenon proposed to favor the processing of internalized exogenous antigens for cross-presentation (Lelouard et al., 2004). It has been proposed that cross-presentation involves early endosomal compartments (Burgdorf et al., 2008; Di Pucchio et al., 2008; Belizaire and Unanue, 2009). During maturation, changes in phagosomal/endosomal routing could potentially also alter the fate of antigens. Blander and Medzhitov (2006b) proposed that the presence of a TLR ligand with an antigen in a phagosome favors MHC-II processing. Only antigens from phagosomes with TLR triggering are efficiently routed to lysosomes where invariant chain processing occurs. This offers a solution to the problem how an antigen-presenting cell would ensure that only harmful antigens and not phagocytosed self-antigens are presented to T cells. For cross-presentation, one could speculate that the effect would be the opposite, i.e., that TLR-dependent shuffling toward lysosomal degradation would impair efficient cross-presentation. It has also been reported that TLR signaling influences phagosome maturation in macrophages (Blander and Medzhitov, 2004). However, Russell and colleague were unable to detect TLR2 or TLR4-dependent regulation of phagosome maturation (Yates and Russell, 2005). Potential

explanations for the discrepancy have been discussed by the authors (Blander and Medzhitov, 2006a; Russell and Yates, 2007). Another study showed that TLR stimulation recruits components of the autophagy pathway to phagosomes, resulting in enhanced phagosome maturation (Sanjuan et al., 2007). The latter three studies on TLR-dependent phagosome maturation have used macrophages and not all findings may apply to DCs.

Finally, maturation signals alone do not determine the cross-presentation ability of a DC. There are many subsets of peripheral DCs that mature after encounter of antigen and migrate to lymph nodes, yet only a specialized subset, defined by the expression of $CD103^+$ (Bedoui et al., 2009; Henri et al., 2010) can cross-present with high efficiency. A particular transcriptional profile is likely to be responsible for this, because migratory $CD103^+$ DCs are very similar to cross-presenting lymph node-resident $CD8a^+$ DCs, sharing the marker XCR1 (Crozat et al., 2011).

CONCLUDING REMARKS

Understanding how maturation affects cross-presentation *in vitro* and *in vivo* is important for vaccination strategies and other immunotherapies, where the induction of maturation is a prerequisite for eliciting an effective T cell response. In addition, dissecting which factors influence the ability to cross-present during maturation will advance our understanding of the molecular process of cross-presentation. Studying maturation *in vitro* allows one to work with one cell type under defined conditions, where the modification of a single parameter, such as the addition of a TLR ligand, changes the outcome of cross-presentation. However, any advances obtained from *in vitro* systems need to be verified in more complex *in vivo* settings, a step where knockout animals have proven to be an invaluable tool. Models using infectious agents that can subvert antigen processing pathways will also contribute to our understanding. Last but not least, having the necessary reagents and readouts to follow antigens other than OVA is a major requirement for further progress.

REFERENCES

Ackerman, A. L., and Cresswell, P. (2003). Regulation of MHC class I transport in human dendritic cells and the dendritic-like cell line KG-1. *J. Immunol.* 170, 4178–4188.

Ackerman, A. L., Giodini, A., and Cresswell, P. (2006). A role for the endoplasmic reticulum protein retrotranslocation machinery during crosspresentation by dendritic cells. *Immunity* 25, 607–617.

Ackerman, A. L., Kyritsis, C., Tampe, R., and Cresswell, P. (2003). Early phagosomes in dendritic cells form a cellular compartment sufficient for cross presentation of exogenous antigens. *Proc. Natl. Acad. Sci. U.S.A.* 100, 12889–12894.

Bagai, R., Valujskikh, A., Canaday, D. H., Bailey, E., Lalli, P. N., Harding, C. V., and Heeger, P. S. (2005). Mouse endothelial cells cross-present lymphocyte-derived antigen on class I MHC via a TAP1- and proteasome-dependent pathway. *J. Immunol.* 174, 7711–7715.

Bedoui, S., Whitney, P. G., Waithman, J., Eidsmo, L., Wakim, L., Caminschi, I., Allan, R. S., Wojtasiak, M., Shortman, K., Carbone, F. R., Brooks, A. G., and Heath, W. R. (2009). Cross-presentation of viral and self antigens by skin-derived $CD103^+$ dendritic cells. *Nat. Immunol.* 10, 488–495.

Belizaire, R., and Unanue, E. R. (2009). Targeting proteins to distinct subcellular compartments reveals unique requirements for MHC class I and II presentation. *Proc. Natl. Acad. Sci. U.S.A.*106, 17463–17468.

Berwin, B., Hart, J. P., Rice, S., Gass, C., Pizzo, S. V., Post, S. R., and Nicchitta, C. V. (2003). Scavenger receptor-A mediates gp96/GRP94 and calreticulin internalization by antigen-presenting cells. *EMBO J.* 22, 6127–6136.

Bevan, M. J. (1976). Cross-priming for a secondary cytotoxic response to minor H antigens with H-2 congenic cells which do not cross-react in the cytotoxic assay. *J. Exp. Med.* 143, 1283–1288.

Blander, J. M., and Medzhitov, R. (2004). Regulation of phagosome maturation by signals from Toll-like receptors. *Science* 304, 1014–1018.

Blander, J. M., and Medzhitov, R. (2006a). On regulation of phagosome maturation and antigen presentation. *Nat. Immunol.* 7, 1029–1035.

Blander, J. M., and Medzhitov, R. (2006b). Toll-dependent selection of microbial antigens for presentation by dendritic cells. *Nature* 440, 808–812.

Bonifaz, L., Bonnyay, D., Mahnke, K., Rivera, M., Nussenzweig, M. C., and Steinman, R. M. (2002). Efficient targeting of protein antigen to the dendritic cell receptor DEC-205 in the steady state leads to antigen presentation on major histocompatibility complex class I products and peripheral $CD8^+$ T cell tolerance. *J. Exp. Med.* 196, 1627–1638.

Bozzacco, L., Trumpfheller, C., Siegal, F. P., Mehandru, S., Markowitz, M., Carrington, M., Nussenzweig, M. C., Piperno, A. G., and Steinman, R. M. (2007). DEC-205 receptor on dendritic cells mediates presentation of HIV gag protein to $CD8^+$ T cells in a spectrum of human MHC I haplotypes. *Proc. Natl. Acad. Sci. U.S.A.* 104, 1289–1294.

Braun, S., Matuschewski, K., Rape, M., Thoms, S., and Jentsch, S. (2002). Role of the ubiquitin-selective CDC48(UFD1/NPL4) chaperone (segregase) in ERAD of OLE1 and other substrates. *EMBO J.* 21, 615–621.

Burgdorf, S., Lukacs-Kornek, V., and Kurts, C. (2006). The mannose receptor mediates uptake of soluble but not of cell-associated antigen for cross-presentation. *J. Immunol.* 176, 6770–6776.

Burgdorf, S., Scholz, C., Kautz, A., Tampe, R., and Kurts, C. (2008). Spatial and mechanistic separation of cross-presentation and endogenous antigen presentation. *Nat. Immunol.* 9, 558–566.

Cebrian, I., Visentin, G., Blanchard, N., Jouve, M., Bobard, A., Moita, C., Enninga, J., Moita, L. F., Amigorena, S., and Savina, A. (2011). Sec22b regulates phagosomal maturation and antigen crosspresentation by dendritic cells. *Cell* 147, 1355–1368.

Cella, M., Engering, A., Pinet, V., Pieters, J., and Lanzavecchia, A. (1997). Inflammatory stimuli induce accumulation of MHC class II complexes on dendritic cells. *Nature* 388, 782–787.

Crozat, K., Tamoutounour, S., Vu Manh, T. P., Fossum, E., Luche, H., Ardouin, L., Guilliams, M., Azukizawa, H., Bogen, B., Malissen, B., Henri, S., and Dalod, M. (2011). Cutting edge: expression of XCR1 defines mouse lymphoid-tissue resident and migratory dendritic cells of the $CD8alpha^+$ type. *J. Immunol.* 187, 4411–4415.

Datta, S. K., Redecke, V., Prilliman, K. R., Takabayashi, K., Corr, M., Tallant, T., Didonato, J., Dziarski, R., Akira, S., Schoenberger, S. P., and Raz, E. (2003). A subset of Toll-like receptor ligands induces cross-presentation by bone marrow-derived dendritic cells. *J. Immunol.* 170, 4102–4110.

Delamarre, L., Holcombe, H., and Mellman, I. (2003). Presentation of exogenous antigens on major histocompatibility complex (MHC) class I and MHC class II molecules is differentially regulated during dendritic cell maturation. *J. Exp. Med.* 198, 111–122.

Delamarre, L., Pack, M., Chang, H., Mellman, I., and Trombetta, E. S. (2005). Differential lysosomal proteolysis in antigen-presenting cells determines antigen fate. *Science* 307, 1630–1634.

Delneste, Y., Magistrelli, G., Gauchat, J., Haeuw, J., Aubry, J., Nakamura, K., Kawakami-Honda, N., Goetsch, L., Sawamura, T., Bonnefoy, J., and Jeannin, P. (2002). Involvement of LOX-1 in dendritic cell-mediated antigen cross-presentation. *Immunity* 17, 353–362.

den Haan, J. M., and Bevan, M. J. (2002). Constitutive versus activation-dependent cross-presentation of immune complexes by CD8(+) and CD8(−) dendritic cells in vivo. *J. Exp. Med.* 196, 817–827.

Di Pucchio, T., Chatterjee, B., Smed-Sorensen, A., Clayton, S., Palazzo, A., Montes, M., Xue, Y., Mellman, I., Banchereau, J., and Connolly, J. E. (2008). Direct proteasome-independent cross-presentation of viral antigen by plasmacytoid dendritic cells on major histocompatibility complex class I. *Nat. Immunol.* 9, 551–557.

Dong, M., Bridges, J. P., Apsley, K., Xu, Y., and Weaver, T. E. (2008). ERdj4 and ERdj5 are required for endoplasmic reticulum-associated protein degradation of misfolded surfactant

protein C. *Mol. Biol. Cell* 19, 2620–2630.

Drutman, S. B., and Trombetta, E. S. (2010). Dendritic cells continue to capture and present antigens after maturation in vivo. *J. Immunol.* 185, 2140–2146.

Fonteneau, J. F., Kavanagh, D. G., Lirvall, M., Sanders, C., Cover, T. L., Bhardwaj, N., and Larsson, M. (2003). Characterization of the MHC class I cross-presentation pathway for cell-associated antigens by human dendritic cells. *Blood* 102, 4448–4455.

Gagnon, E., Duclos, S., Rondeau, C., Chevet, E., Cameron, P. H., Steele-Mortimer, O., Paiement, J., Bergeron, J. J., and Desjardins, M. (2002). Endoplasmic reticulum-mediated phagocytosis is a mechanism of entry into macrophages. *Cell* 110, 119–131.

Gil-Torregrosa, B. C., Lennon-Dumenil, A. M., Kessler, B., Guermonprez, P., Ploegh, H. L., Fruci, D., Van Endert, P., and Amigorena, S. (2004). Control of cross-presentation during dendritic cell maturation. *Eur. J. Immunol.* 34, 398–407.

Giodini, A., and Cresswell, P. (2008). Hsp90-mediated cytosolic refolding of exogenous proteins internalized by dendritic cells. *EMBO J.* 27, 201–211.

Giodini, A., Rahner, C., and Cresswell, P. (2009). Receptor-mediated phagocytosis elicits cross-presentation in nonprofessional antigen-presenting cells. *Proc. Natl. Acad. Sci. U.S.A.* 106, 3324–3329.

Goldszmid, R. S., Coppens, I., Lev, A., Caspar, P., Mellman, I., and Sher, A. (2009). Host ER-parasitophorous vacuole interaction provides a route of entry for antigen cross-presentation in *Toxoplasma gondii*-infected dendritic cells. *J. Exp. Med.* 206, 399–410.

Graham, D. B., Stephenson, L. M., Lam, S. K., Brim, K., Lee, H. M., Bautista, J., Gilfillan, S., Akilesh, S., Fujikawa, K., and Swat, W. (2007). An ITAM-signaling pathway controls cross-presentation of particulate but not soluble antigens in dendritic cells. *J. Exp. Med.* 204, 2889–2897.

Grotzke, J. E., Harriff, M. J., Siler, A. C., Nolt, D., Delepine, J., Lewinsohn, D. A., and Lewinsohn, D. M. (2009). The Mycobacterium tuberculosis phagosome is a HLA-I processing competent organelle. *PLoS Pathog.* 5, e1000374. doi: 10.1371/journal.ppat.1000374

Guermonprez, P., Saveanu, L., Kleijmeer, M., Davoust, J., Van Endert, P., and Amigorena, S. (2003). ER-phagosome fusion defines an MHC class I cross-presentation compartment in dendritic cells. *Nature* 425, 397–402.

Heath, W. R., Belz, G. T., Behrens, G. M., Smith, C. M., Forehan, S. P., Parish, I. A., Davey, G. M., Wilson, N. S., Carbone, F. R., and Villadangos, J. A. (2004). Cross-presentation, dendritic cell subsets, and the generation of immunity to cellular antigens. *Immunol. Rev.* 199, 9–26.

Henri, S., Poulin, L. F., Tamoutounour, S., Ardouin, L., Guilliams, M., De Bovis, B., Devilard, E., Viret, C., Azukizawa, H., Kissenpfennig, A., and Malissen, B. (2010). CD207+ CD103+ dermal dendritic cells cross-present keratinocyte-derived antigens irrespective of the presence of Langerhans cells. *J. Exp. Med.* 207, 189–206.

Henri, S., Siret, C., Machy, P., Kissenpfennig, A., Malissen, B., and Leserman, L. (2007). Mature DC from skin and skin-draining LN retain the ability to acquire and efficiently present targeted antigen. *Eur. J. Immunol.* 37, 1184–1193.

Hildner, K., Edelson, B. T., Purtha, W. E., Diamond, M., Matsushita, H., Kohyama, M., Calderon, B., Schraml, B. U., Unanue, E. R., Diamond, M. S., Schreiber, R. D., Murphy, T. L., and Murphy, K. M. (2008). Batf3 deficiency reveals a critical role for CD8alpha+ dendritic cells in cytotoxic T cell immunity. *Science* 322, 1097–1100.

Houde, M., Bertholet, S., Gagnon, E., Brunet, S., Goyette, G., Laplante, A., Princiotta, M. F., Thibault, P., Sacks, D., and Desjardins, M. (2003). Phagosomes are competent organelles for antigen cross-presentation. *Nature* 425, 402–406.

Ichiyanagi, T., Imai, T., Kajiwara, C., Mizukami, S., Nakai, A., Nakayama, T., and Udono, H. (2010). Essential role of endogenous heat shock protein 90 of dendritic cells in antigen cross-presentation. *J. Immunol.* 185, 2693–2700.

Imai, J., Hasegawa, H., Maruya, M., Koyasu, S., and Yahara, I. (2005). Exogenous antigens are processed through the endoplasmic reticulum-associated degradation (ERAD) in cross-presentation by dendritic cells. *Int. Immunol.* 17, 45–53.

Imai, T., Kato, Y., Kajiwara, C., Mizukami, S., Ishige, I., Ichiyanagi, T., Hikida, M., Wang, J. Y., and Udono, H. (2011). Heat shock protein 90 (HSP90) contributes to cytosolic translocation of extracellular antigen for cross-presentation by dendritic cells. *Proc. Natl. Acad. Sci. U.S.A.* 108, 16363–16368.

Jancic, C., Savina, A., Wasmeier, C., Tolmachova, T., El-Benna, J., Dang, P. M., Pascolo, S., Gougerot-Pocidalo, M. A., Raposo, G., Seabra, M. C., and Amigorena, S. (2007). Rab27a regulates phagosomal pH and NADPH oxidase recruitment to dendritic cell phagosomes. *Nat. Cell Biol.* 9, 367–378.

Jarosch, E., Taxis, C., Volkwein, C., Bordallo, J., Finley, D., Wolf, D. H., and Sommer, T. (2002). Protein dislocation from the ER requires polyubiquitination and the AAA-ATPase Cdc48. *Nat. Cell Biol.* 4, 134–139.

Jung, S., Unutmaz, D., Wong, P., Sano, G., De Los Santos, K., Sparwasser, T., Wu, S., Vuthoori, S., Ko, K., Zavala, F., Pamer, E. G., Littman, D. R., and Lang, R. A. (2002). In vivo depletion of CD11c+ dendritic cells abrogates priming of CD8+ T cells by exogenous cell-associated antigens. *Immunity* 17, 211–220.

Kamath, A. T., Henri, S., Battye, F., Tough, D. F., and Shortman, K. (2002). Developmental kinetics and lifespan of dendritic cells in mouse lymphoid organs. *Blood* 100, 1734–1741.

Kirschke, H., Wiederanders, B., Bromme, D., and Rinne, A. (1989). Cathepsin S from bovine spleen. Purification, distribution, intracellular localization and action on proteins. *Biochem. J.* 264, 467–473.

Klechevsky, E., Flamar, A. L., Cao, Y., Blanck, J. P., Liu, M., O'Bar, A., Agouna-Deciat, O., Klucar, P., Thompson-Snipes, L., Zurawski, S., Reiter, Y., Palucka, A. K., Zurawski, G., and Banchereau, J. (2010). Cross-priming CD8+ T cells by targeting antigens to human dendritic cells through DCIR. *Blood* 116, 1685–1697.

Koopmann, J. O., Albring, J., Huter, E., Bulbuc, N., Spee, P., Neefjes, J., Hammerling, G. J., and Momburg, F. (2000). Export of antigenic peptides from the endoplasmic reticulum intersects with retrograde protein translocation through the Sec61p channel. *Immunity* 13, 117–127.

Kostova, Z., Tsai, Y. C., and Weissman, A. M. (2007). Ubiquitin ligases, critical mediators of endoplasmic reticulum-associated degradation. *Semin. Cell Dev. Biol.* 18, 770–779.

Kovacsovics-Bankowski, M., and Rock, K. L. (1995). A phagosome-to-cytosol pathway for exogenous antigens presented on MHC class I molecules. *Science* 267, 243–246.

Laroux, F. S., Romero, X., Wetzler, L., Engel, P., and Terhorst, C. (2005). Cutting edge: MyD88 controls phagocyte NADPH oxidase function and killing of gram-negative bacteria. *J. Immunol.* 175, 5596–5600.

Lee, R. J., Liu, C. W., Harty, C., Mccracken, A. A., Latterich, M., Romisch, K., Demartino, G. N., Thomas, P. J., and Brodsky, J. L. (2004). Uncoupling retrotranslocation and degradation in the ER-associated degradation of a soluble protein. *EMBO J.* 23, 2206–2215.

Lelouard, H., Ferrand, V., Marguet, D., Bania, J., Camosseto, V., David, A., Gatti, E., and Pierre, P. (2004). Dendritic cell aggresome-like induced structures are dedicated areas for ubiquitination and storage of newly synthesized defective proteins. *J. Cell Biol.* 164, 667–675.

Lennon-Dumenil, A. M., Bakker, A. H., Maehr, R., Fiebiger, E., Overkleeft, H. S., Rosemblatt, M., Ploegh, H. L., and Lagaudriere-Gesbert, C. (2002). Analysis of protease activity in live antigen-presenting cells shows regulation of the phagosomal proteolytic contents during dendritic cell activation. *J. Exp. Med.* 196, 529–540.

Li, G., Zhou, X., Zhao, G., Schindelin, H., and Lennarz, W. J. (2005). Multiple modes of interaction of the deglycosylation enzyme, mouse peptide *N*-glycanase, with the proteasome. *Proc. Natl. Acad. Sci. U.S.A.* 102, 15809–15814.

Lin, M. L., Zhan, Y., Proietto, A. I., Prato, S., Wu, L., Heath, W. R., Villadangos, J. A., and Lew, A. M. (2008). Selective suicide of cross-presenting CD8+ dendritic cells by cytochrome c injection shows functional heterogeneity within this subset. *Proc. Natl. Acad. Sci. U.S.A.* 105, 3029–3034.

Lipson, C., Alalouf, G., Bajorek, M., Rabinovich, E., Atir-Lande, A., Glickman, M., and Bar-Nun, S. (2008). A proteasomal ATPase contributes to dislocation of endoplasmic reticulum-associated degradation (ERAD) substrates. *J. Biol. Chem.* 283, 7166–7175.

Lutz, M. B., Rovere, P., Kleijmeer, M. J., Rescigno, M., Assmann, C. U., Oorschot, V. M., Geuze, H. J., Trucy, J., Demandolx, D., Davoust, J., and Ricciardi-Castagnoli, P. (1997). Intracellular routes and selective retention of antigens in mildly acidic cathepsin D/lysosome-associated membrane protein-1/MHC class II-positive vesicles in immature dendritic cells. *J. Immunol.* 159, 3707–3716.

Machy, P., Serre, K., and Leserman, L. (2000). Class I-restricted presentation of exogenous antigen acquired by Fcgamma receptor-mediated endocytosis is regulated in dendritic cells. *Eur. J. Immunol.* 30, 848–857.

Mayer, T. U., Braun, T., and Jentsch, S. (1998). Role of the proteasome in membrane extraction of a short-lived ER-transmembrane protein. *EMBO J.* 17, 3251–3257.

Merzougui, N., Kratzer, R., Saveanu, L., and Van Endert, P. (2011). A proteasome-dependent, TAP-independent pathway for cross-presentation of phagocytosed antigen. *EMBO Rep.* 12, 1257–1264.

Molinari, M., Galli, C., Piccaluga, V., Pieren, M., and Paganetti, P. (2002). Sequential assistance of molecular chaperones and transient formation of covalent complexes during protein degradation from the ER. *J. Cell Biol.* 158, 247–257.

Morreale, G., Conforti, L., Coadwell, J., Wilbrey, A. L., and Coleman, M. P. (2009). Evolutionary divergence of valosin-containing protein/cell division cycle protein 48 binding interactions among endoplasmic reticulum-associated degradation proteins. *FEBS J.* 276, 1208–1220.

Muller-Taubenberger, A., Lupas, A. N., Li, H., Ecke, M., Simmeth, E., and Gerisch, G. (2001). Calreticulin and calnexin in the endoplasmic reticulum are important for phagocytosis. *EMBO J.* 20, 6772–6782.

Pfeifer, J. D., Wick, M. J., Roberts, R. L., Findlay, K., Normark, S. J., and Harding, C. V. (1993). Phagocytic processing of bacterial antigens for class I MHC presentation to T cells. *Nature* 361, 359–362.

Platt, C. D., Ma, J. K., Chalouni, C., Ebersold, M., Bou-Reslan, H., Carano, R. A., Mellman, I., and Delamarre, L. (2010). Mature dendritic cells use endocytic receptors to capture and present antigens. *Proc. Natl. Acad. Sci. U.S.A.* 107, 4287–4292.

Rabinovich, E., Kerem, A., Frohlich, K. U., Diamant, N., and Bar-Nun, S. (2002). AAA-ATPase p97/Cdc48p, a cytosolic chaperone required for endoplasmic reticulum-associated protein degradation. *Mol. Cell. Biol.* 22, 626–634.

Regnault, A., Lankar, D., Lacabanne, V., Rodriguez, A., Thery, C., Rescigno, M., Saito, T., Verbeek, S., Bonnerot, C., Ricciardi-Castagnoli, P., and Amigorena, S. (1999a). Fcgamma receptor-mediated induction of dendritic cell maturation and major histocompatibility complex class I-restricted antigen presentation after immune complex internalization. *J. Exp. Med.* 189, 371–380.

Rescigno, M., Citterio, S., Thery, C., Rittig, M., Medaglini, D., Pozzi, G., Amigorena, S., and Ricciardi-Castagnoli, P. (1998). Bacteria-induced neo-biosynthesis, stabilization, and surface expression of functional class I molecules in mouse dendritic cells. *Proc. Natl. Acad. Sci. U.S.A.* 95, 5229–5234.

Russell, D. G., and Yates, R. M. (2007). Toll-like receptors and phagosome maturation. *Nat. Immunol.* 8, 217; Author reply 217–218.

Sancho, D., Joffre, O. P., Keller, A. M., Rogers, N. C., Martinez, D., Hernanz-Falcon, P., Rosewell, I., and Reis e Sousa, C. (2009). Identification of a dendritic cell receptor that couples sensing of necrosis to immunity. *Nature* 458, 899–903.

Sancho, D., Mourao-Sa, D., Joffre, O. P., Schulz, O., Rogers, N. C., Pennington, D. J., Carlyle, J. R., and Reis e Sousa, C. (2008). Tumor therapy in mice via antigen targeting to a novel, DC-restricted C-type lectin. *J. Clin. Invest.* 118, 2098–2110.

Sanjuan, M. A., Dillon, C. P., Tait, S. W., Moshiach, S., Dorsey, F., Connell, S., Komatsu, M., Tanaka, K., Cleveland, J. L., Withoff, S., and Green, D. R. (2007). Toll-like receptor signalling in macrophages links the autophagy pathway to phagocytosis. *Nature* 450, 1253–1257.

Savina, A., Jancic, C., Hugues, S., Guermonprez, P., Vargas, P., Moura, I. C., Lennon-Dumenil, A. M., Seabra, M. C., Raposo, G., and Amigorena, S. (2006). NOX2 controls phagosomal pH to regulate antigen processing during crosspresentation by dendritic cells. *Cell* 126, 205–218.

Savina, A., Peres, A., Cebrian, I., Carmo, N., Moita, C., Hacohen, N., Moita, L. F., and Amigorena, S. (2009). The small GTPase Rac2 controls phagosomal alkalinization and antigen crosspresentation selectively in CD8(+) dendritic cells. *Immunity* 30, 544–555.

Schnorrer, P., Behrens, G. M., Wilson, N. S., Pooley, J. L., Smith, C. M., El-Sukkari, D., Davey, G., Kupresanin, F., Li, M., Maraskovsky, E., Belz, G. T., Carbone, F. R., Shortman, K., Heath, W. R., and Villadangos, J. A. (2006). The dominant role of CD8+ dendritic cells in cross-presentation is not dictated by antigen capture. *Proc. Natl. Acad. Sci. U.S.A.* 103, 10729–10734.

Schulze, A., Standera, S., Buerger, E., Kikkert, M., Van Voorden, S., Wiertz, E., Koning, F., Kloetzel, P. M., and Seeger, M. (2005). The ubiquitin-domain protein HERP forms a complex with components of the endoplasmic reticulum associated degradation pathway. *J. Mol. Biol.* 354, 1021–1027.

Shakushiro, K., Yamasaki, Y., Nishikawa, M., and Takakura, Y. (2004). Efficient scavenger receptor-mediated uptake and cross-presentation of negatively charged soluble antigens by dendritic cells. *Immunology* 112, 211–218.

Shen, L., Sigal, L. J., Boes, M., and Rock, K. L. (2004). Important role of cathepsin S in generating peptides for TAP-independent MHC class I crosspresentation in vivo. *Immunity* 21, 155–165.

Singh, R., and Cresswell, P. (2010). Defective cross-presentation of viral antigens in GILT-free mice. *Science* 328, 1394–1398.

Swanson, J. A., and Hoppe, A. D. (2004). The coordination of signaling during Fc receptor-mediated phagocytosis. *J. Leukoc. Biol.* 76, 1093–1103.

Tacken, P. J., De Vries, I. J., Gijzen, K., Joosten, B., Wu, D., Rother, R. P., Faas, S. J., Punt, C. J., Torensma, R., Adema, G. J., and Figdor, C. G. (2005). Effective induction of naive and recall T-cell responses by targeting antigen to human dendritic cells via a humanized anti-DC-SIGN antibody. *Blood* 106, 1278–1285.

Taylor, M., Navarro-Garcia, F., Huerta, J., Burress, H., Massey, S., Ireton, K., and Teter, K. (2010). Hsp90 is required for transfer of the cholera toxin A1 subunit from the endoplasmic reticulum to the cytosol. *J. Biol. Chem.* 285, 31261–31267.

Trombetta, E. S., Ebersold, M., Garrett, W., Pypaert, M., and Mellman, I. (2003). Activation of lysosomal function during dendritic cell maturation. *Science* 299, 1400–1403.

Ushioda, R., Hoseki, J., Araki, K., Jansen, G., Thomas, D. Y., and Nagata, K. (2008). ERdj5 is required as a disulfide reductase for degradation of misfolded proteins in the ER. *Science* 321, 569–572.

van Montfoort, N., Camps, M. G., Khan, S., Filippov, D. V., Weterings, J. J., Griffith, J. M., Geuze, H. J., Van Hall, T., Verbeek, J. S., Melief, C. J., and Ossendorp, F. (2009). Antigen storage compartments in mature dendritic cells facilitate prolonged cytotoxic T lymphocyte cross-priming capacity. *Proc. Natl. Acad. Sci. U.S.A.* 106, 6730–6735.

Vembar, S. S., and Brodsky, J. L. (2008). One step at a time: endoplasmic reticulum-associated degradation. *Nat. Rev. Mol. Cell Biol.* 9, 944–957.

Villadangos, J. A., Schnorrer, P., and Wilson, N. S. (2005). Control of MHC class II antigen presentation in dendritic cells: a balance between creative and destructive forces. *Immunol. Rev.* 207, 191–205.

Villadangos, J. A., and Shortman, K. (2010). Found in translation: the human equivalent of mouse CD8+ dendritic cells. *J. Exp. Med.* 207, 1131–1134.

Vulcano, M., Dusi, S., Lissandrini, D., Badolato, R., Mazzi, P., Riboldi, E., Borroni, E., Calleri, A., Donini, M., Plebani, A., Notarangelo, L., Musso, T., and Sozzani, S. (2004). Toll receptor-mediated regulation of NADPH oxidase in human dendritic cells. *J. Immunol.* 173, 5749–5756.

Wagner, C. S., and Cresswell, P. (2012). TLR and nucleotide-binding oligomerization domain-like receptor signals differentially regulate exogenous antigen presentation. *J. Immunol.* 188, 686–693.

Weck, M. M., Grunebach, F., Werth, D., Sinzger, C., Bringmann, A., and Brossart, P. (2007). TLR ligands differentially affect uptake and presentation of cellular antigens. *Blood* 109, 3890–3894.

West, M. A., Wallin, R. P., Matthews, S. P., Svensson, H. G., Zaru, R., Ljunggren, H. G., Prescott, A. R., and Watts, C. (2004). Enhanced dendritic cell antigen capture via toll-like receptor-induced actin remodeling. *Science* 305, 1153–1157.

Wilson, N. S., Behrens, G. M., Lundie, R. J., Smith, C. M., Waithman, J., Young, L., Forehan, S. P., Mount, A., Steptoe, R. J., Shortman, K. D., De Koning-Ward, T. F., Belz, G. T., Carbone, F. R., Crabb, B. S., Heath, W. R., and Villadangos, J. A. (2006). Systemic activation of dendritic cells by Toll-like receptor ligands or malaria infection impairs cross-presentation and antiviral immunity. *Nat. Immunol.* 7, 165–172.

Wilson, N. S., and Villadangos, J. A. (2005). Regulation of antigen presentation and cross-presentation in the dendritic cell network: facts, hypothesis, and immunological implications. *Adv. Immunol.* 86, 241–305.

Yates, R. M., Hermetter, A., and Russell, D. G. (2005). The kinetics of phagosome maturation as a function of phagosome/lysosome fusion and acquisition of hydrolytic activity. *Traffic* 6, 413–420.

Yates, R. M., and Russell, D. G. (2005). Phagosome maturation proceeds independently of stimulation of toll-like receptors 2 and 4. *Immunity* 23, 409–417.

Ye, Y., Meyer, H. H., and Rapoport, T. A. (2001). The AAA ATPase Cdc48/p97 and its partners transport proteins from the ER into the cytosol. *Nature* 414, 652–656.

Ye, Y., Shibata, Y., Kikkert, M., Van Voorden, S., Wiertz, E., and Rapoport, T.

A. (2005). Recruitment of the p97 ATPase and ubiquitin ligases to the site of retrotranslocation at the endoplasmic reticulum membrane. *Proc. Natl. Acad. Sci. U.S.A.* 102, 14132–14138.

Zehner, M., Chasan, A. I., Schuette, V., Embgenbroich, M., Quast, T., Kolanus, W., and Burgdorf, S. (2011). Mannose receptor polyubiquitination regulates endosomal recruitment of p97 and cytosolic antigen translocation for cross-presentation. *Proc. Natl. Acad. Sci. U.S.A.* 108, 9933–9938.

Zhong, X., Shen, Y., Ballar, P., Apostolou, A., Agami, R., and Fang, S. (2004). AAA ATPase p97/valosin-containing protein interacts with gp78, a ubiquitin ligase for endoplasmic reticulum-associated degradation. *J. Biol. Chem.* 279, 45676–45684.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 27 March 2012; accepted: 14 May 2012; published online: 04 June 2012.

*Citation: Wagner CS, Grotzke JE and Cresswell P (2012) Intracellular events regulating cross-presentation. Front. Immun. **3**:138. doi: 10.3389/fimmu.2012.00138*

This article was submitted to Frontiers in Antigen Presenting Cell Biology, a specialty of Frontiers in Immunology.

frontiers in
IMMUNOLOGY

REVIEW ARTICLE
published: 03 January 2012
doi: 10.3389/fimmu.2011.00087

Cross-presentation: how to get there – or how to get the ER

***Christoph Kreer*†, *Judith Rauen*†, *Matthias Zehner*† *and Sven Burgdorf* ***

Life and Medical Sciences Institute, University of Bonn, Bonn, Germany

Edited by:
Christian Kurts, Friedrich Wilhelms-Universität Bonn, Germany

Reviewed by:
Peter Michael Kloetzel, Charite-Universitatsmedizin Berlin, Germany
Joke M. M. Den Haan, VU University Medical Center, Netherlands

***Correspondence:**
Sven Burgdorf, Laboratory of Cellular Immunology, Life and Medical Sciences Institute, Carl-Troll-Str. 31, 53105 Bonn, Germany.
e-mail: burgdorf@uni-bonn.de

†*Christoph Kreer, Judith Rauen and Matthias Zehner have contributed equally to this work.*

Antigen cross-presentation enables dendritic cells (DCs) to present extracellular antigens on major histocompatibility complex (MHC) I molecules, a process that plays an important role in the induction of immune responses against viruses and tumors and in the induction of peripheral tolerance. In order to allow intracellular processing for cross-presentation, internalized antigens are targeted by distinct endocytic receptors toward specific endosomal compartments, where they are protected from rapid lysosomal degradation. From these compartments, antigens are processed for loading onto MHC I molecules. Such processing generally includes antigen transport into the cytoplasm, a process that is regulated by members of the ER-associated degradation (ERAD) machinery. After proteasomal degradation in the cytoplasm, antigen-derived peptides have been shown to be re-imported into the same endosomal compartment by endosomal transporter associated with antigen processing, another ER protein, which is recruited toward the endosomes after DC maturation. In our review, we highlight the recent advances on the molecular mechanisms of cross-presentation. We focus on the necessity of such antigen storage compartments and point out important parallels to MHC I-restricted presentation of endogenous antigens. We discuss the composition of such endosomes and the targeting of extracellular antigens into this compartment by specific endocytic receptors. Finally, we highlight recent advances on the recruitment of the cross-presentation machinery, like the members of the MHC I loading complex and the ERAD machinery, from the ER toward these storage compartments, a process that can be induced by antigen encounter or by activation of the dendritic cell after contact with endotoxins.

Keywords: dendritic cells, cross-presentation, antigen storage compartments, endosomes

INTRODUCTION

Adaptive immune responses are induced when dendritic cells (DCs) encounter antigens in the peripheral tissue. Upon antigen recognition, the DC migrates toward the draining lymph node, where it can activate antigen-specific T cells (Mellman and Steinman, 2001). Therefore, the corresponding antigen is internalized by the DC and processed in specialized intracellular compartments. The resulting antigen-derived peptides are subsequently loaded on major histocompatibility complex (MHC) molecules. Whereas antigen loading onto MHC II molecules can lead to the activation of antigen-specific $CD4^+$ T helper cells, peptide loading onto MHC I can activate antigen-specific cytotoxic $CD8^+$ T cells.

In classical antigen presentation, intracellular antigens are degraded by the cytosolic proteasome. The resulting peptides are subsequently transported through the transporter associated with antigen processing (TAP) complex into the ER, where they can be loaded onto MHC I molecules (Purcell and Elliott, 2008). Exogenous proteins are internalized into the dendritic cell by endocytosis and end up in a lysosomal compartment, where they are degraded by lysosomal proteases to be loaded onto MHC II molecules (Trombetta et al., 2003). Apart from these classical presentation pathways, a process termed cross-presentation allows the presentation of extracellular antigens also on MHC I molecules (Bevan, 1976; Kurts et al., 1996).

Cross-presentation has been demonstrated to play an important role in a variety of processes, including the induction of an immune response against viruses that do not infect antigen-presenting cells directly or against tumors of non-hematopoietic origin (Huang et al., 1994; Sigal et al., 1999; den Haan and Bevan, 2001; Heath and Carbone, 2001).

The molecular mechanisms of antigen cross-presentation, however, remain only partially understood. In this review, we highlight some of the recent advances on these underlying mechanisms. We will focus on antigen targeting into specialized storage compartments, on the composition of these compartments and on the recruitment of members of the MHC I loading machinery toward these compartments.

THE IMPORTANCE OF ANTIGEN STORAGE COMPARTMENTS FOR CROSS-PRESENTATION

During cross-presentation, antigen-derived peptides are loaded onto MHC I molecules. Subsequently, these peptide–MHC I-complexes are transported toward the cell membrane, where they can be recognized by antigen-specific T cells. Whereas peptide-loaded MHC II molecules are stable at the cell membrane for several days (Cella et al., 1997), differing information on the half-life of peptide-loaded MHC I molecules can be found in literature (Eberl et al., 1996; Rescigno et al., 1998; Cella et al., 1999; Kukutsch et al., 2000). A direct comparison between the stability

of loaded MHC I molecules and MHC II molecules, however, showed that the half-life of loaded MHC I molecules is markedly decreased compared to peptide-loaded MHC II molecules (van Montfoort et al., 2009). This shorter half-life of peptide-loaded MHC I molecules has important implications for antigen cross-presentation. After antigen internalization, the cross-presenting DC must migrate toward the draining lymph node to activate antigen-specific T cells. Since this process is estimated to take up to 48 h (Martin-Fontecha et al., 2003), a prolonged MHC I-restricted presentation is required for efficient T cell activation, implying that ongoing antigen processing and loading of antigen-derived peptides onto MHC I molecules is indispensible. To ensure continuous peptide loading, it is essential that internalized antigens are not degraded instantly within the endo/lysosomal compartments of the DC, since this would rapidly eliminate putative epitopes for cross-presentation. For these reasons, prolonged cross-presentation depends on antigen storage in endosomal compartments, where they are protected from lysosomal degradation.

Delamarre et al. (2005) demonstrated that DCs express less lysosomal proteases compared to macrophages, resulting in a limited capacity for lysosomal degradation and a slower degradation rate of internalized antigens in DCs. Additionally, antigen stability in DCs is increased by active inhibition of lysosomal acidification, a process that prevents the activation of lysosomal proteases and therefore increases cross-presentation (Hotta et al., 2006). Endosome acidification is mediated by vacuolar ATPase (V-ATPase), which transports protons from the cytosol into the endosome (Nishi and Forgac, 2002). In DCs, this process is antagonized by NOX2-mediated alkalization of the endosome. The NADPH oxidase NOX2 is recruited by Rab27a toward endosomal membranes (Savina et al., 2006), where it produces reactive oxygen species (ROS). Since the production of such ROS within endosomes consumes large amounts of protons, it causes a strong alkalization of the endosome lumen (Savina et al., 2006), which neutralizes V-ATPase-mediated acidification and a neutral endosomal pH can be maintained. As described above, such neutral pH prevents rapid antigen degradation, resulting in enhanced cross-presentation. NOX2-mediated alkalization has been shown to be involved in cross-presentation of particulate antigens in phagosomes (Savina et al., 2006) and of soluble antigens in endosomes (Mantegazza et al., 2008).

The decreased expression of lysosomal proteases in DCs and endosomal alkalization by NOX2 might also be responsible for the high stability of antigens that are internalized by DCs in form of immune complexes. Ferry Ossendorp and colleagues have demonstrated that OVA-containing immune complexes were cross-presented efficiently over a time period of several days (van Montfoort et al., 2009). Importantly, nearly full-length OVA was present for over 3 days in endosomal storage compartments, from where it was steadily processed for cross-presentation.

Taken together, prolonged MHC I-restricted presentation requires antigen deposition in specialized storage compartments, where they are protected from extensive proteasomal or lysosomal degradation and from where continuous processing for loading onto MHC I molecules can take place.

ANTIGEN TARGETING INTO STORAGE COMPARTMENTS FOR CROSS-PRESENTATION BY DISTINCT ENDOCYTIC RECEPTORS

In many studies, efficient antigen cross-presentation was shown to be restricted to distinct subsets of DCs. In particular, the $CD8\alpha^+$ splenic DCs were shown to be much better in cross-presentation under steady state conditions compared to their $CD8\alpha^-$ counterpart in mice (den Haan et al., 2000; Pooley et al., 2001; Schnorrer et al., 2006). Accordingly, cross-presentation capacities in mice lacking $CD8\alpha^+$ DCs were severely reduced (Hildner et al., 2008) and the human counterpart of murine $CD8\alpha^+$ DCs was also demonstrated to have superior cross-presentation capacities (Bachem et al., 2010; Crozat et al., 2010; Jongbloed et al., 2010; Poulin et al., 2010).

Cross-presentation of antigens targeted toward DEC-205, an endocytic receptor that is predominantly expressed on $CD8\alpha^+$ splenic DCs was demonstrated to be much more efficient than antigens targeted toward DCIR2, which is only expressed on $CD8\alpha^-$ DCs (Dudziak et al., 2007). Additionally, specific targeting toward DEC-205 resulted in prolonged cross-presentation for over 2 weeks (Bonifaz et al., 2004). It was postulated that these differences in cross-presentation capacity are due to a reduced overall expression of the cross-presentation machinery in $CD8\alpha^-$ DCs (Schnorrer et al., 2006; Dudziak et al., 2007). Indeed, NOX2-mediated alkalization of phagosomes was demonstrated to be more pronounced in $CD8\alpha^+$ DCs (Savina et al., 2009). More recent studies demonstrate that also $CD8\alpha^-$ DCs possess intrinsic cross-presentation capacities, but that the mechanism by which the DCs internalize the antigen is crucial for its cross-presentation (Kamphorst et al., 2010). This was demonstrated using transgenic mice expressing the human DEC-205 on both $CD8\alpha^+$ and $CD8\alpha^-$ DC subsets. Antigen targeting toward this receptor resulted in similar levels of cross-presentation in both $CD8\alpha^+$ and $CD8\alpha^-$ DCs, indicating that also $CD8\alpha^-$ DCs are potent cross-presenters if the antigen is internalized via DEC-205 but not via DCIR2, demonstrating an important role for the endocytic receptor itself in cross-presentation.

In accordance to these findings, we previously demonstrated a clear correlation between the mechanism of antigen internalization and its presentation (Burgdorf et al., 2007). We could show that antigens internalized by DCs via fluid phase pinocytosis or scavenger receptor-mediated endocytosis were rapidly targeted toward lysosomal structures, where they were degraded instantly and processed for presentation on MHC II molecules. If, however, antigens were internalized via mannose receptor (MR)-mediated endocytosis, they were not targeted toward lysosomes but rather routed into a distinct endosomal subset, which maintained all characteristics of early endosomes for a prolonged time. Importantly, from these endosomes, MR-internalized antigens were processed exclusively for cross-presentation.

These observations emphasize the importance of the endocytic receptor for cross-presentation and point out that the endocytic receptor on the DC that makes contact to an antigen already determines its fate in terms of presentation. Additionally, targeting antigens intended for cross-presentation into a separate pool of endosomes might enable enhanced endosomal stability, which is

essential for prolonged cross-presentation, without affecting overall lysosomal activity, which is essential for simultaneous MHC II-restricted presentation.

Interestingly, the MR has also been proposed to directly inhibit lysosomal maturation (Shimada et al., 2006; Sweet et al., 2010), because phagosomes containing MR-internalized glycopeptidolipids displayed impaired phagosome–lysosome fusion. Such alterations were only observed if the glycopeptidolipids were endocytosed by the MR, which then was present in the glycopeptidolipid-containing phagosomes. In both publications, it was postulated that these effects might be due to MR-dependent signaling inside the DC, resulting in an overall impairment of phagosome–lysosome fusion. Another possibility would be that, similar to the observations on the role of the MR in cross-presentation, the MR targets the glycopeptidolipids into a separate endosomal compartment, which does not undergo normal lysosomal maturation. Further studies will reveal whether signaling via the MR additionally alters endosomal trafficking within DCs.

As described above, the different cross-presentation capacities of $CD8\alpha^+$ and $CD8\alpha^-$ splenic DCs might to a large extend be due to the expression of different endocytic receptors. This notion might also explain observations demonstrating that certain yeast antigens and antigens targeted to the neonatal Fc receptor are cross-presented to a higher extend by the $CD8\alpha^-$ subset (Backer et al., 2008; Baker et al., 2011). Future experiments will show whether $CD8\alpha^-$ DCs bear specific receptors for these antigens, enabling their cross-presentation.

The importance of distinct endocytic receptors for cross-presentation is further supported by experiments of the group of Peter Cresswell. They demonstrated that expression of Fc receptors, whose engagement has been shown to lead to potent cross-presentation (van Montfoort et al., 2009), in the human 293 T embryonic kidney cell line enables this cell line to cross-present extracellular antigens (Giodini et al., 2009), pointing out the possibility that nearly every cell possesses intrinsic cross-presentation capacities if the cell expresses a suited receptor.

Although the decisive role of the endocytic receptor for cross-presentation is indubitable, this might only be one half of the story. Increasing evidence points out that also the nature of interaction between the endocytic receptor and the antigen has an important impact on antigen routing and presentation. First, it has been demonstrated that receptor cross-linking by multivalent antigens alters antigen targeting within the DC. It has been shown that dectin-1, an endocytic receptor associated with cross-presentation (Weck et al., 2008), targets monovalent β-glucans into non-lysosomal compartments. If, however, dectin-1 is cross-linked by the multivalent β-glucan zymosan, these antigens are targeted toward lysosomal structures (Herre et al., 2004), demonstrating that antigen valence can regulate antigen trafficking and degradation. Second, it has been shown that the region of the endocytic receptor that recognizes the antigen is of crucial importance. Antigen targeting using antibodies specific for the carbohydrate recognition domain of DC-SIGN has been shown to efficiently deliver such antigens to lysosomal compartments for MHC II presentation (Tacken et al., 2005). A recent study by the same group demonstrated however, that antigen targeting toward the neck region of DC-SIGN results in prolonged antigen retention in early endosomal compartments and in reduced lysosomal trafficking (Tacken et al., 2011). Importantly, these antigens were efficiently cross-presented, demonstrating that different regions of a single endocytic receptor can target antigens to different processing and presentation pathways.

These findings might also provide an explanation for the observation that antigen targeting toward the MR, which targets OVA specifically toward cross-presentation (Burgdorf et al., 2007) as described above, can induce antigen-specific $CD4^+$ T cell responses (Dasgupta et al., 2007; He et al., 2007; McKenzie et al., 2007). In these studies, antigens were targeted toward the MR by conjugation to a MR-specific antibody, which might alter MR-mediated antigen targeting by receptor cross-linking. Alternatively, these antibodies might target other regions of the MR, resulting in different antigen processing and presentation.

In summary, efficient cross-presentation requires antigen recognition by distinct regions of specific endocytic receptors, which target the internalized antigens toward antigen storage compartments, from where they can be processed for loading onto MHC I molecules.

MOLECULAR MECHANISMS OF CROSS-PRESENTATION

THE VACUOLAR VERSUS THE PHAGOSOME-TO-CYTOSOL PATHWAY

Despite intensive investigations, the molecular mechanisms governing antigen processing and loading onto MHC I molecules for cross-presentation are not fully resolved yet. Importantly, the diversity of experimental evidence obtained by different research groups indicates that multiple pathways can lead to MHC I-restricted presentation of exogenous antigens, depending on the nature of the antigen, the nature of the antigen-presenting cell, and the immunological context of the cross-presentation process (**Figure 1**).

In general, two major pathways are considered to be most relevant for antigen cross-presentation: the vacuolar pathway and the phagosome-to-cytosol pathway (Rock and Shen, 2005).

In the vacuolar pathway, which is also termed TAP-independent cross-presentation, internalized antigens are degraded in endosomal compartments by intra-endosomal proteases such as cathepsin S (Shen et al., 2004). After such degradation, antigenic peptides are loaded within the endosomes onto MHC I molecules, which reach the endosomes from the cell surface during endocytosis. The acid environment in these endosomes might allow already bound peptides to dissociate from the MHC I molecules, enabling the peptides generated within the endosomes to bind MHC I molecules.

Although several studies reported of cross-presentation via the vacuolar pathway (Shen et al., 2004; Bertholet et al., 2006), its physiological significance remains unclear. Therefore, the phagosome-to-cytosol pathway is considered to be the most relevant cross-presentation pathway *in vivo* (Rock and Shen, 2005).

In the phagosome-to-cytosol pathway, internalized antigens need to be transported from the endosomal lumen into the cytoplasm. Such antigen transport is required for consecutive degradation by the cytosolic proteasome, which is essential for cross-presentation by the phagosome-to-cytosol pathway (Kovacsovics-Bankowski and Rock, 1995; Ackerman et al., 2003; Palmowski et al., 2006).

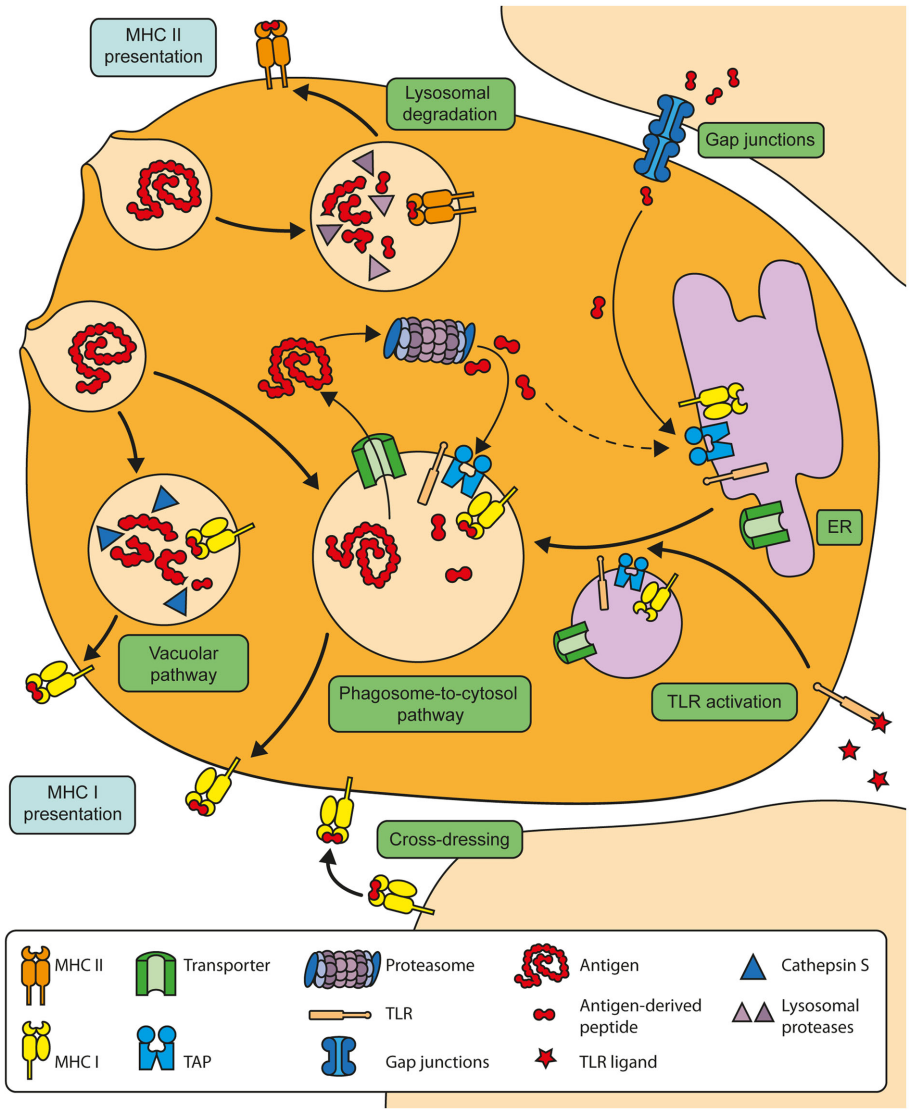


FIGURE 1 | Overview of the molecular mechanisms of cross-presentation. In the vacuolar cross-presentation pathway, extracellular antigens are internalized and degraded in endosomal compartments by Cathepsin S. The resulting peptides are subsequently loaded onto MHC I molecules within the endosomal compartment. In the phagosome-to-cytosol pathway, internalized antigens are transported out of the endosomes into the cytosol for proteasomal degradation. The resulting peptides can be re-imported into the same endosomal compartment by endosomal TAP to be loaded onto MHC I molecules there. The transport of the cross-presentation machinery toward antigen-containing endosomes is induced after stimulation of TLRs. Alternatively, DCs can obtain peptides from neighboring cells via gap junctions. These peptides are thought to subsequently enter the endogenous MHC I-restricted presentation pathway in the ER.

ANTIGEN TRANSPORT FROM ENDOSOMAL COMPARTMENTS INTO THE CYTOSOL AND PROTEASOMAL DEGRADATION

One of the most intriguing questions concerning the molecular mechanisms of antigen cross-presentation is without any doubt how antigens pass the endosomal membrane to reach the cytosol. Although it has been demonstrated that phagosomes containing Cryptococcus neoformans lose membrane integrity (Tucker and Casadevall, 2002) and the presence of sphingosine within the phagosome might influence membrane stability and permeability (Werneburg et al., 2002), it is assumed that antigen transport into the cytoplasm is not due to disruption of the endosomal membrane. Increasing evidence points out that a pore complex spanning the endosomal membrane might rather mediate this process. Antigen translocation has been demonstrated to be size-selective. Although dextranes of 500 kDa and even 2,000 kDa can still be translocated into the cytosol, the efficiency is clearly lower than the transport of 40 kDa-sized dextranes (Rodriguez et al., 1999), which supports the notion that such antigen transport is not simply due to a simple disrupture of the endosomal membrane.

Increasing evidence points out that the ER-associated degradation (ERAD) machinery plays a very central role in this antigen

transport into the cytoplasm (Imai et al., 2005; Ackerman et al., 2006; Giodini and Cresswell, 2008). The ERAD machinery has been studied extensively in the context of protein dislocation at the ER membrane. During dislocation, the ERAD machinery mediates the transport of misfolded proteins from the ER into the cytoplasm for proteasomal degradation, depicting an important function in preventing misfolded proteins from reaching the cell surface. One important member of the ERAD machinery in respect to cross-presentation is Sec61, which is thought to build the pore complex for dislocation of proteins trough the ER membrane. Similar to its role in dislocation, Sec61 has been postulated to build the pore complex through the endosomal membrane for cross-presentation (Ackerman et al., 2006). This hypothesis was based on observations, revealing that DC treatment with exotoxin A, which is assumed to be a specific inhibitor of Sec61, prevented antigen translocation into the cytoplasm and hence cross-presentation (Koopmann et al., 2000). Since such evidence for an involvement of Sec61 in antigen translocation was based on the effect of an inhibitor and therefore indirect, the role of Sec61 in cross-presentation has been questioned (Lin et al., 2008; Segura and Villadangos, 2011). In these studies, it was argued that the size of Sec61, which has been estimated to be about 5–8 Å (Van den Berg et al., 2004), might not be sufficient for antigen translocation. However, this size was calculated for closed or empty Sec61 and it has been postulated that the Sec61 pore complex during protein transport might encompass up to 40–60 Å (Hamman et al., 1997). Additionally, it has been demonstrated that proteins are unfolded during antigen translocation into the cytosol (Giodini and Cresswell, 2008), which might also enable them to transit also through a narrow pore complex.

More direct evidence for an involvement of Sec61 in antigen translocation was provided by experiments, in which Sec61 expression was down-regulated by siRNA (Imai et al., 2005). Such down-regulation prevented cytosolic translocation of the antigen and hence its degradation by the cytosolic proteasome, further supporting an important role of Sec61. However, since Sec61 also interferes with dislocation of MHC I molecules (Wiertz et al., 1996), it cannot be fully excluded that reduced cross-presentation observed in this study was due to an altered expression of MHC I molecules. Therefore, the exact role of Sec61 in antigen translocation for cross-presentation could not be unequivocally determined yet. Additionally, other members of the ERAD machinery, like derlin-1, have also been proposed as candidates to build the pore complex for antigen translocation across the endosomal membrane during cross-presentation (Lilley and Ploegh, 2004; Ye et al., 2004). But as for Sec61, future experiments are needed to reveal a decisive role of these proteins in intracellular antigen transport.

Another member of the ERAD machinery, which has been shown to play an important role in cross-presentation, is the soluble AAA ATPase p97 (Ackerman et al., 2006). P97, which is associated with both Sec61 and derlin-1, is recruited toward the endosomal membrane, where its ATPase activity provides the energy for antigen translocation. Expression of a dominant negative mutant of p97 has been demonstrated to abolish antigen translocation into the cytoplasm and hence cross-presentation (Ackerman et al., 2006; Zehner et al., 2011).

Recent evidence indicates that also Igtp, a protein involved in the generation of lipid bodies, influences antigen cross-presentation (Bougneres et al., 2009). Since Igtp deficiency abolishes cross-presentation but not MHC I-restricted presentation of antigens that were introduced directly into the cytoplasm, Igtp has been postulated as a putative regulator of antigen transport into the cytoplasm (Desjardins, 2009). Whether Igtp and/or lipid bodies indeed play a role in intracellular antigen translocation, however, remains to be elucidated.

After antigen translocation into the cytoplasm, it becomes ubiquitinated and processed by the cytoplasmic proteasome (Kovacsovics-Bankowski and Rock, 1995; Ackerman et al., 2003; Palmowski et al., 2006; Burgdorf et al., 2007, 2008). Importantly, the proteasome constitution in DCs differs from most other cell types. Within DCs, the standard catalytic subunits β1, β2, and β5 are replaced by β1i/LMP2, β2i/MECL-1, and β5i/LMP7 to build the immunoproteasome (Macagno et al., 1999). Such immunoproteasomes display an altered protease activity and cleavage site preference, resulting in the more efficient generation of MHC I epitopes (Kloetzel and Ossendorp, 2004) and the more efficient degradation of poly-ubiquitinated proteins (Seifert et al., 2010). Recent studies additionally reported of proteasomes intermediate between constitutive proteasomes and immunoproteasomes, in which only one or two catalytic subunits were replaced and which displayed an additional cleavage specificity (Guillaume et al., 2010), even enlarging the repertoire of antigens presented on MHC I molecules. The constitutive expression of such immunoproteasomes provides DCs with a unique capacity to generate a broad spectrum of peptides for loading on MHC I molecules.

Since antigens intended for cross-presentation generally enter the DC as proteasome substrates (Norbury et al., 2004), the half-life of the antigen is of crucial importance and epitopes that are degraded shortly after their synthesis are cross-presented very poorly (Wolkers et al., 2004).

LOADING OF ANTIGEN-DERIVED PEPTIDES ON MHC I MOLECULES

Subsequent to proteasomal degradation, cross-presentation requires functional TAP activity (Kovacsovics-Bankowski and Rock, 1995; Huang et al., 1996; Song and Harding, 1996; Norbury et al., 1997; Ackerman et al., 2003, 2006), which led to the hypothesis that proteasome-derived peptides might enter the classical MHC I loading pathway in the ER (Kovacsovics-Bankowski and Rock, 1995). Although direct evidence supporting this hypothesis is missing, it was broadly accepted for years. First evidence that peptide loading for cross-presentation might occur in cellular compartments distinct from the ER was based on the observation that antigen-containing phagosomes contain members of the MHC I loading machinery such as calreticulin, ERp57, tapasin, β2-microglobulin, Sec61, MHC I itself, and functional TAP (Ackerman et al., 2003; Guermonprez et al., 2003; Houde et al., 2003). These observations lead to the assumption that proteasome-derived peptides might be re-imported into the same phagosomal compartment for loading onto MHC I molecules there. Indeed, after TAP-mediated peptide transport into these phagosomes, the generation of peptide-loaded MHC I molecules within these phagosomes could be detected (Guermonprez et al., 2003). Such intra-phagosomal peptide loading was further accomplished by

the recruitment of proteasomes toward the phagosomal membrane (Houde et al., 2003), providing a spatial proximity of all components of the cross-presentation machinery, which might be essential to minimize rapid degradation of proteasome-derived peptides with very limited half-life (Reits et al., 2003) by cytosolic peptidases.

Formal evidence that peptide loading for cross-presentation indeed takes places in antigen-containing endosomes came from experiments that were aimed at inhibiting TAP activity in an endosome-specific fashion (Burgdorf et al., 2008). In this study, the soluble TAP inhibitor US6 (Ackerman et al., 2003), which inhibits TAP activity from its luminal side, was covalently linked to transferrin, resulting in its specific targeting to antigen-containing endosomes. Such endosome-specific targeting abolished TAP activity in endosomes without affecting TAP activity in the ER. By this approach, it was demonstrated that endosomal TAP was absolutely required for cross-presentation and that peptide loading for cross-presentation of MR-internalized antigens does not take place in the ER but occurs in antigen-containing endosomes. Such a spatial separation of endogenous MHC I-restricted antigen presentation and cross-presentation further supports the notion of a strong compartmentalization of MHC I-restricted antigen presentation (Lev et al., 2010), although peptide loading in the ER under certain circumstances cannot be excluded.

After proteasomal degradation and TAP-mediated transport into endosomal compartments, antigen-derived peptides must be trimmed into the suitable size for optimal binding to MHC I molecules, a function that is exerted in the endogenous MHC I presentation pathway by the ER-resident peptidase ERAP. Recent work by the group of Peter Van Endert identified IRAP as an endosome-specific peptidase required for such peptide-trimming in cross-presentation (Saveanu et al., 2009). IRAP, which is specifically targeted toward endosomes by its amino-terminal cytoplasmic tail (Hou et al., 2006), displays a broader pH optimum compared to ERAP, allowing IRAP activity at a slightly acidic endosomal pH (Georgiadou et al., 2010). IRAP activity might ensure that antigen-derived peptides, which were generated by proteasomal degradation in the cytoplasm and re-imported into the endosomes by endosomal TAP, are trimmed to their optimal size for loading on MHC I molecules, providing potent cross-presentation by the phagosome-to-cytosol pathway.

CROSS-PRESENTATION VIA GAP JUNCTIONS-MEDIATED PEPTIDE TRANSFER

In addition to the vacuolar and the phagosome-to-cytosol cross-presentation pathway, it has been demonstrated that DCs can obtain peptides from other cells by gap junctions-mediated cell–cell contact (Neijssen et al., 2005; Mendoza-Naranjo et al., 2007). Such peptides are though to directly enter the endogenous MHC I-restricted presentation pathway via TAP-mediated transport into the ER. Saccheri et al. (2010) demonstrated that infection of melanoma cells with salmonella induced an upregulation of Cx43, which increased the formation of gap junctions with DCs. These gap junctions enabled peptide transfer from the melanoma cell toward the DC, which resulted in the induction of an anti-melanoma immune response (Neijssen et al., 2005; Mendoza-Naranjo et al., 2007; Saccheri et al., 2010). Whether cross-presentation via gap junctions-mediated antigen transfer has a broad physiological relevance, however, remains unclear, especially because cytoplasmic peptides are rapidly degraded by cytosolic peptidases and display a half-life of only a few seconds (Reits et al., 2003).

CROSS-DRESSING OF DCS WITH PEPTIDE–MHC I-COMPLEXES

Independent of cross-presentation of internalized and processed antigens, DCs can also acquire MHC I molecules that are already loaded with antigen-derived peptides from a donor cell, a process that has been termed cross-dressing (Dolan et al., 2006; Smyth et al., 2008). Within this process, the antigen-presenting cell can obtain peptide–MHC I-complexes from a large variety of living or apoptotic donor cells. Presentation of such complexes to antigen-specific T cells does not require further processing by the DC. Transfer of the loaded MHC I molecules has been shown to be mediated by direct cell contact between the DC and the donor cell rather than by transfer of secreted vesicles like exosomes (Dolan et al., 2006; Wakim and Bevan, 2011). Such transfer occurred even at limited antigen concentrations (Smyth et al., 2008) and allows a direct antigen transfer from infected cells to DCs also *in vivo* (Dolan et al., 2006; Wakim and Bevan, 2011). The relevance of cross-dressing compared to direct or cross-presentation by DCs in the control of an infection remains to be analyzed further and will be the topic of intensive future investigations.

INFLUENCE OF DC MATURATION ON CROSS-PRESENTATION AND ON THE RECRUITMENT OF MHC LOADING MACHINERY FROM THE ER TOWARD ENDOSOMES

In the absence of inflammatory stimuli, cross-presentation of internalized antigens, which occurs at moderate efficiency in immature DCs (Burgdorf et al., 2008), leads to T cell tolerance. Once the DC becomes activated by the recognition of microbial substances, its cross-presentation capacities are enhanced. First, in maturing DCs, total antigen uptake is increased (Gil-Torregrosa et al., 2004). Additionally, the composition of the ubiquitin–proteasome system is altered during DC maturation (Ebstein et al., 2009) and overall proteasomal activity is increased (Gil-Torregrosa et al., 2004). Finally, also antigen translocation into the cytoplasm is increased in maturing DCs (Gil-Torregrosa et al., 2004). Since such antigen translocation is mediated by proteins derived from the ER, the transport of these proteins toward the endosomes is an important prerequisite for cross-presentation.

A process termed ER-mediated phagocytosis has been postulated to mediate the transport of ER membrane components to endosomal compartments (Gagnon et al., 2002). In this process, the ER serves as a membrane donor for the developing phagosome, which then contains fragments of both the ER and the plasma membrane. This model, however, has been discussed controversially (Touret et al., 2005) and its physiological significance remains unclear. The same is true for transient fusions between the ER and endosomes after internalization, which has also been proposed to be a putative mechanism for delivery of ER components to endosomal membranes. The existence of such fusion events, however, has never been clearly demonstrated.

Increasing evidence points out that the transport of ER components to endosomes is a process that is controlled very tightly

and that is increased during DC maturation. Goldszmid et al. (2009) demonstrated that the transport of ER components to *Toxoplasma gondii*-containing phagosomes only occurred if living protozoa were present in the phagosome. Additionally, our group demonstrated that TAP is only transported toward the endosomal membrane upon DC stimulation with LPS and due to the activation of the TLR4–MyD88 signaling pathway (Burgdorf et al., 2008). Likewise, the group of Peter Van Endert demonstrated that in unstimulated DCs, TAP is not present in IRAP-containing early endosomes (Saveanu et al., 2009). After phagocytosis of yeast cells, however, a clear TAP translocation from the ER toward the IRAP-containing endosomes was observed. Importantly, ERAP was not recruited to the antigen-containing endosomes, but maintained in the ER. This demonstrates that not all ER components are transported toward the endosome, but a process regulated by microbial substances rather induces the transport of only selected ER proteins toward the endosomal membrane. The selectivity of this transport also implicates that these ER components might not be recruited by ER-mediated endocytosis or by transient fusion events between the ER and the endosomes, since such events would result in an equal transport of all ER components toward the antigen-containing endosomes. These results might rather imply that upon stimulation with microbial substances, selective members of the ER undergo a directed ER-to-endosome transport.

Such TLR ligand-mediated transport from the ER toward endosomes reminds very much of the transport of several TLRs themselves. It has been demonstrated that TLR3, TLR7, and TLR9 in unstimulated DCs are localized in the ER (Latz et al., 2004). Upon DC stimulation with TLR ligands, however, these receptors are rapidly translocated toward early endosomes, from where their signaling takes place (Kagan et al., 2008). It is thinkable that, upon DC stimulation with endotoxin, some ER components involved in antigen presentation are translocated along with the TLRs toward early endosomes, where they can exert their function in cross-presentation. This transport has been shown to occur without passing the golgi and is regulated by the polytopic membrane protein UNC93B1 (Kim et al., 2008). Interestingly, a loss of function mutation of UNC93B1 has a severe influence on antigen presentation and in particular cross-presentation (Tabeta et al., 2006). Whether this loss of cross-presentation capacity indeed is due to an impaired transport of the cross-presentation machinery toward endosomes upon TLR signaling, however, still needs to be investigated.

Additional to the recruitment of ER components by TLR ligands, the endocytic receptor seems to play an important role for the recruitment of soluble ERAD components. In a recent study, we demonstrated that the MR, which targets its antigens specifically toward cross-presentation as described above, plays an important role in the recruitment of p97 (Zehner et al., 2011). During the ERAD process, p97 is recruited toward the ER by binding to poly-ubiquitinated proteins at the ER membrane (Ye et al., 2003). The recruitment of p97 toward the endosomal membrane for cross-presentation seems to be regulated in a very similar way. P97 recruitment for cross-presentation of MR-internalized antigens was regulated by ubiquitination of the MR (Zehner et al., 2011). Ligand binding to the MR induced poly-ubiquitination of its cytoplasmic tail. Without receptor poly-ubiquitination, no p97 recruitment toward the endosomal membrane took place and antigen transport into the cytoplasm and cross-presentation were impaired. These data demonstrate that the endocytic receptor is not only required for antigen targeting into a suited endosomal compartment for cross-presentation as described above, but also is of decisive importance for the antigen to get out of the endosomal compartment to reach the cytosol for proteasomal degradation.

CONCLUSION

In conclusion, it is important to emphasize that there are many different roads to cross-presentation. Whether extracellular antigens are cross-presented via the cytosolic or via the phagosome-to-cytosol pathway might be determined by the physiological conditions of both the antigen-presenting cell and the antigen itself or might even vary for different epitopes of the same antigen.

Furthermore, future experiments are needed to fully understand the molecular mechanisms underlying cross-presentation. Of special interest will be the identification of the pore complex that mediated antigen translocation into the cytoplasm, which without any doubt is one of the most important remaining open questions regarding cross-presentation. In this context, it will be important to unequivocally determine the role of Sec61 and the other postulated candidates in building the transmembrane pore complex.

Another question that will be subject of intense research is the transport of members of the MHC I loading machinery from the ER toward antigen-containing endosomes. Future experiments will show whether such components might be transported along with the different TLRs as postulated in this review, providing an explanation for the dependency of efficient cross-presentation on DC activation by TLR ligands.

ACKNOWLEDGMENTS

This work is supported by the German Research Foundation grant BU2441/1-1 and consortium SFB645 project C1.

REFERENCES

Ackerman, A. L., Giodini, A., and Cresswell, P. (2006). A role for the endoplasmic reticulum protein retrotranslocation machinery during cross-presentation by dendritic cells. *Immunity* 25, 607–617.

Ackerman, A. L., Kyritsis, C., Tampe, R., and Cresswell, P. (2003). Early phagosomes in dendritic cells form a cellular compartment sufficient for cross presentation of exogenous antigens. *Proc. Natl. Acad. Sci. U.S.A.* 100, 12889–12894.

Bachem, A., Guttler, S., Hartung, E., Ebstein, F., Schaefer, M., Tannert, A., Salama, A., Movassaghi, K., Opitz, C., Mages, H. W., Henn, V., Kloetzel, P. M., Gurka, S., and Kroczek, R. A. (2010). Superior antigen cross-presentation and XCR1 expression define human CD11c+CD141+ cells as homologues of mouse CD8+ dendritic cells. *J. Exp. Med.* 207, 1273–1281.

Backer, R., van Leeuwen, F., Kraal, G., and den Haan, J. M. (2008). CD8- dendritic cells preferentially cross-present *Saccharomyces cerevisiae* antigens. *Eur. J. Immunol.* 38, 370–380.

Baker, K., Qiao, S. W., Kuo, T. T., Aveson, V. G., Platzer, B., Andersen, J. T., Sandlie, I., Chen, Z., de Haar, C., Lencer, W. I., Fiebiger, E., and Blumberg, R. S. (2011). Neonatal Fc receptor for IgG (FcRn) regulates cross-presentation of IgG immune complexes by CD8-CD11b+ dendritic cells. *Proc. Natl. Acad. Sci. U.S.A.* 108, 9927–9932.

Bertholet, S., Goldszmid, R., Morrot, A., Debrabant, A., Afrin, F., Collazo-Custodio, C., Houde, M., Desjardins, M., Sher, A., and Sacks, D. (2006). *Leishmania* antigens are presented to CD8+T cells by a transporter associated with antigen processing-independent pathway in vitro and in vivo. *J. Immunol.* 177, 3525–3533.

Bevan, M. J. (1976). Cross-priming for a secondary cytotoxic response to minor H antigens with H-2 congenic cells which do not cross-react in the cytotoxic assay. *J. Exp. Med.* 143, 1283–1288.

Bonifaz, L. C., Bonnyay, D. P., Charalambous, A., Darguste, D. I., Fujii, S., Soares, H., Brimnes, M. K., Moltedo, B., Moran, T. M., and Steinman, R. M. (2004). In vivo targeting of antigens to maturing dendritic cells via the DEC-205 receptor improves T cell vaccination. *J. Exp. Med.* 199, 815–824.

Bougneres, L., Helft, J., Tiwari, S., Vargas, P., Chang, B. H., Chan, L., Campisi, L., Lauvau, G., Hugues, S., Kumar, P., Kamphorst, A. O., Dumenil, A. M., Nussenzweig, M., MacMicking, J. D., Amigorena, S., and Guermonprez, P. (2009). A role for lipid bodies in the cross-presentation of phagocytosed antigens by MHC class I in dendritic cells. *Immunity* 31, 232–244.

Burgdorf, S., Kautz, A., Bohnert, V., Knolle, P. A., and Kurts, C. (2007). Distinct pathways of antigen uptake and intracellular routing in CD4 and CD8 T cell activation. *Science* 316, 612–616.

Burgdorf, S., Scholz, C., Kautz, A., Tampe, R., and Kurts, C. (2008). Spatial and mechanistic separation of cross-presentation and endogenous antigen presentation. *Nat. Immunol.* 9, 558–566.

Cella, M., Engering, A., Pinet, V., Pieters, J., and Lanzavecchia, A. (1997). Inflammatory stimuli induce accumulation of MHC class II complexes on dendritic cells. *Nature* 388, 782–787.

Cella, M., Salio, M., Sakakibara, Y., Langen, H., Julkunen, I., and Lanzavecchia, A. (1999). Maturation, activation, and protection of dendritic cells induced by double-stranded RNA. *J. Exp. Med.* 189, 821–829.

Crozat, K., Guiton, R., Contreras, V., Feuillet, V., Dutertre, C. A., Ventre, E., Vu Manh, T. P., Baranek, T., Storset, A. K., Marvel, J., Boudinot, P., Hosmalin, A., Schwartz-Cornil, I., and Dalod, M. (2010). The XC chemokine receptor 1 is a conserved selective marker of mammalian cells homologous to mouse CD8alpha+ dendritic cells. *J. Exp. Med.* 207, 1283–1292.

Dasgupta, S., Navarrete, A. M., Bayry, J., Delignat, S., Wootla, B., Andre, S., Christophe, O., Nascimbeni, M., Jacquemin, M., Martinez-Pomares, L., Geijtenbeek, T. B., Moris, A., Saint-Remy, J. M., Kazatchkine, M. D., Kaveri, S. V., and Lacroix-Desmazes, S. (2007). A role for exposed mannosylations in presentation of human therapeutic self-proteins to CD4+ T lymphocytes. *Proc. Natl. Acad. Sci. U.S.A.* 104, 8965–8970.

Delamarre, L., Pack, M., Chang, H., Mellman, I., and Trombetta, E. S. (2005). Differential lysosomal proteolysis in antigen-presenting cells determines antigen fate. *Science* 307, 1630–1634.

den Haan, J. M., and Bevan, M. J. (2001). Antigen presentation to CD8+ T cells: cross-priming in infectious diseases. *Curr. Opin. Immunol.* 13, 437–441.

den Haan, J. M., Lehar, S. M., and Bevan, M. J. (2000). CD8(+) but not CD8(-) dendritic cells cross-prime cytotoxic T cells in vivo. *J. Exp. Med.* 192, 1685–1696.

Desjardins, M. (2009). The good fat: a link between lipid bodies and antigen cross-presentation. *Immunity* 31, 176–178.

Dolan, B. P., Gibbs, K. D. Jr., and Ostrand-Rosenberg, S. (2006). Dendritic cells cross-dressed with peptide MHC class I complexes prime CD8+ T cells. *J. Immunol.* 177, 6018–6024.

Dudziak, D., Kamphorst, A. O., Heidkamp, G. F., Buchholz, V. R., Trumpfheller, C., Yamazaki, S., Cheong, C., Liu, K., Lee, H. W., Park, C. G., Steinman, R. M., and Nussenzweig, M. C. (2007). Differential antigen processing by dendritic cell subsets in vivo. *Science* 315, 107–111.

Eberl, G., Widmann, C., and Corradin, G. (1996). The functional half-life of H-2Kd-restricted T cell epitopes on living cells. *Eur. J. Immunol.* 26, 1993–1999.

Ebstein, F., Lange, N., Urban, S., Seifert, U., Kruger, E., and Kloetzel, P. M. (2009). Maturation of human dendritic cells is accompanied by functional remodelling of the ubiquitin-proteasome system. *Int. J. Biochem. Cell Biol.* 41, 1205–1215.

Gagnon, E., Duclos, S., Rondeau, C., Chevet, E., Cameron, P. H., Steele-Mortimer, O., Paiement, J., Bergeron, J. J., and Desjardins, M. (2002). Endoplasmic reticulum-mediated phagocytosis is a mechanism of entry into macrophages. *Cell* 110, 119–131.

Georgiadou, D., Hearn, A., Evnouchidou, I., Chroni, A., Leondiadis, L., York, I. A., Rock, K. L., and Stratikos, E. (2010). Placental leucine aminopeptidase efficiently generates mature antigenic peptides in vitro but in patterns distinct from endoplasmic reticulum aminopeptidase 1. *J. Immunol.* 185, 1584–1592.

Gil-Torregrosa, B. C., Lennon-Dumenil, A. M., Kessler, B., Guermonprez, P., Ploegh, H. L., Fruci, D., van Endert, P., and Amigorena, S. (2004). Control of cross-presentation during dendritic cell maturation. *Eur. J. Immunol.* 34, 398–407.

Giodini, A., and Cresswell, P. (2008). Hsp90-mediated cytosolic refolding of exogenous proteins internalized by dendritic cells. *EMBO J.* 27, 201–211.

Giodini, A., Rahner, C., and Cresswell, P. (2009). Receptor-mediated phagocytosis elicits cross-presentation in nonprofessional antigen-presenting cells. *Proc. Natl. Acad. Sci. U.S.A.* 106, 3324–3329.

Goldszmid, R. S., Coppens, I., Lev, A., Caspar, P., Mellman, I., and Sher, A. (2009). Host ER-parasitophorous vacuole interaction provides a route of entry for antigen cross-presentation in *Toxoplasma gondii*-infected dendritic cells. *J. Exp. Med.* 206, 399–410.

Guermonprez, P., Saveanu, L., Kleijmeer, M., Davoust, J., Van Endert, P., and Amigorena, S. (2003). ER-phagosome fusion defines an MHC class I cross-presentation compartment in dendritic cells. *Nature* 425, 397–402.

Guillaume, B., Chapiro, J., Stroobant, V., Colau, D., Van Holle, B., Parvizi, G., Bousquet-Dubouch, M. P., Theate, I., Parmentier, N., and Van den Eynde, B. J. (2010). Two abundant proteasome subtypes that uniquely process some antigens presented by HLA class I molecules. *Proc. Natl. Acad. Sci. U.S.A.* 107, 18599–18604.

Hamman, B. D., Chen, J. C., Johnson, E. E., and Johnson, A. E. (1997). The aqueous pore through the translocon has a diameter of 40-60 A during cotranslational protein translocation at the ER membrane. *Cell* 89, 535–544.

He, L. Z., Crocker, A., Lee, J., Mendoza-Ramirez, J., Wang, X. T., Vitale, L. A., O'Neill, T., Petromilli, C., Zhang, H. F., Lopez, J., Rohrer, D., Keler, T., and Clynes, R. (2007). Antigenic targeting of the human mannose receptor induces tumor immunity. *J. Immunol.* 178, 6259–6267.

Heath, W. R., and Carbone, F. R. (2001). Cross-presentation in viral immunity and self-tolerance. *Nat. Rev. Immunol.* 1, 126–134.

Herre, J., Marshall, A. S., Caron, E., Edwards, A. D., Williams, D. L., Schweighoffer, E., Tybulewicz, V., Reis e Sousa, C., Gordon, S., and Brown, G. D. (2004). Dectin-1 uses novel mechanisms for yeast phagocytosis in macrophages. *Blood* 104, 4038–4045.

Hildner, K., Edelson, B. T., Purtha, W. E., Diamond, M., Matsushita, H., Kohyama, M., Calderon, B., Schraml, B. U., Unanue, E. R., Diamond, M. S., Schreiber, R. D., Murphy, T. L., and Murphy, K. M. (2008). Batf3 deficiency reveals a critical role for CD8alpha+ dendritic cells in cytotoxic T cell immunity. *Science* 322, 1097–1100.

Hotta, C., Fujimaki, H., Yoshinari, M., Nakazawa, M., and Minami, M. (2006). The delivery of an antigen from the endocytic compartment into the cytosol for cross-presentation is restricted to early immature dendritic cells. *Immunology* 117, 97–107.

Hou, J. C., Suzuki, N., Pessin, J. E., and Watson, R. T. (2006). A specific dileucine motif is required for the GGA-dependent entry of newly synthesized insulin-responsive aminopeptidase into the insulin-responsive compartment. *J. Biol. Chem.* 281, 33457–33466.

Houde, M., Bertholet, S., Gagnon, E., Brunet, S., Goyette, G., Laplante, A., Princiotta, M. F., Thibault, P., Sacks, D., and Desjardins, M. (2003). Phagosomes are competent organelles for antigen cross-presentation. *Nature* 425, 402–406.

Huang, A. Y., Bruce, A. T., Pardoll, D. M., and Levitsky, H. I. (1996). In vivo cross-priming of MHC class I-restricted antigens requires the TAP transporter. *Immunity* 4, 349–355.

Huang, A. Y., Golumbek, P., Ahmadzadeh, M., Jaffee, E., Pardoll, D., and Levitsky, H. (1994). Role of bone marrow-derived cells in presenting MHC class I-restricted tumor antigens. *Science* 264, 961–965.

Imai, J., Hasegawa, H., Maruya, M., Koyasu, S., and Yahara, I. (2005). Exogenous antigens are processed through the endoplasmic reticulum-associated degradation (ERAD) in cross-presentation by dendritic cells. *Int. Immunol.* 17, 45–53.

Jongbloed, S. L., Kassianos, A. J., McDonald, K. J., Clark, G. J., Ju, X., Angel, C. E., Chen, C. J., Dunbar, P. R., Wadley, R. B., Jeet, V., Vulink,

A. J., Hart, D. N., and Radford, K. J. (2010). Human CD141+ (BDCA-3)+ dendritic cells (DCs) represent a unique myeloid DC subset that cross-presents necrotic cell antigens. *J. Exp. Med.* 207, 1247–1260.

Kagan, J. C., Su, T., Horng, T., Chow, A., Akira, S., and Medzhitov, R. (2008). TRAM couples endocytosis of Toll-like receptor 4 to the induction of interferon-beta. *Nat. Immunol.* 9, 361–368.

Kamphorst, A. O., Guermonprez, P., Dudziak, D., and Nussenzweig, M. C. (2010). Route of antigen uptake differentially impacts presentation by dendritic cells and activated monocytes. *J. Immunol.* 185, 3426–3435.

Kim, Y. M., Brinkmann, M. M., Paquet, M. E., and Ploegh, H. L. (2008). UNC93B1 delivers nucleotide-sensing toll-like receptors to endolysosomes. *Nature* 452, 234–238.

Kloetzel, P. M., and Ossendorp, F. (2004). Proteasome and peptidase function in MHC-class-I-mediated antigen presentation. *Curr. Opin. Immunol.* 16, 76–81.

Koopmann, J. O., Albring, J., Huter, E., Bulbuc, N., Spee, P., Neefjes, J., Hammerling, G. J., and Momburg, F. (2000). Export of antigenic peptides from the endoplasmic reticulum intersects with retrograde protein translocation through the Sec61p channel. *Immunity* 13, 117–127.

Kovacsovics-Bankowski, M., and Rock, K. L. (1995). A phagosome-to-cytosol pathway for exogenous antigens presented on MHC class I molecules. *Science* 267, 243–246.

Kukutsch, N. A., Rossner, S., Austyn, J. M., Schuler, G., and Ltz, M. B. (2000). Formation and kinetics of MHC class I-ovalbumin peptide complexes on immature and mature dendritic cells. *J. Invest. Dermatol.* 115, 449–453.

Kurts, C., Heath, W. R., Carbone, F. R., Allison, J., Miller, J. F., and Kosaka, H. (1996). Constitutive class I-restricted exogenous presentation of self antigens in vivo. *J. Exp. Med.* 184, 923–930.

Latz, E., Schoenemeyer, A., Visintin, A., Fitzgerald, K. A., Monks, B. G., Knetter, C. F., Lien, E., Nilsen, N. J., Espevik, T., and Golenbock, D. T. (2004). TLR9 signals after translocating from the ER to CpG DNA in the lysosome. *Nat. Immunol.* 5, 190–198.

Lev, A., Princiotta, M. F., Zanker, D., Takeda, K., Gibbs, J. S., Kumagai, C., Waffarn, E., Dolan, B. P., Burgevin, A., Van Endert, P., Chen, W., Bennink, J. R., and Yewdell, J. W. (2010). Compartmentalized MHC class I antigen processing enhances immunosurveillance by circumventing the law of mass action. *Proc. Natl. Acad. Sci. U.S.A.* 107, 6964–6969.

Lilley, B. N., and Ploegh, H. L. (2004). A membrane protein required for dislocation of misfolded proteins from the ER. *Nature* 429, 834–840.

Lin, M. L., Zhan, Y., Villadangos, J. A., and Lew, A. M. (2008). The cell biology of cross-presentation and the role of dendritic cell subsets. *Immunol. Cell Biol.* 86, 353–362.

Macagno, A., Gilliet, M., Sallusto, F., Lanzavecchia, A., Nestle, F. O., and Groettrup, M. (1999). Dendritic cells up-regulate immunoproteasomes and the proteasome regulator PA28 during maturation. *Eur. J. Immunol.* 29, 4037–4042.

Mantegazza, A. R., Savina, A., Vermeulen, M., Perez, L., Geffner, J., Hermine, O., Rosenzweig, S. D., Faure, F., and Amigorena, S. (2008). NADPH oxidase controls phagosomal pH and antigen cross-presentation in human dendritic cells. *Blood* 112, 4712–4722.

Martin-Fontecha, A., Sebastiani, S., Hopken, U. E., Uguccioni, M., Lipp, M., Lanzavecchia, A., and Sallusto, F. (2003). Regulation of dendritic cell migration to the draining lymph node: impact on T lymphocyte traffic and priming. *J. Exp. Med.* 198, 615–621.

McKenzie, E. J., Taylor, P. R., Stillion, R. J., Lucas, A. D., Harris, J., Gordon, S., and Martinez-Pomares, L. (2007). Mannose receptor expression and function define a new population of murine dendritic cells. *J. Immunol.* 178, 4975–4983.

Mellman, I., and Steinman, R. M. (2001). Dendritic cells: specialized and regulated antigen processing machines. *Cell* 106, 255–258.

Mendoza-Naranjo, A., Saez, P. J., Johansson, C. C., Ramirez, M., Mandakovic, D., Pereda, C., Lopez, M. N., Kiessling, R., Saez, J. C., and Salazar-Onfray, F. (2007). Functional gap junctions facilitate melanoma antigen transfer and cross-presentation between human dendritic cells. *J. Immunol.* 178, 6949–6957.

Neijssen, J., Herberts, C., Drijfhout, J. W., Reits, E., Janssen, L., and Neefjes, J. (2005). Cross-presentation by intercellular peptide transfer through gap junctions. *Nature* 434, 83–88.

Nishi, T., and Forgac, M. (2002). The vacuolar (H+)-ATPases – nature's most versatile proton pumps. *Nat. Rev. Mol. Cell Biol.* 3, 94–103.

Norbury, C. C., Basta, S., Donohue, K. B., Tscharke, D. C., Princiotta, M. F., Berglund, P., Gibbs, J., Bennink, J. R., and Yewdell, J. W. (2004). CD8+ T cell cross-priming via transfer of proteasome substrates. *Science* 304, 1318–1321.

Norbury, C. C., Chambers, B. J., Prescott, A. R., Ljunggren, H. G., and Watts, C. (1997). Constitutive macropinocytosis allows TAP-dependent major histocompatibility complex class I presentation of exogenous soluble antigen by bone marrow-derived dendritic cells. *Eur. J. Immunol.* 27, 280–288.

Palmowski, M. J., Gileadi, U., Salio, M., Gallimore, A., Millrain, M., James, E., Addey, C., Scott, D., Dyson, J., Simpson, E., and Cerundolo, V. (2006). Role of immunoproteasomes in cross-presentation. *J. Immunol.* 177, 983–990.

Pooley, J. L., Heath, W. R., and Shortman, K. (2001). Cutting edge: intravenous soluble antigen is presented to CD4 T cells by CD8- dendritic cells, but cross-presented to CD8 T cells by CD8+ dendritic cells. *J. Immunol.* 166, 5327–5330.

Poulin, L. F., Salio, M., Griessinger, E., Anjos-Afonso, F., Craciun, L., Chen, J. L., Keller, A. M., Joffre, O., Zelenay, S., Nye, E., Le Moine, A., Faure, F., Donckier, V., Sancho, D., Cerundolo, V., Bonnet, D., and Reis e Sousa, C. (2010). Characterization of human DNGR-1+ BDCA3+ leukocytes as putative equivalents of mouse CD8alpha+ dendritic cells. *J. Exp. Med.* 207, 1261–1271.

Purcell, A. W., and Elliott, T. (2008). Molecular machinations of the MHC-I peptide loading complex. *Curr. Opin. Immunol.* 20, 75–81.

Reits, E., Griekspoor, A., Neijssen, J., Groothuis, T., Jalink, K., van Veelen, P., Janssen, H., Calafat, J., Drijfhout, J. W., and Neefjes, J. (2003). Peptide diffusion, protection, and degradation in nuclear and cytoplasmic compartments before antigen presentation by MHC class I. *Immunity* 18, 97–108.

Rescigno, M., Citterio, S., Thery, C., Rittig, M., Medaglini, D., Pozzi, G., Amigorena, S., and Ricciardi-Castagnoli, P. (1998). Bacteria-induced neo-biosynthesis, stabilization, and surface expression of functional class I molecules in mouse dendritic cells. *Proc. Natl. Acad. Sci. U.S.A.* 95, 5229–5234.

Rock, K. L., and Shen, L. (2005). Cross-presentation: underlying mechanisms and role in immune surveillance. *Immunol. Rev.* 207, 166–183.

Rodriguez, A., Regnault, A., Kleijmeer, M., Ricciardi-Castagnoli, P., and Amigorena, S. (1999). Selective transport of internalized antigens to the cytosol for MHC class I presentation in dendritic cells. *Nat. Cell Biol.* 1, 362–368.

Saccheri, F., Pozzi, C., Avogadri, F., Barozzi, S., Faretta, M., Fusi, P., and Rescigno, M. (2010). Bacteria-induced gap junctions in tumors favor antigen cross-presentation and antitumor immunity. *Sci. Transl. Med.* 2, 44–57.

Saveanu, L., Carroll, O., Weimershaus, M., Guermonprez, P., Firat, E., Lindo, V., Greer, F., Davoust, J., Kratzer, R., Keller, S. R., Niedermann, G., and van Endert, P. (2009). IRAP identifies an endosomal compartment required for MHC class I cross-presentation. *Science* 325, 213–217.

Savina, A., Jancic, C., Hugues, S., Guermonprez, P., Vargas, P., Moura, I. C., Lennon-Dumenil, A. M., Seabra, M. C., Raposo, G., and Amigorena, S. (2006). NOX2 controls phagosomal pH to regulate antigen processing during crosspresentation by dendritic cells. *Cell* 126, 205–218.

Savina, A., Peres, A., Cebrian, I., Carmo, N., Moita, C., Hacohen, N., Moita, L. F., and Amigorena, S. (2009). The small GTPase Rac2 controls phagosomal alkalinization and antigen crosspresentation selectively in CD8(+) dendritic cells. *Immunity* 30, 544–555.

Schnorrer, P., Behrens, G. M., Wilson, N. S., Pooley, J. L., Smith, C. M., El-Sukkari, D., Davey, G., Kupresanin, F., Li, M., Maraskovsky, E., Belz, G. T., Carbone, F. R., Shortman, K., Heath, W. R., and Villadangos, J. A. (2006). The dominant role of CD8+ dendritic cells in cross-presentation is not dictated by antigen capture. *Proc. Natl. Acad. Sci. U.S.A.* 103, 10729–10734.

Segura, E., and Villadangos, J. A. (2011). A modular and combinatorial view of the antigen cross-presentation pathway in dendritic cells. *Traffic* 12, 1677–1685.

Seifert, U., Bialy, L. P., Ebstein, F., Bech-Otschir, D., Voigt, A., Schroter, F., Prozorovski, T., Lange, N., Steffen, J., Rieger, M., Kuckelkorn, U., Aktas, O., Kloetzel, P. M., and Krüger, E. (2010). Immunoproteasomes preserve protein homeostasis upon interferon-induced oxidative stress. *Cell* 142, 613–624.

Shen, L., Sigal, L. J., Boes, M., and Rock, K. L. (2004). Important role of cathepsin S in generating peptides for TAP-independent MHC class I

crosspresentation in vivo. *Immunity* 21, 155–165.

Shimada, K., Takimoto, H., Yano, I., and Kumazawa, Y. (2006). Involvement of mannose receptor in glycopeptidolipid-mediated inhibition of phagosome-lysosome fusion. *Microbiol. Immunol.* 50, 243–251.

Sigal, L. J., Crotty, S., Andino, R., and Rock, K. L. (1999). Cytotoxic T-cell immunity to virus-infected non-haematopoietic cells requires presentation of exogenous antigen. *Nature* 398, 77–80.

Smyth, L. A., Harker, N., Turnbull, W., El-Doueik, H., Klavinskis, L., Kioussis, D., Lombardi, G., and Lechler, R. (2008). The relative efficiency of acquisition of MHC: peptide complexes and cross-presentation depends on dendritic cell type. *J. Immunol.* 181, 3212–3220.

Song, R., and Harding, C. V. (1996). Roles of proteasomes, transporter for antigen presentation (TAP), and beta 2-microglobulin in the processing of bacterial or particulate antigens via an alternate class I MHC processing pathway. *J. Immunol.* 156, 4182–4190.

Sweet, L., Singh, P. P., Azad, A. K., Rajaram, M. V., Schlesinger, L. S., and Schorey, J. S. (2010). Mannose receptor-dependent delay in phagosome maturation by *Mycobacterium avium* glycopeptidolipids. *Infect. Immun.* 78, 518–526.

Tabeta, K., Hoebe, K., Janssen, E. M., Du, X., Georgel, P., Crozat, K., Mudd, S., Mann, N., Sovath, S., Goode, J., Shamel, L., Herskovits, A. A., Portnoy, D. A., Cooke, M., Tarantino, L. M., Wiltshire, T., Steinberg, B. E., Grinstein, S., and Beutler, B. (2006). The Unc93b1 mutation 3d disrupts exogenous antigen presentation and signaling via Toll-like receptors 3, 7 and 9. *Nat. Immunol.* 7, 156–164.

Tacken, P. J., de Vries, I. J., Gijzen, K., Joosten, B., Wu, D., Rother, R. P., Faas, S. J., Punt, C. J., Torensma, R., Adema, G. J., and Figdor, C. G. (2005). Effective induction of naive and recall T-cell responses by targeting antigen to human dendritic cells via a humanized anti-DC-SIGN antibody. *Blood* 106, 1278–1285.

Tacken, P. J., Ginter, W., Berod, L., Cruz, L. J., Joosten, B., Sparwasser, T., Figdor, C. G., and Cambi, A. (2011). Targeting DC-SIGN via its neck region leads to prolonged antigen residence in early endosomes, delayed lysosomal degradation, and cross-presentation. *Blood* 118, 4111–4119.

Touret, N., Paroutis, P., Terebiznik, M., Harrison, R. E., Trombetta, S., Pypaert, M., Chow, A., Jiang, A., Shaw, J., Yip, C., Moore, H. P., van der Wel, N., Houben, D., Peters, P. J., de Chastellier, C., Mellman, I., and Grinstein, S. (2005). Quantitative and dynamic assessment of the contribution of the ER to phagosome formation. *Cell* 123, 157–170.

Trombetta, E. S., Ebersold, M., Garrett, W., Pypaert, M., and Mellman, I. (2003). Activation of lysosomal function during dendritic cell maturation. *Science* 299, 1400–1403.

Tucker, S. C., and Casadevall, A. (2002). Replication of *Cryptococcus neoformans* in macrophages is accompanied by phagosomal permeabilization and accumulation of vesicles containing polysaccharide in the cytoplasm. *Proc. Natl. Acad. Sci. U.S.A.* 99, 3165–3170.

Van den Berg, B., Clemons, W. M. Jr., Collinson, I., Modis, Y., Hartmann, E., Harrison, S. C., and Rapoport, T. A. (2004). X-ray structure of a protein-conducting channel. *Nature* 427, 36–44.

van Montfoort, N., Camps, M. G., Khan, S., Filippov, D. V., Weterings, J. J., Griffith, J. M., Geuze, H. J., van Hall, T., Verbeek, J. S., Melief, C. J., and Ossendorp, F. (2009). Antigen storage compartments in mature dendritic cells facilitate prolonged cytotoxic T lymphocyte cross-priming capacity. *Proc. Natl. Acad. Sci. U.S.A.* 106, 6730–6735.

Wakim, L. M., and Bevan, M. J. (2011). Cross-dressed dendritic cells drive memory CD8+ T-cell activation after viral infection. *Nature* 471, 629–632.

Weck, M. M., Appel, S., Werth, D., Sinzger, C., Bringmann, A., Grunebach, F., and Brossart, P. (2008). hDectin-1 is involved in uptake and cross-presentation of cellular antigens. *Blood* 111, 4264–4272.

Werneburg, N. W., Guicciardi, M. E., Bronk, S. F., and Gores, G. J. (2002). Tumor necrosis factor-alpha-associated lysosomal permeabilization is cathepsin B dependent. *Am. J. Physiol. Gastrointest. Liver Physiol.* 283, G947–G956.

Wiertz, E. J., Tortorella, D., Bogyo, M., Yu, J., Mothes, W., Jones, T. R., Rapoport, T. A., and Ploegh, H. L. (1996). Sec61-mediated transfer of a membrane protein from the endoplasmic reticulum to the proteasome for destruction. *Nature* 384, 432–438.

Wolkers, M. C., Brouwenstijn, N., Bakker, A. H., Toebes, M., and Schumacher, T. N. (2004). Antigen bias in T cell cross-priming. *Science* 304, 1314–1317.

Ye, Y., Meyer, H. H., and Rapoport, T. A. (2003). Function of the p97-Ufd1-Npl4 complex in retrotranslocation from the ER to the cytosol: dual recognition of nonubiquitinated polypeptide segments and polyubiquitin chains. *J. Cell Biol.* 162, 71–84.

Ye, Y., Shibata, Y., Yun, C., Ron, D., and Rapoport, T. A. (2004). A membrane protein complex mediates retro-translocation from the ER lumen into the cytosol. *Nature* 429, 841–847.

Zehner, M., Chasan, A. I., Schuette, V., Embgenbroich, M., Quast, T., Kolanus, W., and Burgdorf, S. (2011). Mannose receptor polyubiquitination regulates endosomal recruitment of p97 and cytosolic antigen translocation for cross-presentation. *Proc. Natl. Acad. Sci. U.S.A.* 108, 9933–9938.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 04 November 2011; accepted: 16 December 2011; published online: 03 January 2012.

Citation: Kreer C, Rauen J, Zehner M and Burgdorf S (2012) Cross-presentation: how to get there – or how to get the ER. Front. Immun. ***2****:87. doi: 10.3389/fimmu.2011.00087*

This article was submitted to Frontiers in Antigen Presenting Cell Biology, a specialty of Frontiers in Immunology.

frontiers in
IMMUNOLOGY

REVIEW ARTICLE
published: 30 March 2012
doi: 10.3389/fimmu.2012.00063

The role of heat shock proteins in antigen cross presentation

Ayesha Murshid[1], Jianlin Gong[2] and Stuart K. Calderwood[1]*

[1] *Molecular and Cellular Radiation Oncology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, USA*
[2] *Boston University Medical Center, Boston, MA, USA*

Edited by:
Christian Kurts, Friedrich Wilhelms-Universität Bonn, Germany

Reviewed by:
Emmanuel Wiertz, University Medical Center Utrecht, Netherlands
Willem van Eden, Utrecht University, Netherlands
Jonathan Yewdell, National Institute of Allergy and Infectious Diseases, USA

***Correspondence:**
Stuart K. Calderwood, Beth Israel Deaconess Medical Center, Harvard Medical School, 99 Brookline Avenue, Boston, MA 02215, USA.
e-mail: scalderw@bidmc.harvard.edu

Heat shock proteins (HSPs) are molecular chaperones that bind tumor antigens and mediate their uptake into antigen presenting cells. HSP–antigen complexes are then directed toward either the MHC class I pathway through antigen cross presentation or the conventional class II pathway, leading to activation of T cell subsets. Uptake of HSP-chaperoned polypeptides can involve both receptor-mediated and receptor-independent routes, and mechanisms of antigen sorting between the Class I and II pathways after uptake are currently under investigation. The processes involved in internalization of HSP–antigen complexes differ somewhat from the mechanisms previously determined for (unchaperoned) particulate and free soluble antigens. A number of studies show that HSP-facilitated antigen cross presentation requires uptake of the complexes by scavenger receptors (SR) followed by processing in the proteasome, and loading onto MHC class I molecules. In this review we have examined the roles of HSPs and SR in antigen uptake, sorting, processing, cell signaling, and activation of innate and adaptive immunity.

Keywords: heat shock proteins, antigen cross presentation, CTL response, scavenger receptor, antigen presenting cells, soluble vs. particulate antigen, anti-cancer vaccine, tumor immunity

INTRODUCTION

Heat shock proteins (HSPs) are a class of polypeptides powerfully induced by heat shock that mediate profound levels of stress resistance (Craig, 1985; Ellis, 2007). HSPs are molecular chaperones, binding to (holding) and refolding other cellular polypeptides (clients) with aberrant conformations (Ellis, 2007). There are a number of families of molecular chaperone families (a–d), with members of *class a* (Hsp70, Hsp110, GRP170) and *class c* (Hsp90, Grp94/Gp96) thought to be of particular significance in tumor immunology (Murshid et al., 2011c). (Grp is the abbreviation for *glucose regulated protein* and such proteins are retained in the endoplasmic reticulum, ER.) The molecular chaperone activity of class a and class c chaperones appears to be conferred by two functional domains: a dedicated peptide binding domain that seizes client polypeptides and an ATPase domain (Scheibel et al., 1998; Mayer and Bukau, 2005; Vogel et al., 2006). HSPs are allosteric molecules, one domain reciprocally affecting the other, and when polypeptide moieties bind to the peptide binding domain, ATP is hydrolyzed to ADP and orthophosphate and when ATP binds, associated peptides are discarded. These properties have been intensely studied for Hsp70 and Hsp90 and are largely inferred for the sibling proteins. The ability to hold polypeptide clients appears to correlate with the size of class a chaperones, the smaller class a member (Hsp70) binding to smaller peptides with medium affinity while larger class a chaperones (Hsp110 and Grp170), with very large peptide binding domains, bind tightly to their clients, including peptides and whole proteins (Oh et al., 1999; Park et al., 2006).

Heat shock protein–peptide complexes (HSP.PC) can be used as vaccines to elicit antigen-specific cytotoxic lymphocytes (CTL) responses (Srivastava, 2000; Manjili et al., 2002; Wang et al., 2003; Enomoto et al., 2006; Gong et al., 2010). In order for polypeptides bound to HSPs to activate adaptive immunity, associated antigens must be internalized by antigen presenting cells (APC) and inserted into the antigen presentation pathways. Indeed, HSPs have been shown to be taken up by dendritic cells (DC), the most efficient professional APC (Steinman et al., 1983; Heath and Carbone, 2009). Antigen presentation occurs through a number of pathways. Intracellular proteins were shown to be processed by digestion through the multiple protease activities in the proteasome and antigens presented on the cell surface by *major histocompatibility class I* (MHCI) molecules found in all cells and thus displayed to CD8+ T cells to permit immunosurveillance (Neefjes and Momburg, 1993). By contrast, exogenous antigens after internalization into immune cells are processed in lysosomes and presented on the cell surface by *major histocompatibility class II* (MHCII) molecules restricted to cells of the immune system (Cresswell, 1994). It was subsequently shown that another antigen presentation pathway exists permitting external antigens to enter the MHC class I pathway (Norbury et al., 2002, 2004). This process, *antigen cross presentation* permits external antigens to be presented by APC in the context of MHC class I and activate CD8+ CTL to kill virus infected or malignant cells (Norbury et al., 2002, 2004; **Figure 1**). Antigen cross presentation was shown to be a complex process requiring external antigens to enter cells, penetrate sites for protein processing, and associate with MHC class I molecules in intracellular vesicular structures although many aspects of this process are incompletely understood. We aim to elucidate some of the mechanisms by which antigens bound to HSP interact with APC and mediate antigen

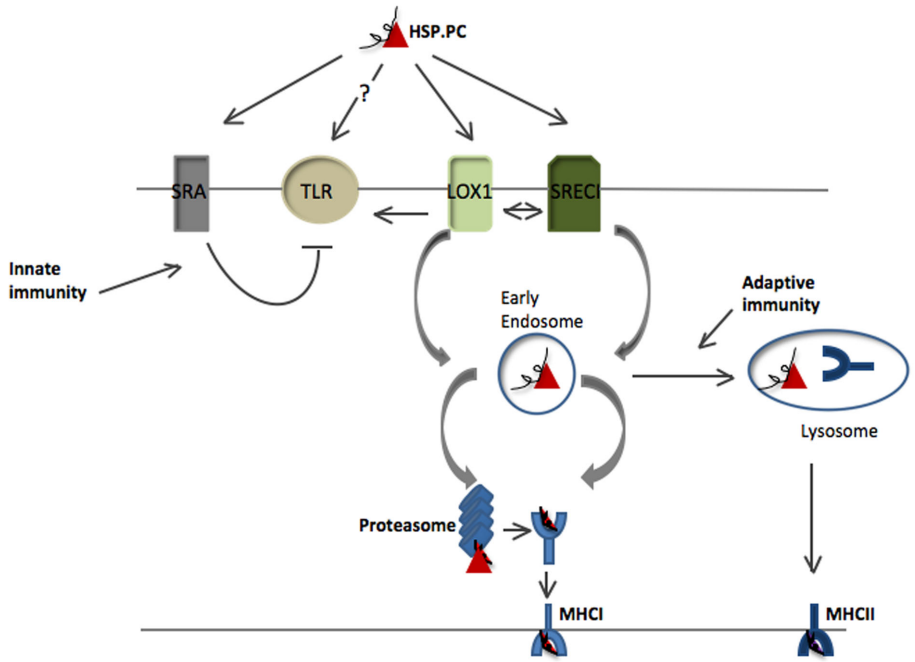


FIGURE 1 | Antigen presentation pathways for HSP-bound polypeptides. HSP.PCs bind to surface receptors on DC including SRECI and LOX-1 and are internalized in complexes with the receptors. Binding to these receptors may also trigger secondary activation of Toll-like receptors (TLR2 or TLR4) that may amplify antigen cross presentation. Alternatively some studies suggest direct binding of HSPs to TLR. HSP binding to cell surface SRA/CD204 is inhibitory to TLR4 activity and likely antagonizes the immune responses induced by HSPs through LOX-1 or SRECI. HSP.PC are internalized by SRECI or LOX-1 into endosomes with the subsequent release of the peptides from the HSP.PC complex. Such peptides are then processed either within endosomes or undergo trafficking to the cytosol where they encounter the proteasomal system. Processed peptides are then loaded either onto MHC class I in the ER or phagosomes or onto MHC class II molecules in lysosomes and presented to, respectively CD8+ T cells and CD4+ T cells. The triage mechanisms directing HSP-chaperoned peptides toward either of these two pathways are currently not known.

cross presentation as compared with the pathways utilized for unchaperoned antigens.

HSPs IN TUMOR IMMUNITY AND ANTI-CANCER VACCINES: COMMON AND UNCOMMON ANTIGENS

When HSPs are extracted from cancer cells under controlled conditions and introduced into tumor bearing hosts they can induce immunity to the parent tumor cells (Srivastava, 2002; Manjili et al., 2003; Wang et al., 2003, 2010; Murshid et al., 2011c). The major paradigm for using tumor derived HSP as vaccines involves their assumed ability to bind to antigenic oligopeptides (Srivastava, 2002; Murshid et al., 2011c). This hypothesis takes into account the chaperone properties of the HSPs mentioned above and their relative abundance inside cells and is supported by a considerable body of data. However some controversy remains, particularly for the ER resident class c chaperone Grp94 (Gp96; Chandawarkar et al., 1999, 2004; Jockheck-Clark et al., 2010). Although some indirect studies suggested that Grp94 could bind to antigenic peptides *in vitro*, these findings have been disputed (Ying and Flatmark, 2006). In addition, this protein has been shown to initiate tumor rejection in the absence of bound peptides, presumably through stimulation of innate immunity (Nicchitta, 2003). However these drawbacks may not apply to the other class *a* and *c* chaperones. Hsp70 has for instance been shown to bind to oligopeptides *in vitro* by phage display and affinity chromatography approaches and *in vivo* by mass spectrometry (Flynn et al., 1989; Fourie et al., 1994; Gragerov and Gottesman, 1994; Grossmann et al., 2004).

Heat shock protein levels are elevated in many tumor types due to the increased requirement for molecular chaperones needed to stabilize the abundant mutant and over-expressed oncoproteins found in cancer (Calderwood et al., 2006; Calderwood and Gong, 2011). The expansion of molecular chaperone clients during stress was shown previously to trigger expression of HSPs due to elevated transcription and selective translation (Calderwood et al., 2010). HSP–oncoprotein complexes thus accumulate in abundant levels in such tumor cells in response to the increased folding burden and can be used to prepare chaperone-based vaccines: this is the major rationale behind using HSPs in tumor immunotherapy (Srivastava and Old, 1988; Kamal et al., 2003; Murshid et al., 2011c). The multiple mutations found in cancer genomes appear to be required to overcome the matrix of regulatory barriers to transformation and progression that characterize normal cells (Vogelstein and Kinzler, 1993; Hanahan and Weinberg, 2000). Alterations such as mutation and overexpression occur in growth factor receptors and downstream signaling pathways and permit cytokine-independent growth in cancer. In addition, mutations in tumor suppressor genes override their inhibitory effects of their gene products on cancer (Hanahan and Weinberg, 2000). However not all mutations are equal. Many cells in the tumor population may be unable to initiate efficient tumor formation

and tumorigenesis appears to be maintained by driver mutations in minority clones of tumor-initiating cells (TIC; Carter et al., 2009; Hanahan and Weinberg, 2011). Thus many of the random mutations accumulating in the large non-TIC population may have passenger status and are unlikely to select for malignancy. A high proportion of the antigenic epitopes in individual cancers could therefore be unique and not shared with other tumors. However, many tumors express common antigens (such as HER2-neu and MUC1 in breast cancer) or re-express embryonic and developmental genes such as Mart1 and gp100 common to many cancers (Engelhard et al., 2002). For tumor immunotherapy in patients, Grp94 and Hsp70 vaccines have been used in an autologous mode, with the patients receiving HSP complexes derived from their own primary tumors for immunotherapy (Srivastava and Old, 1988). This approach was based on experimental studies of chemically induced rodent cancers, in which Grp74 and Hsp70 vaccines were shown to be effective only in an autologous context and were not cross reactive even with tumors from matched animals bearing tumors induced by the same carcinogen (Udono and Srivastava, 1993; Chandawarkar et al., 2004).

Characterizing the antigenic peptide repertoires associated with individual HSPs extracted from tumor cells has however rarely been attempted. Thus although it is suspected that the individual class a and class c HSPs chaperone a wide spectrum of potential antigenic epitopes that may be representative of the cell proteome and that individual HSPs may favor distinct subpopulations of such peptides, these issues have not been comprehensively studied and the question remains open.

PATHWAYS OF ANTIGEN CROSS PRESENTATION: SOLUBLE, PARTICULATE, AND HSP–CHAPERONED ANTIGENS

Antigen presenting cells utilize at least two pathways for sampling external antigens and displaying them to T lymphocytes. These are the MHC class I and MHC class II pathways. However, the mechanisms used for sorting and trafficking of antigens between the MHC class I or class II pathways varies according to the physical nature of the antigen (Inaba and Inaba, 2005). Particulate and soluble antigens are dealt with quite differently.

(a) *Particulate antigens* are taken up by phagocytosis and enter vesicular structures known as phagosomes (Ackerman et al., 2003; Cresswell et al., 2005; Inaba and Inaba, 2005). Triage of antigens between the Class I and Class II pathways then appears to involve close regulation of the intraphagosomal pH. The previously acidic pH of the phagosome is initially increased, favoring antigen processing through the Class I pathway. After some minutes, these phagosomes are re-acidified, permitting antigen processing toward the Class II pathway (Inaba and Inaba, 2005).

Following triage between MHC class I and class II pathways, the cross presentation of particulate antigens involves in itself at least two variant mechanisms. In the *Cytosolic Pathway of Antigen Cross Presentation*, antigens are taken up into phagosomes and, after partial processing in these organelles, exported into the cytoplasm where they are hydrolyzed into short peptides within the proteasome. The processed peptides are then re-imported into the ER by *transporter associated with antigen presentation* (TAP), enter the conventional antigen presentation pathway, and become loaded onto appropriate MHC class I molecules (Rock and Shen, 2005). A variation on this mechanism has been proposed in which the antigen processing machinery of the ER becomes associated with phagosomes and loading onto MHC class I occurs in these organelles (Rock et al., 2005).

A second, alternative pathway for cross presentation of particulate antigens has been described, *The Vacuolar Pathway*, also known as the TAP-independent pathway of cross presentation (Rock and Shen, 2005; Ackerman et al., 2006). This route of antigen presentation is rapid compared to the cytosolic/proteasome pathway and obviates the need for polypeptides to be pumped in and out of the cytoplasm. In this case, antigens are taken up into endosomes and directly processed by endogenous proteases such as the cysteine protease cathepsin S, loaded onto MHC class I molecules recycling from the cell surface and then trafficked back to the surface in recycling endosomes.

(b) *Soluble antigens* appear to be taken up by APC and partitioned toward the MHC class I and MHC class II pathways by different mechanisms compared to particulate antigens. Sorting to the class I and class II routes occurs at the cell surface through either non-specific macropinocytosis or endocytosis, involving surface receptors. Uptake of antigens directed to the antigen cross presentation pathways may involve Fc receptors, and c-type lectins including CLEC9A, DC-SIGN, DEC205, and the mannose receptor 1 (Bonifaz et al., 2002; Burgdorf et al., 2006, 2010; Idoyaga et al., 2011; Tacken et al., 2011). C-type lectins have in common the possession of a recognition domain that determines their carbohydrate specificity and have been studied mostly in regard to interaction with pathogens (Geijtenbeek and Gringhuis, 2009). Cross presentation of internalized antigens next involves the two variant pathways described above for particulate antigens after antigen–receptor complexes are internalized into endosomes.

EXTRACELLULAR HSP–PEPTIDE COMPLEXES AND ANTIGEN CROSS PRESENTATION

The uptake of HSP.PC may involve receptors expressed on the surface of DC and can precede antigen presentation through the Class I and Class II pathways (Jeannin et al., 2005; Theriault et al., 2006; Kurotaki et al., 2007; Murshid et al., 2011b). The receptors involved in this process appear to differ from those that mediate uptake of soluble, unchaperoned antigens, and the c-type lectins involved in the latter process do not seem to be major endocytic receptors for HSP.PC in APC (Theriault et al., 2006). Instead HSPs bind mainly to scavenger receptors (SR) such as SRECI/Scarf1, FEEL-I/Stabilin-1, LOX-1, SRA/CD204, and LRP1/CD91 (Binder et al., 2000; Delneste et al., 2002; Theriault et al., 2006; Murshid et al., 2011b; **Figure 1**). SR were first characterized in studies of uptake of chemically modified low-density lipoproteins (LDL) by endothelial cells and appear to bind a wide variety of other molecules, including HSPs (Murshid et al., 2011b). However, Hsp70 can be bound by some molecules with c-type lectin specificity, including the natural killer (NK) cell receptor NKG2D; LOX-1 belongs to both SR and c-type lectin families (Theriault et al., 2006; Murshid

et al., 2011a). As FEEL-1 is not abundantly expressed in DC we have not further pursued its potential role in HSP-mediated antigen cross presentation (Chu, B., Murshid, A., and Calderwood, S. K., unpublished). The status of LRP1/CD91 in HSP binding and cross presentation is somewhat controversial. This protein has been proposed as a common HSP receptor (Binder et al., 2000; Srivastava, 2002). However, LRP1/CD91 is not abundantly expressed in most DC subtypes and binding of Grp94, calreticulin, and Hsp70 to the receptor was not detected in a number of studies (Delneste et al., 2002; Berwin et al., 2003; Walters and Berwin, 2005; Theriault et al., 2006). SRA/CD204 plays a significant immune suppressive function after binding HSPs and other ligands and there is currently no evidence that it plays a role in antigen cross presentation, although it is capable of mediating internalization of Grp94 and calreticulin (Berwin et al., 2003; Wang et al., 2007). SRECI and LOX-1 each appeared to mediate approximately 50% of the cross presentation of the ovalbumin (Ova) SIINFEKL epitope in murine BMDC (Murshid et al., 2010).

It has recently been shown that GRP94 can lead to antigen cross presentation in a receptor-independent manner, questioning the significance of HSP receptors (Jockheck-Clark et al., 2010). This issue was also recently examined by Murshid et al. (2010) who tested the ability of CHO cells to cross prime B3Z T cell hybridoma cells that respond to Ova peptide SIINFEKL in the context of H2kb. Wild-type CHO cells or CHO engineered to express H2kb on the surface and exposed to Hsp90–Ova or Hsp90-associated with N-terminally extended SIINFEKL failed to cross prime B3Z cells. However, co-expression of H2kb and the receptor SCRECI rendered the CHO cells competent to cross prime the B3Z cells (Murshid et al., 2010). These studies strongly suggest a role for receptors such as SR in cross presentation of HSP-bound antigens, although they cannot rule out a role for non-receptor mechanisms in some cell types (Jockheck-Clark et al., 2010).

The SR were initially shown to be important in HSP binding and CTL activation using a wide range of SR agonists. Compounds that bind SR such as such as maleylated BSA, Oxidized LDL, acetylated BSA, poly-inosine, and fucoidan all reduce Hsp70 binding to DC (Delneste et al., 2002). Class E SR family member LOX-1 was shown to be expressed at high levels in myeloid derived DC and in mouse bone marrow derived DC and it was demonstrated conclusively that this receptor is involved in antigen cross presentation by DC exposed to Hsp70–antigen complexes (Delneste et al., 2002). Indeed, anti-LOX-1 antibodies coupled to Ova could mediate the rejection of Ova expressing tumor cells. Recently, it was also shown that LOX-1 is an Hsp60 receptor and Hsp60-fused Ova can be processed by DC and can be delivered to MHCI molecules to activate CD8+ T cells response, suggesting their role in antigen cross presentation (Xie et al., 2010).

Class F scavenger receptor SRECI binds avidly to Hsp70, Hsp90, Grp94, Hsp110, and Grp170 with or without associated antigens and appears to be a common receptor for HSPs in DC (Manjili et al., 2002; Berwin et al., 2004; Murshid et al., 2010). Hsp90–antigen complexes can be bound and internalized in a range of cell types including DC and packaged into distinct classes of vesicles by SRECI, involving lipid raft localization of the receptor–HSP.PC complex and taken up in a dynamin- and clathrin-independent pathway (Murshid et al., 2010). HSP.PC along with SRECI is endocytosed into tubule like vesicles termed Clathrin-independent carriers (CLIC) or GPI anchored protein-enriched endocytic compartments (GEEC; Doherty and McMahon, 2009; Gupta et al., 2009). Although the significance of entry of HSP–SRECI complexes through the CLIC/GEEC pathway is not entirely clear, it does permit regulation of antigen cross presentation by signal transducing molecules as discussed below. In addition, LOX-1 is internalized through a dynamin-dependent mechanism involving association with caveolae, distinct from the GEEC pathway used by SRECI but is also able to mediate cross presentation of peptides bound to HSPs (Murphy et al., 2008).

It is not clear to what degree the antigen presentation pathways utilized by HSP-chaperoned antigens after endocytosis conform to those used by unchaperoned antigens. For instance, particulate antigen processing toward the class I pathway, involves an elevation in intraphagosomal pH and it is not yet known if such processes are important for HSP-mediated antigen processing and presentation (Inaba and Inaba, 2005). Intravesicular pH changes could be involved in the dissociation of peptides from the HSP, as well as subsequent loading onto MHC class I. Cross presentation in BMDC after Hsp90–Ova peptide exposure was reduced by exposure to chloroquine, an inhibitor of vesicular acidification suggesting that low pH may be required at least for cross presentation through the vesicular pathway (Murshid et al., 2010). So far it has been established that both the *endosome-plasma membrane* and *endosome to cytosol-cell membrane* mechanisms are involved in Hsp70 and Hsp90-mediated antigen cross presentation in BMDC (Murshid et al., 2010). Partition of antigens between these two pathways may depend on the size of the client polypeptide associated with the HSP. Smaller, Ova-derived peptides bound to Hsp90 could be efficiently cross presented through a mechanism resembling the vesicular pathway and such processing was blocked by cysteine protease inhibitors and, primaquine an antagonist of membrane recycling (Murshid et al., 2010). Larger polypeptides associated with Hsp90, such as full length ovalbumin required TAP expression and proteasome function, indicating a key role for the endosome–cytosol pathway in processing these larger antigens (Murshid et al., 2010). HSP chaperoning of peptide antigens may increase the efficiency of antigen processing by protecting peptides from proteolysis during trafficking through the cell compartments and reducing the amounts of antigen required to initiate CD8+ T cell cross priming (Kunisawa and Shastri, 2006). One question that is currently unclear is how far along the journey of antigen cross presentation the HSP-receptor–polypeptide complex remains intact. Hsp90, SRECI, and Ova-derived peptides have been detected co-localizing in endosomal compartments of BMDC (Murshid et al., 2010). In addition, in DC incubated with Hsp90–Ova complexes, internalized Hsp90 and Ova were shown to co-localize with cytosolic proteasomes (Oura et al., 2011). Localization of Ova to proteasomes and TAP-dependent antigen cross presentation could be abolished by introduction of anti-Hsp90 antibodies into cells, indicating a key role for Hsp90 in targeting associated Ova molecules to proteasomes (Oura et al., 2011). It has been shown recently that intracellular Hsp90 plays an essential role in antigen cross presentation by permitting the transport of model antigen Ova out of endosomes and into the cytosol to permit processing by the proteasome (Ichiyanagi et al., 2010;

Imai et al., 2011). By contrast with free Ova, cross presentation of Hsp90–chaperoned Ova appeared to be independent of endogenous Hsp90, suggesting that exogenous Hsp90 may facilitate antigen transport across the endosomal membrane independently of the endogenous chaperone (Oura et al., 2011). Ultimately, peptides dissociate from the HSP prior to processing and presentation and receptors such as SREC1 are likely recycled to the cell surface. Fluorescently labeled Hsp90 was observed for approximately 60 min in murine BMDC, after binding and internalization, before fluorescence became undetectable (Murshid, A., unpublished data). Hsp90 taken up from the extracellular medium and entering endosomes may be broken down by intravesicular proteases or lysosomal hydrolases.

ROLE OF INTRACELLULAR MOLECULAR CHAPERONES IN CROSS PRESENTATION OF ANTIGENS IN CELLULAR MATERIALS ENGULFED BY APC

Tumor cells or virally infected cells may trigger immunity either directly when MHC class I antigen complexes on the surface interact with CD8+ T cells or when such cells die, are engulfed by APC and epitopes are cross presented to the CD8+ cells (Wolkers et al., 2001). There has been some suggestion that HSPs might play a role in the second process, in cross presentation of antigens sequestered by molecular chaperones by APC after engulfment of cell bodies. HSP-chaperoned peptides appear to exist in detectable quantities in cells and could be significant in this latter process. Indeed it was suggested by Binder et al. (2000) that essentially all antigenic epitopes in cells may be bound to molecular chaperones (Binder and Srivastava, 2005). However in a study of Ova epitope presentation in cell lysates, free unprocessed proteins appeared to be the dominant form of antigens for cross priming, far exceeding the activities of free or HSP-chaperoned peptides (Shen and Rock, 2004). Most peptides generated by proteolysis of cytoplasmic proteins have a very short half-life, surviving for only a few seconds, although a minority can survive for longer periods by association with TAP (Reits et al., 2003). There has been some suggestion that HSPs could chaperone such peptides in the cytoplasm and protect them from cytoplasmic aminopeptidases (Srivastava, 2002). Intracellular chaperones may acquire peptide antigens during endogenous antigen processing (Kunisawa and Shastri, 2003, 2006). Indeed Shastri et al. have shown that Hsp90a is essential in stabilizing large peptide intermediates generated by the proteasome from Ova and for presentation through the MHC class I pathway (Kunisawa and Shastri, 2006). In addition, a minority of viral epitopes that can bind Hsp90α appear to be stabilized and are important in cross priming (Lev et al., 2008). However the exact place of HSPs in cytoplasmic antigen processing is uncertain. When peptides are taken up by TAP into the ER they could potentially encounter other molecular chaperones, including the Hsp70 paralog Grp78 and the Hsp90 paralog Grp94, molecules with suspected immune functions. It is not clear whether Grp78 binds peptides or participates in antigen presentation although it has known immune functions in the chaperoning of IgG (Kozutsumi et al., 1989). In addition, Grp94 does not appear to participate in cross priming of anti-viral CD8+ cells *in vivo* (Nicchitta, 2003; Lev et al., 2009). Another ER chaperone shown *in vitro* to bind avidly to polypeptide clients is Grp170 (Park et al., 2006). However it is not clear whether ER resident Grp170 can acquire peptide antigens *in vivo* or can play a role in antigen presentation, although its high avidity for peptides and proteins suggests investigation of such a possibility.

RELEASE OF HSPs FROM CELLS INTO THE EXTRACELLULAR ENVIRONMENT

Heat shock proteins can also be released from cells, tissues, and access the extracellular environment, although their functions outside the cell are largely unknown and much debated (Pockley et al., 2008). HSPs can be released from tumor cells or macrophages into the extracellular microenvironment through mechanisms involving secretory lysosomes, very similar to the processes used by cells to release interleukin-1β (Mambula and Calderwood, 2006b). This pathway of non-canonical HSP secretion occurs at a gradual basal rate and can be triggered in cells by stresses such as fever range heat or by exposing macrophages to live *E. coli* bacteria (Mambula and Calderwood, 2006b; Mambula, S. S., Murshid, A., and Calderwood, S. K., in preparation). HSPs released in this way could potentially export antigenic peptides acquired during intracellular antigen processing as cargo, interact with APC and trigger cross priming of CD8+ subsets. However, it is not clear how stable antigen binding would be under these circumstances. It is known that for *hspa* family chaperones such as Hsp70, peptide binding is relatively stable when ADP rather than ATP is bound to the chaperone. Hsp70 is also released from necrotic cells and under these circumstances might be thought to bind associated polypeptides, as intracellular ATP levels would decline rapidly in necrosis and the chaperone, in its ADP-bound form would bind peptides more effectively (Mambula and Calderwood, 2006a). Hsp70 released from cells undergoing necrotic death has been shown to lead to a strong T cell mediated anti-tumor immune response (Daniels et al., 2004). In addition, it was shown that Grp170, engineered for secretion into the extracellular environment initiated tumor rejection due to activation of NK and CD8+ cells, indicating the potential of this approach (Wang et al., 2003).

In addition to secretion in free form, HSPs have been shown to be released from cells after being packaged in exosomes. Exosomes derived from multivesicular bodies in DC contain Hsp70 and are known to play a major role in cross priming of T lymphocytes (Chaput et al., 2006). Thus Hsp70 in exosomes could potentially play a significant role in immunity. However, exosomes from tumors (EL4 Thymoma) which contain Hsp70 on the vesicular surface were shown to be immunosuppressive through interaction with myeloid derived suppressor cells (MDSC; Chalmin et al., 2010). Relatively little work has been carried out in this interesting area and we anticipate further studies.

EXTRACELLULAR HSPs AND THE CLASS II PATHWAY

Cross presentation of HSP-associated antigens, although essential would not be sufficient to fully program CTL and in the absence of other signals, CTL would ultimately fail to actively proliferate and could undergo programmed cell death. For a sturdy CTL response, DC would process and present antigens via the MHC class II pathway and thus activate CD4+ T helper cells. It has

been shown that HSPs can chaperone external antigens through the MHC class II pathway and activate CD4+ T cells (Gong et al., 2009). As mentioned above, unchaperoned antigens, after uptake into phagosomes, traverse separate trafficking routes in DC that direct them to compartments in which association with MHC class I or II molecules can occur. It is not clear which mechanisms are involved in the partitioning of HSP-chaperoned antigens into these pathways. We have shown however that SRECI is involved in HSP-mediated CD4+ T cell activation and our preliminary studies indicate that the early endocytic and regulatory stages of this process, such as uptake of HSP.PC through SRECI, involve similar pathways to those in Class I presentation (Gong et al., 2009; Murshid, A., and Calderwood, S. K., in preparation). Activation of CTL is thought to require DC licensing, in which an individual DC interacts with both CD4+ and CD8+ T cells (Bennett et al., 1997, 1998). Initial interaction of CD40 ligand on the CD4+ T cell surface with CD40 on the DC sets in motion a series of events including the induction of co-stimulatory molecules such as CD80 (B7-1), CD86 (B7-2) that permit subsequent CD8+ T cells activity (Kurts et al., 2010; **Figure 1**). Co-stimulatory molecules are recognized by counter receptors such as CD28 expressed on T cells and reinforce the signals induced in CD8+ T cells by MHCI–peptide complex-T cell receptor binding (van der Merwe et al., 1997; Rudd et al., 2009; **Figure 1**). Most HSPs are likely to bind to a wide spectrum of intracellular antigens, containing both MHC class I and class II epitopes and may favor the reactions involved in DC licensing.

HSP ASSOCIATION WITH PATTERN RECOGNITION RECEPTORS

Cytotoxic lymphocytes activation can also be reinforced by signals induced by *pathogen-associated molecular patterns* (PAMPs). During infection, PAMPs bind to several classes of pattern recognition receptors (PRR) such as such as Toll-like receptors (TLR) and trigger innate immune responses in APC (Yamamoto and Takeda, 2010). This type of signaling activates a number of transcription factors, most notably *nuclear factor-κB* (NFκB) that can stimulate transcription of genes encoding inflammatory cytokines and co-stimulatory molecules such as B7-1 and B7-2 (Parra et al., 1995; Rudd et al., 2009). Innate immune activation through these pathways can thus reinforce CTL programming through pathways overlapping those induced by CD40L–CD40 interactions between CD4+ T cells and DC (Maurer et al., 2002; Schulz et al., 2005; Najar and Dutz, 2007; **Figure 1**). HSPs may potentially function as DAMPs (damage associated molecular patterns) and induce innate immunity under sterile conditions through interaction with PRR (Chase et al., 2007; Wheeler et al., 2009). Indeed, there is an evidence for HSPs interacting with PRR such as TLR2 and TLR4 (Gong et al., 2009; **Figure 1**). It was shown that SRECI-dependent tumor immunity induced by Hsp70 vaccines requires TLR signaling and knockout of the TLR signaling intermediate Myd88 or the receptors TLR2 and TLR4 obliterates tumor immunity (Gong et al., 2009). In addition to affecting innate immune transcription, TLR signaling also mediates a number of stages in antigen cross presentation and positive HSP interaction with TLRs may thus reinforce antigen cross presentation (Amigorena and Savina, 2010). As CTL programming involves the interaction of three rare cell types, including DC, CD8+ T cells, and CD4+ T cells, this confluence may be strongly influenced by the release of inflammatory CCL cytokines from DC. It may be notable that Hsp70 has been shown to trigger release of CCL cytokines in a TLR4 dependent manner and that mycobacterial Hsp70 binds directly to CCL receptor CCR5 (Whittall et al., 2006; Chen et al., 2009).

HSP–SCAVENGER RECEPTOR INTERACTIONS AND TRANSMEMBRANE SIGNALING

In addition to internalizing bound peptides, ligand-bound HSPs may play significant roles in cell signaling pathways that impact immunity. LOX-1 and SRECI can both interact with TLR2 to induce innate immune signaling and transcription (Jeannin et al., 2005). Although little is known regarding HSP–LOX-1 signaling, binding of LOX-1 to other ligands, such as modified LDL induces MAP kinase activity, NF-κB, and pro-inflammatory signaling through the small intracellular domain of the receptor (Chen et al., 2002). However, when HSPs interact with class I scavenger receptor SRA/CD204, innate immune signaling through TLR4, NFκB, and MAP kinase is reduced, and CD4+ T cell activation is inhibited (Yi et al., 2011). Knockout of the SRA/CD204 gene, although decreasing Hsp110 binding to DC led to a profoundly increased ability to stimulate melanoma antigen gp100-specific naïve T cells, compared to wild-type mice (Qian et al., 2011). HSP–SR interactions may thus send both pro-immune and inhibitory signals resulting in a tightly regulated system. Signaling through SRECI appears to reinforce antigen cross presentation and the appearance of SRECI in lipid rafts after binding Hsp90 may be a key stage in this process (Murshid et al., 2010). Lipid rafts are cholesterol- and sphingolipid-rich membrane microdomains that can concentrate molecules involved in cell signaling (Lingwood et al., 2009). Although lacking the glycerophosphoinositide anchor domain motifs found in many raft-associated membrane proteins, SRECI contains other motifs that would permit it to associate with lipid rafts (Murshid et al., 2011a). Indeed, Hsp90–SRECI complexes associate with the small GTPase Cdc42 and non-receptor tyrosine kinase Src, molecules tightly associated with lipid rafts (Murshid et al., 2010). These associations appear to be important in regulating antigen cross presentation of Hsp90-associated antigens in DC.

Lipid rafts also concentrate intermediates in the TLR4 signaling pathway in response to innate immune stimuli (Triantafilou et al., 2002). Indeed, preliminary studies show significant co-localization of Hsp90–SRECI complexes with TLR4 (Murshid, A., and Calderwood, S. K., in preparation). HSP-triggered signaling through SRECI and LOX-1 may thus be involved both in amplifying antigen cross presentation and in stimulating innate immunity and these receptors may operate in concert to thwart the inhibitory effects of HSP–SRA/CD204 signaling. It may be significant that LOX-1, with essentially no sequence similarity to SRECI associates with TLR2 after ligand binding and mediates immune responses in a similar way to SRECI (Jeannin et al., 2005). The similarity in immune properties and protein interaction partners between SRECI and LOX-1 despite sequence homology may suggest functional interaction on the cell surface after HSP binding.

CONCLUSION

On theoretical grounds, HSPs might be seen as ideal sources of tumor antigens due to their high abundance in cancer and their molecular chaperone functions. However, the nature of the polypeptides chaperoned by HSPs which carry the "antigenic load" of the HSP.PC is less clear. Most cytoplasmic peptides appear to be in free, unchaperoned form unless acquired by TAP, although Hsp90 appears to be an exception to this rule and appears to be capable of binding to peptides exiting the proteasome. However, Hsp70 and Hsp90 both associate with intact proteins *in vivo* during protein folding and quality control, and the antigenic nature of extracellular HSPs extracted from tumors may thus at least partially reflect stable chaperone binding to antigenic proteins.

It seems clear that HSPs can mediate cross presentation of acquired tumor antigens by APC. This process may involve receptor-mediated uptake by SR LOX-1 or SRECI or receptor-independent uptake. If small peptides are bound to HSPs they can then be presented to CD8+ cells through the rapid vacuolar pathway of antigen cross presentation. Hsp90 appears to be able to chaperone whole proteins such as ovalbumin across the plasma membrane, into endosomes, and direct them to the proteasome for processing and presentation by MHC class I molecules.

In addition to directing antigens toward the Class I pathway, HSPs can chaperone antigens toward the Class II pathway of antigen presentation. This effect may be significant in recruiting antigen-specific Th1 helper cells to mediate DC licensing and amplify CTL processing.

Association of HSP–antigen complexes with SR SRECI and LOX-1 may help to amplify cross presentation of antigens by triggering signal transduction pathways emanating from lipid rafts. HSP-bound SR can associate with lipid rafts and activate signaling through small GTPase Cdc42, tyrosine kinase c-sec, and induce activity of PRR such as TLR2. There also appear to be HSP-mediated regulatory signals and binding to SRA/CD204 can inhibit immunity.

There remains a need to identify a number of unresolved cell biological and biochemical aspects of HSP.PC-mediated immunity. These include further investigation of the signals initiated when HSPs bind to cell surface receptors, the kinetics of compartmentalization of the chaperoned peptides and the routes of trafficking and processing of peptides after release from HSP. Advanced technology studies of the trafficking of single cell molecules may open up this field. Elucidating the mechanisms of antigen cross presentation of HSP-bound antigens via MHCI and the signaling events associated with this immunogenic effect may allow development of superior HSP-based vaccines and immunotherapy protocols.

ACKNOWLEDGMENTS

This work was supported by NIH research grants RO-1CA047407, R01CA119045, and RO-1CA094397.

REFERENCES

Ackerman, A. L., Giodini, A., and Cresswell, P. (2006). A role for the endoplasmic reticulum protein retrotranslocation machinery during crosspresentation by dendritic cells. *Immunity* 25, 607–617.

Ackerman, A. L., Kyritsis, C., Tampe, R., and Cresswell, P. (2003). Early phagosomes in dendritic cells form a cellular compartment sufficient for cross presentation of exogenous antigens. *Proc. Natl. Acad. Sci. U.S.A.* 100, 12889–12894.

Amigorena, S., and Savina, A. (2010). Intracellular mechanisms of antigen cross presentation in dendritic cells. *Curr. Opin. Immunol.* 22, 109–117.

Bennett, S. R., Carbone, F. R., Karamalis, F., Flavell, R. A., Miller, J. F., and Heath, W. R. (1998). Help for cytotoxic-T-cell responses is mediated by CD40 signalling. *Nature* 393, 478–480.

Bennett, S. R., Carbone, F. R., Karamalis, F., Miller, J. F., and Heath, W. R. (1997). Induction of a CD8+ cytotoxic T lymphocyte response by cross-priming requires cognate CD4+ T cell help. *J. Exp. Med.* 186, 65–70.

Berwin, B., Delneste, Y., Lovingood, R. V., Post, S. R., and Pizzo, S. V. (2004). SREC-I, a type F scavenger receptor, is an endocytic receptor for calreticulin. *J. Biol. Chem.* 279, 51250–51257.

Berwin, B., Hart, J. P., Rice, S., Gass, C., Pizzo, S. V., Post, S. R., and Nicchitta, C. V. (2003). Scavenger receptor-A mediates gp96/GRP94 and calreticulin internalization by antigen-presenting cells. *EMBO J.* 22, 6127–6136.

Binder, R. J., Han, D. K., and Srivastava, P. K. (2000). CD91: a receptor for heat shock protein gp96. *Nat. Immunol.* 1, 151–155.

Binder, R. J., and Srivastava, P. K. (2005). Peptides chaperoned by heat-shock proteins are a necessary and sufficient source of antigen in the cross-priming of CD8+ T cells. *Nat. Immunol.* 6, 593–599.

Bonifaz, L., Bonnyay, D., Mahnke, K., Rivera, M., Nussenzweig, M. C., and Steinman, R. M. (2002). Efficient targeting of protein antigen to the dendritic cell receptor DEC-205 in the steady state leads to antigen presentation on major histocompatibility complex class I products and peripheral CD8+ T cell tolerance. *J. Exp. Med.* 196, 1627–1638.

Burgdorf, S., Lukacs-Kornek, V., and Kurts, C. (2006). The mannose receptor mediates uptake of soluble but not of cell-associated antigen for cross-presentation. *J. Immunol.* 176, 6770–6776.

Burgdorf, S., Schuette, V., Semmling, V., Hochheiser, K., Lukacs-Kornek, V., Knolle, P. A., and Kurts, C. (2010). Steady-state cross-presentation of OVA is mannose receptor-dependent but inhibitable by collagen fragments. *Proc. Natl. Acad. Sci. U.S.A.* 107, E48–E49.

Calderwood, S. K., and Gong, J. (2011). Molecular chaperones in mammary cancer growth and breast tumor therapy. *J. Cell. Biochem.* doi: 10.002/jcb.23461. [Epub ahead of print].

Calderwood, S. K., Khaleque, M. A., Sawyer, D. B., and Ciocca, D. R. (2006). Heat shock proteins in cancer: chaperones of tumorigenesis. *Trends Biochem. Sci.* 31, 164–172.

Calderwood, S. K., Xie, Y., Wang, X., Khaleque, M. A., Chou, S. D., Murshid, A., Prince, T., and Zhang, Y. (2010). Signal transduction pathways leading to heat shock transcription. *Sign. Transduct. Insights* 2, 13–24.

Carter, H., Chen, S., Isik, L., Tyekucheva, S., Velculescu, V. E., Kinzler, K. W., Vogelstein, B., and Karchin, R. (2009). Cancer-specific high-throughput annotation of somatic mutations, computational prediction of driver missense mutations. *Cancer Res.* 69, 6660–6667.

Chalmin, F., Ladoire, S., Mignot, G., Vincent, J., Bruchard, M., Remy-Martin, J. P., Boireau, W., Rouleau, A., Simon, B., Lanneau, D., De Thonel, A., Multhoff, G., Hamman, A., Martin, F., Chauffert, B., Solary, E., Zitvogel, L., Garrido, C., Ryffel, B., Borg, C., Apetoh, L., Rébé, C., and Ghiringhelli, F. (2010). Membrane-associated Hsp72 from tumor-derived exosomes mediates STAT3-dependent immunosuppressive function of mouse and human myeloid-derived suppressor cells. *J. Clin. Invest.* 120, 457–471.

Chandawarkar, R. Y., Wagh, M. S., Kovalchin, J. T., and Srivastava, P. (2004). Immune modulation with high-dose heat-shock protein gp96: therapy of murine autoimmune diabetes and encephalomyelitis. *Int. Immunol.* 16, 615–624.

Chandawarkar, R. Y., Wagh, M. S., and Srivastava, P. K. (1999). The dual nature of specific immunological activity of tumor-derived gp96 preparations. *J. Exp. Med.* 189, 1437–1442.

Chaput, N., Flament, C., Viaud, S., Taieb, J., Roux, S., Spatz, A., André, F., LePecq, J. B., Boussac, M., Garin, J., Amigorena, S., Théry, C., and Zitvogel, L. (2006). Dendritic cell derived-exosomes, biology and clinical implementations. *J. Leukoc. Biol.* 80, 471–478.

Chase, M. A., Wheeler, D. S., Lierl, K. M., Hughes, V. S., Wong, H. R., and Page, K. (2007). Hsp72 induces inflammation and regulates cytokine production in airway epithelium through a TLR4- and NF-kappaB-dependent mechanism. *J. Immunol.* 179, 6318–6324.

Chen, M., Masaki, T., and Sawamura, T. (2002). LOX-1, the receptor for oxidized low-density lipoprotein identified from endothelial cells, implications in endothelial dysfunction and atherosclerosis. *Pharmacol. Ther.* 95, 89–100.

Chen, T., Guo, J., Han, C., Yang, M., and Cao, X. (2009). Heat shock protein 70, released from heat-stressed tumor cells, initiates antitumor immunity by inducing tumor cell chemokine production and activating dendritic cells via TLR4 pathway. *J. Immunol.* 182, 1449–1459.

Craig, E. A. (1985). The heat shock response. *CRC Crit. Rev. Biochem.* 18, 239–280.

Cresswell, P. (1994). Assembly, transport, and function of MHC class II molecules. *Annu. Rev. Immunol.* 12, 259–293.

Cresswell, P., Ackerman, A. L., Giodini, A., Peaper, D. R., and Wearsch, P. A. (2005). Mechanisms of MHC class I-restricted antigen processing and cross-presentation. *Immunol. Rev.* 207, 145–157.

Daniels, G. A., Sanchez-Perez, L., Diaz, R. M., Kottke, T., Thompson, J., Lai, M., Gough, M., Karim, M., Bushell, A., Chong, H., Melcher, A., Harrington, K., and Vile, R. G. (2004). A simple method to cure established tumors by inflammatory killing of normal cells. *Nat. Biotechnol.* 22, 1125–1132.

Delneste, Y., Magistrelli, G., Gauchat, J., Haeuw, J., Aubry, J., Nakamura, K., Kawakami-Honda, N., Goetsch, L., Sawamura, T., Bonnefoy, J., and Jeannin, P. (2002). Involvement of LOX-1 in dendritic cell-mediated antigen cross-presentation. *Immunity* 17, 353–362.

Doherty, G. J., and McMahon, H. T. (2009). Mechanisms of endocytosis. *Annu. Rev. Biochem.* 78, 857–902.

Ellis, R. J. (2007). Protein misassembly: macromolecular crowding and molecular chaperones. *Adv. Exp. Med. Biol.* 594, 1–13.

Engelhard, V. H., Bullock, T. N., Colella, T. A., Sheasley, S. L., and Mullins, D. W. (2002). Antigens derived from melanocyte differentiation proteins: self-tolerance, autoimmunity, and use for cancer immunotherapy. *Immunol. Rev.* 188, 136–146.

Enomoto, Y., Bharti, A., Khaleque, A. A., Song, B., Liu, C., Apostolopoulos, V., Xing, P. X., Calderwood, S. K., and Gong, J. (2006). Enhanced immunogenicity of heat shock protein 70 peptide complexes from dendritic cell-tumor fusion cells. *J. Immunol.* 177, 5946–5955.

Flynn, G. C., Chappell, T. G., and Rothman, J. E. (1989). Peptide binding and release by proteins implicated as catalysts of protein assembly. *Science* 245, 385–390.

Fourie, A. M., Sambrook, J. F., and Gething, M. J. (1994). Common and divergent peptide binding specificities of hsp70 molecular chaperones. *J. Biol. Chem.* 269, 30470–30478.

Geijtenbeek, T. B., and Gringhuis, S. I. (2009). Signalling through C-type lectin receptors: shaping immune responses. *Nat. Rev. Immunol.* 9, 465–479.

Gong, J., Zhang, Y., Durfee, J., Weng, D., Liu, C., Koido, S., Song, B., Apostolopoulos, V., and Calderwood, S. K. (2010). A heat shock protein 70-based vaccine with enhanced immunogenicity for clinical use. *J. Immunol.* 184, 488–496.

Gong, J., Zhu, B., Murshid, A., Adachi, H., Song, B., Lee, A., Liu, C., and Calderwood, S. K. (2009). T cell activation by heat shock protein 70 vaccine requires TLR signaling and scavenger receptor expressed by endothelial cells-1. *J. Immunol.* 183, 3092–3098.

Gragerov, A., and Gottesman, M. E. (1994). Different peptide binding specificities of hsp70 family members. *J. Mol. Biol.* 241, 133–135.

Grossmann, M. E., Madden, B. J., Gao, F., Pang, Y. P., Carpenter, J. E., McCormick, D., and Young, C. Y. (2004). Proteomics shows Hsp70 does not bind peptide sequences indiscriminately in vivo. *Exp. Cell Res.* 297, 108–117.

Gupta, G. D., Swetha, M. G., Kumari, S., Lakshminarayan, R., Dey, G., and Mayor, S. (2009). Analysis of endocytic pathways in *Drosophila* cells reveals a conserved role for GBF1 in internalization via GEECs. *PLoS ONE* 4, e6768. doi:10.1371/journal.pone.0006768

Hanahan, D., and Weinberg, R. A. (2000). The hallmarks of cancer. *Cell* 100, 57–70.

Hanahan, D., and Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. *Cell* 144, 646–674.

Heath, W. R., and Carbone, F. R. (2009). Dendritic cell subsets in primary and secondary T cell responses at body surfaces. *Nat. Immunol.* 10, 1237–1244.

Ichiyanagi, T., Imai, T., Kajiwara, C., Mizukami, S., Nakai, A., Nakayama, T., and Udono, H. (2010). Essential role of endogenous heat shock protein 90 of dendritic cells in antigen cross-presentation. *J. Immunol.* 185, 2693–2700.

Idoyaga, J., Lubkin, A., Fiorese, C., Lahoud, M. H., Caminschi, I., Huang, Y., Rodriguez, A., Clausen, B. E., Park, C. G., Trumpfheller, C., and Steinman, R. M. (2011). Comparable T helper 1 (Th1) and CD8 T-cell immunity by targeting HIV gag p24 to CD8 dendritic cells within antibodies to Langerin, DEC205, and Clec9A. *Proc. Natl. Acad. Sci. U.S.A.* 108, 2384–2389.

Imai, T., Kato, Y., Kajiwara, C., Mizukami, S., Ishige, I., Ichiyanagi, T., Hikida, M., Wang, J. Y., and Udono, H. (2011). Heat shock protein 90 (HSP90) contributes to cytosolic translocation of extracellular antigen for cross-presentation by dendritic cells. *Proc. Natl. Acad. Sci. U.S.A.* 108, 16363–16368.

Inaba, K., and Inaba, M. (2005). Antigen recognition and presentation by dendritic cells. *Int. J. Hematol.* 81, 181–187.

Jeannin, P., Bottazzi, B., Sironi, M., Doni, A., Rusnati, M., Presta, M., Maina, V., Magistrelli, G., Haeuw, J. F., Hoeffel, G., Thieblemont, N., Corvaia, N., Garlanda, C., Delneste, Y., and Mantovani, A. (2005). Complexity and complementarity of outer membrane protein A recognition by cellular and humoral innate immunity receptors. *Immunity* 22, 551–560.

Jockheck-Clark, A. R., Bowers, E. V., Totonchy, M. B., Neubauer, J., Pizzo, S. V., and Nicchitta, C. V. (2010). Re-examination of CD91 function in GRP94 (glycoprotein 96) surface binding, uptake, and peptide cross-presentation. *J. Immunol.* 185, 6819–6830.

Kamal, A., Thao, L., Sensintaffar, J., Zhang, L., Boehm, M. F., Fritz, L. C., and Burrows, F. J. (2003). A high-affinity conformation of Hsp90 confers tumour selectivity on Hsp90 inhibitors. *Nature* 425, 407–410.

Kozutsumi, Y., Normington, K., Press, E., Slaughter, C., Sambrook, J., and Gething, M. J. (1989). Identification of immunoglobulin heavy chain binding protein as glucose-regulated protein 78 on the basis of amino acid sequence, immunological cross-reactivity, and functional activity. *J. Cell Sci. Suppl.* 11, 115–137.

Kunisawa, J., and Shastri, N. (2003). The group II chaperonin TRiC protects proteolytic intermediates from degradation in the MHC class I antigen processing pathway. *Mol. Cell* 12, 565–576.

Kunisawa, J., and Shastri, N. (2006). Hsp90alpha chaperones large C-terminally extended proteolytic intermediates in the MHC class I antigen processing pathway. *Immunity* 24, 523–534.

Kurotaki, T., Tamura, Y., Ueda, G., Oura, J., Kutomi, G., Hirohashi, Y., Sahara, H., Torigoe, T., Hiratsuka, H., Sunakawa, H., Hirata, K., and Sato, N. (2007). Efficient cross-presentation by heat shock protein 90-peptide complex-loaded dendritic cells via an endosomal pathway. *J. Immunol.* 179, 1803–1813.

Kurts, C., Robinson, B. W., and Knolle, P. A. (2010). Cross-priming in health and disease. *Nat. Rev. Immunol.* 10, 403–414.

Lev, A., Dimberu, P., Das, S. R., Maynard, J. C., Nicchitta, C. V., Bennink, J. R., and Yewdell, J. W. (2009). Efficient cross-priming of antiviral CD8+ T cells by antigen donor cells is GRP94 independent. *J. Immunol.* 183, 4205–4210.

Lev, A., Takeda, K., Zanker, D., Maynard, J. C., Dimberu, P., Waffarn, E., Gibbs, J., Netzer, N., Princiotta, M. F., Neckers, L., Picard, D., Nicchitta, C. V., Chen, W., Reiter, Y., Bennink, J. R., and Yewdell, J. W. (2008). The exception that reinforces the rule: crosspriming by cytosolic peptides that escape degradation. *Immunity* 28, 787–798.

Lingwood, D., Kaiser, H. J., Levental, I., and Simons, K. (2009). Lipid rafts as functional heterogeneity in cell membranes. *Biochem. Soc. Trans.* 37(Pt 5), 955–960.

Mambula, S. S., and Calderwood, S. K. (2006a). Heat induced release of Hsp70 from prostate carcinoma cells involves both active secretion and passive release from necrotic cells. *Int. J. Hyperthermia* 22, 575–585.

Mambula, S. S., and Calderwood, S. K. (2006b). Heat shock protein 70 is secreted from tumor cells by a nonclassical pathway involving lysosomal endosomes. *J. Immunol.* 177, 7849–7857.

Manjili, M. H., Henderson, R., Wang, X. Y., Chen, X., Li, Y., Repasky, E., Kazim, L., and Subjeck, J. R. (2002). Development of a recombinant HSP110-HER-2/neu vaccine using the chaperoning properties of HSP110. *Cancer Res.* 62, 1737–1742.

Manjili, M. H., Wang, X. Y., Chen, X., Martin, T., Repasky, E. A., Henderson, R., and Subjeck, J. R. (2003). HSP110-HER2/neu chaperone complex vaccine induces protective immunity against spontaneous

mammary tumors in HER-2/neu transgenic mice. *J. Immunol.* 171, 4054–4061.

Maurer, T., Heit, A., Hochrein, H., Ampenberger, F., O'Keeffe, M., Bauer, S., Lipford, G. B., Vabulas, R. M., and Wagner, H. (2002). CpG-DNA aided cross-presentation of soluble antigens by dendritic cells. *Eur. J. Immunol.* 32, 2356–2364.

Mayer, M. P., and Bukau, B. (2005). Hsp70 chaperones: cellular functions and molecular mechanism. *Cell. Mol. Life Sci.* 62, 670–684.

Murphy, J. E., Vohra, R. S., Dunn, S., Holloway, Z. G., Monaco, A. P., Homer-Vanniasinkam, S., Walker, J. H., and Ponnambalam, S. (2008). Oxidised LDL internalisation by the LOX-1 scavenger receptor is dependent on a novel cytoplasmic motif and is regulated by dynamin-2. *J. Cell. Sci.* 121(Pt 13), 2136–2147.

Murshid, A., Gong, J., and Calderwood, S. K. (2010). Heat shock protein 90 mediates efficient antigen cross presentation through the scavenger receptor expressed by endothelial cells-I. *J. Immunol.* 185, 2903–2917.

Murshid, A., Gong, J., and Calderwood, S. K. (2011a). "Molecular chaperone receptors: binding and trafficking of molecular chaperones by class F and class G scavenger receptors," in *Cellular Trafficking of Molecular Chaperones in Health and Disease*, eds B. Henderson and A. G. Pockley. (in press).

Murshid, A., Theriault, J., Gong, J., and Calderwood, S. K. (2011b). Investigating receptors for extracellular heat shock proteins. *Methods Mol. Biol.* 787, 289–302.

Murshid, A., Gong, J., Stevenson, M. A., and Calderwood, S. K. (2011c). Heat shock proteins and cancer vaccines: developments in the past decade and chaperoning in the decade to come. *Expert Rev. Vaccines* 10, 1553–1568.

Najar, H. M., and Dutz, J. P. (2007). Topical TLR9 agonists induce more efficient cross-presentation of injected protein antigen than parenteral TLR9 agonists do. *Eur. J. Immunol.* 37, 2242–2256.

Neefjes, J. J., and Momburg, F. (1993). Cell biology of antigen presentation. *Curr. Opin. Immunol.* 5, 27–34.

Nicchitta, C. V. (2003). Re-evaluating the role of heat-shock protein-peptide interactions in tumour immunity. *Nat. Rev. Immunol.* 3, 427–432.

Norbury, C. C., Basta, S., Donohue, K. B., Tscharke, D. C., Princiotta, M. F., Berglund, P., Gibbs, J., Bennink, J. R., and Yewdell, J. W. (2004). CD8+ T cell cross-priming via transfer of proteasome substrates. *Science* 304, 1318–1321.

Norbury, C. C., Malide, D., Gibbs, J. S., Bennink, J. R., and Yewdell, J. W. (2002). Visualizing priming of virus-specific CD8+ T cells by infected dendritic cells in vivo. *Nat. Immunol.* 3, 265–271.

Oh, H. J., Easton, D., Murawski, M., Kaneko, Y., and Subjeck, J. R. (1999). The chaperoning activity of hsp110. Identification of functional domains by use of targeted deletions. *J. Biol. Chem.* 274, 15712–15718.

Oura, J., Tamura, Y., Kamiguchi, K., Kutomi, G., Sahara, H., Torigoe, T., Himi, T., and Sato, N. (2011). Extracellular heat shock protein 90 plays a role in translocating chaperoned antigen from endosome to proteasome for generating antigenic peptide to be cross-presented by dendritic cells. *Int. Immunol.* 23, 223–237.

Park, J. E., Facciponte, J., Chen, X., MacDonald, I., Repasky, E. A., Manjili, M. H., Wang, X. Y., and Subjeck, J. R. (2006). Chaperoning function of stress protein grp170, a member of the hsp70 superfamily, is responsible for its immunoadjuvant activity. *Cancer Res.* 66, 1161–1168.

Parra, E., Varga, M., Sigvardsson, M., Hedlund, G., Kalland, T., Leanderson, T., Sjögren, H. O., and Dohlsten, M. (1995). Costimulation of human CD4+ T cells with LFA-3 and B7 induce distinct effects on AP-1 and NF-kappa B transcription factors. *J. Immunol.* 155, 1132–1140.

Pockley, A. G., Muthana, M., and Calderwood, S. K. (2008). The dual immunoregulatory roles of stress proteins. *Trends Biochem. Sci.* 33, 71–79.

Qian, J., Yi, H., Guo, C., Yu, X., Zuo, D., Chen, X., Kane, J. M. III, Repasky, E. A., Subjeck, J. R., and Wang, X. Y. (2011). CD204 suppresses large heat shock protein-facilitated priming of tumor antigen gp100-specific T cells and chaperone vaccine activity against mouse melanoma. *J. Immunol.* 187, 2905–2914.

Reits, E., Griekspoor, A., Neijssen, J., Groothuis, T., Jalink, K., van Veelen, P., Janssen, H., Calafat, J., Drijfhout, J. W., and Neefjes, J. (2003). Peptide diffusion, protection, and degradation in nuclear and cytoplasmic compartments before antigen presentation by MHC class I. *Immunity* 18, 97–108.

Rock, K. L., Hearn, A., Chen, C. J., and Shi, Y. (2005). Natural endogenous adjuvants. *Springer Semin. Immunopathol.* 26, 231–246.

Rock, K. L., and Shen, L. (2005). Cross-presentation: underlying mechanisms and role in immune surveillance. *Immunol. Rev.* 207, 166–183.

Rudd, C. E., Taylor, A., and Schneider, H. (2009). CD28 and CTLA-4 coreceptor expression and signal transduction. *Immunol. Rev.* 229, 12–26.

Scheibel, T., Weikl, T., and Buchner, J. (1998). Two chaperone sites in Hsp90 differing in substrate specificity and ATP dependence. *Proc. Natl. Acad. Sci. U.S.A.* 95, 1495–1499.

Schulz, O., Diebold, S. S., Chen, M., Naslund, T. I., Nolte, M. A., Alexopoulou, L., Azuma, Y. T., Flavell, R. A., Liljeström, P., and Reis e Sousa, C. (2005). Toll-like receptor 3 promotes cross-priming to virus-infected cells. *Nature* 433, 887–892.

Shen, L., and Rock, K. L. (2004). Cellular protein is the source of cross-priming antigen in vivo. *Proc. Natl. Acad. Sci. U.S.A.* 101, 3035–3040.

Srivastava, P. (2002). Interaction of heat shock proteins with peptides and antigen presenting cells: chaperoning of the innate and adaptive immune responses. *Annu. Rev. Immunol.* 20, 395–425.

Srivastava, P. K. (2000). Immunotherapy of human cancer, lessons from mice. *Nat. Immunol.* 1, 363–366.

Srivastava, P. K., and Old, L. J. (1988). Individually distinct transplantation antigens of chemically induced mouse tumors. *Immunol. Today* 9, 78–83.

Steinman, R. M., Gutchinov, B., Witmer, M. D., and Nussenzweig, M. C. (1983). Dendritic cells are the principal stimulators of the primary mixed leukocyte reaction in mice. *J. Exp. Med.* 157, 613–627.

Tacken, P. J., Ginter, W., Berod, L., Cruz, L. J., Joosten, B., Sparwasser, T., Figdor, C. G., and Cambi, A. (2011). Targeting DC-SIGN via its neck region leads to prolonged antigen residence in early endosomes, delayed lysosomal degradation, and cross-presentation. *Blood* 118, 4111–4119.

Theriault, J. R., Adachi, H., and Calderwood, S. K. (2006). Role of scavenger receptors in the binding and internalization of heat shock protein 70. *J. Immunol.* 177, 8604–8611.

Triantafilou, M., Miyake, K., Golenbock, D. T., and Triantafilou, K. (2002). Mediators of innate immune recognition of bacteria concentrate in lipid rafts and facilitate lipopolysaccharide-induced cell activation. *J. Cell. Sci.* 115(Pt 12), 2603–2611.

Udono, H., and Srivastava, P. K. (1993). Heat shock protein 70-associated peptides elicit specific cancer immunity. *J. Exp. Med.* 178, 1391–1396.

van der Merwe, P. A., Bodian, D. L., Daenke, S., Linsley, P., and Davis, S. J. (1997). CD80 (B7-1) binds both CD28 and CTLA-4 with a low affinity and very fast kinetics. *J. Exp. Med.* 185, 393–403.

Vogel, M., Mayer, M. P., and Bukau, B. (2006). Allosteric regulation of Hsp70 chaperones involves a conserved interdomain linker. *J. Biol. Chem.* 281, 38705–38711.

Vogelstein, B., and Kinzler, K. W. (1993). The multistep nature of cancer. *Trends Genet.* 9, 138–141.

Walters, J. J., and Berwin, B. (2005). Differential CD91 dependence for calreticulin and *Pseudomonas* exotoxin-A endocytosis. *Traffic* 6, 1173–1182.

Wang, X. Y., Facciponte, J., Chen, X., Subjeck, J. R., and Repasky, E. A. (2007). Scavenger receptor-A negatively regulates antitumor immunity. *Cancer Res.* 67, 4996–5002.

Wang, X. Y., Kazim, L., Repasky, E. A., and Subjeck, J. R. (2003). Immunization with tumor-derived ER chaperone grp170 elicits tumor-specific CD8+ T-cell responses and reduces pulmonary metastatic disease. *Int. J. Cancer* 105, 226–231.

Wang, X. Y., Sun, X., Chen, X., Facciponte, J., Repasky, E. A., Kane, J., and Subjeck, J. R. (2010). Superior antitumor response induced by large stress protein chaperoned protein antigen compared with peptide antigen. *J. Immunol.* 184, 6309–6319.

Wheeler, D. S., Chase, M. A., Senft, A. P., Poynter, S. E., Wong, H. R., and Page, K. (2009). Extracellular Hsp72, an endogenous DAMP, is released by virally infected airway epithelial cells and activates neutrophils via Toll-like receptor (TLR)-4. *Respir. Res.* 10, 31.

Whittall, T., Wang, Y., Younson, J., Kelly, C., Bergmeier, L., Peters, B., Singh, M., and Lehner, T. (2006). Interaction between the CCR5 chemokine receptors and microbial HSP70. *Eur. J. Immunol.* 36, 2304–2314.

Wolkers, M. C., Stoetter, G., Vyth-Dreese, F. A., and Schumacher, T. N. (2001). Redundancy of direct priming and cross-priming

in tumor-specific CD8+ T cell responses. *J. Immunol.* 167, 3577–3584.

Xie, J., Zhu, H., Guo, L., Ruan, Y., Wang, L., Sun, L., Zhou, L., Wu, W., Yun, X., Shen, A., and Gu, J. (2010). Lectin-like oxidized low-density lipoprotein receptor-1 delivers heat shock protein 60-fused antigen into the MHC class I presentation pathway. *J. Immunol.* 185, 2306–2313.

Yamamoto, M., and Takeda, K. (2010). Current views of toll-like receptor signaling pathways. *Gastroenterol. Res. Pract.* 2010, 240365.

Yi, H., Guo, C., Yu, X., Gao, P., Qian, J., Zuo, D., Manjili, M. H., Fisher, P. B., Subjeck, J. R., and Wang, X. Y. (2011). Targeting the immunoregulator SRA/CD204 potentiates specific dendritic cell vaccine-induced T-cell response and antitumor immunity. *Cancer Res.* 71, 6611–6620.

Ying, M., and Flatmark, T. (2006). Binding of the viral immunogenic octapeptide VSV8 to native glucose-regulated protein Grp94 (gp96) and its inhibition by the physiological ligands ATP and Ca2+. *FEBS J.* 273, 513–522.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 09 December 2011; accepted: 14 March 2012; published online: 30 March 2012.

*Citation: Murshid A, Gong J and Calderwood SK (2012) The role of heat shock proteins in antigen cross presentation. Front. Immun. **3**:63. doi: 10.3389/fimmu.2012.00063*

This article was submitted to Frontiers in Antigen Presenting Cell Biology, a specialty of Frontiers in Immunology.

frontiers in
IMMUNOLOGY

REVIEW ARTICLE
published: 27 February 2012
doi: 10.3389/fimmu.2012.00026

Transcriptional regulation of dendritic cell diversity

Michaël Chopin, Rhys S. Allan and Gabrielle T. Belz*

Division of Molecular Immunology, The Walter and Eliza Hall Institute of Medical Research, Melbourne, VIC, Australia

Edited by:
Christian Kurts, Friedrich Wilhelms-Universität Bonn, Germany

Reviewed by:
Oliver Pabst, Hannover Medical School, Germany
Natalio Garbi, German Cancer Research Center DKFZ, Germany

Correspondence:
Gabrielle T. Belz, Division of Molecular Immunology, Walter and Eliza Hall Institute of Medical Research, 1G Royal Parade, Parkville, Melbourne, VIC 3052, Australia.
e-mail: belz@wehi.edu.au

Dendritic cells (DCs) are specialized antigen presenting cells that are exquisitely adapted to sense pathogens and induce the development of adaptive immune responses. They form a complex network of phenotypically and functionally distinct subsets. Within this network, individual DC subsets display highly specific roles in local immunosurveillance, migration, and antigen presentation. This division of labor amongst DCs offers great potential to tune the immune response by harnessing subset-specific attributes of DCs in the clinical setting. Until recently, our understanding of DC subsets has been limited and paralleled by poor clinical translation and efficacy. We have now begun to unravel how different DC subsets develop within a complex multilayered system. These findings open up exciting possibilities for targeted manipulation of DC subsets. Furthermore, ground-breaking developments overcoming a major translational obstacle – identification of similar DC populations in mouse and man – now sets the stage for significant advances in the field. Here we explore the determinants that underpin cellular and transcriptional heterogeneity within the DC network, how these influence DC distribution and localization at steady-state, and the capacity of DCs to present antigens via direct or cross-presentation during pathogen infection.

Keywords: dendritic cells, transcription factors, differentiation, immunity

INTRODUCTION

Dendritic cells (DCs) are a heterogeneous population of rare hematopoietic cells that are present in most tissues and are essential to the induction of both immunity and tolerance (Steinman and Witmer, 1978; Birnberg et al., 2008; Ohnmacht et al., 2009). They are organized as a specialized "network" that enables them to sample antigens from their environment which are then presented to other lymphocytes. As such, this elegant arm of the immune system is dedicated to shaping the immune response to peripheral antigens.

Dendritic cells are distinct from other immune cells as they are equipped with molecular machinery that enables them to very efficiently take up, process, and present antigens on major histocompatibility (MHC) class I and II molecules to T cells. In addition, they are equiped with a range of pathogen sensing molecules such as toll-like receptors (TLRs), nucleotide-binding oligomerization domain proteins, retinoid-inducible gene 1-like receptors, and C-type lectins that allow them to detect pathogen products and sense inflammation. Signaling through these receptors triggers migration of DCs from peripheral tissues to secondary lymphoid organs bringing DCs carrying antigens into close association with T cells. This pathway provides the critical link between the external environment (the major entry point for pathogens) and the sites where organized immune responses are induced, the lymph nodes (Randolph et al., 2008).

Abbreviations: APCs, antigen presenting cells; DCs, dendritic cells; HSV, herpes simplex virus; IL, interleukin; LP, lamina propria; MHC, major histocompatibility complex; n.d., not determined.

In recent years a number of distinct DC subsets have been defined. These subsets have been based largely on long standing criteria that relies on the expression of specific cell-surface markers. More recently, understanding the development and contributions of these DC subsets to immunity has been broadened significantly by insights to the ontogeny of the different subsets and the transcription factors that guide their development. Here we will highlight recent studies deciphering the transcriptional regulation that underpins DC heterogeneity which is critical in defense against pathogen infection.

ARCHITECTURE OF THE DC NETWORK

On the October 3, 2011, DCs took center stage in the scientific world with the awarding of the Nobel Prize for Physiology or Medicine to Ralph Steinman together with Bruce Beutler and Jules Hoffmann for revolutionizing our understanding of the immune system by discovering key principles for its activation. Hoffman pioneered the discovery of sensing molecules in fruit flies enabling them to combat infection (Lemaitre et al., 1996), while Beutler uncovered the homologous receptors in mice that could detect pathogen products (Poltorak et al., 1998). More than a decade earlier, Ralph Steinman had discovered a rare cell type, the DC, within the immune system that had a unique capacity to efficiently activate immune cells (Steinman and Cohn, 1973). Since then, it has been shown that DCs express a wide range of innate receptors that enable pathogen sensing and the initiation of protective immunity.

Cardinal features of DCs are (i) their ability to efficiently take up and present self and pathogen-derived antigens to other cells of the immune system such as T cells and B cells, and (ii) their capacity to migrate from peripheral tissues such as skin and mucosa to

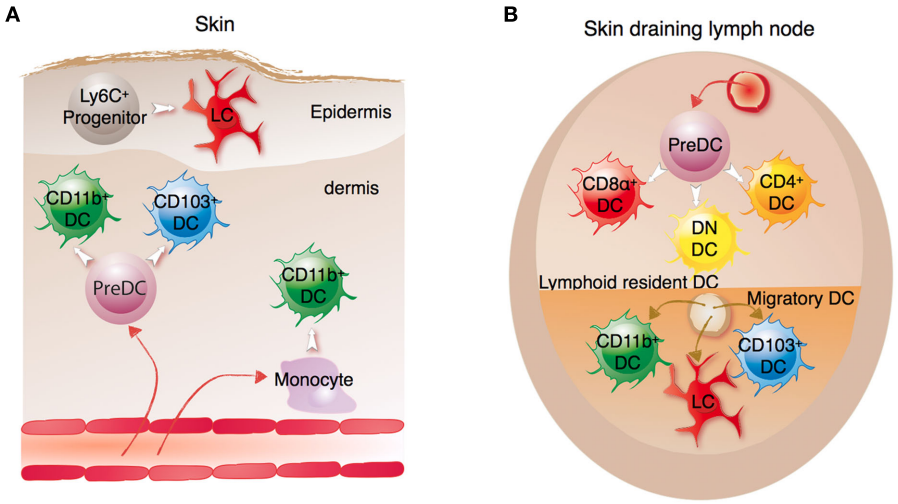


FIGURE 1 | Architecture of the DC network. The DC network is composed of multiple DC subsets that are broadly divided into cDCs, pDCs, MoDCs, and LCs which have distinct anatomical localizations in tissues. **(A)** Migratory DCs in peripheral tissues sample antigen from the periphery and then migrate through the lymphatic vessels to the afferent lymph node to present captured-processed antigens to the T cell within the T cell zone. In peripheral tissues, such as the skin, three main DC subsets are found. Dermal DCs are composed of the $CD103^+CD11b^-$ DC and $CD11b^+$ DC. Both arise from a pre-DC that homed to the tissue. Under conditions of inflammation, some $CD11b^+$ DC can be derived from a monocytic precursor. In addition to the dermal DCs, the epidermis of the skin is populated by the LCs which are derived from a $Ly6C^+$ progenitor. **(B)** In secondary lymphoid tissues such as spleen, $CD4^+$, $CD8\alpha^+$, and $CD4^-CD8\alpha^-$ or double negative (DN) DCs are found. These subsets are also found in the draining lymph nodes which also receive the influx of the migratory $CD11b^+$, CD103+$CD11b^-$ DCs, and LCs arriving from the peripheral tissues.

secondary lymphoid tissues where they can activate lymphocytes and initiate the immune response. This migratory behavior is pivotal and provides a critical cellular link between the external environment where pathogens might enter the body and the secondary lymphoid tissues where immune responses are initiated.

A signature of DCs is their heterogeneity. The DC network is composed of multiple subtypes of DCs that vary in their origin, anatomical localization, lifespan, and function (**Figure 1**). Unraveling the developmental history of these subtypes has been complicated in part by the rarity of DCs in tissues (∼1% of cells) and their short lifespan. This was compounded by the early difficulties in establishing the growth factors and culture conditions necessary for generating large numbers *in vitro*. However, recent studies have made significant progress in clarifying a number of these steps.

Simplistically, four major populations of DCs have been described (**Figure 1**), namely the conventional DCs (cDCs), plasmacytoid DCs (pDCs), Langerhans cells (LCs), and the monocyte-derived DCs (MoDCs).

CONVENTIONAL DCs

Conventional DCs can be divided into two main groups of cells. They are (i) the *migratory* DCs and (ii) the *lymphoid tissue-resident* DCs (**Figure 1**).

Migratory DCs reside in peripheral tissues such as the skin and mucosa where they efficiently sample environmental antigens and then migrate from to the regional lymph node in afferent lymphatics to present antigens to T cells. They are composed of the dermal or interstitial DCs and can be divided into the $CD11b^+$ and $CD11b^-$ DCs (**Figure 1A**). These DCs may also express the integrin αE, also known as CD103. CD103 is expressed on $CD11b^-$ DCs and can be found in a variety of others tissues. However, in intestinal tissues, CD103 is expressed on $CD11b^+$ DCs. Despite a similarity in expression of surface molecules by these two DC subsets, the transcriptional machinery regulating these two populations is distinctly different as discussed below.

The second category of cDCs is composed of several subsets of DCs that are known as *tissue-resident DCs*. In contrast to their migratory counterparts, they do not circulate through peripheral tissues and thus can only process antigens found within the tissue in which they are localized (**Figure 1B**). To overcome this potentially limited access to antigen, migratory DCs can transfer antigens to lymphoid resident DCs who via the process of *cross-presentation*, provide an alternate strategy for the amplification of $CD8^+$ T cell responses (Belz et al., 2004a; Allan et al., 2006).

Tissue-resident DCs are delineated by the expression of the surface molecules CD4 and CD8α and are found in secondary lymphoid organs such as the thymus, spleen, and lymph nodes. Three subsets have been defined which are (i) the $CD4^+$ DCs, (ii) the $CD8\alpha^+$ DCs, and (iii) the $CD4^-CD8\alpha^-$ (double negative, DN) DCs. These subsets develop *in situ* from a common precursor generated in the bone marrow that homes to the lymphoid organs where they undergo further differentiation into mature DC subsets (Naik et al., 2006). Although there are a number of shared functions between these subsets, an interesting division of labor has emerged: $CD8\alpha^+$ DCs are highly efficient in direct and cross-presentation of soluble, cell-associated (**Table 1**), and pathogen-derived antigens to $CD8^+$ T cells. Although $CD4^+$ DCs and $CD4^-CD8\alpha^-$ DCs can also present MHC class I-restricted antigens in some settings (Kim and Braciale, 2009; Lukens et al.,

Table 1 | Features of cross-presenting antigen presenting cells.

	DC Subset			
Molecule	**CD8α$^+$ DCs**	**CD103$^+$ DC peripheral tissues**	**Monocyte-derived DCs**	**Reference**
XCR1	+	+	n.d.	Dorner et al. (2009)
Clec9a	+	+	−	Caminschi et al. (2008), Sancho et al. (2009), Hashimoto et al. (2011)
Mannose receptor	+	n.d.	n.d.	Burgdorf et al. (2006)
CD36	+	+	+	Albert et al. (1998), Belz et al. (2002b), Desch et al. (2011)
E-cadherin	n.d.	n.d.	+	Siddiqui et al. (2010)
TLR3	+	+	n.d.	Schulz et al. (2005), Jelinek et al. (2011)
Cytochrome *c*/Apaf-1 mediated death	+	n.d.	n.d.	Lin et al. (2008)
Migratory	No	Yes	n.t.	del Rio et al. (2010)
Involved in pathogen infection	Yes	Yes	Yes	Belz et al. (2004b), Allan et al. (2006), Bedoui et al. (2009)
Cross-presentation	Constitutive soluble and cell-associated	Constitutive soluble and cell-associated	Induced in inflammation (TLR4)	den Haan et al. (2000), Jackson et al. (2011)

n.d., Not determined.

2009). They are more potent in presenting MHC II antigens to CD4$^+$ T cells (Allenspach et al., 2008; Mount et al., 2008).

CD8α$^+$ and CD103$^+$ DCs – drivers of cross-presentation

The CD8α$^+$ and CD103$^+$ DCs are cDCs that are of special interest due to their shared functional attributes in driving immune responses to pathogen infections, their capacity to cross-present antigens, and the potential to harness the human equivalents of these subsets for clinical use (Steinman, 2010). What distinguishes these two subsets functionally and transcriptionally has thus been an area of intense investigation.

The CD8α$^+$ DC subset. CD8α$^+$ DCs are distinct from other conventional murine DC subsets by their unique surface expression of a CD8αα homodimer. This non-migratory, lymphoid tissue-resident DC has been shown to be key drivers of cross-presentation to a range of experimental pathogen antigens both *in vitro* and *in vivo*. CD8α$^+$ DCs have been found to be critical for cross-presentation of self-antigens resulting in the induction of immune tolerance (Belz et al., 2002a; Heath et al., 2004). This subset was also identified as the main subset involved in presenting pathogen-derived antigens (Allan et al., 2003; Belz et al., 2004a,b, 2005; Lemos et al., 2004; Neuenhahn et al., 2006; Edelson et al., 2011). A number of mechanisms have been proposed to explain their constitutive ability to cross-present antigens. These include the expression of a number of surface receptors, such as CD36 and Clec9a, that allow them to uptake dead or dying cells, specialized intracellular pathways, and regulation of the pH in the phagosome (**Table 1**). This antigen presenting function and their production of interleukin-12 (IL-12) distinguish the CD8α$^+$ DCs from their CD8α$^-$ counterparts. Although initially cross-presentation by CD8α$^+$ DCs was thought to be a unique property restricted solely to this subset, it is now clear that other DC subsets can act as potent cross-presenting cells (i.e., CD103$^+$ DC subset and MoDCs, **Table 1**).

The CD103$^+$ DC subset. This unusual DC subset that appeared to be related to CD8α$^+$ DCs was first described in the influenza infection and was characterized by the lack of expression of CD8α and CD11b (CD8α$^-$CD11b$^-$; Belz et al., 2004b). It was found in a number of lymph nodes including the inguinal, brachial, superficial cervical, mediastinal, mesenteric, hepatic, and renal nodes. This DC subset presents viral antigens very efficiently suggesting that CD8α$^-$CD11b$^-$ DCs may play an important role in T cell mediated immunity. It was postulated that these cells could traffic to the lymph nodes, thereby providing a critical link between antigen sampling in peripheral tissues and antigen presentation in the lymph node. Furthermore it was speculated that these cells would be capable of cross-presentation. Following this early report, it was discovered that these DCs expressed the molecules integrin α_E (CD103) and langerin (Sung et al., 2006). Following on from the establishment of the importance of CD8α$^+$ DC in promoting viral immunity (Allan et al., 2003), CD103$^+$ (langerin$^+$) dermal DCs, and lung CD103$^+$ DCs have emerged as a potent migratory DC able to process and load self- and viral antigens onto MHC class I molecules (Sung et al., 2006; Bedoui et al., 2009).

PLASMACYTOID DCs

Plasmacytoid DCs express several key molecules that distinguish them from the cDC subsets. These include the sialic acid-binding immunoglobulin like lectin H (Siglec-H), Lilra4 (also known as ILT7), bone marrow stromal antigen-2 (BST2, also known as tetherin) and blood DC antigen-2 (BDCA-2, also known as CLEC4C), and the CD45RA isoform (Reizis et al., 2011). The hallmark property of pDCs is their capacity to rapidly secrete type I interferons (IFN-α) during viral infection. This is in part attributable to their expression of TLR7 and TLR9 that enables sensing of nucleic acids during viral infection. The antigen presentation potency of pDCs is poor and although this can be enhanced during maturation (Colonna et al., 2004), their low expression of MHC and co-stimulatory molecules compared to their cDCs counterparts

provide some explanation for their inefficiency in priming of T cells.

LANGERHANS CELLS

Langerhans cells are a unique DC subset found within the epidermis of the skin and mucosa and contain large tennis racquet shaped granules known as Birbeck granules of which langerin is a crucial component. Initially, it was thought that langerin expression was restricted solely to LCs, but it has more recently been discovered that DC subsets located in the lamina propria of skin, bronchi, and mucosa also express langerin (Bursch et al., 2007; Ginhoux et al., 2007; Poulin et al., 2007). Despite sharing the expression of langerin, other langerin$^+$ DCs can arise along a different developmental pathway distinct from LCs (Merad et al., 2008). LCs develop from a local LY6C$^+$ myelo-monocytic precursor cell population, and their development is dependent on TGF-β signaling (Chorro et al., 2009; Nagao et al., 2009), while langerin$^+$ DC develop from a bone marrow precursor that is dependent on Fms-like tyrosine kinase 3 (Flt3) signaling. LCs are exquisitely positioned to provide the front line of defense of the immune system against external invading pathogens. As LCs are the only cells that express MHC II in the epidermis under non-inflammatory conditions and since they are able to migrate through the dermis to the skin-draining lymph nodes, LCs have been believed to be critical to promote immunity, i.e., after skin infection.

This classical paradigm has been recently challenged. LCs are largely resistant to γ-irradiation, a characteristic that has been taken advantage of in deciphering the immunological relevance of LC over dermal DC in chimeric mice. This feature has allowed the generation of elegant chimeric mice in which LCs could be of host origin while dermal and other DCs were of donor origin. Thus the individual function of LCs could be addressed. LCs were found to be unable to initiate T cell dependent immunity when challenged with a herpes simplex virus (HSV) type 1 skin infection (Allan et al., 2003). Similarly, LCs from the vaginal mucosa are not able to present HSV type 2-derived antigens to CD4$^+$ T cells (Zhao et al., 2003). Although the biological function of LCs *in vivo* still raises much debate, it is now clearer that a major role of LCs is in maintaining immune tolerance (Shklovskaya et al., 2011).

MONOCYTE-DERIVED DCs

Under non-inflammatory conditions, monocytes can give rise to CD11b$^+$ MoDCs in non-lymphoid organ, and their development is in part dependent on MCSF-R signaling (Bogunovic et al., 2009; Ginhoux et al., 2009). However the contribution of monocytes to the DC pool is by far more important under inflammatory condition. Pathogen recognition by CD11c$^-$MHCII$^-$ blood monocytes leads to their differentiation into CD11c$^+$MHCII$^+$ MoDCs that express GR1/LY6C pointing to their monocytic origin (Leon et al., 2004; Hohl et al., 2009; Nakano et al., 2009), becoming thereafter the dominant DC population. Until recently, the contributions of MoDCs to pathogen infections have been largely ignored. Recent detailed analyses of their antigen-capturing, processing, and presentation capacity revealed that they are strikingly efficient at both direct and cross-presentation of antigens and at least under certain conditions as good as CD8α and CD103$^+$ DCs (Cheong et al., 2010). Under inflammatory conditions, MoDCs have been found to be the main population presenting antigens in the T cell area (Ingersoll et al., 2011). Hence, blood monocytes serve as an emergency reservoir of antigen presenting cells that efficiently recognize pathogens and their associated danger signals and rapidly induce specialized antigen presentation machinery. Based on the short kinetic of differentiation observed *in vivo*, it is likely that so called "DC genes" are poised to enable rapid differentiation into MoDCs to efficiently counter pathogen attack. The characterization of new markers that clearly identify MoDCs from cDCs in inflamed tissues will open the door for a better understanding of the molecular mechanisms that drive monocyte differentiation into MoDCs (Cheong et al., 2010; Siddiqui et al., 2010).

DC DEVELOPMENT

Ontogeny

The hematopoietic system is hierarchically organized (**Figure 2**). Long-term repopulating hematopoietic stem cells (HSC) constantly self renew but can also give rise to short-term repopulating cells that have lost much of their self-renewal capacity. These precursors differentiate further into the multi-lineage progenitor (MPP). MPP can give rise to both the common-lymphoid progenitor (CLP) and the common myeloid progenitor (CMP). Both CLP and CMP can differentiate into several DC subsets (Manz

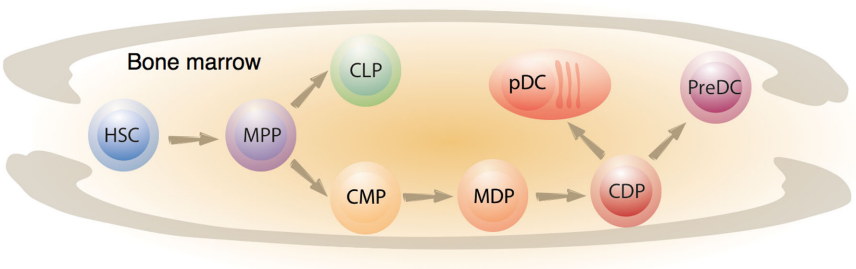


FIGURE 2 | Ontogeny of DC precursors. Short-term HSCs commit into multipotent progenitors that give rise to either a common-lymphoid progenitor (CLP) or a common myeloid progenitor (CMP). A population that lies downstream of CMP has been found to differentiate either into DC or macrophages, and was therefore named the macrophage-dendritic cell progenitor (MDP). Full commitment to the DC lineage is acquired at the CDP stage (common DC progenitor) where a CDP can either differentiate into plasmacytoid DCs (pDCs) or into a pre-DC. The latter will further differentiate into mature conventional DCs (cDCs) in the peripheral tissues, or secondary lymphoid organs.

et al., 2001). This dual origin of DCs has provoked controversy in the field. However, recent studies have clarified the origin of DCs. Under steady-state conditions, the two main populations of DCs, the cDCs and pDCs, have both been shown to arise from a common dendritic cell precursor (also called the CDP) found in the bone marrow. CDPs are devoid of macrophage potential and exclusively give rise to DCs *in vitro* and *in vivo* (Naik et al., 2007; Onai et al., 2007). Once generated CDPs develop either into pDCs in the bone marrow, or give rise to pre-cDCs that are able to migrate from bone marrow to lymphoid and non-lymphoid organs to further mature into cDCs (Naik et al., 2006). The CDP is thought to originate from a bipotential macrophage/DC progenitor (MDP) that can generate either DCs or macrophages (Fogg et al., 2006).

EXTRACELLULAR CUES DRIVING DC DEVELOPMENT

Development of early DC precursors is directed by the interplay between extrinsic cytokine signals such as Flt3, macrophage colony-stimulating factor (M-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), and transcription factors PU.1, Ikaros, and Gfi-1. A key determinant of the development of DC precursors at steady-state is the Flt3:Flt3L pathway. Mice that lack Flt3L, or its receptor Flt3, have a severe reduction in conventional and pDC subsets (McKenna, 2001; Waskow et al., 2008). Conditional expression, or exogenous administration of Flt3L, results in a dramatic expansion of DCs in secondary lymphoid tissues (Maraskovsky et al., 1996; Manfra et al., 2003). Thus, Flt3 and Flt3L are pivotal in driving DC ontogeny. Coordinate regulation of Flt3 signaling is mediated through Stat3 but is dispensable for differentiation of DCs via GM-CSF signaling which is important for driving the differentiation of monocyte-derived DC development in an inflammatory setting (Laouar et al., 2003; Onai et al., 2006).

The growth factor M-CSF is well known to promote the development of macrophages and monocytes. The first example for a function of M-CSF in DC development was uncovered by Ginhoux et al. (2006), who found that mice lacking the M-CSFR failed to generate LCs, highlighting the critical role of M-CSFR/M-CSF axis in the generation of this unique DC subset. Although this pathway is thought to be dispensable for the generation of cDC and pDC, recently, a novel role for M-CSF in DC development has been uncovered. pDCs and cDCs can be generated from *Flt3L*$^{-/-}$ bone marrow cells when cultured in presence of M-CSF (Fancke et al., 2008). In addition the injection of M-CSF into mice leads to increased pDCs and cDCs numbers. Nevertheless, the effect of M-CSF on steady-state DCs (except from the LC) is modest when compared with the influence of Flt3 in steady-state DC development. The M-CSFR/M-CSF axis would be predicted to be more critical in the generation of MoDCs during a pathogen attack, but this pathway remains to be fully elucidated.

TRANSCRIPTION FACTORS DRIVING DC DEVELOPMENT

TRANSCRIPTION FACTORS REGULATING EARLY DC DEVELOPMENT

The differentiation of hematopoietic precursor cells into different DC lineages is a well orchestrated process controlled in large part by transcription factors that are modulated by extracellular cues such as cytokines. The transcriptional networks that guide the development of B and T cells, particularly in early hematopoiesis, are relatively well defined. In contrast, the transcription factors that specifically regulate DC differentiation have only now begun to be elucidated. This has been facilitated by the use of lineage-specific knockout mice and elegant lineage tracing approaches.

These transcription factors can be broadly divided into those that are required or act early in DC development in DC progenitors (Gfi-1, Pu.1), and those whose major actions affect DC subset specification late in DC development (E2.2, Spi-B, IRF8, Id2, Nfil3, Batf3).

Gfi-1

Gfi-1 is a zinc-finger critical for DC ontogeny. Mice lacking Gfi-1 show a global reduction in DC numbers but increased frequency of LCs (Rathinam et al., 2005). Interestingly, *Gfi-1*$^{-/-}$ bone marrow progenitors cultured *in vitro* in presence of Flt3L or GM-CSF fail to produce DC and instead develop into macrophages. Gfi-1 antagonizes PU.1 activity through direct protein interaction and it seems plausible that the aberrant macrophage potential observed in *Gfi-1*$^{-/-}$ bone marrow progenitors reflects failed repression of PU.1 binding to its positive regulatory elements, resulting in a macrophage rather than a DC fate (Spooner et al., 2009).

PU.1

PU.1 (encoded by the gene *Sfpi1*) belongs to the Ets family of transcription factors. Until recently, its role in DCs has been unclear due to disparate studies – the first reporting a deficit in DC differentiation (Anderson et al., 2000) and the second reporting apparently normal DC development in the absence of PU.1 (Guerriero et al., 2000). These studies could not distinguish whether PU.1 played a role at the stage of DC commitment or whether its main action was in multipotent progenitors. Conditional deletion of PU.1 at different stages of DC development highlighted the requirement of PU.1 for DC commitment as differentiation was abrogated in absence of PU.1 (Carotta et al., 2010). Furthermore, PU.1 lies upstream of FLT3 and GM-CSFR and is required for the development of DCs via both pathways. Thus, PU.1 is a central player in the generation of both steady-state DCs and presumably inflammatory DCs although the complex interactions of PU.1 in coordinating DC differentiation remain to be fully explored.

TRANSCRIPTION FACTORS REGULATING LATE DC DIFFERENTIATION

While a number of transcription factors delineate the development of precursor cells that ultimately give rise to DCs, specification of individual DC subset identity appears to be a late event that is guided a handful of transcription factors (see **Table 2**). These can be broadly divided into three pathways of regulation – those transcription factors that predominantly influence (i) the pDCs (namely E2-2 and Spi-B), (ii) the CD8α^{+} and CD103^{+} lineages (IRF8, Id2, EB4BP, and Batf3), and (iii) the non-CD8α^{+} DC lineages (IRF2, IRF4).

Regulation of pDCs by E2-2

E proteins constitute a family of basic helix-loop-helix (bHLH) transcription factor whose function have been most clearly defined in B lymphocytes (de Pooter and Kee, 2010). The expression of E proteins is modulated by inhibitor of DNA-binding (ID) proteins which can bind E proteins to prevent their binding to DNA targets. To date three mammalian E proteins have been described

Table 2 | Transcription factors guiding steady-state DC subset development.

Transcription factor	Transcription factor family	Phenotype	Reference
PU.1 (SPI1, Sfpi1)	Ets-domain transcription factor binds to PU box sequences	Required for development of all DC subsets	Anderson et al. (2000), Guerriero et al. (2000), Carotta et al. (2010)
Irf2	Interferon regulatory factor, inhibits the IRF1-mediated activation of IFNα/β	Alters pDC ratios; reduction in $CD8^-$ DCs and LCs	Ichikawa et al. (2004)
Irf4	Interferon regulatory factor	Required for non-$CD8\alpha^+$ DC lineage development	Suzuki et al. (2004), Tamura et al. (2005)
Irf8 (ICSBP)	Interferon regulatory factor	Required for pDC and most cDC development	Schiavoni et al. (2002), Aliberti et al. (2003), Tsujimura et al. (2003), Schiavoni et al. (2004), Tailor et al. (2008)
Gfi-1	Zinc-finger protein, repressor	50% reduction in cDC and pDC subsets and increased LCs	Rathinam et al. (2005)
Id2	Inhibitor of DNA-binding family containing helix-loop-helix domains (HLH)	Required for development of $CD103^+$ DCs and $CD8\alpha^+$ DCs in PLN and spleen; not required for DCs in MLNs.	Hacker et al. (2003), Ginhoux et al. (2009), Jackson et al. (2011)
E4BP4 (NFIL3)	PAR-related basic leucine zipper (bZIP) transcription factor	Required for development of $CD8\alpha^+$ DCs	Kashiwada et al. (2011)
E2-2 (Tcf4)	E protein containing basic helix-loop-helix domains (bHLH)	Required for development of pDCs and their maintenance	Cisse et al. (2008), Ghosh et al. (2010)
Stat3	Signal transducer and activator of transcription	Significant reduction in cDCs	Laouar et al. (2003)
Stat5a/b	Signal transducer and activator of transcription	Inhibits pDC development by interacting with *Irf8,* reduced cDC and pDC subsets	Esashi et al. (2008)
Ikaros (Ikzf1)	Zinc-finger DNA-binding protein	Absence of most DCs. pDCs specifically lost in hypomorphic mutant	Wu et al. (1997), Allman et al. (2006)
Batf3	bZIP, heterodimerizes with Jun	Failure to develop $CD103^+$ DCs; impaired survival of precursor $CD8\alpha^+$ DCs	Hildner et al. (2008), Edelson et al. (2010), Jackson et al. (2011)
RelB	Rel homology domain family, interacts with NFκB family	Loss of $CD8^-$ DCs	Burkly et al. (1995), Wu et al. (1998)
SpiB	Ets-domain transcription factor	Required for human pDC differentiation	Schotte et al. (2004)

(E2a, HEB, E2-2) that act on their targeted sequence (CACCTG E-box) either as homodimers or heterodimers. The first indication that E proteins played important roles in pDC differentiation came from the study by Spits et al. (2000), where the overexpression of Id proteins, which sequester E proteins, impaired *in vitro* pDC development but left cDC differentiation intact. Subsequent analysis uncovered that E2-2, encoded by the gene *Tcf4,* was a key determinant of pDC differentiation. Germline or conditional deletion of E2-2 led to a complete loss of the pDC and abolished the ability of mice to respond to unmethylated DNA (Cisse et al., 2008). Through the binding of E2-2 to many pDC signature genes (*Irf8, Irf7, SpiB, BDCA-2*) it has been proposed as a master regulator of the pDC compartment. In line with this hypothesis, the depletion of E2-2 in mature pDC led to a predominance of cDC-like cells implying that E2-2 plays a pivotal role in maintaining pDC cell fate (Ghosh et al., 2010). However, in this setting it is difficult to separate the contribution of expansion of the pre-existing cDCs as molecular analyses showed enrichment of the conventional DC genes rather than a pure signature. Nevertheless, pDCs are particularly sensitive to E2-2 concentration as both E2-2-deficient mice and rare patients with haploinsufficiency (Pitt–Hopkins syndrome) show impaired pDC formation and function (Cisse et al., 2008).

Spi-B

A second transcription factor that influences the development of pDCs is Spi-B, a member of Ets family transcription factor (Schotte et al., 2004). In contrast to the loss of PU.1, deficiency of Spi-B affects only the pDC compartment. Interestingly, over expression of Spi-B in human pDCs impairs Id2 expression and consequently E2-2 activity is enhanced suggesting that Id2 is a key regulator of the cDC/pDC balance (Nagasawa et al., 2008). However, the capacity for E2-2 to promote pDC differentiation was abolished in these DC when Spi-B expression was ablated showing that Spi-B and E2-2 are jointly necessary to efficiently drive development of human precursors into the pDC lineage (Nagasawa et al., 2008).

THE IRF8–ID2–BATF3 AXIS REGULATES CLASSICAL CROSS-PRESENTING DCs

IRF8

Interferon regulatory factor 8 (also called the interferon consensus sequence-binding protein, or ICSBP) regulates the development of both cDCs and pDCs and is highly expressed on the CDP. Mice deficient in IRF8 lack many mature DC subsets including $CD8\alpha^+$ DCs and LCs (Schiavoni et al., 2002, 2004). In addition, IRF8 controls functional features such as TLR9 and IFN-α production in pDCs and IL-12 production in $CD8\alpha^+$ DCs (Tailor et al., 2008). *Irf8*$^{-/-}$ mice are more susceptible to viral infection but also develop a myeloproliferative syndrome characterized by overproduction of granulocytes (Holtschke et al., 1996). This suggests that while IRF8 is important for conventional DC development, it may also be required for the generation or maintenance of the upstream MDP progenitor that can give rise to monocytes, cDCs, and pDCs.

Id2

The expression of Id2 is highest in $CD8\alpha^+$ and $CD103^+CD11b^-$ DCs but it is nevertheless broadly expressed by all conventional DC subsets (Ginhoux et al., 2009; Jackson et al., 2011). Loss of Id2 results in the failure of these two subsets, $CD8\alpha^+$ and $CD103^+$ DCs, to develop in skin-draining lymph nodes and spleen. $CD103^+CD11b^+$ DCs found in the lymph nodes draining the gut together with $CD4^+$ and $CD4^-CD8\alpha^-$ DCs in lymphoid tissues, appear to develop normally (Hacker et al., 2003; Ginhoux et al., 2009; Edelson et al., 2010). Through its action in regulating E protein binding, Id2 appears to plays a pivotal role in the maintenance of conventional DC identity. Multiple E proteins are expressed by DCs so precisely how these are regulated, and which E proteins (E2A, E2-2, or HEB) are the critical targets of Id2 is unclear. Understanding the balance between E proteins and Id proteins in cDCs will be required to understand why Id2 plays such an key role in $CD8\alpha^+$ and $CD103^+CD11b^-$ DCs and how differentiation of such subsets might be enhanced.

Nfil3 (also known as E4BP4)

E4BP4 is a mammalian basic leucine zipper (bZIP) transcription factor that is required for the development of NK cells, and is a key regulator of cytokine production in other hematopoietic lineages (Gascoyne et al., 2009; Kamizono et al., 2009; Kashiwada et al., 2011; Kobayashi et al., 2011). In NK cells E4BP4 acts in a dose dependent manner downstream of the IL-15 receptor and regulates Id2 expression (Gascoyne et al., 2009; Kamizono et al., 2009). Thus, it has been found that induction of E4BP4 is important for the development of $CD8\alpha^+$ DCs (Kashiwada et al., 2011). *E4BP4*$^{-/-}$ mice exhibit impaired IL-12 production upon TLR3 activation, and failed to cross-prime $CD8^+$ T cells against cell-associated antigens. CDPs deficient in E4BP4 displayed reduced BATF3 expression and enforced expression in these cells rescued $CD8\alpha^+$ DCs development *in vitro* (Kashiwada et al., 2011). Thus, E4BP4 is emerging as an important regulator of conventional DC development.

Batf3

Batf3 is a bZIP transcription factor (also known as Jun dimerization protein p21SNFT) that represses NFAT–AP1 activity by competing with FOS for JUN dimerization (Dorsey et al., 1995; Echlin et al., 2000). It provoked enormous interest in the DC field when it was shown to play a critical role in DC differentiation. Indeed, it was the first description of a transcription factor that appeared to have an exclusive role in the development of the $CD8\alpha^+$ DC subset. The fact that deletion of Batf3 resulted in increased susceptibility to various pathogen infections such as West Nile virus, influenza virus, *Listeria monocytogenes*, and *Toxoplasma gondii* (Hildner et al., 2008; Desch et al., 2011; Edelson et al., 2011; Mashayekhi et al., 2011) and critically impaired cross-priming was striking (Hildner et al., 2008). More recently it has been described to be involved in the development of migratory cross-presenting $CD103^+CD11b^-$ DCs in peripheral lymphoid tissues (Edelson et al., 2010), but not in gut lymphoid tissue $CD103^+CD11b^+$ DCs (Bar-On et al., 2010). However, although *Batf3*$^{-/-}$ mice show a reduction in the frequency of $CD8\alpha^+$ DCs, particularly in the spleen, it is now clear that $CD8\alpha^+$ DCs are indeed present in the absence of Batf3 (Edelson et al., 2011; Jackson et al., 2011). Thus in contrast to the early suggestions, Batf3 appears to exclusively regulate the development of $CD103^+$ DC while $CD8\alpha^+$ DC precursors develop although they seem unable to cross-present exogenous antigens to $CD8^+$ T cells. This suggests that some stages in DC maturation may be perturbed in the absence of Batf3.

$CD8\alpha^-$ DC, MONOCYTE-DERIVED DC LINEAGES, AND TRANSCRIPTIONAL REGULATION

Until recently, the capacity to cross-present antigens was a feature attributed almost exclusively to $CD8\alpha^+$ and $CD103^+$ DCs. This distinction gained some foothold with the notion that particular DC subsets possess unique molecular machinery intrinsic to these subsets exclusively enabling them to cross-present (Schnorrer et al., 2006). However, in other settings, it was clear that non-$CD8\alpha^+$ lineage cells could also perform such functions. This was poignantly highlighted by the systematic characterization of the capacity for MoDCs to efficiently cross-present antigens, particularly in the inflammatory setting, demonstrated that induction of cross-presenting machinery could occur in different DC subsets when subjected to specific stimuli (Cheong et al., 2010).

Non-$CD8\alpha^+$ DC lineages are composed of DN and $CD4^+$ DCs together with MoDCs. Much less is known about the transcription factors that regulate the fate decisions of these subsets although it is clear that at least the conventional subsets in this group are significantly influenced by IRF2 (Hida et al., 2000; Honda et al., 2004; Ichikawa et al., 2004; Arakura et al., 2007), IRF4 (Suzuki et al., 2004; Tamura et al., 2005), and RelB (Burkly et al., 1995; Wu et al., 1998). At least some of these pathways are also likely to regulate MoDCs. This could potentially happen through the role of IRF4 in coordinating signals from GM-CSF stimulation through the NF-κB pathway (Gilliet et al., 2002). It will be important to dissect the network of transcription factors that drive not only $CD8\alpha^-$ DC development but also function. This latter feature seems particularly important where a property such as cross-presentation is inducible and transcription factors such as Id2 and Batf3 are broadly expressed in the non-$CD8\alpha^-$ DC lineages but not obviously required for the development of these subsets. They may indeed be important for maturation or localization of DCs.

NOMENCLATURE, DC SUBSETS, AND THE TOOLS NECESSARY FOR PRECISE DEFINITIONS

Key steps in understanding DC subsets has come from detailed phenotypic characterization made possible through the development of novel antibodies combined with the power of flow cytometry. These descriptions have been significantly extended by elucidation of the anatomical localization of particular DC subset, the precursors from which they arise and the identification of unique or restricted functions that can now be attributed to particular DC subsets. For example, CD8α$^+$ and CD103$^+$ DCs are the dominant DC subsets that cross-present soluble and cell-associated antigens and present viral antigens to CD8$^+$ T cells (den Haan and Bevan, 2002; Allan et al., 2003; Belz et al., 2004a,b, 2005; Bedoui et al., 2009). While a number of seminal studies that have identified a new DC subset or function are critical to the development of the field, they have not always led to clarification of the contributions of DCs, or DC subsets, to immunoregulation of responses. This arises largely because surface markers or receptors are expressed by multiple DC subsets and in many instances, a broader range of immune cells. Thus, with regard to the capacity to cross-present antigens, MoDCs have recently also been discovered to be very efficient in shuttling antigen into this pathway, particularly in an inflammatory setting (Cheong et al., 2010).

One approach to this problem is to focus on the molecular wiring of cells – that is, the transcription factors – to develop a definitive map of DC identity (Satpathy et al., 2011). This approach indeed offers a way forward. However, deletion of specific DC populations is premised on the notion that presence or absence of a transcription factor results in a complete loss or alternately, exaggeration of DC subsets or their functions. Loss of some transcription factors such as Irf4, Irf8, and Id2 seem to demonstrate relatively definitive outcomes with complete loss of some DC subsets. Certainly, mice engineered to allow conditional deletion of a gene in a cell-specific manner (e.g., driven by CD11c) allows analysis of the intrinsic requirement for factors allows delineation of phenotypes that may arise due to extrinsic signals. Similar approaches have also been used where a cell-specific promoter drives conditional deletion of a cell type. A key example of this is the development of mice in which CD11c$^+$ cells can be deleted by treating them with diptheria toxin (CD11c–DTR mice; Jung et al., 2002). This provides an elegant model for examination of cDCs but it was quickly realized that other cell types (such as inflamed lung epithelial cells) expressed CD11c and therefore generation of bone marrow chimeric mice was necessary to fully utilize this model without confounding effects (Sapoznikov and Jung, 2008).

In biology, this "black and white" model seldom reflects reality and it is not clear that DC differentiation into subsets is a purely linear process. Loss of the ability to detect a DC subset depends mainly on the same tools we have used to identify them in the first instance. These approaches do not generally distinguish between two important stages of developmental arrest – firstly, complete ablation of a lineage, and secondly, the formation of the immediate precursors of that lineage that then fail to receive the necessary signals for DC progenitors to develop into fully matured cells in the peripheral tissues. The former situation identifies factors that are definitively required for the formation of a DC subset; the latter allows detailed dissection of the factors that influence the maturation and survival of DC subsets in the periphery. Distinguishing between these two cases is facilitated by the use of genetically modified mice in which a transcription factor is linked to a fluorescent reporter allowing rare precursor cells to be identified when most of the population does not survive to maturity. This was first identified in mice expressing green fluorescent protein linked to Ror(c)t, an important transcription factor for the development of lymphoid-tissue inducer (LTi) cells (Schmutz et al., 2009; Chappaz and Finke, 2010). Treatment of these reporter mice with IL-7/IL-7R mAb complexes revealed that progenitor cells were in fact formed in the absence of Ror(c)t but they did not develop into cells that could colonize and form secondary lymphoid tissues due to impaired IL-7 signaling. Similarly, Batf3 was initially described (Hildner et al., 2008) to unequivocally regulate the development of CD8α$^+$ DCs and was later discovered to also regulate CD103$^+$ DCs (Edelson et al., 2010). By analyzing the expression patterns of ID2 in *Batf3*$^{-/-}$ mice using the *Id2*$^{GFP/GFP}$ strain (Jackson et al., 2011), it was discovered that both *in vitro* and *in vivo* CD8α$^+$ DC precursors do develop in the absence of Batf3 although their persistence, particularly in spleen, was diminished (Edelson et al., 2011; Jackson et al., 2011). These two examples highlight that careful dissection of phenotypes is critical and that complementary methods to analyze the expression of transcription factors and consequences of their loss provides a real strength in tackling the difficult questions of the future.

Overall, the factors that determine differentiation patterns of individual DC subsets with unique functions are complex. Establishment of the features that define an individual DC subset's identity, their maintenance and differentiation is unlikely to be simplistic and rely on a single phenotypic marker or transcription factor. A more likely scenario is that DC subset development is determined by combinatorial interactions between transcription factors to guide fate decisions (Lin et al., 2010; Wilson et al., 2010). Analyses of genome wide DNA-binding data will be critical for understanding precisely how these different transcription factors work together to define the different populations of DCs that are necessary to provide protective immunity.

KEY QUESTIONS AND FUTURE DIRECTIONS

Our current understanding of DC commitment and differentiation at a steady-state and during pathogen infection is limited, especially when compared to the extensive understanding of regulatory networks that guide fate decisions in B cells and CD4$^+$ T cells. In addition, the mechanisms that guide the emergence inflammatory DCs and the induction of protective cross-presenting machinery offer a fertile field for discovery in the future. Collectively, understanding the complex and multilayered circuitry that integrates DC location, expression of surface molecules, components of the antigen processing and presentation machinery together with and understanding of the molecular wiring, and potential rewiring of DC subsets will be essential. This information will lead to a more complete picture of the roles of DCs and the DC network in maintaining immune tolerance and immunity and may prove pivotal to improvements in the clinical efficacy of therapeutic approaches. Solving the following, and other, critical questions in the field of DC biology will be instrumental in progressing discoveries in the field.

1. Do individual transcription factors, or alternately, combinatorial transcriptional programs involving networks of transcription factors determine DC subset specification?
2. How do DC subsets that emerge during inflammatory setting reflect those identified at steady-state?
3. How is the constitutive cross-presenting machinery that maintains tolerance through presentation of self-antigens at steady-state, and is dominant in CD8α$^+$ and CD103$^+$ DCs during viral infection, induced in multiple DC subsets during inflammation or pathogen challenge?
4. Can this inductive process be harnessed and how?
5. How does the MDP engage the "DC program"?
6. How does DC lifespan affect immunity?
7. How is developmental plasticity is maintained to ensure immunity?

The challenge now is to develop the skill sets, tools, and technologies that will enable us to embark on dissection of these core features of DCs to open the pathway for targeted use of this cell type.

ACKNOWLEDGMENTS

The authors thank Y. Zhan, M. Pang, S. Nutt, and A. Kallies for helpful discussions and critical reading of the manuscript. This work is supported by the National Health and Medical Research Council (NHMRC) of Australia. Gabrielle T. Belz is supported by a Sylvia and Charles Viertel Foundation Fellowship and Rhys S. Allan is supported by an NHMRC International Postdoctoral Fellowship. This work was made possible through Victorian State Government Operational Infrastructure Support and Australian Government NHMRC IRIIS.

REFERENCES

Albert, M. L., Pearce, S. F., Francisco, L. M., Sauter, B., Roy, P., Silverstein, R. L., and Bhardwaj, N. (1998). Immature dendritic cells phagocytose apoptotic cells via alphavbeta5 and CD36, and cross-present antigens to cytotoxic T lymphocytes. *J. Exp. Med.* 188, 1359–1368.

Aliberti, J., Schulz, O., Pennington, D. J., Tsujimura, H., Reis E Sousa, C., Ozato, K., and Sher, A. (2003). Essential role for ICSBP in the in vivo development of murine CD8alpha + dendritic cells. *Blood* 101, 305–310.

Allan, R. S., Smith, C. M., Belz, G. T., Van Lint, A. L., Wakim, L. M., Heath, W. R., and Carbone, F. R. (2003). Epidermal viral immunity induced by CD8alpha+ dendritic cells but not by Langerhans cells. *Science* 301, 1925–1928.

Allan, R. S., Waithman, J., Bedoui, S., Jones, C. M., Villadangos, J. A., Zhan, Y., Lew, A. M., Shortman, K., Heath, W. R., and Carbone, F. R. (2006). Migratory dendritic cells transfer antigen to a lymph node-resident dendritic cell population for efficient CTL priming. *Immunity* 25, 153–162.

Allenspach, E. J., Lemos, M. P., Porrett, P. M., Turka, L. A., and Laufer, T. M. (2008). Migratory and lymphoid-resident dendritic cells cooperate to efficiently prime naive CD4 T cells. *Immunity* 29, 795–806.

Allman, D., Dalod, M., Asselin-Paturel, C., Delale, T., Robbins, S. H., Trinchieri, G., Biron, C. A., Kastner, P., and Chan, S. (2006). Ikaros is required for plasmacytoid dendritic cell differentiation. *Blood* 108, 4025–4034.

Anderson, K. L., Perkin, H., Surh, C. D., Venturini, S., Maki, R. A., and Torbett, B. E. (2000). Transcription factor PU.1 is necessary for development of thymic and myeloid progenitor-derived dendritic cells. *J. Immunol.* 164, 1855–1861.

Arakura, F., Hida, S., Ichikawa, E., Yajima, C., Nakajima, S., Saida, T., and Taki, S. (2007). Genetic control directed toward spontaneous IFN-alpha/IFN-beta responses and downstream IFN-gamma expression influences the pathogenesis of a murine psoriasis-like skin disease. *J. Immunol.* 179, 3249–3257.

Bar-On, L., Birnberg, T., Lewis, K. L., Edelson, B. T., Bruder, D., Hildner, K., Buer, J., Murphy, K. M., Reizis, B., and Jung, S. (2010). CX3CR1+ CD8alpha+ dendritic cells are a steady-state population related to plasmacytoid dendritic cells. *Proc. Natl. Acad. Sci. U.S.A.* 107, 14745–14750.

Bedoui, S., Whitney, P. G., Waithman, J., Eidsmo, L., Wakim, L., Caminschi, I., Allan, R. S., Wojtasiak, M., Shortman, K., Carbone, F. R., Brooks, A. G., and Heath, W. R. (2009). Cross-presentation of viral and self antigens by skin-derived CD103+ dendritic cells. *Nat. Immunol.* 10, 488–495.

Belz, G. T., Behrens, G. M., Smith, C. M., Miller, J. F., Jones, C., Lejon, K., Fathman, C. G., Mueller, S. N., Shortman, K., Carbone, F. R., and Heath, W. R. (2002a). The CD8alpha(+) dendritic cell is responsible for inducing peripheral self- tolerance to tissue-associated antigens. *J. Exp. Med.* 196, 1099–1104.

Belz, G. T., Vremec, D., Febbraio, M., Corcoran, L., Shortman, K., Carbone, F. R., and Heath, W. R. (2002b). CD36 is differentially expressed by CD8+ splenic dendritic cells but is not required for cross-presentation in vivo. *J. Immunol.* 168, 6066–6070.

Belz, G. T., Shortman, K., Bevan, M. J., and Heath, W. R. (2005). CD8alpha+ dendritic cells selectively present MHC class I-restricted noncytolytic viral and intracellular bacterial antigens in vivo. *J. Immunol.* 175, 196–200.

Belz, G. T., Smith, C. M., Eichner, D., Shortman, K., Karupiah, G., Carbone, F. R., and Heath, W. R. (2004a). Cutting edge: conventional CD8 alpha+ dendritic cells are generally involved in priming CTL immunity to viruses. *J. Immunol.* 172, 1996–2000.

Belz, G. T., Smith, C. M., Kleinert, L., Reading, P., Brooks, A., Shortman, K., Carbone, F. R., and Heath, W. R. (2004b). Distinct migrating and nonmigrating dendritic cell populations are involved in MHC class I-restricted antigen presentation after lung infection with virus. *Proc. Natl. Acad. Sci. U.S.A.* 101, 8670–8675.

Birnberg, T., Bar-on, L., Sapoznikov, A., Caton, M. L., Cervantes-Barragan, L., Makia, D., Krauthgamer, R., Brenner, O., Ludewig, B., Brockschnieder, D., Riethmacher, D., Reizis, B., and Jung, S. (2008). Lack of conventional dendritic cells is compatible with normal development and T cell homeostasis, but causes myeloid proliferative syndrome. *Immunity* 29, 986–997.

Bogunovic, M., Ginhoux, F., Helft, J., Shang, L., Hashimoto, D., Greter, M., Liu, K., Jakubzick, C., Ingersoll, M. A., Leboeuf, M., Stanley, E. R., Nussenzweig, M., Lira, S. A., Randolph, G. J., and Merad, M. (2009). Origin of the lamina propria dendritic cell network. *Immunity* 31, 513–525.

Burgdorf, S., Lukacs-Kornek, V., and Kurts, C. (2006). The mannose receptor mediates uptake of soluble but not of cell-associated antigen for cross-presentation. *J. Immunol.* 176, 6770–6776.

Burkly, L., Hession, C., Ogata, L., Reilly, C., Marconi, L. A., Olson, D., Tizard, R., Cate, R., and Lo, D. (1995). Expression of relB is required for the development of thymic medulla and dendritic cells. *Nature* 373, 531–536.

Bursch, L. S., Wang, L., Igyarto, B., Kissenpfennig, A., Malissen, B., Kaplan, D. H., and Hogquist, K. A. (2007). Identification of a novel population of Langerin+ dendritic cells. *J. Exp. Med.* 204, 3147–3156.

Caminschi, I., Proietto, A. I., Ahmet, F., Kitsoulis, S., Shin Teh, J., Lo, J. C., Rizzitelli, A., Wu, L., Vremec, D., Van Dommelen, S. L., Campbell, I. K., Maraskovsky, E., Braley, H., Davey, G. M., Mottram, P., Van De Velde, N., Jensen, K., Lew, A. M., Wright, M. D., Heath, W. R., Shortman, K., and Lahoud, M. H. (2008). The dendritic cell subtype-restricted C-type lectin Clec9A is a target for vaccine enhancement. *Blood* 112, 3264–3273.

Carotta, S., Dakic, A., D'Amico, A., Pang, S. H., Greig, K. T., Nutt, S. L., and Wu, L. (2010). The transcription factor PU.1 controls dendritic cell development and Flt3 cytokine receptor expression in a dose-dependent manner. *Immunity* 32, 628–641.

Chappaz, S., and Finke, D. (2010). The IL-7 signaling pathway regulates lymph node development independent of peripheral lymphocytes. *J. Immunol.* 184, 3562–3569.

Cheong, C., Matos, I., Choi, J. H., Dandamudi, D. B., Shrestha, E., Longhi, M. P., Jeffrey, K. L., Anthony, R. M., Kluger, C., Nchinda, G., Koh, H., Rodriguez, A., Idoyaga, J., Pack, M., Velinzon, K., Park, C. G., and Steinman, R. M. (2010). Microbial stimulation fully differentiates monocytes to DC-SIGN/CD209(+) dendritic cells for immune T cell areas. *Cell* 143, 416–429.

Chorro, L., Sarde, A., Li, M., Woollard, K. J., Chambon, P., Malissen, B., Kissenpfennig, A., Barbaroux, J. B., Groves, R., and Geissmann, F. (2009). Langerhans cell (LC) proliferation mediates neonatal development, homeostasis, and inflammation-associated expansion of the epidermal LC network. *J. Exp. Med.* 206, 3089–3100.

Cisse, B., Caton, M. L., Lehner, M., Maeda, T., Scheu, S., Locksley, R., Holmberg, D., Zweier, C., Den Hollander, N. S., Kant, S. G., Holter, W., Rauch, A., Zhuang, Y., and Reizis, B. (2008). Transcription factor E2-2 is an essential and specific regulator of plasmacytoid dendritic cell development. *Cell* 135, 37–48.

Colonna, M., Trinchieri, G., and Liu, Y. J. (2004). Plasmacytoid dendritic cells in immunity. *Nat. Immunol.* 5, 1219–1226.

de Pooter, R. F., and Kee, B. L. (2010). E proteins and the regulation of early lymphocyte development. *Immunol. Rev.* 238, 93–109.

del Rio, M. L., Bernhardt, G., Rodriguez-Barbosa, J. I., and Forster, R. (2010). Development and functional specialization of CD103+ dendritic cells. *Immunol. Rev.* 234, 268–281.

den Haan, J. M., and Bevan, M. J. (2002). Constitutive versus activation-dependent cross-presentation of immune complexes by CD8(+) and CD8(-) dendritic cells in vivo. *J. Exp. Med.* 196, 817–827.

den Haan, J. M., Lehar, S. M., and Bevan, M. J. (2000). CD8(+) but not CD8(-) dendritic cells cross-prime cytotoxic T cells in vivo. *J. Exp. Med.* 192, 1685–1696.

Desch, A. N., Randolph, G. J., Murphy, K., Gautier, E. L., Kedl, R. M., Lahoud, M. H., Caminschi, I., Shortman, K., Henson, P. M., and Jakubzick, C. V. (2011). CD103+ pulmonary dendritic cells preferentially acquire and present apoptotic cell-associated antigen. *J. Exp. Med.* 208, 1789–1797.

Dorner, B. G., Dorner, M. B., Zhou, X., Opitz, C., Mora, A., Guttler, S., Hutloff, A., Mages, H. W., Ranke, K., Schaefer, M., Jack, R. S., Henn, V., and Kroczek, R. A. (2009). Selective expression of the chemokine receptor XCR1 on cross-presenting dendritic cells determines cooperation with CD8+ T cells. *Immunity* 31, 823–833.

Dorsey, M. J., Tae, H. J., Sollenberger, K. G., Mascarenhas, N. T., Johansen, L. M., and Taparowsky, E. J. (1995). B-ATF: a novel human bZIP protein that associates with members of the AP-1 transcription factor family. *Oncogene* 11, 2255–2265.

Echlin, D. R., Tae, H. J., Mitin, N., and Taparowsky, E. J. (2000). B-ATF functions as a negative regulator of AP-1 mediated transcription and blocks cellular transformation by Ras and Fos. *Oncogene* 19, 1752–1763.

Edelson, B. T., Bradstreet, T. R., Hildner, K., Carrero, J. A., Frederick, K. E., Kc, W., Belizaire, R., Aoshi, T., Schreiber, R. D., Miller, M. J., Murphy, T. L., Unanue, E. R., and Murphy, K. M. (2011). CD8alpha(+) dendritic cells are an obligate cellular entry point for productive infection by Listeria monocytogenes. *Immunity* 35, 236–248.

Edelson, B. T., Kc, W., Juang, R., Kohyama, M., Benoit, L. A., Klekotka, P. A., Moon, C., Albring, J. C., Ise, W., Michael, D. G., Bhattacharya, D., Stappenbeck, T. S., Holtzman, M. J., Sung, S. S., Murphy, T. L., Hildner, K., and Murphy, K. M. (2010). Peripheral CD103+ dendritic cells form a unified subset developmentally related to CD8alpha+ conventional dendritic cells. *J. Exp. Med.* 207, 823–836.

Esashi, E., Wang, Y. H., Perng, O., Qin, X. F., Liu, Y. J., and Watowich, S. S. (2008). The signal transducer STAT5 inhibits plasmacytoid dendritic cell development by suppressing transcription factor IRF8. *Immunity* 28, 509–520.

Fancke, B., Suter, M., Hochrein, H., and O'Keeffe, M. (2008). M-CSF: a novel plasmacytoid and conventional dendritic cell poietin. *Blood* 111, 150–159.

Fogg, D. K., Sibon, C., Miled, C., Jung, S., Aucouturier, P., Littman, D. R., Cumano, A., and Geissmann, F. (2006). A clonogenic bone marrow progenitor specific for macrophages and dendritic cells. *Science* 311, 83–87.

Gascoyne, D. M., Long, E., Veiga-Fernandes, H., De Boer, J., Williams, O., Seddon, B., Coles, M., Kioussis, D., and Brady, H. J. (2009). The basic leucine zipper transcription factor E4BP4 is essential for natural killer cell development. *Nat. Immunol.* 10, 1118–1124.

Ghosh, H. S., Cisse, B., Bunin, A., Lewis, K. L., and Reizis, B. (2010). Continuous expression of the transcription factor e2-2 maintains the cell fate of mature plasmacytoid dendritic cells. *Immunity* 33, 905–916.

Gilliet, M., Boonstra, A., Paturel, C., Antonenko, S., Xu, X. L., Trinchieri, G., O'Garra, A., and Liu, Y. J. (2002). The development of murine plasmacytoid dendritic cell precursors is differentially regulated by FLT3-ligand and granulocyte/macrophage colony-stimulating factor. *J. Exp. Med.* 195, 953–958.

Ginhoux, F., Collin, M. P., Bogunovic, M., Abel, M., Leboeuf, M., Helft, J., Ochando, J., Kissenpfennig, A., Malissen, B., Grisotto, M., Snoeck, H., Randolph, G., and Merad, M. (2007). Blood-derived dermal langerin+ dendritic cells survey the skin in the steady state. *J. Exp. Med.* 204, 3133–3146.

Ginhoux, F., Liu, K., Helft, J., Bogunovic, M., Greter, M., Hashimoto, D., Price, J., Yin, N., Bromberg, J., Lira, S. A., Stanley, E. R., Nussenzweig, M., and Merad, M. (2009). The origin and development of nonlymphoid tissue CD103+ DCs. *J. Exp. Med.* 206, 3115–3130.

Ginhoux, F., Tacke, F., Angeli, V., Bogunovic, M., Loubeau, M., Dai, X. M., Stanley, E. R., Randolph, G. J., and Merad, M. (2006). Langerhans cells arise from monocytes in vivo. *Nat. Immunol.* 7, 265–273.

Guerriero, A., Langmuir, P. B., Spain, L. M., and Scott, E. W. (2000). PU.1 is required for myeloid-derived but not lymphoid-derived dendritic cells. *Blood* 95, 879–885.

Hacker, C., Kirsch, R. D., Ju, X. S., Hieronymus, T., Gust, T. C., Kuhl, C., Jorgas, T., Kurz, S. M., Rose-John, S., Yokota, Y., and Zenke, M. (2003). Transcriptional profiling identifies Id2 function in dendritic cell development. *Nat. Immunol.* 4, 380–386.

Hashimoto, D., Miller, J., and Merad, M. (2011). Dendritic cell and macrophage heterogeneity in vivo. *Immunity* 35, 323–335.

Heath, W. R., Belz, G. T., Behrens, G. M., Smith, C. M., Forehan, S. P., Parish, I. A., Davey, G. M., Wilson, N. S., Carbone, F. R., and Villadangos, J. A. (2004). Cross-presentation, dendritic cell subsets, and the generation of immunity to cellular antigens. *Immunol. Rev.* 199, 9–26.

Hida, S., Ogasawara, K., Sato, K., Abe, M., Takayanagi, H., Yokochi, T., Sato, T., Hirose, S., Shirai, T., Taki, S., and Taniguchi, T. (2000). CD8(+) T cell-mediated skin disease in mice lacking IRF-2, the transcriptional attenuator of interferon-alpha/beta signaling. *Immunity* 13, 643–655.

Hildner, K., Edelson, B. T., Purtha, W. E., Diamond, M., Matsushita, H., Kohyama, M., Calderon, B., Schraml, B. U., Unanue, E. R., Diamond, M. S., Schreiber, R. D., Murphy, T. L., and Murphy, K. M. (2008). Batf3 deficiency reveals a critical role for CD8alpha+ dendritic cells in cytotoxic T cell immunity. *Science* 322, 1097–1100.

Hohl, T. M., Rivera, A., Lipuma, L., Gallegos, A., Shi, C., Mack, M., and Pamer, E. G. (2009). Inflammatory monocytes facilitate adaptive CD4 T cell responses during respiratory fungal infection. *Cell Host Microbe* 6, 470–481.

Holtschke, T., Lohler, J., Kanno, Y., Fehr, T., Giese, N., Rosenbauer, F., Lou, J., Knobeloch, K. P., Gabriele, L., Waring, J. F., Bachmann, M. F., Zinkernagel, R. M., Morse, H. C. III, Ozato, K., and Horak, I. (1996). Immunodeficiency and chronic myelogenous leukemia-like syndrome in mice with a targeted mutation of the ICSBP gene. *Cell* 87, 307–317.

Honda, K., Mizutani, T., and Taniguchi, T. (2004). Negative regulation of IFN-alpha/beta signaling by IFN regulatory factor 2 for homeostatic development of dendritic cells. *Proc. Natl. Acad. Sci. U.S.A.* 101, 2416–2421.

Ichikawa, E., Hida, S., Omatsu, Y., Shimoyama, S., Takahara, K., Miyagawa, S., Inaba, K., and Taki, S. (2004). Defective development of splenic and epidermal CD4+ dendritic cells in mice deficient for IFN regulatory factor-2. *Proc. Natl. Acad. Sci. U.S.A.* 101, 3909–3914.

Ingersoll, M. A., Platt, A. M., Potteaux, S., and Randolph, G. J. (2011). Monocyte trafficking in acute and chronic inflammation. *Trends Immunol.* 32, 470–477.

Jackson, J. T., Hu, Y., Liu, R., Masson, F., D'Amico, A., Carotta, S., Xin, A., Camilleri, M. J., Mount, A. M., Kallies, A., Wu, L., Smyth, G. K., Nutt, S. L., and Belz, G. T. (2011). Id2 expression delineates differential checkpoints in the genetic program of CD8alpha+ and CD103+ dendritic cell lineages. *EMBO J.* 30, 2690–2704.

Jelinek, I., Leonard, J. N., Price, G. E., Brown, K. N., Meyer-Manlapat, A., Goldsmith, P. K., Wang, Y., Venzon, D., Epstein, S. L., and Segal, D. M. (2011). TLR3-specific double-stranded RNA oligonucleotide adjuvants induce dendritic cell cross-presentation, CTL responses, and antiviral protection. *J. Immunol.* 186, 2422–2429.

Jung, S., Unutmaz, D., Wong, P., Sano, G., De Los Santos, K., Sparwasser, T., Wu, S., Vuthoori, S., Ko, K., Zavala, F., Pamer, E. G., Littman, D. R., and Lang, R. A. (2002). In vivo depletion of CD11c+ dendritic cells abrogates priming of CD8+ T cells by

exogenous cell-associated antigens. *Immunity* 17, 211–220.

Kamizono, S., Duncan, G. S., Seidel, M. G., Morimoto, A., Hamada, K., Grosveld, G., Akashi, K., Lind, E. F., Haight, J. P., Ohashi, P. S., Look, A. T., and Mak, T. W. (2009). Nfil3/E4bp4 is required for the development and maturation of NK cells in vivo. *J. Exp. Med.* 206, 2977–2986.

Kashiwada, M., Cassel, S. L., Colgan, J. D., and Rothman, P. B. (2011). NFIL3/E4BP4 controls type 2 T helper cell cytokine expression. *EMBO J.* 30, 2071–2082.

Kim, T. S., and Braciale, T. J. (2009). Respiratory dendritic cell subsets differ in their capacity to support the induction of virus-specific cytotoxic CD8+ T cell responses. *PLoS ONE* 4, e4204. doi:10.1371/journal.pone.0004204

Kobayashi, T., Matsuoka, K., Sheikh, S. Z., Elloumi, H. Z., Kamada, N., Hisamatsu, T., Hansen, J. J., Doty, K. R., Pope, S. D., Smale, S. T., Hibi, T., Rothman, P. B., Kashiwada, M., and Plevy, S. E. (2011). NFIL3 is a regulator of IL-12 p40 in macrophages and mucosal immunity. *J. Immunol.* 186, 4649–4655.

Laouar, Y., Welte, T., Fu, X. Y., and Flavell, R. A. (2003). STAT3 is required for Flt3L-dependent dendritic cell differentiation. *Immunity* 19, 903–912.

Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J. M., and Hoffmann, J. A. (1996). The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in *Drosophila* adults. *Cell* 86, 973–983.

Lemos, M. P., Esquivel, F., Scott, P., and Laufer, T. M. (2004). MHC class II expression restricted to CD8alpha+ and CD11b+ dendritic cells is sufficient for control of Leishmania major. *J. Exp. Med.* 199, 725–730.

Leon, B., Martinez Del Hoyo, G., Parrillas, V., Vargas, H. H., Sanchez-Mateos, P., Longo, N., Lopez-Bravo, M., and Ardavin, C. (2004). Dendritic cell differentiation potential of mouse monocytes: monocytes represent immediate precursors of CD8- and CD8+ splenic dendritic cells. *Blood* 103, 2668–2676.

Lin, M. L., Zhan, Y., Proietto, A. I., Prato, S., Wu, L., Heath, W. R., Villadangos, J. A., and Lew, A. M. (2008). Selective suicide of cross-presenting CD8+ dendritic cells by cytochrome c injection shows functional heterogeneity within this subset. *Proc. Natl. Acad. Sci. U.S.A.* 105, 3029–3034.

Lin, Y. C., Jhunjhunwala, S., Benner, C., Heinz, S., Welinder, E., Mansson, R., Sigvardsson, M., Hagman, J., Espinoza, C. A., Dutkowski, J., Ideker, T., Glass, C. K., and Murre, C. (2010). A global network of transcription factors, involving E2A, EBF1 and Foxo1, that orchestrates B cell fate. *Nat. Immunol.* 11, 635–643.

Lukens, M. V., Kruijsen, D., Coenjaerts, F. E., Kimpen, J. L., and Van Bleek, G. M. (2009). Respiratory syncytial virus-induced activation and migration of respiratory dendritic cells and subsequent antigen presentation in the lung-draining lymph node. *J. Virol.* 83, 7235–7243.

Manfra, D. J., Chen, S. C., Jensen, K. K., Fine, J. S., Wiekowski, M. T., and Lira, S. A. (2003). Conditional expression of murine Flt3 ligand leads to expansion of multiple dendritic cell subsets in peripheral blood and tissues of transgenic mice. *J. Immunol.* 170, 2843–2852.

Manz, M. G., Traver, D., Miyamoto, T., Weissman, I. L., and Akashi, K. (2001). Dendritic cell potentials of early lymphoid and myeloid progenitors. *Blood* 97, 3333–3341.

Maraskovsky, E., Brasel, K., Teepe, M., Roux, E. R., Lyman, S. D., Shortman, K., and Mckenna, H. J. (1996). Dramatic increase in the numbers of functionally mature dendritic cells in Flt3 ligand-treated mice: multiple dendritic cell subpopulations identified. *J. Exp. Med.* 184, 1953–1962.

Mashayekhi, M., Sandau, M. M., Dunay, I. R., Frickel, E. M., Khan, A., Goldszmid, R. S., Sher, A., Ploegh, H. L., Murphy, T. L., Sibley, L. D., and Murphy, K. M. (2011). CD8alpha(+) dendritic cells are the critical source of interleukin-12 that controls acute infection by *Toxoplasma gondii* tachyzoites. *Immunity* 35, 249–259.

McKenna, H. J. (2001). Role of hematopoietic growth factors/flt3 ligand in expansion and regulation of dendritic cells. *Curr. Opin. Hematol.* 8, 149–154.

Merad, M., Ginhoux, F., and Collin, M. (2008). Origin, homeostasis and function of Langerhans cells and other langerin-expressing dendritic cells. *Nat. Rev. Immunol.* 8, 935–947.

Mount, A. M., Smith, C. M., Kupresanin, F., Stoermer, K., Heath, W. R., and Belz, G. T. (2008). Multiple dendritic cell populations activate CD4+ T cells after viral stimulation. *PLoS ONE* 3, e1691. doi:10.1371/journal.pone.0001691

Nagao, K., Ginhoux, F., Leitner, W. W., Motegi, S., Bennett, C. L., Clausen, B. E., Merad, M., and Udey, M. C. (2009). Murine epidermal Langerhans cells and langerin-expressing dermal dendritic cells are unrelated and exhibit distinct functions. *Proc. Natl. Acad. Sci. U.S.A.* 106, 3312–3317.

Nagasawa, M., Schmidlin, H., Hazekamp, M. G., Schotte, R., and Blom, B. (2008). Development of human plasmacytoid dendritic cells depends on the combined action of the basic helix-loop-helix factor E2-2 and the Ets factor Spi-B. *Eur. J. Immunol.* 38, 2389–2400.

Naik, S. H., Metcalf, D., Van Nieuwenhuijze, A., Wicks, I., Wu, L., O'Keeffe, M., and Shortman, K. (2006). Intrasplenic steady-state dendritic cell precursors that are distinct from monocytes. *Nat. Immunol.* 7, 663–671.

Naik, S. H., Sathe, P., Park, H. Y., Metcalf, D., Proietto, A. I., Dakic, A., Carotta, S., O'Keeffe, M., Bahlo, M., Papenfuss, A., Kwak, J. Y., Wu, L., and Shortman, K. (2007). Development of plasmacytoid and conventional dendritic cell subtypes from single precursor cells derived in vitro and in vivo. *Nat. Immunol.* 8, 1217–1226.

Nakano, H., Lin, K. L., Yanagita, M., Charbonneau, C., Cook, D. N., Kakiuchi, T., and Gunn, M. D. (2009). Blood-derived inflammatory dendritic cells in lymph nodes stimulate acute T helper type 1 immune responses. *Nat. Immunol.* 10, 394–402.

Neuenhahn, M., Kerksiek, K. M., Nauerth, M., Suhre, M. H., Schiemann, M., Gebhardt, F. E., Stemberger, C., Panthel, K., Schroder, S., Chakraborty, T., Jung, S., Hochrein, H., Russmann, H., Brocker, T., and Busch, D. H. (2006). CD8alpha+ dendritic cells are required for efficient entry of *Listeria monocytogenes* into the spleen. *Immunity* 25, 619–630.

Ohnmacht, C., Pullner, A., King, S. B., Drexler, I., Meier, S., Brocker, T., and Voehringer, D. (2009). Constitutive ablation of dendritic cells breaks self-tolerance of CD4 T cells and results in spontaneous fatal autoimmunity. *J. Exp. Med.* 206, 549–559.

Onai, N., Obata-Onai, A., Schmid, M. A., Ohteki, T., Jarrossay, D., and Manz, M. G. (2007). Identification of clonogenic common Flt3+M-CSFR+ plasmacytoid and conventional dendritic cell progenitors in mouse bone marrow. *Nat. Immunol.* 8, 1207–1216.

Onai, N., Obata-Onai, A., Tussiwand, R., Lanzavecchia, A., and Manz, M. G. (2006). Activation of the Flt3 signal transduction cascade rescues and enhances type I interferon-producing and dendritic cell development. *J. Exp. Med.* 203, 227–238.

Poltorak, A., He, X., Smirnova, I., Liu, M. Y., Van Huffel, C., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., Freudenberg, M., Ricciardi-Castagnoli, P., Layton, B., and Beutler, B. (1998). Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* 282, 2085–2088.

Poulin, L. F., Henri, S., De Bovis, B., Devilard, E., Kissenpfennig, A., and Malissen, B. (2007). The dermis contains langerin+ dendritic cells that develop and function independently of epidermal Langerhans cells. *J. Exp. Med.* 204, 3119–3131.

Randolph, G. J., Ochando, J., and Partida-Sanchez, S. (2008). Migration of dendritic cell subsets and their precursors. *Annu. Rev. Immunol.* 26, 293–316.

Rathinam, C., Geffers, R., Yucel, R., Buer, J., Welte, K., Moroy, T., and Klein, C. (2005). The transcriptional repressor Gfi1 controls STAT3-dependent dendritic cell development and function. *Immunity* 22, 717–728.

Reizis, B., Bunin, A., Ghosh, H. S., Lewis, K. L., and Sisirak, V. (2011). Plasmacytoid dendritic cells: recent progress and open questions. *Annu. Rev. Immunol.* 29, 163–183.

Sancho, D., Joffre, O. P., Keller, A. M., Rogers, N. C., Martinez, D., Hernanz-Falcon, P., Rosewell, I., and Reis E Sousa, C. (2009). Identification of a dendritic cell receptor that couples sensing of necrosis to immunity. *Nature* 458, 899–903.

Sapoznikov, A., and Jung, S. (2008). Probing in vivo dendritic cell functions by conditional cell ablation. *Immunol. Cell Biol.* 86, 409–415.

Satpathy, A. T., Murphy, K. M., and Kc, W. (2011). Transcription factor networks in dendritic cell development. *Semin. Immunol.* 23, 388–397.

Schiavoni, G., Mattei, F., Borghi, P., Sestili, P., Venditti, M., Morse, H. C. III, Belardelli, F., and Gabriele, L. (2004). ICSBP is critically involved in the normal development and trafficking of Langerhans cells and dermal dendritic cells. *Blood* 103, 2221–2228.

Schiavoni, G., Mattei, F., Sestili, P., Borghi, P., Venditti, M., Morse, H. C. III, Belardelli, F., and Gabriele, L. (2002). ICSBP is essential for the development of mouse type I interferon-producing cells and for the generation and activation of CD8alpha(+) dendritic cells. *J. Exp. Med.* 196, 1415–1425.

Schmutz, S., Bosco, N., Chappaz, S., Boyman, O., Acha-Orbea, H., Ceredig, R., Rolink, A. G., and Finke, D. (2009). Cutting edge: IL-7 regulates the peripheral pool of

adult ROR gamma+ lymphoid tissue inducer cells. *J. Immunol.* 183, 2217–2221.

Schnorrer, P., Behrens, G. M., Wilson, N. S., Pooley, J. L., Smith, C. M., El-Sukkari, D., Davey, G., Kupresanin, F., Li, M., Maraskovsky, E., Belz, G. T., Carbone, F. R., Shortman, K., Heath, W. R., and Villadangos, J. A. (2006). The dominant role of CD8+ dendritic cells in cross-presentation is not dictated by antigen capture. *Proc. Natl. Acad. Sci. U.S.A.* 103, 10729–10734.

Schotte, R., Nagasawa, M., Weijer, K., Spits, H., and Blom, B. (2004). The ETS transcription factor Spi-B is required for human plasmacytoid dendritic cell development. *J. Exp. Med.* 200, 1503–1509.

Schulz, O., Diebold, S. S., Chen, M., Naslund, T. I., Nolte, M. A., Alexopoulou, L., Azuma, Y. T., Flavell, R. A., Liljestrom, P., and Reis E Sousa, C. (2005). Toll-like receptor 3 promotes cross-priming to virus-infected cells. *Nature* 433, 887–892.

Shklovskaya, E., O'Sullivan, B. J., Ng, L. G., Roediger, B., Thomas, R., Weninger, W., and Fazekas De St Groth, B. (2011). Langerhans cells are precommitted to immune tolerance induction. *Proc. Natl. Acad. Sci. U.S.A.* 108, 18049–18054.

Siddiqui, K. R., Laffont, S., and Powrie, F. (2010). E-cadherin marks a subset of inflammatory dendritic cells that promote T cell-mediated colitis. *Immunity* 32, 557–567.

Spits, H., Couwenberg, F., Bakker, A. Q., Weijer, K., and Uittenbogaart, C. H. (2000). Id2 and Id3 inhibit development of CD34(+) stem cells into predendritic cell (pre-DC)2 but not into pre-DC1. Evidence for a lymphoid origin of pre-DC2. *J. Exp. Med.* 192, 1775–1784.

Spooner, C. J., Cheng, J. X., Pujadas, E., Laslo, P., and Singh, H. (2009). A recurrent network involving the transcription factors PU.1 and Gfi1 orchestrates innate and adaptive immune cell fates. *Immunity* 31, 576–586.

Steinman, R. M. (2010). Some active areas of DC research and their medical potential. *Eur. J. Immunol.* 40, 2085–2088.

Steinman, R. M., and Cohn, Z. A. (1973). Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. *J. Exp. Med.* 137, 1142–1162.

Steinman, R. M., and Witmer, M. D. (1978). Lymphoid dendritic cells are potent stimulators of the primary mixed leukocyte reaction in mice. *Proc. Natl. Acad. Sci. U.S.A.* 75, 5132–5136.

Sung, S. S., Fu, S. M., Rose, C. E. Jr., Gaskin, F., Ju, S. T., and Beaty, S. R. (2006). A major lung CD103 (alphaE)-beta7 integrin-positive epithelial dendritic cell population expressing Langerin and tight junction proteins. *J. Immunol.* 176, 2161–2172.

Suzuki, S., Honma, K., Matsuyama, T., Suzuki, K., Toriyama, K., Akitoyo, I., Yamamoto, K., Suematsu, T., Nakamura, M., Yui, K., and Kumatori, A. (2004). Critical roles of interferon regulatory factor 4 in CD11bhighCD8alpha- dendritic cell development. *Proc. Natl. Acad. Sci. U.S.A.* 101, 8981–8986.

Tailor, P., Tamura, T., Morse, H. C. III, and Ozato, K. (2008). The BXH2 mutation in IRF8 differentially impairs dendritic cell subset development in the mouse. *Blood* 111, 1942–1945.

Tamura, T., Tailor, P., Yamaoka, K., Kong, H. J., Tsujimura, H., O'Shea, J. J., Singh, H., and Ozato, K. (2005). IFN regulatory factor-4 and -8 govern dendritic cell subset development and their functional diversity. *J. Immunol.* 174, 2573–2581.

Tsujimura, H., Tamura, T., and Ozato, K. (2003). Cutting edge: IFN consensus sequence binding protein/IFN regulatory factor 8 drives the development of type I IFN-producing plasmacytoid dendritic cells. *J. Immunol.* 170, 1131–1135.

Waskow, C., Liu, K., Darrasse-Jeze, G., Guermonprez, P., Ginhoux, F., Merad, M., Shengelia, T., Yao, K., and Nussenzweig, M. (2008). The receptor tyrosine kinase Flt3 is required for dendritic cell development in peripheral lymphoid tissues. *Nat. Immunol.* 9, 676–683.

Wilson, N. K., Foster, S. D., Wang, X., Knezevic, K., Schutte, J., Kaimakis, P., Chilarska, P. M., Kinston, S., Ouwehand, W. H., Dzierzak, E., Pimanda, J. E., De Bruijn, M. F., and Gottgens, B. (2010). Combinatorial transcriptional control in blood stem/progenitor cells: genome-wide analysis of ten major transcriptional regulators. *Cell Stem Cell* 7, 532–544.

Wu, L., D'Amico, A., Winkel, K. D., Suter, M., Lo, D., and Shortman, K. (1998). RelB is essential for the development of myeloid-related CD8alpha- dendritic cells but not of lymphoid-related CD8alpha+ dendritic cells. *Immunity* 9, 839–847.

Wu, L., Nichogiannopoulou, A., Shortman, K., and Georgopoulos, K. (1997). Cell-autonomous defects in dendritic cell populations of Ikaros mutant mice point to a developmental relationship with the lymphoid lineage. *Immunity* 7, 483–492.

Zhao, X., Deak, E., Soderberg, K., Linehan, M., Spezzano, D., Zhu, J., Knipe, D. M., and Iwasaki, A. (2003). Vaginal submucosal dendritic cells, but not Langerhans cells, induce protective Th1 responses to herpes simplex virus-2. *J. Exp. Med.* 197, 153–162.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 05 December 2011; accepted: 08 February 2012; published online: 27 February 2012.

*Citation: Chopin M, Allan RS and Belz GT (2012) Transcriptional regulation of dendritic cell diversity. Front. Immun. **3**:26. doi: 10.3389/fimmu.2012.00026*

This article was submitted to Frontiers in Antigen Presenting Cell Biology, a specialty of Frontiers in Immunology.

frontiers in
IMMUNOLOGY

REVIEW ARTICLE
published: 14 March 2012
doi: 10.3389/fimmu.2012.00041

Cross-presentation of cell-associated antigens by mouse splenic dendritic cell populations

Robert I. Thacker and Edith M. Janssen*

Division of Molecular Immunology, Cincinnati Children's Hospital Research Foundation, University of Cincinnati College of Medicine, Cincinnati, OH, USA

Edited by:
Christian Kurts, Friedrich-Wilhelms-Universität Bonn, Germany

Reviewed by:
Daniel Robert Engel, University Clinic of Bonn, Germany
Carlos Ardavin, Centro Nacional de Biotecnologia/Consejo Superior de Investigaciones Científicas, Spain

***Correspondence:**
Edith M. Janssen, Division of Molecular Immunology, Cincinnati Children's Hospital Research Foundation, University of Cincinnati College of Medicine, Room S5.419, 3333 Burnet Avenue, Cincinnati, OH 45229, USA.
e-mail: edith.janssen@cchmc.org

Cross-presentation of cell-associated antigens (Ag) plays an important role in the induction of anti-tumor responses, autoimmune diseases, and transplant rejection. While several dendritic cell (DC) populations can induce pro-inflammatory $CD8^+$ T cell responses to cell-associated Ag during infection, in the absence of infection, cross-priming of naïve $CD8^+$ T cells is highly restricted. Comparison of the main splenic DC populations in mice – including the classic, cross-presenting CD8α DC and the recently described merocytic DC (mcDC) – reveals that cross-priming DCs display a distinct phenotype in cell-associated Ag uptake, endosomal/lysosomal trafficking, lysosomal acidification, and Ag persistence compared to non-cross-priming DC populations. Although the CD8α DC and mcDC subsets utilize similar processing pathways to cross-present cell-associated Ag, cross-priming by CD8α DCs is associated with IL-12 production, while the superior priming of the mcDC is critically dependent on type I IFN production. This discussion illustrates how subtle differences in internal processing pathways and their signaling sequelae significantly affect the duration of Ag cross-presentation and cytokine production by DCs, thereby shaping the ensuing $CD8^+$ T cell response.

Keywords: dendritic cell, cross-presentation, cell-associated antigen, type I IFN, antigen processing

INTRODUCTION

Every day millions of cells die in the human body, producing cellular corpses and material that must be disposed of. Dead cells originating from the body's surfaces can simply be sloughed off with little or no consequence. In contrast, cells that die within tissues must be removed, a task typically undertaken by phagocytic cells of the immune system. This system has dual purpose. If the cell death is necrotic, due to viral or bacterial infection, the clearance of diseased cells assists in removing the insult and activating specific immunity against the offending cell-associated Ag. If the cell death is a part of natural tissue homeostasis, i.e., apoptotic cell death, the clearance of dead cells can function to maintain peripheral tolerance and prevent autoimmune disease. Within this context apoptotic cell death is historically considered an immunologically silent event.

Though seemingly simple in concept, continuing research on apoptosis and the clearance of apoptotic cells has revealed the complexity of this system. As a result, a multitude of factors have been identified that influence whether tolerance or immunity is established against cell-associated Ag upon uptake of apoptotic cells. These factors include, but are not limited to, the type of cell that is dying, how death was induced, in which tissue the death occurred, the recognition and uptake by phagocytic cells, the type of phagocyte involved in the uptake, and the resulting micro-environment (Poon et al., 2010). For example, cells treated with irradiation or chemotherapy become apoptotic but tend to be immunogenic (Ronchetti et al., 1999; Janssen et al., 2006; Green et al., 2009; Reboulet et al., 2010; Ferguson et al., 2011). In this context immunity probably results from the irradiation or chemical induced release of damage associated molecular proteins (DAMPS) such as high mobility group box 1 (HMGB1), uric acid/mono-sodium urate crystals, heat shock proteins, and nucleotide structures from the dying cell (Green et al., 2009; Poon et al., 2010). These signals, similar to those released during necrotic cell death, provoke immunity instead of tolerance. Though important, signals released by the dying cell do not fully explain immunologic outcome. Studies wherein identically treated cells induce tolerance if injected intravenously, yet immunity if injected subcutaneously illustrated how the location of cell death and, more importantly, the type of antigen presenting cell (APC) performing the uptake crucially affect immunity or tolerance. Subsequent studies correlated this induction of immunity or tolerance specifically with the dendritic cell (DC) subset that took up and processed the injected cells (Belz et al., 2002; Ferguson et al., 2002; Iyoda et al., 2002; Green et al., 2009).

DENDRITIC CELLS AND THE PRESENTATION OF CELL-ASSOCIATED ANTIGENS

In the evolution of the vertebrate immune system, DCs have filled the role of premier APC. All APCs characteristically take up, process and present exogenous antigens to $CD4^+$ T cells within the context of MHC class II molecules. Uniquely, DCs are additionally able to shuttle a portion of "eaten" antigens into the MHC class I restricted pathway, a pathway that in all other cell types is reserved for presentation of endogenous proteins. In DCs, this cross-presentation process allows exogenous Ag, including cell-associated Ag originating from dead and dying cells, to be effectively presented to $CD8^+$ T cells. Seminal work over the last decade addressing uptake and processing of cell-associated Ag by phagocytes has elucidated common

mechanisms utilized by cross-presenting DC subsets that influence cross-presentation and the resulting immune response. Successful cross-presentation is characterized by specific uptake, distinct endosomal/lysosomal trafficking, delayed lysosomal acidification, and Ag persistence compared to non-cross-priming DC populations. Also, the cytokine profile by which each DC subset responds to uptake of dying cells influences these processes and the final potency of the Ag-specific response.

MOUSE SPLENIC DC SUBSETS DURING STEADY STATE

As a result of ongoing research, the system by which DCs are classified continues to change. Currently, as new subsets are discovered, characterization often places them into one of two groups: steady state conventional DCs (cDCs) or non-conventional DCs (Kushwah and Hu, 2011). This initial classification is based on lineage, function, and location, with each subset being identified by the presence or absence of different cell surface markers. Steady state cDCs, as the name suggests, are present and function continually, even during inflammation, and include subsets found in the lymphoid organs as well as migratory subsets present in the tissues. Non-conventional DCs are mostly comprised of monocyte-derived DCs subsets, populations which are highly enriched during inflammation and thus are often referred to as inflammatory DCs (Shortman and Liu, 2002; Heath et al., 2004; Shortman and Naik, 2007; Liu et al., 2009; Kushwah and Hu, 2011). DCs found in non-lymphoid tissue regardless of whether formally categorized as steady state conventional or monocyte-derived non-conventional, are typically classified by the tissue in which they are found and the presence or absence of CD103, CD11b, langerin (for skin associated subsets), or the chemokine receptor CX3CR1 (Kushwah and Hu, 2011). Though some of these subsets are capable of cross-presentation, this review focuses on those subsets present in the spleen. We refer those interested to several publications that more completely dissect the lineages, functionality and surface expression of various markers in these other DC populations (Shortman and Liu, 2002; Heath et al., 2004; Shortman and Naik, 2007; Liu et al., 2009; del Rio et al., 2010; Liu and Nussenzweig, 2010; Shortman and Heath, 2010; Kushwah and Hu, 2011). Plasmacytoid DCs (pDCs), a subset also present in the spleen during steady state, are functionally distinct from both cDCs and non-conventional subsets, but possess a common precursor with cDCs subsets. In spite of this connected lineage, functional differences between cDCs and pDCs complicates the exact placement of the latter subset and, thus, has led to controversy. As a result, some researchers place pDCs with non-conventional DCs while others place them within a distinct group called pre-DCs or within their own category (Shortman and Liu, 2002; Shortman and Naik, 2007; Liu and Nussenzweig, 2010; Kushwah and Hu, 2011).

Conventional DCs and pDCs present in spleen and lymph nodes are distinguished by differential expression of CD11c, B220, and PDCA-1. Splenic cDCs lack B220 and PDCA-1 (**Figure 1A**) and can be further divided into four subpopulations characterized by the presence or lack of various markers (**Table 1**): (1) CD8α DCs ($CD8\alpha^+$, $CD4^-$, $CD11b^-$); (2) CD11b DCs ($CD8^-$, $CD4^-$, $CD11b^+$); (3) CD4 DCs ($CD8^-$, $CD4^+$, $CD11b^+$); or (4) merocytic DCs (mcDCs)/$CD8^-$, $CD4^-$, $CD11b^-$ DC (**Figure 1A**; Janssen et al., 2006; Reboulet et al., 2010; Shortman and Heath, 2010; Hennies et al., 2011; Kushwah and Hu, 2011). We have investigated, and thus will discuss in this review, the uptake of cell-associated Ag under steady state conditions and their cross-presentation within four splenic DC subgroups – CD8α DCs, CD11b DCs (which includes the $CD4^+$ subset), mcDC/$CD8^-$, $CD4^-$, $CD11b^-$ DC, and pDCs.

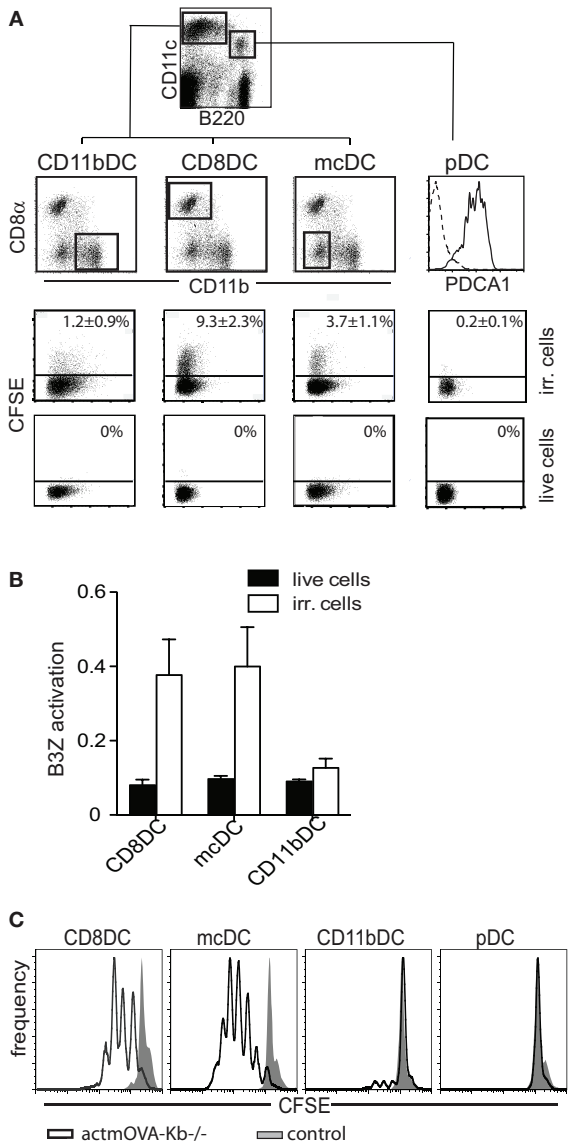


FIGURE 1 | Differential uptake of dying cells and subsequent cross-priming by splenic DC populations. (A) Live or irradiated CFSE-labeled OVA-expressing splenocytes were injected (i.v.) into mice. After 2 h uptake CFSE-labeled material was analyzed in indicated splenic DC subsets. **(B)** Mice were i.v. injected with irradiated OVA-expressing K^b-deficient splenocytes (Ehst et al., 2003). Cross-presentation of H-$2K^b$ restricted $OVA_{257–264}$ was assessed by activation of an $OVA_{257–264}$-specific T cell hybridoma, B3Z (Karttunen et al., 1992). **(C)** Characteristic cross-priming – as determined by CFSE dilution – of $OVA_{257–264}$-specific $CD8^+$ T cells by purified DCs upon *in vitro* culture with apoptotic OVA-expressing K^b-deficient cells.

Table 1 | Characteristics of splenic DC subsets.

	$CD8^+$ DC	$CD11b^+$ DC	$CD8^-$ $CD4^-$ mcDC	pDC	Reference
Itgax/CD11c	+++	+++	+++	++	Hashimoto et al. (2011)
itgam/CD11b	−	+++	+	−	Vremec et al. (2000), Vremec and Shortman (1997)
Sirpa/CD172a	−/+	+++	−/+	+/−	Lahoud et al. (2006)
CD4	−	++	−	−	Crowley et al. (1989), Vremec et al. (2000)
CD8a	+++	−	−	−	Shortman and Heath (2010)
itgae/CD103	++	−	−	−	Bedoui et al. (2009b), McLellan et al. (2002), Qiu et al. (2009)
CD205	++	−	−/+	−	Kraal et al. (1986), Shrimpton et al. (2009)
XCR1	++	−	+	−	Crozat et al. (2011), Robbins et al. (2008)
IRF8	++	−	++	++	Aliberti et al. (2003), Schiavoni et al. (2002), Tailor et al. (2008)
IRF4	−/+	++	−/+	−/+	Hashimoto et al. (2011)
MHC II	+++	+++	+++	++	Wilson et al. (2003)
CD80	+	+	+	−	Shortman and Heath (2010), Wilson et al. (2003)
CD86	++	+	++	+/−	Shortman and Heath (2010)
CD40	+	++	+	+	Shortman and Heath (2010)
TLR3	+++	+	+++	−	Edwards et al. (2003)
TLR7	−	+	−	++	Edwards et al. (2003)
TLR9	+	+	+	++	Edwards et al. (2003)
Clec9a	+++	+	+++	+	Sancho et al. (2008)
CLec12a	+++	+	nd	nd	Lahoud et al. (2009)
Havcr1/tim1	−	−	−	+++	Kobayashi et al. (2007)
Havcr2/tim3	+++	+++	+++	−	Nakayama et al. (2009)
Tim 4	+	+/−	+	+	Albacker et al. (2010), Kobayashi et al. (2007)
Treml2	+	+	++	+++	Hemmi et al. (2009)
Treml4	+++	++	++	−	Hemmi et al. (2009)
CD36	+++	++	+++	+/−	Albert et al. (1998)
MR	−	+	−	−	Burgdorf et al. (2006), Burgdorf et al. (2008), Sallusto et al. (1995)
Lox1	+	−	+	−	Delneste et al. (2002), Erwig and Henson (2008), Oka et al. (1998)
FcγR2b	+	++	+	+	Amigorena (2002), Rodriguez et al. (1999)
Cystatin C	++	+	++	+	El-Sukkari et al. (2003)
NOX2 gpphox91	+/−	++	+	nd	Savina et al. (2006)
CYTOKINE INDUCTION UPON UPTAKE OF APOPTOTIC CELLS					
IL-12	−	−	−	−	Morelli et al. (2003)
IL-10	−	++	−	−	Hennies et al. (2011)
TGFβ	+/−	++	−	−	Hennies et al. (2011), Yamazaki et al. (2008)
Type I IFN	−	−	++	−	Janssen et al. (2006), Lorenzi et al. (2011)

Data compiled from indicated literature and unpublished DNA arrays.
nd, not done.

CD8α DCs

The CD8α DC, classically considered to be the major cross-presenting DC subset in the mouse spleen, is located in the T cell zone of the spleen and has repeatedly been shown to effectively cross-present beads, soluble Ag, and cell-associated Ag (**Figures 1B,C**; den Haan et al., 2000; Pooley et al., 2001; Heath et al., 2004; Belz et al., 2005; Schnorrer et al., 2006). The development of CD8α DCs is dependent on Flt3L-STAT3 signaling. While cDC development in general requires the transcription factors Ikaros, and PU.1 (Wu et al., 1997; Anderson et al., 2000; Guerriero et al., 2000; Allman et al., 2006; Wu and Liu, 2007), the CD8α DC lineage commitment is also dependent on Batf3, IRF-8, and Id2 (Schiavoni et al., 2002; Hacker et al., 2003; Hildner et al., 2008). This DC subset, in addition to being $CD8\alpha^+$, also expresses DEC205, XCR1, and Clec9a (Vremec et al., 2000; Sancho et al., 2009; Shortman and Heath, 2010; Crozat et al., 2011). Depending on age and strain of mouse, up to 70% of CD8α DCs co-express CD103, which has been suggested to represent a developmental stage or activation state within the CD8α DC population or a $CD8^+$ subset with distinct functionality (Pribila et al., 2004; Qiu et al., 2009; del Rio et al., 2010; Shortman and Heath, 2010). CD8α DCs take up dead cells more readily than other splenic DC subsets (**Figure 1A**; Iyoda et al., 2002; Schulz and Reis e Sousa, 2002; Schnorrer et al., 2006) and have been implicated in the induction and maintenance of $CD8^+$ T cell tolerance to cell-associated Ag in models of autoimmunity and transplantation (Kurts et al., 1996, 1998; Hawiger et al., 2001; Belz et al., 2002; Bonifaz et al., 2002; Scheinecker et al., 2002; Shortman and Heath, 2010). This subset's importance in the cross-presentation of cell-associated Ag is further supported by a dramatic reduction of anti-tumor immunity

in mice deficient in Batf3, a gene crucial for the development of the CD8α DC precursor (Hildner et al., 2008).

CD11b DCs

Splenic CD11b DCs reside in the marginal zone of the spleen and predominantly co-express CD4, DCIR2, and Sirp-α (Crowley et al., 1989; Pulendran et al., 1997; Maldonado-Lopez and Moser, 2001; Lahoud et al., 2006). CD11b DC development is governed by transcription factors IRF2/4 (Honda et al., 2004; Ichikawa et al., 2004; Suzuki et al., 2004) and RelB (Burkly et al., 1995; Weih et al., 1995; Wu et al., 1998).

While CD11b DCs display great potential for phagocytosis of proteins, beads/particles, and bacteria, their capacity for cross-presentation under steady state conditions is poor. Moreover, CD11b DCs display weak phagocytosis of apoptotic cells and no role has been described for these cells in cross-presentation to cell-associated Ag under steady state conditions (**Figures 1A–C**; den Haan et al., 2000; Pooley et al., 2001; Iyoda et al., 2002; Schulz and Reis e Sousa, 2002; Morelli et al., 2003; Schnorrer et al., 2006).

PLASMACYTOID DENDRITIC CELLS

Splenic pDCs are defined by strong expression of both B220 and PDCA-1 and are predominantly located in the T cell area and red pulp. While there is some discussion on the exact delineation of pDC with regard to shared precursors with other cDC, research has shown the requirement for the transcription factors E2-2, IRF8, and Spi-B (Schiavoni et al., 2002; Schotte et al., 2004; Cisse et al., 2008). Although pDCs are poor at taking up cell-associated Ag, depletion studies have shown that pDCs are critical in the induction of tolerance after intravenous injection of apoptotic cells. Such tolerance, however, does not require direct pDC–apoptotic cell interactions, but rather soluble mediators from marginal zone macrophages (Bonnefoy et al., 2011).

MEROCYTIC DCs/CD8$^-$, CD4$^-$, CD11b$^-$ DC

Over the recent years various laboratories have identified splenic DCs that lack the conventional markers (CD8α$^-$, CD11b$^-$, CD4$^-$; **Figures 1A–C**; Hochrein et al., 2001; Naik et al., 2005; Janssen et al., 2006; Vremec et al., 2007; Bedoui et al., 2009a; Katz et al., 2010; Reboulet et al., 2010; Hennies et al., 2011). Generally, these populations are relatively small and only comprise <1–10% of the DC in a naïve steady state spleen. Several of these DC populations have been shown to cross-present antigens in protein (Vremec et al., 2007) or cell-associated form (Bedoui et al., 2009a; Reboulet et al., 2010; Hennies et al., 2011). Flt3L treatment of mice significantly increases the frequency of these DCs, and cells with similar features can be generated by *in vitro* Flt3L bone-marrow cultures (Bedoui et al., 2009a; Reboulet et al., 2010; Hennies et al., 2011).

During the course of our work we have named the CD8α$^-$ CD11b$^-$ CD4$^-$ DC in the naïve spleen mcDCs due to the smaller particles (meros = particle) characteristically taken up by these cells (**Figure 2C**) and will use this name throughout this review (Reboulet et al., 2010). Like CD8α DCs, mcDCs express XCR1, Clec9a, and are Sirp-α negative, but in contrast are DEC205$^-$, CD103$^-$, and CD11b$^{-/dull}$ (**Table 1**). It has been suggested that this marker negative subset is a precursor to the CD8α DCs (Janssen et al., 2006; Bedoui et al., 2009a), a hypothesis supported by the presence of Clec9a and CD24, surface molecules shown to be present on the immediate precursors of CD8α DCs that lack CD8 and DEC205 expression (Sathe and Shortman, 2008; Shortman and Heath, 2010; Kushwah and Hu, 2011). CD8α$^-$ CD11b$^-$ CD4$^-$ DC obtained from Flt3L treated mice readily convert into CD8α DCs upon transfer (Bedoui et al., 2009a). However, only a small fraction of CD8α$^-$ CD11b$^-$ CD4$^-$ DC/mcDC from naïve spleens convert to CD8α DCs (Reboulet et al., 2010) suggesting that mcDCs are either "long-term" CD8α DC precursors that are relatively resistant to conversion or a stable population that possesses unique functionality and marker expression compared to other known precursors.

Merocytic DCs take up cellular material from dead and dying cells, though be it less than CD8α DCs (**Figure 1A**). Though mcDCs take up less apoptotic cell material they show extended cross-priming of CD8$^+$ T cells due to prolonged storage of cell-associated Ag (Reboulet et al., 2010). Importantly, mcDCs prime both CD4$^+$ and CD8$^+$ T cells to cell-associated Ag (**Figures 1B,C**; Janssen et al., 2006; Reboulet et al., 2010). CD4$^+$ T cell activation is important in the induction of immunity against cell-associated Ag as CD8$^+$ T cells become tolerant without sufficient CD4$^+$ T cell help (Janssen et al., 2003, 2005; Griffith et al., 2007). CD8$^+$ T cells primed by mcDC to cell-associated Ag show greater capacity for primary expansion, cytokine production, and memory formation on a per cell basis than those primed by CD8α DC (Janssen et al., 2006; Katz et al., 2010; Reboulet et al., 2010; Hennies et al., 2011).

Merocytic DC have been associated with the breaking of tolerance and acceleration of immune responses to cell-associated Ags. Treatment of tumor bearing mice with mcDC previously exposed to irradiated tumor cells, resulted in tumor suppression and increased host survival through the activation of naïve tumor-specific CD8$^+$T cells as well as the reinvigoration of tumor-specific T cells that had been rendered non-responsive by the tumor *in vivo* (Reboulet et al., 2010). Dysregulation of the mcDC compartment has also been associated with the development of autoimmunity; mcDCs are more numerous and more biologically active in the non-obese diabetic (NOD) mouse model of type I diabetes and absolute numbers correlate with disease development and progression. Transfer of mcDCs – loaded with irradiated islet cells– transferred diabetes in young NOD recipients. Moreover, when purified from the pancreatic lymph nodes of overtly diabetic NOD mice, mcDCs break peripheral tolerance to beta antigens *in vivo* and induce the rapid onset of T cell-mediated type I diabetes in young NOD mice (Katz et al., 2010).

RECOGNITION AND DIFFERENTIAL UPTAKE OF DEAD AND DYING CELLS

Though little is known regarding the influence of clathrin-mediated uptake vs. phagocytosis or macropinocytosis on cross-presentation pathways of cell-associated Ag, uptake and cross-presentation of cellular material is largely thought to be receptor mediated (Erwig and Henson, 2008).

RECOGNITION THROUGH PHAGOCYTIC RECEPTORS

As a cell becomes apoptotic, the steady state "don't eat me" signals of viable cells are lost and replaced through a series of

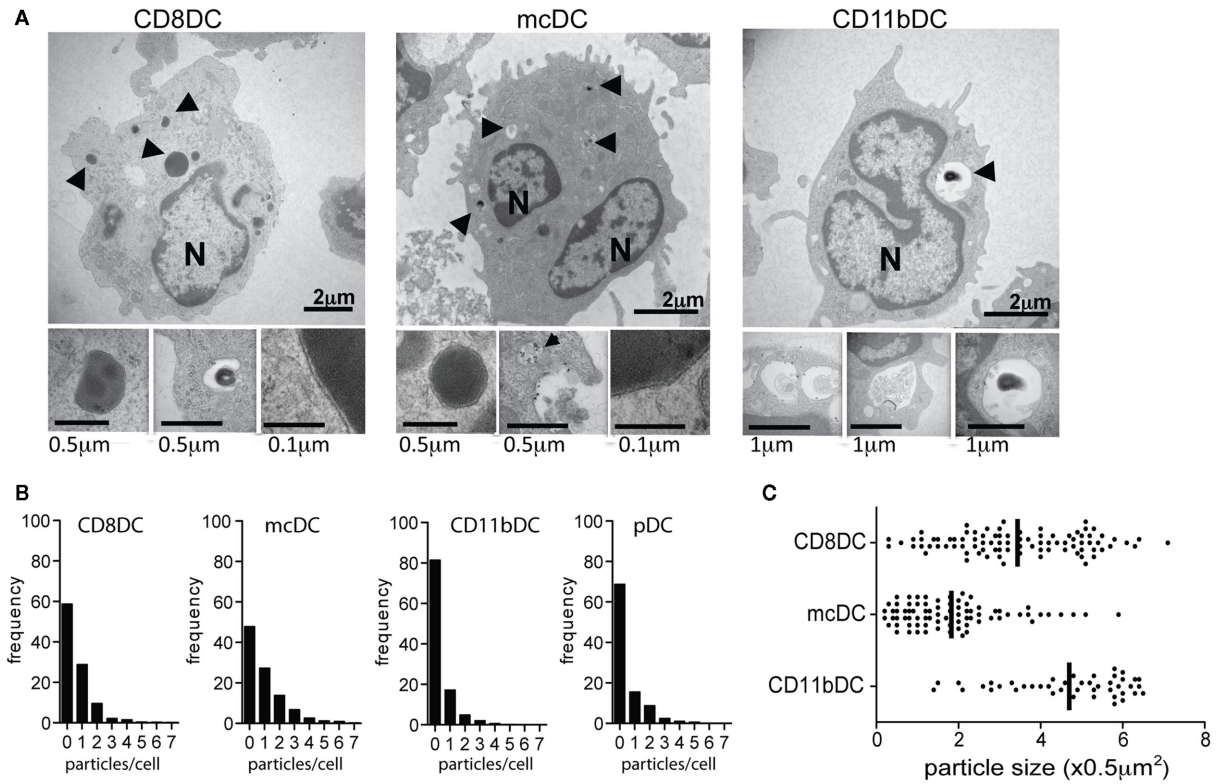


FIGURE 2 | Splenic DC subsets differentially take up cellular particles influencing particle frequency and size. (A) Transmission electron microscopy (TEM) images characterizing uptake of irradiated splenocytes by purified DC subsets after 4 h co-culture: N, nucleus; arrows, apoptotic material. **(B,C)** ImageStream analysis of the frequency and size of internalized CFSE-labeled particles after 20 h co-culture with CFSE-labeled irradiated cells.

morphological and biochemical changes (Elward and Gasque, 2003; Erwig and Henson, 2008; Poon et al., 2010). The most prominent and perhaps best-characterized change is the exposure of phosphatidylserine (PS) on the surface of the dying cell. Once PS is exposed, it can be recognized by a number of bridging molecules including milk fat globular-EGF factor 8 protein (MFG-E8; Borisenko et al., 2004; Hanayama et al., 2004), growth arrest-specific 6 (Gas6; Ishimoto et al., 2000; Scott et al., 2001), β2-glycoprotein I (β2-GPI; Balasubramanian et al., 1997), and serum Protein S (Erwig and Henson, 2008; Krysko and Vandenabeele, 2008; Poon et al., 2010). Many of these bridging molecules then facilitate recognition by receptors on the surface of the phagocyte, including the integrins αvβ3 or αvβ5 (Borisenko et al., 2004; Hanayama et al., 2004; Erwig and Henson, 2008). Additionally, the apoptotic cell and other bridging molecules like TSP-1 are recognized by phagocytic receptors such as Tim-1, Tim-3, and Tim-4 (Kobayashi et al., 2007; Nakayama et al., 2009; Albacker et al., 2010), CD36 (Albert et al., 1998), Treml2 and Treml4 (Hemmi et al., 2009), DEC205 (Shrimpton et al., 2009), class A scavenger receptors (Platt et al., 2000), Lox-1 (Oka et al., 1998), and various C-type lectins including Clec9a (DNGR1; Krysko and Vandenabeele, 2008; Sancho et al., 2009; Poon et al., 2010; Shortman and Heath, 2010). While the seemingly overabundance of receptors involved in dying cell uptake stresses the importance of apoptotic cell removal in the maintenance of immune homeostasis, it is becoming apparent that these molecules are not merely redundant, but may distinctly influence APC behavior, and, thus, guide specific responses toward cell-associated antigens under various settings (Bratton and Henson, 2008; Erwig and Henson, 2008; Krysko and Vandenabeele, 2008).

Cross-presentation, but not necessarily cross-priming, of cell-associated Ag is generally enhanced when uptake is mediated by DEC205 (Bonifaz et al., 2002), Clec9a (Sancho et al., 2008, 2009), Lox-1(Delneste et al., 2002), or Fc gamma R (Regnault et al., 1999; Rodriguez et al., 1999; Amigorena, 2002; den Haan and Bevan, 2002; Kalergis and Ravetch, 2002; Flinsenberg et al., 2011). Uptake via the mannose receptor has also been implicated in cross-presentation pathways, but its relevance has thus far only been shown within the context of soluble Ag, not cell-associated Ag (Burgdorf et al., 2006, 2008). Comparison of the transcriptome of the four DC subsets suggests the most similarity between mcDCs and CD8α DCs with regard to the expression of molecules involved in the recognition and uptake of dying cells. However, some receptors are still restricted to one subset – as is the case for DEC205 and CD8α DCs – or have differential expression/different levels of expression, resulting in unique patterns for each subset (**Table 1**).

Consistent with receptor-mediated uptake leading to cross-presentation, both CD8α DCs and mcDCs appear to take up cellular material via a classical receptor-mediated phagocytic process. Transmission electron microscopy (TEM)of CD8α DCs and mcDCs exposed to dying cells predominantly shows the presence of small particles of phagocytosed material tightly surrounded by

a double membrane (**Figure 2A**). In contrast, CD11b DCs appear to use a more macropinocytic mechanism resulting in the uptake of larger particles and inclusion of extracellular solutes and fluids (**Figures 2A,C**).

PARTICLE SIZE AND FREQUENCY

Our studies and the work of others indicate that the method of uptake, i.e., receptor-mediated phagocytosis vs. macropinocytosis, influences the particle size taken up by APCs (Rejman et al., 2004). While particle size determines the total amount of Ag available to the cell, a growing body of literature indicates that particle size also affects intracellular trafficking, the kinetics of phagosomal pH, and thereby cross-presentation (Fifis et al., 2004; Rejman et al., 2004; Tran and Shen, 2009). Cross-presentation has been shown to be enhanced when Ag are bound onto particles between the range of 0.5 and 3 μ (Tran and Shen, 2009). However, it is likely that the optimal size for cross-presentation will be affected by the composition of the particle, the receptors involved in the uptake and the nature of the cell.

Transmission electron microscopy combined with ImageStream technology, a flow cytometric based method that allows for quantitative image analysis on vast number of cells, confirmed that cross-presenting splenic DCs differentially take up material from dead and dying cells as measured by total particles per cell and the overall particle size. CD8α DCs and mcDCs not only take up particles of dying cells more readily, but also preferentially take up smaller particles than CD11b DCs (**Figures 2B,C**), a size differential that most likely facilitates the entrance of cell-associated Ag into cross-presentation pathways (Fifis et al., 2004; Rejman et al., 2004; Tran and Shen, 2009).

Interestingly, upon exposure to dead and dying cells, mcDCs generally take up a comparable number of particles/cell to CD8α DCs, but these particles are typically smaller in size (**Figures 2B,C**; Reboulet et al., 2010). While the mcDCs takes up a lower "net amount" of Ag, the smaller particle size might expedite the export to the cytosol which would facilitate Ag processing (Rodriguez et al., 1999; Rock et al., 2010).

ANTIGEN TRAFFICKING, PROCESSING, AND LOADING

ANTIGEN TRAFFICKING AND VESICLE ACIDIFICATION

Upon uptake, cellular material from dying cells is found within early phagosomes – or sorting endosomes – characterized by the presence of the early endosomal markers EEA-1, Rab5, PI(3)*P*, syntaxin 13, transferrin, and vesicle-associated membrane protein 3(VAMP-3; Vieira et al., 2002; Peng and Elkon, 2011). The phagosome is transformed into a phagolysosome through a progressive maturation process that is dependent on the sequential fusion of endosomes and lysosomes with the internalized phagosome. Most recently this maturation process and antigen cross-presentation was shown to be regulated by the SNARE protein Sec22b through its control of ER-resident protein recruitment to phagosomes (Cebrian et al., 2011). Late endosomes/late phagosomes are associated with Rab7, Rab9, mannose 6-phosphate receptor, syntaxin7, LAMP-1 and LAMP-2 (Vieira et al., 2002). The final product, phagolysosomes, express LAMPs but have lost most of the earlier endosomal markers. In addition, the phagolysosomes possess a number of complementary degradative properties, including a very low pH, hydrolytic enzymes for particle digestion, defensins and other bactericidal peptides, and the ability to generate toxic oxidative compounds (Amigorena and Savina, 2010; Rock et al., 2010).

Cellular material taken up by CD11b DCs rapidly ends up in fully matured phagolysosomes (**Figures 3A–C**; Savina et al., 2006, 2009; Reboulet et al., 2010; Peng and Elkon, 2011). Colocalization studies show an association of phagocytosed materials with LAMP-1+ organelles and pulse-chase experiments show the degradation of >80% of the material in less than 20 h (**Figures 3A,B**). As a result, Ag are quickly processed and either

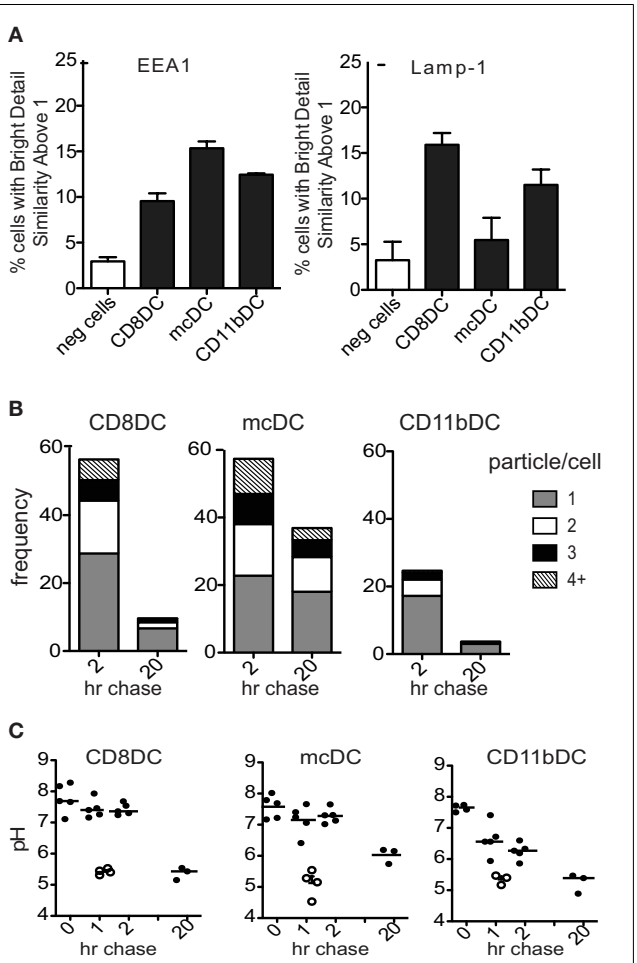


FIGURE 3 | Unique trafficking of phagocytosed material in cross-presenting DCs. (A) ImageStream analysis of the colocalization between internalized Violet labeled-irradiated cells and PE-labeled EEA-1 or LAMP-1 at 4 h ($n > 950$ events/group). Colocalization was based upon Bright Detail Similarity score between the two markers. Scores of 0–1 represent minimal colocalization. As the markers of interest become more colocalized the score increases to reflect this similarity. **(B)** ImageStream analysis of the frequency of CFSE-containing DCs and the number of CFSE+ particles per DC 2 h and 20 h after the removal of irradiated CFSE-labeled cells. Decreases in particle frequency and number/cell were attributed to acidification of the endosome and the subsequent CFSE-quenching. **(C)** Differences in lysosomal acidification rate between DC populations as determined by flow cytometric analysis of dual-labeled pH-indicating beads.

shuttled into MHC class II restricted pathways or simply degraded and disposed of. In contrast, cross-presenting DC subsets take up material from dying cells and hold these particles in immature endosomes for an extended period of time (**Figures 3A–C**). While CD8α DCs have degraded most of the material after 20 h, digestion is much slower than in CD11b DCs. Phagocytosed cellular particles in mcDCs are more colocalized with EEA-1 and less with LAMP-1 after 4 h of co-incubation with irradiated cells, indicating slower endosomal maturation (**Figure 3A**). Moreover, pulse-chase studies showed the persistence of materials over a 20-h time span (**Figure 3B**; Reboulet et al., 2010). This persistence of Ag in the mcDCs has been shown to prolong the cross-presentation of specific cell-associated Ag and thereby increase T cell priming (Savina et al., 2006; Reboulet et al., 2010; Peng and Elkon, 2011).

As endosomal acidification causes the robust activation of lysosomal proteases and the subsequent destruction of Ag, acidification is considered to be poorly compatible with cross-presentation. Forced lysosomal acidification dramatically reduces cross-presentation while prevention of acidification has been shown to enhance cross-presentation (Savina et al., 2006; Amigorena and Savina, 2010; Reboulet et al., 2010). Though it was previously described that DCs have relative ineffective acidification of their lysosomes, the mechanism under pinning the sustained phagosome alkalinization was only recently unraveled. Studies by laboratory of Mellman and Amigorena indicate that this results from an incomplete assembly of V-ATPase in DC lysosomes and the Rab27-mediated recruitment of the NADPH NOX2. The NOX2-mediated generation of reactive oxygen species (ROS) in endocytic compartments causes the consumption of protons, followed by the active alkalinization of these compartments (Trombetta et al., 2003; Savina and Amigorena, 2007; Savina et al., 2009; Rock et al., 2010).

Acidification studies indicate that endosomes with cell-associated material in both CD8α DCs and mcDCs maintain a similar high/neutral pH for several hours post uptake of cell-associated antigens, which correlates with Ag persistence. Compared to CD8α DCs, mcDCs show decreased lysosomal acidification over a prolonged period of time, resulting in a less acidic endosomal compartment after 20 h (**Figures 3B,C**; Reboulet et al., 2010).

In both populations the treatment with diphenylene iodonium (DPI) – an inhibitor of flavin-containing enzymes such as NOX2 – accelerates lysosomal acidification, prevents Ag persistence, and rapidly decreases the cross-presenting capacity of both CD8α DCs and mcDCs, emphasizing the importance of endosomal acidification in their cross-presentation (Reboulet et al., 2010).

The mechanisms that govern the prolonged Ag persistence in mcDC remain unclear as the biogenesis of phagolysosomes still involves many poorly understood processes. Transcriptome analysis of CD8α DCs and mcDCs showed ≈20-fold higher expression of Cybb (NOX2) in mcDCs. In addition, differential expression of various R- and Q-SNAREs (soluble *N*-ethyl maleimide sensitive-factor attachment protein receptors), sorting nexins, and V-ATPases that have been suggested to play a role in vesicle transport and fusion are seen (Vieira et al., 2002; Cebrian et al., 2011). However, as most of these processes depend on active recruitment of these proteins to endosomal/lysosomal membranes, differences in expression levels might not be indicative of their degree of involvement. It is more likely that the nature of the phagocytosed particle – including size – and the receptors involved in their uptake dictate phagosome maturation (Peng and Elkon, 2011).

PROCESSING AND MHC I LOADING

As intact internalized Ag fill the cell, there are two proposed pathways by which they are cross-presented: the vacuolar and cytosolic pathways. The vacuolar pathway hypothesizes that cross-presented Ag are fully processed within the endosomes. The aminopeptidase IRAP facilitates the production of MHC class I-specific peptides that bind to the MHC molecule within the endosome. This pathway appears to be cathepsin S dependent and TAP independent (Shen et al., 2004; Chen and Jondal, 2008; Rock et al., 2010). In contrast, the cytosolic pathway requires minimally processed antigen to escape into the cytosol. Once in the cytosol, the Ag is processed by the proteasome, and generated peptides are shuttled into the lumen of the ER via sec61 or into phagosomes that have recruited ER components. The ER associated aminopeptidase ERAP actively clips the peptides to the proper length and TAP facilitates the loading into MHC class I (Rock et al., 2010).

Multiple studies indicate a dominant role for the cytosolic pathway in the processing of cell-associated Ag by cross-presenting DC subsets (**Figure 4**; Shen et al., 2004; Rock et al., 2010). Smaller particles, like those taken up by mcDCs and CD8α DCs (**Figure 2C**), are more rapidly and efficiently exported to the cytosol, a process that would drive the cytosolic pathway of cross-presentation (Rodriguez et al., 1999). Also, lactacystin and brefeldin A, inhibitors of the proteasome and Golgi transport, respectively, completely inhibit the ability of both CD8α DCs and mcDCs to activate Ag-specific $CD8^+$ T cells against cell-associated Ag (**Figure 4**). A recent report by Cebrian et al. (2011) implicates the SNARE protein Sec22b as an essential element of the cytosolic pathway. Depletion of Sec22b inhibits the recruitment of ER-resident proteins to the phagosome and phagolysosomal

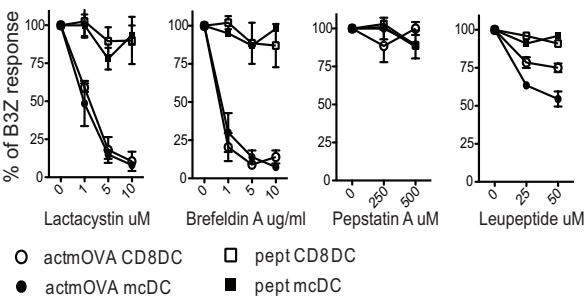


FIGURE 4 | Proteasomal inhibitors effectively block cross-presentation in both CD8α DC and mcDC. Splenic DC were incubated with irradiated actmOVA cells in the presence of indicated inhibitors. After 20 h, samples were fixed, sorted, and cultured with OVA-specific B3Z T cells to assess the cross-presentation of cell-associated antigens. Cells pulsed with $OVA_{257–264}$ prior to fixation were used to demonstrate priming capacity. Responses are normalized to control treatment.

fusion was enhanced. As a result, antigens are rapidly degraded instead of being transported to the cytosol. These combined effects of Sec22b loss drastically reduce the cross-presentation of soluble and surface bound OVA as well as parasite and bacterial associated antigens. Interestingly, the presentation of both MHC class II and endogenous MHC class I restricted peptides is unaffected demonstrating significant separation between these processing pathways (Cebrian et al., 2011). Taken together, these data support the hypothesis that Ag must reach the cytosol, and eventually become associated with ER-derived proteins, to be cross-presented.

The maintenance of a more neutral pH correlates with delayed or reduced lysosomal protease activation. Cross-presenting DCs characteristically express lower levels of proteases and higher levels of protease inhibitors as compared to other APCs (Lennon-Dumenil et al., 2002; Trombetta et al., 2003). Pepstatin A, an inhibitor of acid proteases involved in lysosomal maturation and acidification, has no effect on the DCs capacity to cross-present. This demonstrates that protease mediated peptide production plays a minimal role in cross-presentation (**Figure 4**; Rock et al., 2010). Interestingly, leupeptin, an inhibitor of cysteine proteases essential for the vacuolar pathway, partially inhibits cross-presentation, but affected the mcDCs more than the CD8α DC subset. This suggests that mcDCs may utilize the vacuolar pathway for the processing of cell-associated Ag more than other subsets. In support of this, removal of either ERAP or IRAP results in only a 50% reduction in cross-presentation, suggesting the utilization of both pathways by cross-presenting DCs (Firat et al., 2007; Blanchard et al., 2008; Saveanu et al., 2009; Rock et al., 2010).

EFFECT OF AUTOCRINE CYTOKINE PRODUCTION

The cytokines produced by DCs in the context of cellular death and clearance drastically influence Ag processing, presentation and, subsequently, the capacity of the DC to prime T cells against cell-associated Ag (Voll et al., 1997; Fadok et al., 1998; Janssen et al., 2006; Chung et al., 2007; Green et al., 2009). While the induction of anti-inflammatory cytokines – including IL-10 and TGF-β-upon uptake of apoptotic cells is relatively poor, various studies indicate that DC concurrently reduce their capacity to produce pro inflammatory cytokines (IL-1α/β, IL-6, IL-12, TNFα; Stuart et al., 2002; Morelli et al., 2003). This altered cytokine production profile has been suggested to become entrenched in the APC and to affect subsequent spontaneous and induced cytokine production (Stuart et al., 2002; Morelli et al., 2003; Kim et al., 2004).

Upon exposure to apoptotic cells, CD11b DCs induce the anti-inflammatory cytokines IL-10 and TGF-β (Hennies et al., 2011). CD8α DCs readily express the pro-inflammatory cytokines IL-12 and Type I IFN under inflammatory conditions (Hochrein et al., 2001; Heath et al., 2004; Naik et al., 2005), but demonstrate minimal induction of these and other cytokines, including IL-10 and TGF-β, in response to apoptotic cells (Morelli et al., 2003; Hennies et al., 2011; Janssen, unpublished). This is particularly interesting as CD8α DCs produce TGF-βduring steady state (Yamazaki et al., 2008), a cytokine heavily implicated in the induction and maintenance of peripheral tolerance (Erwig and Henson, 2007; Green et al., 2009). In contrast, mcDCs express the pro-inflammatory cytokines IL-1β and type I IFN upon exposure to dying cells (**Table 1**; Hennies et al., 2011).

IL-10 and TGF-β have potent immunosuppressive properties and promote the induction of tolerance. Both have been shown to reduce Ag presentation by regulating the transcription of the class I heavy chain, β_2M, tapasin, TAP, and components of the proteasome (Geiser et al., 1993; Ma and Niederkorn, 1995; Koppelman et al., 1997; Nandan and Reiner, 1997; Salazar-Onfray et al., 1997; Zeidler et al., 1997; Strobl and Knapp, 1999; Francois et al., 2009). The pro-inflammatory cytokine IL-12 is a critical mediator of $CD8^+$ T cell activation as it drives the necessary help of $CD4^+$ T cells toward a Th1 phenotype (Trinchieri, 2003; Trinchieri et al., 2003; Chang et al., 2004; Del Vecchio et al., 2007; Lee et al., 2007). In spite of this, IL-12 seems to have little autocrine effect on Ag processing by CD8α DCs (Grohmann et al., 1999; Janssen, unpublished) and its induction has been suggested to require additional signals, including TLR engagement, CD40L, IL-4 or IFN-γ (Hochrein et al., 2000; Hochrein et al., 2001; Reis e Sousa et al., 1997; Schulz et al., 2000). These additional stimuli – or their sequelae – positively affect the expression of the proteasomal subunits, TAP1 and TAP2, calnexin, calreticulin, tapasin, NOX2, and MHC class I.

Merocytic DCs produce pro-inflammatory cytokines in response to apoptotic cells without the apparent need for additional signals. While IL-1β is traditionally incorporated in maturation cocktails in the generation of human DCs, its does not significantly affect cross-presentation or cross-priming by mcDCs. mcDCs lacking IL-1RI or MyD88 display $CD8^+$ T cell priming capacity similar to WT mcDCs (Janssen, unpublished). In contrast, type I IFN production by the mcDCs, and resulting autocrine signaling, is critical for this subset's enhanced cross-presentation and activation of $CD8^+$ T cells against cell-associated Ag (Reboulet et al., 2010). Type I IFNs have been shown to affect the expression of various components of the Ag processing and loading machinery, including proteasome subunits, TAP, tapasin, calreticulin, NOX2, MHC, and various SNAREs (Cho et al., 2002; Tosello et al., 2009; Lattanzi et al., 2011). The importance of autocrine type I IFN production was illustrated in experiments where transfer of apoptotic cell-exposed mcDCs that lacked the type I IFN receptor (*ifnar*) showed significantly reduced priming of endogenous $CD8^+$ T cells to cell-associated Ag compared to WT mcDCs. As CD8α DCs do not produce type I IFN upon apoptotic cell uptake (Hennies et al., 2011), the presence or absence of the type I IFNα/βR on CD8α DCs did not affect their priming capacity (**Figure 5A**). Further *in vitro* experiments showed that the reduction in priming correlated with reduced expression of the specific MHC-peptide complexes on the *ifnar*-mcDCs. No significant differences were observed in total MHC class I levels, suggesting that type I IFNs interfered relatively early in the process of Ag presentation.

Image stream analysis of WT and *ifnar*−/− CD8α DCs and mcDCs exposed to dying cells *in vitro* indicated that type I IFN sensing did not affect the capacity for phagocytosis by either subset with regard to the frequency of phagocytosing cells, the number of particles per cell, or particle size (**Figures 5B,C**). However, pulse-chase studies indicated the absence of type I IFN

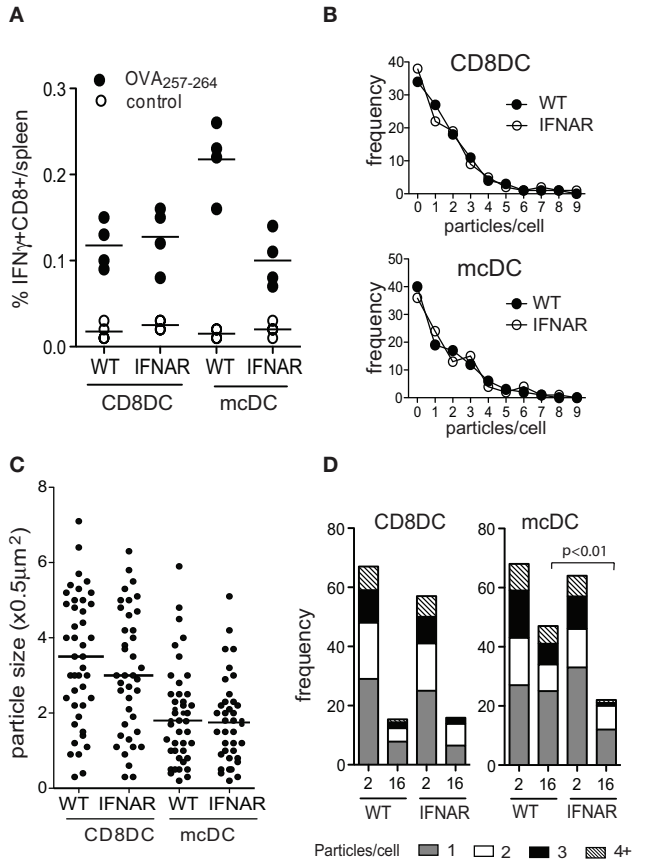


FIGURE 5 | Autocrine type I IFN signaling by mcDC affects CFSE loss on phagocytosed cellular particles suggesting changes in the lysosomal acidification rate. (A) CD8α DCs and mcDCs from indicated strains were exposed to irradiated actmOVA cells *in vitro*, purified and transferred into WT recipients. Seven days later the endogenous $CD8^+$ T cell response was analyzed. **(B,C)** ImageStream analysis comparing the frequency and size of internalized CFSE-labeled particles in CD8α DCs and mcDCs from WT and *ifnar−/−* mice. **(D)** Loss of delayed acidification in *ifnar−/−* mcDC as determined by ImageStream analysis using CFSE-labeled irradiated cells and a pulse-chase approach.

sensing accelerated CFSE loss, suggesting increased endosomal acidification, and significantly increased degradation of endosomal materials in mcDCs (**Figure 5D**). This is in line with recent findings of Lorenzi et al. (2011), who showed that pretreatment of CD8α DCs with recombinant type I IFN, which is otherwise absent in these cultures, significantly increased Ag retention after engulfment of apoptotic cells. Increased retention correlated with decreased endosomal acidification and resulted in enhanced cross-presentation of cell-associated Ag (Lorenzi et al., 2011). Interestingly, two recent *in vivo* studies demonstrated a critical role for type I IFN sensing in DC in tumor rejection models. The authors showed that mice lacking *ifnar* in DC failed to reject highly immunogenic tumor cells and that CD8α DCs from these mice displayed defects in antigen cross-presentation to $CD8^+$ T cells (Diamond et al., 2011; Fuertes et al., 2011).

The concept of type I IFNs affecting endosomal pH and regulating Ag retention provides an intriguing concept that could explain why so many DC populations that fail to cross-present under steady state conditions are capable of doing so under inflammatory conditions associated with type I IFNs (Di Pucchio et al., 2008; Segura et al., 2009; Kamphorst et al., 2010; de Brito et al., 2011).

OF MICE AND MEN

While it is possible to perform extensive analysis on mouse DCs through the use of transgenic mice and the ability to remove specific organs, human DC studies are hampered by the limited availability of human lymphoid tissue and differences in DC surface markers. However, recent research indicates the existence of various human counterparts that – albeit phenotypically different – have functional similarities to mouse DCs. While the details on cross-presentation by human DCs are addressed elsewhere in this issue, it is noteworthy that cross-priming has been observed by human pDCs and the "CD8α DC"-like DCs that expresses BDCA3, XCR1, DNGR1/Clec9A (Hoeffel et al., 2007; Dorner et al., 2009; Bachem et al., 2010; Henri et al., 2010; Crozat et al., 2011). Both cell types seem to have the capacity to actively internalize small particles of dead cell material. In addition, human DCs – like mouse DCs – require the regulation of phagosomal and endosomal pH for efficient cross-priming (Hoeffel et al., 2007; Amigorena and Savina, 2010). Whether these human DCs also encompass the counterpart of the human mcDCs is unclear. If human mcDCs would behave like mouse mcDCs, they would be associated with lymphoid tissue and very rare in blood. In this case it is interesting to note that most experiments using blood-derived or *in vitro* generated pDCs/BDCA3-DCs required inclusion of type I IFN inducing ligands to reveal their cross-priming ability.

CONCLUDING REMARKS

Under steady state conditions, cross-presentation of cell-associated Ag is a continuous process that is imperative for the maintenance of peripheral tolerance. While great strides have been made in the elucidation of the mechanisms that govern cross-presentation and subsequent cross-priming, there are still many questions to be answered. Little is known about the proteins that orchestrate vesicle composition and trafficking or the signals involved in the recruitment of these proteins. It is likely that these processes are influenced by the composition and "state of decay" of the dying cells, the receptors involved in uptake, and the nature and maturation state of the DC. Moreover, *in vivo*, signals in trans provided by bystander cells can significantly affect intrinsic mechanisms of cross-presentation by DCs. Although elucidation of these processes may be a daunting task, increased mechanistic insight into these pathways will have tremendous therapeutic potential in the fields of autoimmune disease, transplantation, and cancer.

ACKNOWLEDGMENTS

The authors would like to thank Dr. C. Karp for critically reading the manuscript. This work is supported by NIH grant CA138617 (NCI) and AI079545 (NIAID) to Edith M. Janssen.

REFERENCES

Albacker, L. A., Karisola, P., Chang, Y. J., Umetsu, S. E., Zhou, M., Akbari, O., Kobayashi, N., Baumgarth, N., Freeman, G. J., Umetsu, D. T., and DeKruyff, R. H. (2010). TIM-4, a receptor for phosphatidylserine, controls adaptive immunity by regulating the removal of antigen-specific T cells. *J. Immunol.* 185, 6839–6849.

Albert, M. L., Pearce, S. F., Francisco, L. M., Sauter, B., Roy, P., Silverstein, R. L., and Bhardwaj, N. (1998). Immature dendritic cells phagocytose apoptotic cells via alphavbeta5 and CD36, and cross-present antigens to cytotoxic T lymphocytes. *J. Exp. Med.* 188, 1359–1368.

Aliberti, J., Schulz, O., Pennington, D. J., Tsujimura, H., Reis e Sousa, C., Ozato, K., and Sher, A. (2003). Essential role for ICSBP in the in vivo development of murine CD8alpha+ dendritic cells. *Blood* 101, 305–310.

Allman, D., Dalod, M., Asselin-Paturel, C., Delale, T., Robbins, S. H., Trinchieri, G., Biron, C. A., Kastner, P., and Chan, S. (2006). Ikaros is required for plasmacytoid dendritic cell differentiation. *Blood* 108, 4025–4034.

Amigorena, S. (2002). Fc gamma receptors and cross-presentation in dendritic cells. *J. Exp. Med.* 195, F1–F3.

Amigorena, S., and Savina, A. (2010). Intracellular mechanisms of antigen cross presentation in dendritic cells. *Curr. Opin. Immunol.* 22, 109–117.

Anderson, K. L., Perkin, H., Surh, C. D., Venturini, S., Maki, R. A., and Torbett, B. E. (2000). Transcription factor PU.1 is necessary for development of thymic and myeloid progenitor-derived dendritic cells. *J. Immunol.* 164, 1855–1861.

Bachem, A., Guttler, S., Hartung, E., Ebstein, F., Schaefer, M., Tannert, A., Salama, A., Movassaghi, K., Opitz, C., Mages, H. W., Henn, V., Kloetzel, P. M., Gurka, S., and Kroczek, R. A. (2010). Superior antigen cross-presentation and XCR1 expression define human CD11c+ CD141+ cells as homologues of mouse CD8+ dendritic cells. *J. Exp. Med.* 207, 1273–1281.

Balasubramanian, K., Chandra, J., and Schroit, A. J. (1997). Immune clearance of phosphatidylserine-expressing cells by phagocytes. The role of beta2-glycoprotein I in macrophage recognition. *J. Biol. Chem.* 272, 31113–31117.

Bedoui, S., Prato, S., Mintern, J., Gebhardt, T., Zhan, Y., Lew, A. M., Heath, W. R., Villadangos, J. A., and Segura, E. (2009a). Characterization of an immediate splenic precursor of CD8+ dendritic cells capable of inducing antiviral T cell responses. *J. Immunol.* 182, 4200–4207.

Bedoui, S., Whitney, P. G., Waithman, J., Eidsmo, L., Wakim, L., Caminschi, I., Allan, R. S., Wojtasiak, M., Shortman, K., Carbone, F. R., Brooks, A. G., and Heath, W. R. (2009b). Cross-presentation of viral and self antigens by skin-derived CD103+ dendritic cells. *Nat. Immunol.* 10, 488–495.

Belz, G. T., Behrens, G. M., Smith, C. M., Miller, J. F., Jones, C., Lejon, K., Fathman, C. G., Mueller, S. N., Shortman, K., Carbone, F. R., and Heath, W. R. (2002). The CD8alpha(+) dendritic cell is responsible for inducing peripheral self-tolerance to tissue-associated antigens. *J. Exp. Med.* 196, 1099–1104.

Belz, G. T., Shortman, K., Bevan, M. J., and Heath, W. R. (2005). CD8alpha+ dendritic cells selectively present MHC class I-restricted noncytolytic viral and intracellular bacterial antigens in vivo. *J. Immunol.* 175, 196–200.

Blanchard, N., Gonzalez, F., Schaeffer, M., Joncker, N. T., Cheng, T., Shastri, A. J., Robey, E. A., and Shastri, N. (2008). Immunodominant, protective response to the parasite *Toxoplasma gondii* requires antigen processing in the endoplasmic reticulum. *Nat. Immunol.* 9, 937–944.

Bonifaz, L., Bonnyay, D., Mahnke, K., Rivera, M., Nussenzweig, M. C., and Steinman, R. M. (2002). Efficient targeting of protein antigen to the dendritic cell receptor DEC-205 in the steady state leads to antigen presentation on major histocompatibility complex class I products and peripheral CD8+ T cell tolerance. *J. Exp. Med.* 196, 1627–1638.

Bonnefoy, F., Perruche, S., Couturier, M., Sedrati, A., Sun, Y., Tiberghien, P., Gaugler, B., and Saas, P. (2011). Plasmacytoid dendritic cells play a major role in apoptotic leukocyte-induced immune modulation. *J. Immunol.* 186, 5696–5705.

Borisenko, G. G., Iverson, S. L., Ahlberg, S., Kagan, V. E., and Fadeel, B. (2004). Milk fat globule epidermal growth factor 8 (MFG-E8) binds to oxidized phosphatidylserine: implications for macrophage clearance of apoptotic cells. *Cell Death Differ.* 11, 943–945.

Bratton, D. L., and Henson, P. M. (2008). Apoptotic cell recognition: will the real phosphatidylserine receptor(s) please stand up? *Curr. Biol.* 18, R76–R79.

Burgdorf, S., Lukacs-Kornek, V., and Kurts, C. (2006). The mannose receptor mediates uptake of soluble but not of cell-associated antigen for cross-presentation. *J. Immunol.* 176, 6770–6776.

Burgdorf, S., Scholz, C., Kautz, A., Tampe, R., and Kurts, C. (2008). Spatial and mechanistic separation of cross-presentation and endogenous antigen presentation. *Nat. Immunol.* 9, 558–566.

Burkly, L., Hession, C., Ogata, L., Reilly, C., Marconi, L. A., Olson, D., Tizard, R., Cate, R., and Lo, D. (1995). Expression of relB is required for the development of thymic medulla and dendritic cells. *Nature* 373, 531–536.

Cebrian, I., Visentin, G., Blanchard, N., Jouve, M., Bobard, A., Moita, C., Enninga, J., Moita, L. F., Amigorena, S., and Savina, A. (2011). Sec22b regulates phagosomal maturation and antigen crosspresentation by dendritic cells. *Cell* 147, 1355–1368.

Chang, J., Cho, J. H., Lee, S. W., Choi, S. Y., Ha, S. J., and Sung, Y. C. (2004). IL-12 priming during in vitro antigenic stimulation changes properties of CD8 T cells and increases generation of effector and memory cells. *J. Immunol.* 172, 2818–2826.

Chen, L., and Jondal, M. (2008). Brefeldin A inhibits vesicular MHC class I processing in resting but not in CpG- and disruption-activated DC. *Mol. Immunol.* 46, 158–165.

Cho, H. J., Hayashi, T., Datta, S. K., Takabayashi, K., Van Uden, J. H., Horner, A., Corr, M., and Raz, E. (2002). IFN-alpha beta promote priming of antigen-specific CD8+ and CD4+ T lymphocytes by immunostimulatory DNA-based vaccines. *J. Immunol.* 168, 4907–4913.

Chung, E. Y., Liu, J., Homma, Y., Zhang, Y., Brendolan, A., Saggese, M., Han, J., Silverstein, R., Selleri, L., and Ma, X. (2007). Interleukin-10 expression in macrophages during phagocytosis of apoptotic cells is mediated by homeodomain proteins Pbx1 and Prep-1. *Immunity* 27, 952–964.

Cisse, B., Caton, M. L., Lehner, M., Maeda, T., Scheu, S., Locksley, R., Holmberg, D., Zweier, C., den Hollander, N. S., Kant, S. G., Holter, W., Rauch, A., Zhuang, Y., and Reizis, B. (2008). Transcription factor E2-2 is an essential and specific regulator of plasmacytoid dendritic cell development. *Cell* 135, 37–48.

Crowley, M., Inaba, K., Witmer-Pack, M., and Steinman, R. M. (1989). The cell surface of mouse dendritic cells: FACS analyses of dendritic cells from different tissues including thymus. *Cell. Immunol.* 118, 108–125.

Crozat, K., Tamoutounour, S., Vu Manh, T. P., Fossum, E., Luche, H., Ardouin, L., Guilliams, M., Azukizawa, H., Bogen, B., Malissen, B., Henri, S., and Dalod, M. (2011). Cutting edge: expression of XCR1 defines mouse lymphoid-tissue resident and migratory dendritic cells of the CD8{alpha}+ type. *J. Immunol.* 187, 4411–4415.

de Brito, C., Tomkowiak, M., Ghittoni, R., Caux, C., Leverrier, Y., and Marvel, J. (2011). CpG promotes cross-presentation of dead cell-associated antigens by pre-CD8alpha+ dendritic cells [corrected]. *J. Immunol.* 186, 1503–1511.

del Rio, M. L., Bernhardt, G., Rodriguez-Barbosa, J. I., and Forster, R. (2010). Development and functional specialization of CD103+ dendritic cells. *Immunol. Rev.* 234, 268–281.

Del Vecchio, M., Bajetta, E., Canova, S., Lotze, M. T., Wesa, A., Parmiani, G., and Anichini, A. (2007). Interleukin-12: biological properties and clinical application. *Clin. Cancer Res.* 13, 4677–4685.

Delneste, Y., Magistrelli, G., Gauchat, J., Haeuw, J., Aubry, J., Nakamura, K., Kawakami-Honda, N., Goetsch, L., Sawamura, T., Bonnefoy, J., and Jeannin, P. (2002). Involvement of LOX-1 in dendritic cell-mediated antigen cross-presentation. *Immunity* 17, 353–362.

den Haan, J. M., and Bevan, M. J. (2002). Constitutive versus activation-dependent cross-presentation of immune complexes by CD8(+) and CD8(−) dendritic cells in vivo. *J. Exp. Med.* 196, 817–827.

den Haan, J. M., Lehar, S. M., and Bevan, M. J. (2000). CD8(+) but not CD8(−) dendritic cells cross-prime cytotoxic T cells in vivo. *J. Exp. Med.* 192, 1685–1696.

Di Pucchio, T., Chatterjee, B., Smed-Sorensen, A., Clayton, S., Palazzo, A., Montes, M., Xue, Y., Mellman, I., Banchereau, J., and Connolly, J. E. (2008). Direct proteasome-independent cross-presentation of viral antigen by plasmacytoid dendritic cells on major histocompatibility complex class I. *Nat. Immunol.* 9, 551–557.

Diamond, M. S., Kinder, M., Matsushita, H., Mashayekhi, M., Dunn, G. P., Archambault, J. M., Lee, H., Arthur, C. D., White, J. M., Kalinke, U., Murphy, K. M., and Schreiber, R. D. (2011). Type I interferon is selectively required by dendritic cells for immune rejection of tumors. *J. Exp. Med.* 208, 1989–2003.

Dorner, B. G., Dorner, M. B., Zhou, X., Opitz, C., Mora, A., Guttler, S., Hutloff, A., Mages, H. W., Ranke, K., Schaefer, M., Jack, R. S., Henn, V., and Kroczek, R. A. (2009). Selective expression of the chemokine receptor XCR1 on cross-presenting dendritic cells determines cooperation with CD8+ T cells. *Immunity* 31, 823–833.

Edwards, A. D., Diebold, S. S., Slack, E. M., Tomizawa, H., Hemmi, H., Kaisho, T., Akira, S., and Reis e Sousa, C. (2003). Toll-like receptor expression in murine DC subsets: lack of TLR7 expression by CD8 alpha+ DC correlates with unresponsiveness to imidazoquinolines. *Eur. J. Immunol.* 33, 827–833.

Ehst, B. D., Ingulli, E., and Jenkins, M. K. (2003). Development of a novel transgenic mouse for the study of interactions between CD4 and CD8 T cells during graft rejection. *Am. J. Transplant.* 3, 1355–1362.

El-Sukkari, D., Wilson, N. S., Hakansson, K., Steptoe, R. J., Grubb, A., Shortman, K., and Villadangos, J. A. (2003). The protease inhibitor cystatin C is differentially expressed among dendritic cell populations, but does not control antigen presentation. *J. Immunol.* 171, 5003–5011.

Elward, K., and Gasque, P. (2003). "Eat me" and "don't eat me" signals govern the innate immune response and tissue repair in the CNS: emphasis on the critical role of the complement system. *Mol. Immunol.* 40, 85–94.

Erwig, L. P., and Henson, P. M. (2007). Immunological consequences of apoptotic cell phagocytosis. *Am. J. Pathol.* 171, 2–8.

Erwig, L. P., and Henson, P. M. (2008). Clearance of apoptotic cells by phagocytes. *Cell Death Differ.* 15, 243–250.

Fadok, V. A., Bratton, D. L., Konowal, A., Freed, P. W., Westcott, J. Y., and Henson, P. M. (1998). Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF. *J. Clin. Invest.* 101, 890–898.

Ferguson, T. A., Choi, J., and Green, D. R. (2011). Armed response: how dying cells influence T-cell functions. *Immunol. Rev.* 241, 77–88.

Ferguson, T. A., Herndon, J., Elzey, B., Griffith, T. S., Schoenberger, S., and Green, D. R. (2002). Uptake of apoptotic antigen-coupled cells by lymphoid dendritic cells and cross-priming of CD8(+) T cells produce active immune unresponsiveness. *J. Immunol.* 168, 5589–5595.

Fifis, T., Gamvrellis, A., Crimeen-Irwin, B., Pietersz, G. A., Li, J., Mottram, P. L., McKenzie, I. F., and Plebanski, M. (2004). Size-dependent immunogenicity: therapeutic and protective properties of nano-vaccines against tumors. *J. Immunol.* 173, 3148–3154.

Firat, E., Saveanu, L., Aichele, P., Staeheli, P., Huai, J., Gaedicke, S., Nil, A., Besin, G., Kanzler, B., van Endert, P., and Niedermann, G. (2007). The role of endoplasmic reticulum-associated aminopeptidase 1 in immunity to infection and in cross-presentation. *J. Immunol.* 178, 2241–2248.

Flinsenberg, T. W., Compeer, E. B., Boelens, J. J., and Boes, M. (2011). Antigen cross-presentation: extending recent laboratory findings to therapeutic intervention. *Clin. Exp. Immunol.* 165, 8–18.

Francois, M., Romieu-Mourez, R., Stock-Martineau, S., Boivin, M. N., Bramson, J. L., and Galipeau, J. (2009). Mesenchymal stromal cells cross-present soluble exogenous antigens as part of their antigen-presenting cell properties. *Blood* 114, 2632–2638.

Fuertes, M. B., Kacha, A. K., Kline, J., Woo, S. R., Kranz, D. M., Murphy, K. M., and Gajewski, T. F. (2011). Host type I IFN signals are required for antitumor CD8+ T cell responses through CD8{alpha}+ dendritic cells. *J. Exp. Med.* 208, 2005–2016.

Geiser, A. G., Letterio, J. J., Kulkarni, A. B., Karlsson, S., Roberts, A. B., and Sporn, M. B. (1993). Transforming growth factor beta 1 (TGF-beta 1) controls expression of major histocompatibility genes in the postnatal mouse: aberrant histocompatibility antigen expression in the pathogenesis of the TGF-beta 1 null mouse phenotype. *Proc. Natl. Acad. Sci. U.S.A.* 90, 9944–9948.

Green, D. R., Ferguson, T., Zitvogel, L., and Kroemer, G. (2009). Immunogenic and tolerogenic cell death. *Nat. Rev. Immunol.* 9, 353–363.

Griffith, T. S., Kazama, H., VanOosten, R. L., Earle, J. K. Jr., Herndon, J. M., Green, D. R., and Ferguson, T. A. (2007). Apoptotic cells induce tolerance by generating helpless CD8+ T cells that produce TRAIL. *J. Immunol.* 178, 2679–2687.

Grohmann, U., Bianchi, R., Belladonna, M. L., Vacca, C., Silla, S., Ayroldi, E., Fioretti, M. C., and Puccetti, P. (1999). IL-12 acts selectively on CD8 alpha-dendritic cells to enhance presentation of a tumor peptide in vivo. *J. Immunol.* 163, 3100–3105.

Guerriero, A., Langmuir, P. B., Spain, L. M., and Scott, E. W. (2000). PU.1 is required for myeloid-derived but not lymphoid-derived dendritic cells. *Blood* 95, 879–885.

Hacker, C., Kirsch, R. D., Ju, X. S., Hieronymus, T., Gust, T. C., Kuhl, C., Jorgas, T., Kurz, S. M., Rose-John, S., Yokota, Y., and Zenke, M. (2003). Transcriptional profiling identifies Id2 function in dendritic cell development. *Nat. Immunol.* 4, 380–386.

Hanayama, R., Tanaka, M., Miyasaka, K., Aozasa, K., Koike, M., Uchiyama, Y., and Nagata, S. (2004). Autoimmune disease and impaired uptake of apoptotic cells in MFG-E8-deficient mice. *Science* 304, 1147–1150.

Hashimoto, D., Miller, J., and Merad, M. (2011). Dendritic cell and macrophage heterogeneity in vivo. *Immunity* 35, 323–335.

Hawiger, D., Inaba, K., Dorsett, Y., Guo, M., Mahnke, K., Rivera, M., Ravetch, J. V., Steinman, R. M., and Nussenzweig, M. C. (2001). Dendritic cells induce peripheral T cell unresponsiveness under steady state conditions in vivo. *J. Exp. Med.* 194, 769–779.

Heath, W. R., Belz, G. T., Behrens, G. M., Smith, C. M., Forehan, S. P., Parish, I. A., Davey, G. M., Wilson, N. S., Carbone, F. R., and Villadangos, J. A. (2004). Cross-presentation, dendritic cell subsets, and the generation of immunity to cellular antigens. *Immunol. Rev.* 199, 9–26.

Hemmi, H., Idoyaga, J., Suda, K., Suda, N., Kennedy, K., Noda, M., Aderem, A., and Steinman, R. M. (2009). A new triggering receptor expressed on myeloid cells (Trem) family member, Trem-like 4, binds to dead cells and is a DNAX activation protein 12-linked marker for subsets of mouse macrophages and dendritic cells. *J. Immunol.* 182, 1278–1286.

Hennies, C. M., Reboulet, R. A., Garcia, Z., Nierkens, S., Wolkers, M. C., and Janssen, E. M. (2011). Selective expansion of merocytic dendritic cells and CD8DCs confers antitumour effect of Fms-like tyrosine kinase 3-ligand treatment in vivo. *Clin. Exp. Immunol.* 163, 381–391.

Henri, S., Poulin, L. F., Tamoutounour, S., Ardouin, L., Guilliams, M., de Bovis, B., Devilard, E., Viret, C., Azukizawa, H., Kissenpfennig, A., and Malissen, B. (2010). CD207+ CD103+ dermal dendritic cells cross-present keratinocyte-derived antigens irrespective of the presence of Langerhans cells. *J. Exp. Med.* 207, 189–206.

Hildner, K., Edelson, B. T., Purtha, W. E., Diamond, M., Matsushita, H., Kohyama, M., Calderon, B., Schraml, B. U., Unanue, E. R., Diamond, M. S., Schreiber, R. D., Murphy, T. L., and Murphy, K. M. (2008). Batf3 deficiency reveals a critical role for CD8alpha+ dendritic cells in cytotoxic T cell immunity. *Science* 322, 1097–1100.

Hochrein, H., O'Keeffe, M., Luft, T., Vandenabeele, S., Grumont, R. J., Maraskovsky, E., and Shortman, K. (2000). Interleukin (IL)-4 is a major regulatory cytokine governing bioactive IL-12 production by mouse and human dendritic cells. *J. Exp. Med.* 192, 823–833.

Hochrein, H., Shortman, K., Vremec, D., Scott, B., Hertzog, P., and O'Keeffe, M. (2001). Differential production of IL-12, IFN-alpha, and IFN-gamma by mouse dendritic cell subsets. *J. Immunol.* 166, 5448–5455.

Hoeffel, G., Ripoche, A. C., Matheoud, D., Nascimbeni, M., Escriou, N., Lebon, P., Heshmati, F., Guillet, J. G., Gannage, M., Caillat-Zucman, S., Casartelli, N., Schwartz, O., De la Salle, H., Hanau, D., Hosmalin, A., and Maranon, C. (2007). Antigen crosspresentation by human plasmacytoid dendritic cells. *Immunity* 27, 481–492.

Honda, K., Mizutani, T., and Taniguchi, T. (2004). Negative regulation of IFN-alpha/beta signaling by IFN regulatory factor 2 for homeostatic development of dendritic cells. *Proc. Natl. Acad. Sci. U.S.A.* 101, 2416–2421.

Ichikawa, E., Hida, S., Omatsu, Y., Shimoyama, S., Takahara, K., Miyagawa, S., Inaba, K., and Taki, S. (2004). Defective development of splenic and epidermal CD4+ dendritic cells in mice deficient for IFN regulatory factor-2. *Proc. Natl. Acad. Sci. U.S.A.* 101, 3909–3914.

Ishimoto, Y., Ohashi, K., Mizuno, K., and Nakano, T. (2000). Promotion of the uptake of PS liposomes and apoptotic cells by a product of growth arrest-specific gene, gas6. *J. Biochem.* 127, 411–417.

Iyoda, T., Shimoyama, S., Liu, K., Omatsu, Y., Akiyama, Y., Maeda, Y., Takahara, K., Steinman, R. M., and Inaba, K. (2002). The CD8+ dendritic cell subset selectively endocytoses dying cells in culture and in vivo. *J. Exp. Med.* 195, 1289–1302.

Janssen, E., Tabeta, K., Barnes, M. J., Rutschmann, S., McBride, S., Bahjat, K. S., Schoenberger, S. P., Theofilopoulos, A. N., Beutler, B., and Hoebe, K. (2006). Efficient T cell activation via a Toll-Interleukin

1 Receptor-independent pathway. *Immunity* 24, 787–799.

Janssen, E. M., Droin, N. M., Lemmens, E. E., Pinkoski, M. J., Bensinger, S. J., Ehst, B. D., Griffith, T. S., Green, D. R., and Schoenberger, S. P. (2005). CD4+ T-cell help controls CD8+ T-cell memory via TRAIL-mediated activation-induced cell death. *Nature* 434, 88–93.

Janssen, E. M., Lemmens, E. E., Wolfe, T., Christen, U., von Herrath, M. G., and Schoenberger, S. P. (2003). CD4+ T cells are required for secondary expansion and memory in CD8+ T lymphocytes. *Nature* 421, 852–856.

Kalergis, A. M., and Ravetch, J. V. (2002). Inducing tumor immunity through the selective engagement of activating Fcgamma receptors on dendritic cells. *J. Exp. Med.* 195, 1653–1659.

Kamphorst, A. O., Guermonprez, P., Dudziak, D., and Nussenzweig, M. C. (2010). Route of antigen uptake differentially impacts presentation by dendritic cells and activated monocytes. *J. Immunol.* 185, 3426–3435.

Karttunen, J., Sanderson, S., and Shastri, N. (1992). Detection of rare antigen-presenting cells by the lacZ T-cell activation assay suggests an expression cloning strategy for T-cell antigens. *Proc. Natl. Acad. Sci. U.S.A.* 89, 6020–6024.

Katz, J. D., Ondr, J. K., Opoka, R. J., Garcia, Z., and Janssen, E. M. (2010). Cutting edge: merocytic dendritic cells break T cell tolerance to beta cell antigens in nonobese diabetic mouse diabetes. *J. Immunol.* 185, 1999–2003.

Kim, S., Elkon, K. B., and Ma, X. (2004). Transcriptional suppression of interleukin-12 gene expression following phagocytosis of apoptotic cells. *Immunity* 21, 643–653.

Kobayashi, N., Karisola, P., Pena-Cruz, V., Dorfman, D. M., Jinushi, M., Umetsu, S. E., Butte, M. J., Nagumo, H., Chernova, I., Zhu, B., Sharpe, A. H., Ito, S., Dranoff, G., Kaplan, G. G., Casasnovas, J. M., Umetsu, D. T., Dekruyff, R. H., and Freeman, G. J. (2007). TIM-1 and TIM-4 glycoproteins bind phosphatidylserine and mediate uptake of apoptotic cells. *Immunity* 27, 927–940.

Koppelman, B., Neefjes, J. J., de Vries, J. E., and de Waal Malefyt, R. (1997). Interleukin-10 down-regulates MHC class II alphabeta peptide complexes at the plasma membrane of monocytes by affecting arrival and recycling. *Immunity* 7, 861–871.

Kraal, G., Breel, M., Janse, M., and Bruin, G. (1986). Langerhans' cells, veiled cells, and interdigitating cells in the mouse recognized by a monoclonal antibody. *J. Exp. Med.* 163, 981–997.

Krysko, D. V., and Vandenabeele, P. (2008). From regulation of dying cell engulfment to development of anti-cancer therapy. *Cell Death Differ.* 15, 29–38.

Kurts, C., Heath, W. R., Carbone, F. R., Allison, J., Miller, J. F., and Kosaka, H. (1996). Constitutive class I-restricted exogenous presentation of self antigens in vivo. *J. Exp. Med.* 184, 923–930.

Kurts, C., Heath, W. R., Kosaka, H., Miller, J. F., and Carbone, F. R. (1998). The peripheral deletion of autoreactive CD8+ T cells induced by cross-presentation of self-antigens involves signaling through CD95 (Fas, Apo-1). *J. Exp. Med.* 188, 415–420.

Kushwah, R., and Hu, J. (2011). Complexity of dendritic cell subsets and their function in the host immune system. *Immunology* 133, 409–419.

Lahoud, M. H., Proietto, A. I., Ahmet, F., Kitsoulis, S., Eidsmo, L., Wu, L., Sathe, P., Pietersz, S., Chang, H. W., Walker, I. D., Maraskovsky, E., Braley, H., Lew, A. M., Wright, M. D., Heath, W. R., Shortman, K., and Caminschi, I. (2009). The C-type lectin Clec12A present on mouse and human dendritic cells can serve as a target for antigen delivery and enhancement of antibody responses. *J. Immunol.* 182, 7587–7594.

Lahoud, M. H., Proietto, A. I., Gartlan, K. H., Kitsoulis, S., Curtis, J., Wettenhall, J., Sofi, M., Daunt, C., O'Keeffe, M., Caminschi, I., Satterley, K., Rizzitelli, A., Schnorrer, P., Hinohara, A., Yamaguchi, Y., Wu, L., Smyth, G., Handman, E., Shortman, K., and Wright, M. D. (2006). Signal regulatory protein molecules are differentially expressed by CD8$^-$ dendritic cells. *J. Immunol.* 177, 372–382.

Lattanzi, L., Rozera, C., Marescotti, D., D'Agostino, G., Santodonato, L., Cellini, S., Belardelli, F., Gavioli, R., and Ferrantini, M. (2011). IFN-alpha boosts epitope cross-presentation by dendritic cells via modulation of proteasome activity. *Immunobiology* 216, 537–547.

Lee, J. B., Lee, K. A., and Chang, J. (2007). Phenotypic changes induced by IL-12 priming regulate effector and memory CD8 T cell differentiation. *Int. Immunol.* 19, 1039–1048.

Lennon-Dumenil, A. M., Bakker, A. H., Maehr, R., Fiebiger, E., Overkleeft, H. S., Rosemblatt, M., Ploegh, H. L., and Lagaudriere-Gesbert, C. (2002). Analysis of protease activity in live antigen-presenting cells shows regulation of the phagosomal proteolytic contents during dendritic cell activation. *J. Exp. Med.* 196, 529–540.

Liu, K., and Nussenzweig, M. C. (2010). Origin and development of dendritic cells. *Immunol. Rev.* 234, 45–54.

Liu, K., Victora, G. D., Schwickert, T. A., Guermonprez, P., Meredith, M. M., Yao, K., Chu, F. F., Randolph, G. J., Rudensky, A. Y., and Nussenzweig, M. (2009). In vivo analysis of dendritic cell development and homeostasis. *Science* 324, 392–397.

Lorenzi, S., Mattei, F., Sistigu, A., Bracci, L., Spadaro, F., Sanchez, M., Spada, M., Belardelli, F., Gabriele, L., and Schiavoni, G. (2011). Type I IFNs control antigen retention and survival of CD8alpha(+) dendritic cells after uptake of tumor apoptotic cells leading to cross-priming. *J. Immunol.* 186, 5142–5150.

Ma, D., and Niederkorn, J. Y. (1995). Transforming growth factor-beta down-regulates major histocompatibility complex class I antigen expression and increases the susceptibility of uveal melanoma cells to natural killer cell-mediated cytolysis. *Immunology* 86, 263–269.

Maldonado-Lopez, R., and Moser, M. (2001). Dendritic cell subsets and the regulation of Th1/Th2 responses. *Semin. Immunol.* 13, 275–282.

McLellan, A. D., Kapp, M., Eggert, A., Linden, C., Bommhardt, U., Brocker, E. B., Kammerer, U., and Kampgen, E. (2002). Anatomic location and T-cell stimulatory functions of mouse dendritic cell subsets defined by CD4 and CD8 expression. *Blood* 99, 2084–2093.

Morelli, A. E., Larregina, A. T., Shufesky, W. J., Zahorchak, A. F., Logar, A. J., Papworth, G. D., Wang, Z., Watkins, S. C., Falo, L. D. Jr., and Thomson, A. W. (2003). Internalization of circulating apoptotic cells by splenic marginal zone dendritic cells: dependence on complement receptors and effect on cytokine production. *Blood* 101, 611–620.

Naik, S. H., Proietto, A. I., Wilson, N. S., Dakic, A., Schnorrer, P., Fuchsberger, M., Lahoud, M. H., O'Keeffe, M., Shao, Q. X., Chen, W. F., Villadangos, J. A., Shortman, K., and Wu, L. (2005). Cutting edge: generation of splenic CD8+ and CD8$^-$ dendritic cell equivalents in Fms-like tyrosine kinase 3 ligand bone marrow cultures. *J. Immunol.* 174, 6592–6597.

Nakayama, M., Akiba, H., Takeda, K., Kojima, Y., Hashiguchi, M., Azuma, M., Yagita, H., and Okumura, K. (2009). Tim-3 mediates phagocytosis of apoptotic cells and cross-presentation. *Blood* 113, 3821–3830.

Nandan, D., and Reiner, N. E. (1997). TGF-beta attenuates the class II transactivator and reveals an accessory pathway of IFN-gamma action. *J. Immunol.* 158, 1095–1101.

Oka, K., Sawamura, T., Kikuta, K., Itokawa, S., Kume, N., Kita, T., and Masaki, T. (1998). Lectin-like oxidized low-density lipoprotein receptor 1 mediates phagocytosis of aged/apoptotic cells in endothelial cells. *Proc. Natl. Acad. Sci. U.S.A.* 95, 9535–9540.

Peng, Y., and Elkon, K. B. (2011). Autoimmunity in MFG-E8-deficient mice is associated with altered trafficking and enhanced cross-presentation of apoptotic cell antigens. *J. Clin. Invest.* 121, 2221–2241.

Platt, N., Suzuki, H., Kodama, T., and Gordon, S. (2000). Apoptotic thymocyte clearance in scavenger receptor class A-deficient mice is apparently normal. *J. Immunol.* 164, 4861–4867.

Pooley, J. L., Heath, W. R., and Shortman, K. (2001). Cutting edge: intravenous soluble antigen is presented to CD4 T cells by CD8− dendritic cells, but cross-presented to CD8 T cells by CD8+ dendritic cells. *J. Immunol.* 166, 5327–5330.

Poon, I. K., Hulett, M. D., and Parish, C. R. (2010). Molecular mechanisms of late apoptotic/necrotic cell clearance. *Cell Death Differ.* 17, 381–397.

Pribila, J. T., Itano, A. A., Mueller, K. L., and Shimizu, Y. (2004). The alpha 1 beta 1 and alpha E beta 7 integrins define a subset of dendritic cells in peripheral lymph nodes with unique adhesive and antigen uptake properties. *J. Immunol.* 172, 282–291.

Pulendran, B., Lingappa, J., Kennedy, M. K., Smith, J., Teepe, M., Rudensky, A., Maliszewski, C. R., and Maraskovsky, E. (1997). Developmental pathways of dendritic cells in vivo: distinct function, phenotype, and localization of dendritic cell subsets in FLT3 ligand-treated mice. *J. Immunol.* 159, 2222–2231.

Qiu, C. H., Miyake, Y., Kaise, H., Kitamura, H., Ohara, O., and Tanaka, M. (2009). Novel subset of CD8{alpha}+ dendritic cells localized in the marginal zone is responsible for tolerance to cell-associated antigens. *J. Immunol.* 182, 4127–4136.

Reboulet, R. A., Hennies, C. M., Garcia, Z., Nierkens, S., and Janssen, E. M. (2010). Prolonged antigen storage endows merocytic dendritic cells

with enhanced capacity to prime anti-tumor responses in tumor-bearing mice. *J. Immunol.* 185, 3337–3347.

Regnault, A., Lankar, D., Lacabanne, V., Rodriguez, A., Thery, C., Rescigno, M., Saito, T., Verbeek, S., Bonnerot, C., Ricciardi-Castagnoli, P., and Amigorena, S. (1999). Fcgamma receptor-mediated induction of dendritic cell maturation and major histocompatibility complex class I-restricted antigen presentation after immune complex internalization. *J. Exp. Med.* 189, 371–380.

Reis e Sousa, C., Hieny, S., Scharton-Kersten, T., Jankovic, D., Charest, H., Germain, R. N., and Sher, A. (1997). In vivo microbial stimulation induces rapid CD40 ligand-independent production of interleukin 12 by dendritic cells and their redistribution to T cell areas. *J. Exp. Med.* 186, 1819–1829.

Rejman, J., Oberle, V., Zuhorn, I. S., and Hoekstra, D. (2004). Size-dependent internalization of particles via the pathways of clathrin- and caveolae-mediated endocytosis. *Biochem. J.* 377, 159–169.

Robbins, S. H., Walzer, T., Dembele, D., Thibault, C., Defays, A., Bessou, G., Xu, H., Vivier, E., Sellars, M., Pierre, P., Sharp, F. R., Chan, S., Kastner, P., and Dalod, M. (2008). Novel insights into the relationships between dendritic cell subsets in human and mouse revealed by genome-wide expression profiling. *Genome Biol.* 9, R17.

Rock, K. L., Farfan-Arribas, D. J., and Shen, L. (2010). Proteases in MHC class I presentation and cross-presentation. *J. Immunol.* 184, 9–15.

Rodriguez, A., Regnault, A., Kleijmeer, M., Ricciardi-Castagnoli, P., and Amigorena, S. (1999). Selective transport of internalized antigens to the cytosol for MHC class I presentation in dendritic cells. *Nat. Cell Biol.* 1, 362–368.

Ronchetti, A., Rovere, P., Iezzi, G., Galati, G., Heltai, S., Protti, M. P., Garancini, M. P., Manfredi, A. A., Rugarli, C., and Bellone, M. (1999). Immunogenicity of apoptotic cells in vivo: role of antigen load, antigen-presenting cells, and cytokines. *J. Immunol.* 163, 130–136.

Salazar-Onfray, F., Charo, J., Petersson, M., Freland, S., Noffz, G., Qin, Z., Blankenstein, T., Ljunggren, H. G., and Kiessling, R. (1997). Down-regulation of the expression and function of the transporter associated with antigen processing in murine tumor cell lines expressing IL-10. *J. Immunol.* 159, 3195–3202.

Sallusto, F., Cella, M., Danieli, C., and Lanzavecchia, A. (1995). Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products. *J. Exp. Med.* 182, 389–400.

Sancho, D., Joffre, O. P., Keller, A. M., Rogers, N. C., Martinez, D., Hernanz-Falcon, P., Rosewell, I., and Reis e Sousa, C. (2009). Identification of a dendritic cell receptor that couples sensing of necrosis to immunity. *Nature* 458, 899–903.

Sancho, D., Mourao-Sa, D., Joffre, O. P., Schulz, O., Rogers, N. C., Pennington, D. J., Carlyle, J. R., and Reis e Sousa, C. (2008). Tumor therapy in mice via antigen targeting to a novel, DC-restricted C-type lectin. *J. Clin. Invest.* 118, 2098–2110.

Sathe, P., and Shortman, K. (2008). The steady-state development of splenic dendritic cells. *Mucosal Immunol.* 1, 425–431.

Saveanu, L., Carroll, O., Weimershaus, M., Guermonprez, P., Firat, E., Lindo, V., Greer, F., Davoust, J., Kratzer, R., Keller, S. R., Niedermann, G., and van Endert, P. (2009). IRAP identifies an endosomal compartment required for MHC class I cross-presentation. *Science* 325, 213–217.

Savina, A., and Amigorena, S. (2007). Phagocytosis and antigen presentation in dendritic cells. *Immunol. Rev.* 219, 143–156.

Savina, A., Jancic, C., Hugues, S., Guermonprez, P., Vargas, P., Moura, I. C., Lennon-Dumenil, A. M., Seabra, M. C., Raposo, G., and Amigorena, S. (2006). NOX2 controls phagosomal pH to regulate antigen processing during crosspresentation by dendritic cells. *Cell* 126, 205–218.

Savina, A., Peres, A., Cebrian, I., Carmo, N., Moita, C., Hacohen, N., Moita, L. F., and Amigorena, S. (2009). The small GTPase Rac2 controls phagosomal alkalinization and antigen crosspresentation selectively in CD8(+) dendritic cells. *Immunity* 30, 544–555.

Scheinecker, C., McHugh, R., Shevach, E. M., and Germain, R. N. (2002). Constitutive presentation of a natural tissue autoantigen exclusively by dendritic cells in the draining lymph node. *J. Exp. Med.* 196, 1079–1090.

Schiavoni, G., Mattei, F., Sestili, P., Borghi, P., Venditti, M., Morse, H. C. III, Belardelli, F., and Gabriele, L. (2002). ICSBP is essential for the development of mouse type I interferon-producing cells and for the generation and activation of CD8alpha(+) dendritic cells. *J. Exp. Med.* 196, 1415–1425.

Schnorrer, P., Behrens, G. M., Wilson, N. S., Pooley, J. L., Smith, C. M., El-Sukkari, D., Davey, G., Kupresanin, F., Li, M., Maraskovsky, E., Belz, G. T., Carbone, F. R., Shortman, K., Heath, W. R., and Villadangos, J. A. (2006). The dominant role of CD8+ dendritic cells in cross-presentation is not dictated by antigen capture. *Proc. Natl. Acad. Sci. U.S.A.* 103, 10729–10734.

Schotte, R., Nagasawa, M., Weijer, K., Spits, H., and Blom, B. (2004). The ETS transcription factor Spi-B is required for human plasmacytoid dendritic cell development. *J. Exp. Med.* 200, 1503–1509.

Schulz, O., Edwards, A. D., Schito, M., Aliberti, J., Manickasingham, S., Sher, A., and Reis e Sousa, C. (2000). CD40 triggering of heterodimeric IL-12 p70 production by dendritic cells in vivo requires a microbial priming signal. *Immunity* 13, 453–462.

Schulz, O., and Reis e Sousa, C. (2002). Cross-presentation of cell-associated antigens by CD8alpha+ dendritic cells is attributable to their ability to internalize dead cells. *Immunology* 107, 183–189.

Scott, R. S., McMahon, E. J., Pop, S. M., Reap, E. A., Caricchio, R., Cohen, P. L., Earp, H. S., and Matsushima, G. K. (2001). Phagocytosis and clearance of apoptotic cells is mediated by MER. *Nature* 411, 207–211.

Segura, E., Albiston, A. L., Wicks, I. P., Chai, S. Y., and Villadangos, J. A. (2009). Different cross-presentation pathways in steady-state and inflammatory dendritic cells. *Proc. Natl. Acad. Sci. U.S.A.* 106, 20377–20381.

Shen, L., Sigal, L. J., Boes, M., and Rock, K. L. (2004). Important role of cathepsin S in generating peptides for TAP-independent MHC class I crosspresentation in vivo. *Immunity* 21, 155–165.

Shortman, K., and Heath, W. R. (2010). The CD8+ dendritic cell subset. *Immunol. Rev.* 234, 18–31.

Shortman, K., and Liu, Y. J. (2002). Mouse and human dendritic cell subtypes. *Nat. Rev. Immunol.* 2, 151–161.

Shortman, K., and Naik, S. H. (2007). Steady-state and inflammatory dendritic-cell development. *Nat. Rev. Immunol.* 7, 19–30.

Shrimpton, R. E., Butler, M., Morel, A. S., Eren, E., Hue, S. S., and Ritter, M. A. (2009). CD205 (DEC-205): a recognition receptor for apoptotic and necrotic self. *Mol. Immunol.* 46, 1229–1239.

Strobl, H., and Knapp, W. (1999). TGF-beta1 regulation of dendritic cells. *Microbes Infect.* 1, 1283–1290.

Stuart, L. M., Lucas, M., Simpson, C., Lamb, J., Savill, J., and Lacy-Hulbert, A. (2002). Inhibitory effects of apoptotic cell ingestion upon endotoxin-driven myeloid dendritic cell maturation. *J. Immunol.* 168, 1627–1635.

Suzuki, S., Honma, K., Matsuyama, T., Suzuki, K., Toriyama, K., Akitoyo, I., Yamamoto, K., Suematsu, T., Nakamura, M., Yui, K., and Kumatori, A. (2004). Critical roles of interferon regulatory factor 4 in CD11bhighCD8alpha- dendritic cell development. *Proc. Natl. Acad. Sci. U.S.A.* 101, 8981–8986.

Tailor, P., Tamura, T., Morse, H. C. III, and Ozato, K. (2008). The BXH2 mutation in IRF8 differentially impairs dendritic cell subset development in the mouse. *Blood* 111, 1942–1945.

Tosello, V., Zamarchi, R., Merlo, A., Gorza, M., Piovan, E., Mandruzzato, S., Bronte, V., Wang, X., Ferrone, S., Amadori, A., and Zanovello, P. (2009). Differential expression of constitutive and inducible proteasome subunits in human monocyte-derived DC differentiated in the presence of IFN-alpha or IL-4. *Eur. J. Immunol.* 39, 56–66.

Tran, K. K., and Shen, H. (2009). The role of phagosomal pH on the size-dependent efficiency of cross-presentation by dendritic cells. *Biomaterials* 30, 1356–1362.

Trinchieri, G. (2003). Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat. Rev. Immunol.* 3, 133–146.

Trinchieri, G., Pflanz, S., and Kastelein, R. A. (2003). The IL-12 family of heterodimeric cytokines: new players in the regulation of T cell responses. *Immunity* 19, 641–644.

Trombetta, E. S., Ebersold, M., Garrett, W., Pypaert, M., and Mellman, I. (2003). Activation of lysosomal function during dendritic cell maturation. *Science* 299, 1400–1403.

Vieira, O. V., Botelho, R. J., and Grinstein, S. (2002). Phagosome maturation: aging gracefully. *Biochem. J.* 366, 689–704.

Voll, R. E., Herrmann, M., Roth, E. A., Stach, C., Kalden, J. R., and Girkontaite, I. (1997). Immunosuppressive effects of apoptotic cells. *Nature* 390, 350–351.

Vremec, D., O'Keeffe, M., Hochrein, H., Fuchsberger, M., Caminschi, I., Lahoud, M., and Shortman, K. (2007). Production of interferons by

dendritic cells, plasmacytoid cells, natural killer cells, and interferon-producing killer dendritic cells. *Blood* 109, 1165–1173.

Vremec, D., Pooley, J., Hochrein, H., Wu, L., and Shortman, K. (2000). CD4 and CD8 expression by dendritic cell subtypes in mouse thymus and spleen. *J. Immunol.* 164, 2978–2986.

Vremec, D., and Shortman, K. (1997). Dendritic cell subtypes in mouse lymphoid organs: cross-correlation of surface markers, changes with incubation, and differences among thymus, spleen, and lymph nodes. *J. Immunol.* 159, 565–573.

Weih, F., Carrasco, D., Durham, S. K., Barton, D. S., Rizzo, C. A., Ryseck, R. P., Lira, S. A., and Bravo, R. (1995). Multiorgan inflammation and hematopoietic abnormalities in mice with a targeted disruption of RelB, a member of the NF-kappa B/Rel family. *Cell* 80, 331–340.

Wilson, N. S., El-Sukkari, D., Belz, G. T., Smith, C. M., Steptoe, R. J., Heath, W. R., Shortman, K., and Villadangos, J. A. (2003). Most lymphoid organ dendritic cell types are phenotypically and functionally immature. *Blood* 102, 2187–2194.

Wu, L., D'Amico, A., Winkel, K. D., Suter, M., Lo, D., and Shortman, K. (1998). RelB is essential for the development of myeloid-related CD8alpha- dendritic cells but not of lymphoid-related CD8alpha+ dendritic cells. *Immunity* 9, 839–847.

Wu, L., and Liu, Y. J. (2007). Development of dendritic-cell lineages. *Immunity* 26, 741–750.

Wu, L., Nichogiannopoulou, A., Shortman, K., and Georgopoulos, K. (1997). Cell-autonomous defects in dendritic cell populations of Ikaros mutant mice point to a developmental relationship with the lymphoid lineage. *Immunity* 7, 483–492.

Yamazaki, S., Dudziak, D., Heidkamp, G. F., Fiorese, C., Bonito, A. J., Inaba, K., Nussenzweig, M. C., and Steinman, R. M. (2008). CD8+ CD205+ splenic dendritic cells are specialized to induce Foxp3+ regulatory T cells. *J. Immunol.* 181, 6923–6933.

Zeidler, R., Eissner, G., Meissner, P., Uebel, S., Tampe, R., Lazis, S., and Hammerschmidt, W. (1997). Down-regulation of TAP1 in B lymphocytes by cellular and Epstein-Barr virus-encoded interleukin-10. *Blood* 90, 2390–2397.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 09 December 2011; accepted: 19 February 2012; published online: 14 March 2012.

*Citation: Thacker RI and Janssen EM (2012) Cross-presentation of cell-associated antigens by mouse splenic dendritic cell populations. Front. Immun. **3**:41. doi: 10.3389/fimmu.2012.00041*

This article was submitted to Frontiers in Antigen Presenting Cell Biology, a specialty of Frontiers in Immunology.

frontiers in
IMMUNOLOGY

MINI REVIEW ARTICLE
published: 28 February 2012
doi: 10.3389/fimmu.2012.00031

Into the intracellular logistics of cross-presentation

Jacques Neefjes* and Charlotte Sadaka*

Division of Cell Biology, The Netherlands Cancer Institute, Amsterdam, Netherlands

Edited by:
Christian Kurts, Friedrich Wilhelms-Universität Bonn, Germany

Reviewed by:
Gabrielle Belz, Walter and Eliza Hall Institute of Medical Research, Australia
Peter M. Van Endert, Université Paris Descartes, France

****Correspondence:***
Jacques Neefjes and Charlotte Sadaka, Division of Cell Biology, The Netherlands Cancer Institute, Plesmanlaan 121, 1066CX Amsterdam, Netherlands.
e-mail: j.neefjes@nki.nl; c.sadaka@nki.nl

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).

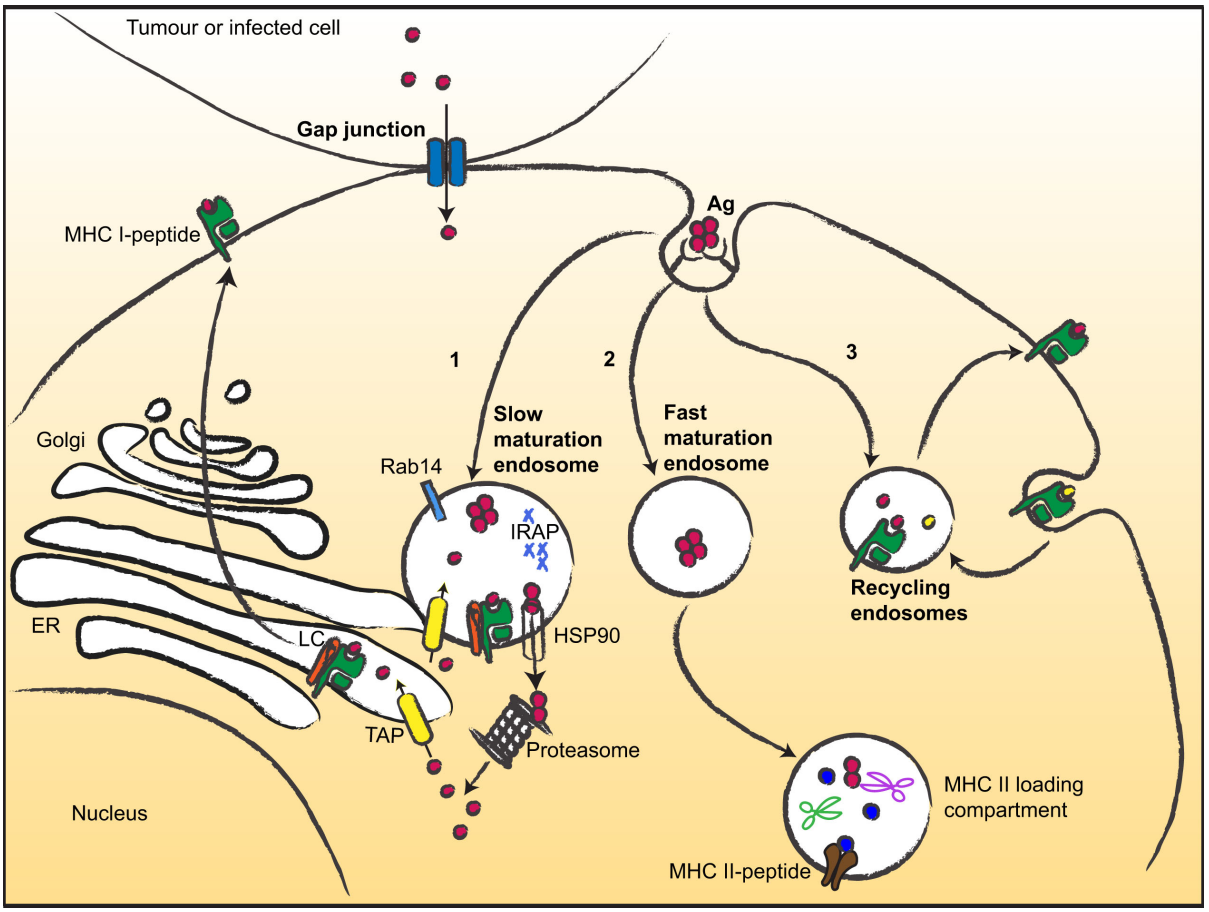


FIGURE 1 | Multiple ways for MHC I cross-presentation. After uptake, antigens can follow different routes and be targeted to (1) slow maturating endosomes (or phagosome) displaying low proteolytic activity. Then, antigens can escape to the cytosol (via HSP90) and are degraded by the proteasome into peptides 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.

ACKNOWLEDGMENTS

This work was supported by European Research Council (ERC) and Netherlands Organization for Scientific Research (NWO) grants to Jacques Neefjes. Robbert Spaapen, Sander van Kasteren, and Shalin Naik are warmly acknowledged for their critical reading of the review.

REFERENCES

Ackerman, A. L., Kyritsis, C., Tampe, R., and Cresswell, P. (2005). Access of soluble antigens to the endoplasmic reticulum can explain cross-presentation by dendritic cells. *Nat. Immunol.* 6, 107–113.

Asano, K., Nabeyama, A., Miyake, Y., Qiu, C. H., Kurita, A., Tomura, M., Kanagawa, O., Fujii, S., and Tanaka, M. (2011). CD169-positive macrophages dominate antitumor immunity by crosspresenting dead cell-associated antigens. *Immunity* 34, 85–95.

Bachem, A., Guttler, S., Hartung, E., Ebstein, F., Schaefer, M., Tannert, A., Salama, A., Movassaghi, K., Opitz, C., Mages, H. W., Henn, V., Kloetze, P. M., Gurka, S., and Kroczek, R. A. (2010). Superior antigen cross-presentation and XCR1 expression define human CD11c+CD141+ cells as homologues of mouse CD8+ dendritic cells. *J. Exp. Med.* 207, 1273–1281.

Bedoui, S., Whitney, P. G., Waithman, J., Eidsmo, L., Wakim, L., Caminschi, I., Allan, R. S., Wojtasiak, M., Shortman, K., Carbone, F. R., Brooks, A. G., and Heath, W. R. (2009). Cross-presentation of viral and self antigens by skin-derived CD103+ dendritic cells. *Nat. Immunol.* 10, 488–495.

Brossart, P., and Bevan, M. J. (1997). Presentation of exogenous protein antigens on major histocompatibility complex class I molecules by dendritic cells: pathway of presentation and regulation by cytokines. *Blood* 90, 1594–1599.

Burgdorf, S., Kautz, A., Bohnert, V., Knolle, P. A., and Kurts, C. (2007). Distinct pathways of antigen uptake and intracellular routing in CD4 and CD8 T cell activation. *Science* 316, 612–616.

Burgdorf, S., Lukacs-Kornek, V., and Kurts, C. (2006). The mannose receptor mediates uptake of soluble but not of cell-associated antigen for cross-presentation. *J. Immunol.* 176, 6770–6776.

Burgdorf, S., Scholz, C., Kautz, A., Tampe, R., and Kurts, C. (2008). Spatial and mechanistic separation of cross-presentation and endogenous antigen presentation. *Nat. Immunol.* 9, 558–566.

Caminschi, I., Proietto, A. I., Ahmet, F., Kitsoulis, S., Shin Teh, J., Lo, J. C., Rizzitelli, A., Wu, L., Vremec, D., van Dommelen, S. L., Campbell, I. K., Maraskovsky, E., Braley, H., Davey, G. M., Mottram, P., van de Velde, N., Jensen, K., Lew, A. M., Wright, M. D., Heath, W. R., Shortman, K., and Lahoud, M. H. (2008). The dendritic cell subtype-restricted C-type lectin Clec9A is a target for vaccine enhancement. *Blood* 112, 3264–3273.

Cebrian, I., Visentin, G., Blanchard, N., Jouve, M., Bobard, A., Moita, C., Enninga, J., Moita, L. F., Amigorena, S., and Savina, A. (2011). Sec22b regulates phagosomal maturation and antigen crosspresentation by dendritic cells. *Cell* 147, 1355–1368.

Dantuma, N. P., Groothuis, T. A., Salomons, F. A., and Neefjes, J. (2006). A dynamic ubiquitin equilibrium couples proteasomal activity to chromatin remodeling. *J. Cell Biol.* 173, 19–26.

de Wit, J., Souwer, Y., Jorritsma, T., Klaasse Bos, H., ten Brinke, A., Neefjes, J., and van Ham, S. M. (2010). Antigen-specific B cells reactivate an effective cytotoxic T cell response against phagocytosed *Salmonella* through cross-presentation. *PLoS ONE* 5, e13016. doi:10.1371/journal.pone.0013016

del Rio, M. L., Rodriguez-Barbosa, J. I., Kremmer, E., and Forster, R. (2007). CD103- and CD103+ bronchial lymph node dendritic cells are specialized in presenting and cross-presenting innocuous antigen to CD4+ and CD8+ T cells. *J. Immunol.* 178, 6861–6866.

Delamarre, L., Pack, M., Chang, H., Mellman, I., and Trombetta, E. S. (2005). Differential lysosomal proteolysis in antigen-presenting cells determines antigen fate. *Science* 307, 1630–1634.

den Haan, J. M., and Bevan, M. J. (2002). Constitutive versus activation-dependent cross-presentation of immune complexes by CD8(+) and CD8(−) dendritic cells in vivo. *J. Exp. Med.* 196, 817–827.

den Haan, J. M., Lehar, S. M., and Bevan, M. J. (2000). CD8(+) but not CD8(−) dendritic cells cross-prime cytotoxic T cells in vivo. *J. Exp. Med.* 192, 1685–1696.

Di Pucchio, T., Chatterjee, B., Smed-Sorensen, A., Clayton, S., Palazzo, A., Montes, M., Xue, Y., Mellman, I., Banchereau, J., and Connolly, J. E. (2008). Direct proteasome-independent cross-presentation of viral antigen by plasmacytoid dendritic cells on major histocompatibility complex class I. *Nat. Immunol.* 9, 551–557.

Faure, F., Mantegazza, A., Sadaka, C., Sedlik, C., Jotereau, F., and Amigorena, S. (2009). Long-lasting cross-presentation of tumor antigen in human DC. *Eur. J. Immunol.* 39, 380–390.

Fonteneau, J. F., Kavanagh, D. G., Lirvall, M., Sanders, C., Cover, T. L., Bhardwaj, N., and Larsson, M. (2003). Characterization of the MHC class I cross-presentation pathway for cell-associated antigens by human dendritic cells. *Blood* 102, 4448–4455.

Gagnon, E., Duclos, S., Rondeau, C., Chevet, E., Cameron, P. H., Steele-Mortimer, O., Paiement, J., Bergeron, J. J., and Desjardins, M. (2002). Endoplasmic reticulum-mediated phagocytosis is a mechanism of entry into macrophages. *Cell* 110, 119–131.

Gromme, M., Uytdehaag, F. G., Janssen, H., Calafat, J., van Binnendijk, R. S., Kenter, M. J., Tulp, A., Verwoerd, D., and Neefjes, J. (1999). Recycling MHC class I molecules and endosomal peptide loading. *Proc. Natl. Acad. Sci. U.S.A.* 96, 10326–10331.

Guermonprez, P., Saveanu, L., Kleijmeer, M., Davoust, J., Van Endert, P., and Amigorena, S. (2003). ER-phagosome fusion defines an MHC class I cross-presentation compartment in dendritic cells. *Nature* 425, 397–402.

Haug, G., Leemhuis, J., Tiemann, D., Meyer, D. K., Aktories, K., and Barth, H. (2003). The host cell chaperone Hsp90 is essential for translocation of the binary Clostridium botulinum C2 toxin into the cytosol. *J. Biol. Chem.* 278, 32266–32274.

Herrera, O. B., Golshayan, D., Tibbott, R., Salcido Ochoa, F., James, M. J., Marelli-Berg, F. M., and Lechler, R. I. (2004). A novel pathway of alloantigen presentation by dendritic cells. *J. Immunol.* 173, 4828–4837.

Hildner, K., Edelson, B. T., Purtha, W. E., Diamond, M., Matsushita, H., Kohyama, M., Calderon, B., Schraml, B. U., Unanue, E. R., Diamond, M. S., Schreiber, R. D., Murphy, T. L., and Murphy, K. M. (2008). Batf3 deficiency reveals a critical role for CD8alpha+ dendritic cells in cytotoxic T cell immunity. *Science* 322, 1097–1100.

Houde, M., Bertholet, S., Gagnon, E., Brunet, S., Goyette, G., Laplante, A., Princiotta, M. F., Thibault, P., Sacks, D., and Desjardins, M. (2003). Phagosomes are competent organelles for antigen cross-presentation. *Nature* 425, 402–406.

Imai, T., Kato, Y., Kajiwara, C., Mizukami, S., Ishige, I., Ichiyanagi, T., Hikida, M., Wang, J. Y., and Udono, H. (2011). Heat shock protein 90 (HSP90) contributes to cytosolic translocation of extracellular antigen for cross-presentation by dendritic cells. *Proc. Natl. Acad. Sci. U.S.A.* 108, 16363–16368.

Jancic, C., Savina, A., Wasmeier, C., Tolmachova, T., El-Benna, J., Dang, P. M., Pascolo, S., Gougerot-Pocidalo, M. A., Raposo, G., Seabra, M. C., and Amigorena, S. (2007). Rab27a regulates phagosomal pH and NADPH oxidase recruitment to dendritic cell phagosomes. *Nat. Cell Biol.* 9, 367–378.

Jongbloed, S. L., Kassianos, A. J., McDonald, K. J., Clark, G. J., Ju, X., Angel, C. E., Chen, C. J., Dunbar, P. R., Wadley, R. B., Jeet, V., Vulink, A. J., Hart, D. N., and Radford, K. J. (2010). Human CD141+ (BDCA-3)+ dendritic cells (DCs) represent a unique myeloid DC subset that cross-presents necrotic cell antigens. *J. Exp. Med.* 207, 1247–1260.

Kuijl, C., Savage, N. D., Marsman, M., Tuin, A. W., Janssen, L., Egan, D. A., Ketema, M., van den Nieuwendijk, R., van den Eeden, S. J., Geluk, A., Poot, A., van der Marel, G., Beijersbergen, R. L., Overkleeft, H., Ottenhoff, T. H., and Neefjes, J. (2007). Intracellular bacterial growth is controlled by a kinase network around PKB/AKT1. *Nature* 450, 725–730.

Lakadamyali, M., Rust, M. J., and Zhuang, X. (2006). Ligands for clathrin-mediated endocytosis are differentially sorted into distinct populations of early endosomes. *Cell* 124, 997–1009.

Luber, C. A., Cox, J., Lauterbach, H., Fancke, B., Selbach, M., Tschopp, J., Akira, S., Wiegand, M., Hochrein, H., O'Keeffe, M., and Mann, M. (2010). Quantitative proteomics reveals subset-specific viral recognition in dendritic cells. *Immunity* 32, 279–289.

Merzougui, N., Kratzer, R., Saveanu, L., and van Endert, P. (2011). A proteasome-dependent, TAP-independent pathway for cross-presentation of phagocytosed antigen. *EMBO Rep.* 12, 1257–1264.

Neefjes, J., Jongsma, M. L., Paul, P., and Bakke, O. (2011). Towards a systems understanding of MHC class I and MHC class II antigen presentation. *Nat. Rev. Immunol.* 11, 823–836.

Neefjes, J. J., Verkerk, J. M., Broxterman, H. J., van der Marel, G. A., van Boom, J. H., and Ploegh, H. L. (1988). Recycling glycoproteins do not return to the cis-Golgi. *J. Cell Biol.* 107, 79–87.

Neijssen, J., Herberts, C., Drijfhout, J. W., Reits, E., Janssen, L., and Neefjes, J. (2005). Cross-presentation by intercellular peptide transfer through gap junctions. *Nature* 434, 83–88.

Neijssen, J., Pang, B., and Neefjes, J. (2007). Gap junction-mediated intercellular communication in the immune system. *Prog. Biophys. Mol. Biol.* 94, 207–218.

Oh, J. Z., Kurche, J. S., Burchill, M. A., and Kedl, R. M. (2011). TLR7 enables cross-presentation by multiple dendritic cell subsets through a type I IFN-dependent pathway. *Blood* 118, 3028–3038.

Oura, J., Tamura, Y., Kamiguchi, K., Kutomi, G., Sahara, H., Torigoe, T., Himi, T., and Sato, N. (2011). Extracellular heat shock protein 90 plays a role in translocating chaperoned antigen from endosome to proteasome for generating antigenic peptide to be cross-presented by dendritic cells. *Int. Immunol.* 23, 223–237.

Pang, B., and Neefjes, J. (2010). Coupled for cross-presentation in tumor immunotherapy. *Sci. Transl. Med.* 2, 44ps40.

Pang, B., Neijssen, J., Qiao, X., Janssen, L., Janssen, H., Lippuner, C., and Neefjes, J. (2009). Direct antigen presentation and gap junction mediated cross-presentation during apoptosis. *J. Immunol.* 183, 1083–1090.

Poulin, L. F., Salio, M., Griessinger, E., Anjos-Afonso, F., Craciun, L., Chen, J. L., Keller, A. M., Joffre, O., Zelenay, S., Nye, E., Le Moine, A., Faure, F., Donckier, V., Sancho, D., Cerundolo, V., Bonnet, D., and Sousa, C. (2010). Characterization of human DNGR-1+ BDCA3+ leukocytes as putative equivalents of mouse CD8alpha+ dendritic cells. *J. Exp. Med.* 207, 1261–1271.

Robbins, S. H., Walzer, T., Dembele, D., Thibault, C., Defays, A., Bessou, G., Xu, H., Vivier, E., Sellars, M., Pierre, P., Sharp, F. R., Chan, S., Kastner, P., and Dalod, M. (2008). Novel insights into the relationships between dendritic cell subsets in human and mouse revealed by genome-wide expression profiling. *Genome Biol.* 9, R17.

Rodriguez, A., Regnault, A., Kleijmeer, M., Ricciardi-Castagnoli, P., and Amigorena, S. (1999). Selective transport of internalized antigens to the cytosol for MHC class I presentation in dendritic cells. *Nat. Cell Biol.* 1, 362–368.

Saccheri, F., Pozzi, C., Avogadri, F., Barozzi, S., Faretta, M., Fusi, P., and Rescigno, M. (2010). Bacteria-induced gap junctions in tumors favor antigen cross-presentation and antitumor immunity. *Sci. Transl. Med.* 2, 44ra57.

Sancho, D., Joffre, O. P., Keller, A. M., Rogers, N. C., Martinez, D., Hernanz-Falcon, P., Rosewell, I., and Reis e Sousa, C. (2009). Identification of a dendritic cell receptor that couples sensing of necrosis to immunity. *Nature* 458, 899–903.

Saveanu, L., Carroll, O., Weimershaus, M., Guermonprez, P., Firat, E., Lindo, V., Greer, F., Davoust, J., Kratzer, R., Keller, S. R., Niedermann, G., and van Endert, P. (2009). IRAP identifies an endosomal compartment required for MHC class I cross-presentation. *Science* 325, 213–217.

Savina, A., Jancic, C., Hugues, S., Guermonprez, P., Vargas, P., Moura, I. C., Lennon-Dumenil, A. M., Seabra, M. C., Raposo, G., and Amigorena, S. (2006). NOX2 controls phagosomal pH to regulate antigen processing during crosspresentation by dendritic cells. *Cell* 126, 205–218.

Schuurhuis, D. H., Ioan-Facsinay, A., Nagelkerken, B., van Schip, J. J., Sedlik, C., Melief, C. J., Verbeek, J. S., and Ossendorp, F. (2002). Antigen-antibody immune complexes empower dendritic cells to efficiently prime specific CD8+ CTL responses in vivo. *J. Immunol.* 168, 2240–2246.

Segura, E., Albiston, A. L., Wicks, I. P., Chai, S. Y., and Villadangos, J. A. (2009). Different cross-presentation pathways in steady-state and inflammatory dendritic cells. *Proc. Natl. Acad. Sci. U.S.A.* 106, 20377–20381.

Segura, E., Kapp, E., Gupta, N., Wong, J., Lim, J., Ji, H., Heath, W. R., Simpson, R., and Villadangos, J. A. (2010). Differential expression of pathogen-recognition molecules between dendritic cell subsets revealed by plasma membrane proteomic analysis. *Mol. Immunol.* 47, 1765–1773.

Segura, E., and Villadangos, J. A. (2011). A modular and combinatorial view of the antigen cross-presentation pathway in dendritic cells. *Traffic* 12, 1677–1685.

Shen, L., Sigal, L. J., Boes, M., and Rock, K. L. (2004). Important role of cathepsin S in generating peptides for TAP-independent MHC class I crosspresentation in vivo. *Immunity* 21, 155–165.

Tacken, P. J., Ginter, W., Berod, L., Cruz, L. J., Joosten, B., Sparwasser, T., Figdor, C. G., and Cambi, A. (2011). Targeting DC-SIGN via its neck region leads to prolonged antigen residence in early endosomes, delayed lysosomal degradation, and cross-presentation. *Blood* 118, 4111–4119.

Taylor, M., Navarro-Garcia, F., Huerta, J., Burress, H., Massey, S., Ireton, K., and Teter, K. (2010). Hsp90 is required for transfer of the cholera toxin A1 subunit from the endoplasmic reticulum to the cytosol. *J. Biol. Chem.* 285, 31261–31267.

van Montfoort, N., Camps, M. G., Khan, S., Filippov, D. V., Weterings, J. J., Griffith, J. M., Geuze, H. J., van Hall, T., Verbeek, J. S., Melief, C. J., and Ossendorp, F. (2009). Antigen storage compartments in mature dendritic cells facilitate prolonged cytotoxic T lymphocyte cross-priming capacity. *Proc. Natl. Acad. Sci. U.S.A.* 106, 6730–6735.

Wakim, L. M., and Bevan, M. J. (2011). Cross-dressed dendritic cells drive memory CD8+ T-cell activation after viral infection. *Nature* 471, 629–632.

Weck, M. M., Appel, S., Werth, D., Sinzger, C., Bringmann, A., Grunebach, F., and Brossart, P. (2008). hDectin-1 is involved in uptake and cross-presentation of cellular antigens. *Blood* 111, 4264–4272.

Win, S. J., Ward, V. K., Dunbar, P. R., Young, S. L., and Baird, M. A. (2011). Cross-presentation of epitopes on virus-like particles via the MHC I receptor recycling pathway. *Immunol. Cell Biol.* 89, 681–688.

Wolfers, J., Lozier, A., Raposo, G., Regnault, A., Thery, C., Masurier, C., Flament, C., Pouzieux, S., Faure, F., Tursz, T., Angevin, E., Amigorena, S., and Zitvogel, L. (2001). Tumor-derived exosomes are a source of shared tumor rejection antigens for CTL cross-priming. *Nat. Med.* 7, 297–303.

Zehner, M., Chasan, A. I., Schuette, V., Embgenbroich, M., Quast, T., Kolanus, W., and Burgdorf, S. (2011). Mannose receptor polyubiquitination regulates endosomal recruitment of p97 and cytosolic antigen translocation for cross-presentation. *Proc. Natl. Acad. Sci. U.S.A.* 108, 9933–9938.

Zou, L., Zhou, J., Zhang, J., Li, J., Liu, N., Chai, L., Li, N., Liu, T., Li, L., Xie, Z., Liu, H., Wan, Y., and Wu, Y. (2009). The GTPase Rab3b/3c-positive recycling vesicles are involved in cross-presentation in dendritic cells. *Proc. Natl. Acad. Sci. U.S.A.* 106, 15801–15806.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 14 December 2011; accepted: 12 February 2012; published online: 28 February 2012.

Citation: Neefjes J and Sadaka C (2012) Into the intracellular logistics of cross-presentation. Front. Immun. ***3****:31. doi: 10.3389/fimmu.2012.00031*

This article was submitted to Frontiers in Antigen Presenting Cell Biology, a specialty of Frontiers in Immunology.

frontiers in
IMMUNOLOGY

REVIEW ARTICLE
published: 07 March 2012
doi: 10.3389/fimmu.2012.00037

Antigen processing and remodeling of the endosomal pathway: requirements for antigen cross-presentation

Ewoud Bernardus Compeer, Thijs Willem Hendrik Flinsenberg, Susanna Geertje van der Grein and Marianne Boes*

Department of Pediatric Immunology, University Medical Center Utrecht/Wilhelmina Children's Hospital, Utrecht, Netherlands

Edited by:
Christian Kurts, Friedrich Wilhelms-Universität Bonn, Germany

Reviewed by:
Veronika Lukacs-Kornek, Harvard Medical School, USA
Ted Howard Hansen, Washington University School of Medicine, USA
Luis Ferreira Moita, Instituto de Medicina Molecular, Portugal

***Correspondence:**
Marianne Boes, Department of Pediatric Immunology, Cellular Immunology laboratory, University Medical Center Utrecht/Wilhelmina Children's Hospital, Utrecht University, Lundlaan 6, Utrecht, 3508 AB, Netherlands.
e-mail: m.l.boes@umcutrecht.nl

Cross-presentation of endocytosed antigen as peptide/class I major histocompatibility complex complexes plays a central role in the elicitation of $CD8^+$ T cell clones that mediate anti-viral and anti-tumor immune responses. While it has been clear that there are specific subsets of professional antigen presenting cells capable of antigen cross-presentation, identification of mechanisms involved is still ongoing. Especially amongst dendritic cells (DC), there are specialized subsets that are highly proficient at antigen cross-presentation. We here present a focused survey on the cell biological processes in the endosomal pathway that support antigen cross-presentation. This review highlights DC-intrinsic mechanisms that facilitate the cross-presentation of endocytosed antigen, including receptor-mediated uptake, maturation-induced endosomal sorting of membrane proteins, dynamic remodeling of endosomal structures and cell surface-directed endosomal trafficking. We will conclude with the description of pathogen-induced deviation of endosomal processing, and discuss how immune evasion strategies pertaining endosomal trafficking may preclude antigen cross-presentation.

Keywords: cross-presentation, endosomal recycling compartment, Rab GTPase, microtubule-organizing center, endosomal remodeling

INTRODUCTION

Major histocompatibility complex (MHC) restriction of T lymphocytes was first reported by Doherty and Zinkernagel (1975). They provided experimental proof that T cells can only respond to peptide antigens when they are presented in complex with host-derived MHC molecules, existing in a class I and class II variant. $CD8^+$ T cells can recognize peptide/class I MHC complexes, whereas $CD4^+$ T cells can respond to peptide/class II MHC complexes. Soon thereafter, Bevan (1976) showed that the functional dichotomy of endogenous antigen presentation on class I MHC and exogenous antigen on class II MHC is not absolute. He demonstrated that minor histocompatibility antigens from transplanted cells (e.g., exogenous antigen) could prime cytotoxic $CD8^+$ T cells in a host class I MHC-restricted manner and named this process cross-priming. More recent work showed that injected naive antigen-specific $CD8^+$ T cells accumulate in the lymph nodes that drain tissues expressing a membrane-bound self-antigen in a class I MHC-dependent manner. $CD8^+$ T cells can thus survey processed self-antigen delivered from non-lymphoid tissues, without leaving lymphoid organs (Kurts et al., 1996). In following years, the capability to present exogenous antigens via class I MHC was shown to be relevant for numerous cell-associated antigens in various settings, including viral, self, and tumor-associated antigens (Heath and Carbone, 2001). Antigen presentation that results in $CD8^+$ T cell activation is now named cross-priming (Bevan, 1976), whereas T cell deletion or induction of anergy is called cross-tolerance (Albert et al., 2001; Bonifaz et al., 2002). Collectively the presentation of exogenous cell-associated antigens via class I MHC molecules to $CD8^+$ T cells is called cross-presentation.

Under homeostatic conditions, cross-presentation of self-antigens harbors the risk of autoreactivity and is therefore strictly controlled. Under these circumstances, antigen cross-presentation is mostly confined to a specific subset of dendritic cells (DC), notably $CD8\alpha^+$ DC in mice (den Haan et al., 2000) and $CD141^+$ DC in human (Bachem et al., 2010; Crozat et al., 2010; Jongbloed et al., 2010; Poulin et al., 2010). $CD8\alpha^+$ $DEC205^+$ mouse DC not only excel in antigen cross-presentation, but are also specialized in the uptake of dying cells (Dudziak et al., 2007). Receptor-based antigen capture and cross-presentation was shown using a DEC205 antibody to which protein antigen was chemically coupled (Bonifaz et al., 2002; Bozzacco et al., 2007). Antigen internalized via DEC205 targeting results in the continuous, steady state capture and processing of antigen into peptide/class I MHC complexes in a manner that results in tolerogenic $CD8^+$ T cell responses (Bonifaz et al., 2002). Besides DC, macrophages (Kovacsovics-Bankowski et al., 1993) and liver sinusoidal endothelial cells (Limmer et al., 2000) can also cross-present antigens in the steady state. In contrast, more cell types can cross-present antigen during inflammation. Mouse DC (Jung et al., 2002), macrophages (Kovacsovics-Bankowski et al., 1993), neutrophils (Beauvillain et al., 2007) and under specific conditions even B cells (Heit et al., 2004), have been demonstrated to cross-present antigen *in vivo*. Additionally, cell types that can cross-present antigen *in vitro* include basophils (Kim et al., 2009), γδ T cells (Brandes et al., 2009), mast cells (Stelekati et al., 2009), and endothelial cells (Bagai et al., 2005).

Although multiple cells can be involved in cross-priming *in vivo*, DC are especially important for this process, as shown

by abrogated $CD8^+$ T cell priming after depletion of $CD11c^+$ cells (predominantly DC) in mice (Jung et al., 2002). Hence, this review focuses mainly on DC, for most cell-intrinsic mechanisms involved in cross-presentation of endocytosed antigen are described in this cell type.

In the last 15 years, at least 50 DC vaccination-based trials, aimed in part at harnessing effective $CD8^+$ T cell responses were performed with overall minimal success (reviewed in Rosenberg et al., 2004). This review aims at providing insight in molecular mechanisms that are pivotal to cross-presentation.

ANTIGEN PROCESSING COMPARTMENT FOR CROSS-PRESENTATION

Antigen recognition can trigger receptor-mediated endocytosis and can bring forth the ability of cells to cross-present the receptor-bound antigen (Sancho et al., 2009; Bachem et al., 2010; Jongbloed et al., 2010). In contrast to complement-opsonized antigen, immunoglobulin (Ig)-opsonized antigens are delivered in an endosomal compartment that favors cross-presentation by murine DC (Kim et al., 2008). Because Ig-opsonized antigen is predominantly endocytosed via Fc receptor and complement-opsonized antigen via complement receptor, this study indicates that antigen recognition dictates antigen delivery in distinct endosomal compartments. Ultimately, this may favor either the class I or class II MHC presentation pathway (reviewed in Flinsenberg et al., 2011).

Aside from antigen recognition, antigen size plays a role in its handling by phagocytes: particulate antigens that are larger than roughly 0.5 μm are internalized by phagocytosis, whereas smaller antigens are brought in by pinocytosis (Pratten and Lloyd, 1986; Mant et al., 2012). One major route of antigen internalization that yields cross-presentation seems to be phagocytosis, as particulate antigens are often more efficiently targeted for cross-presentation in comparison to their soluble counterparts (Graham et al., 2010). Thus, DC internalize antigens via distinct routes that are dictated by the structure of the antigen (i.e., particulate or soluble, size) and possible involvement of a recognizing endocytic receptor.

Receptor-mediated endocytosis is considered to be a highly efficient process that permits the selective retrieval of macromolecules present in the extracellular fluid (Brown et al., 1976). Such uptake depends on structural proteins that mediate the formation of lipid vacuoles, e.g., clathrin or caveolin (Mayor and Pagano, 2007). Within minutes of internalization from the plasma membrane into the endosomal pathway, antigen is located in vesicular compartments named early endosomes/phagosomes, characterized by a near neutral pH (pH 7.5; Savina et al., 2006) and presence of the small GTPase Rab5 (Simonsen et al., 1998). Endosomal maturation causes the fusion of early endosomes (EE) with late compartments, accompanied by transition of Rab5 expression to Rab7-positivity. The endosomal compartment is now renamed to late endosomes (LE) with their hallmark of a lowered pH of the endosomal lumen (pH 5.5). Further maturation of the LE leads to fusion with lysosomes in which the acidic environment (pH 4.7) and lysosomal proteases and hydrolases with low pH optima can mediate full degradation of luminal content (i.e., internalized antigen, but also cellular components for turnover). Especially DC harbor cellular mechanisms that prevent the rapid maturation-induced acidification of endosomal compartments, thereby allowing protein antigen fragments to remain intact for a prolonged time (as proteolytic activity by pH-sensitive proteases is restrained). Thereby, DC can cross-present antigen-derived peptides more efficiently than other phagocytes (Trombetta et al., 2003; Savina et al., 2006). Micro-organisms exploit these mechanisms to prevent their display as peptide/class I MHC complexes as immune evasion strategies (i.e., Mycobacteria and *Salmonella*; Jantsch et al., 2011; Johansen et al., 2011), as will be discussed in paragraph 6.

Peptides of 8–10 amino acids in length fit within the antigen-binding groove of class I MHC molecules, leading to a stable formation of peptide/class I MHC complexes (Urban et al., 1994; Rammensee, 1995). The proteasome is the foremost contributor to cleaved peptides for the classical class I MHC presentation pathway. Therefore its role in cross-presentation was assessed. The use of proteasome-selective inhibitors clarified the existence of both proteasome-dependent (Kovacsovics-Bankowski and Rock, 1995) and independent antigen processing (Shen et al., 2004; Saveanu et al., 2009) in distinct cross-presentation model systems. To date, two main routes leading to cross-presentation have broad experimental support: the cytosolic and vacuolar pathway. The cytosolic pathway proposes that endocytosed antigen is transported into the cytosol for proteasome/cytosolic peptidase-mediated degradation, whereas the vacuolar pathway relies on proteases for antigen processing within endosomes.

The cytosolic pathway model is supported by phagosome-to-cytosol translocation of OVA-beads (Kovacsovics-Bankowski and Rock, 1995), OVA-IgG and HRP-IgG immune complexes in murine cells (Rodriguez et al., 1999). Lin et al. (2008) demonstrated that cross-presentation competent $CD8\alpha^+$ and not the incapable $CD8\alpha^-$ DC were sensitive for exogenously added cytochrome-*c* (cytochrome-*c* induces apoptosis when cytosolic concentrations are elevated). Antigen translocation from phagosome-to-cytosol involves processes that are antigen-specific, have antigen size-restrictions, may involve the reduction and unfolding of protein antigen and are Sec61 complex mediated (Rodriguez et al., 1999; Ackerman et al., 2006; Singh and Cresswell, 2010). In addition, cytosolic transfer of apoptotic peptides by neighboring cells and interacting dendritic cells can occur via gap-junctions into the cross-presenting cell (Pang et al., 2009).

The processing of antigen that is translocated into the cytosol involves the proteasome, as well as amino- and carboxy-terminal peptidases (Levy et al., 2002; Shen et al., 2011). The transporter associated with antigen processing (TAP) translocates the peptides into the endoplasmic reticulum (ER) which thereby enter the conventional class I MHC pathway (Ackerman et al., 2005), or back into the phagosomal pathway in an MyD88-dependent manner (Ackerman et al., 2003; Houde et al., 2003; Burgdorf et al., 2008). All necessary components to enable peptide trimming, loading, and translocation appear present and functional in early phagosomes (Houde et al., 2003; Ackerman et al., 2005). It was proposed that phagosome–ER fusion occurs to deliver the necessary components to the phagosome (Guermonprez et al., 2003). It now appears that rather than complete phagosome–ER fusion, which was disputed (Touret et al., 2005), only selective ER-derived components are delivered to phagosomes (Burgdorf et al., 2008; Cebrian et al., 2011). The SNARE Sec22b is shown to

recruit ER-resident proteins to phagosomes that are necessary for phagosome-to-cytosol translocation (Cebrian et al., 2011).

Several groups demonstrated that peptide generation for cross-presentation may occur independent of the proteasome (Shen et al., 2004), while requiring endosomal acidification (Gromme et al., 1999; Di Pucchio et al., 2008). The proposed vacuolar pathway does not require phagosome-to-cytosol translocation, but relies on endosomal proteases for generation of antigenic peptides (Gromme et al., 1999; Di Pucchio et al., 2008). Shen et al. showed that cell-associated OVA can be degraded by both cathepsin S in the endosomal pathway or the cytosolic proteasome within one population of DC. This indicates that the proteasome-independent vacuolar pathway may co-exist with the cytosolic pathways. This possibility is supported by reports demonstrating that plasmacytoid DC cross-present in both proteasome-dependent and independent pathways (Hoeffel et al., 2007; Di Pucchio et al., 2008). In summary, antigen processing for cross-presentation depends on distinct proteolytic enzymes and may occur in the endosomal compartment as well as the cytosol.

These studies strengthen the concept that both antigen recognition and its physical characteristics affect antigen sorting into the given processing pathways, thereby influencing antigen presentation. Immunization studies showed that appropriate endosomal sorting is essential for efficient cross-presentation. Immunization with bead-coupled OVA caused $CD8^+$ T cell responses and proliferation in an Fcγ receptor and DAP12-dependent manner. Cross-presentation of soluble OVA was independent of Fcγ receptors and DAP12 (Graham et al., 2007).

CLASS I MHC IN THE ENDOSOMAL COMPARTMENT

Early studies showed that TAP-dependent cross-presentation is sensitive to Brefeldin A through its ability to block ER-to-Golgi transport. These data fueled the initial proposal that peptide loading occurs in the ER (Kovacsovics-Bankowski and Rock, 1995; Fonteneau et al., 2003). However, the identification of Brefeldin A-independent antigen cross-presentation (Pfeifer et al., 1993; Belizaire and Unanue, 2009) and the discovery that components for peptide loading are present in phagosomes (Houde et al., 2003; Ackerman et al., 2005) suggest that peptide loading onto class I MHC may occur also outside of the ER. Class I MHC molecules are distributed in endosomal compartments, as shown in human melanoma epithelial cells (Mel JuSo cells) and lymphoblastoid cells (B-LCLs; Gromme et al., 1999; Kleijmeer et al., 2001). In contrast to endosomal class II MHC molecules, that can directly transit from the Golgi system to the endosomal pathway via association with the invariant chain chaperone, a major route for endosomal localization of class I MHC involves internalization from the plasma membrane. Peptide-class I MHC interactions are destabilized in late endosomal compartments (pH around 5.0), thereby facilitating peptide loading (Gromme et al., 1999). Further support came from TAP inhibition studies in which TAP function in the early and recycling endosomal (transferrin-positive) compartment was selectively disrupted (Burgdorf et al., 2008). Endosomal peptide loading would contribute to rapid cross-presentation of a selective set of endocytosed antigen-derived peptides, while decreasing the risk for competition with endogenous peptides that are assembled into peptide/class I MHC complexes in the ER.

For efficient endosomal peptide/class I MHC loading, class I MHC molecules must be delivered into the peptide loading compartment. Class I MHC molecules are constitutively internalized (Vega and Strominger, 1989). Mutational analysis of the cytoplasmic domain of class I MHC molecules identified several key residues that are essential for internalization (Lizee et al., 2003; Basha et al., 2008). An evolutionary-conserved tyrosine residue mediates the delivery into lysosomes (Lizee et al., 2003). This tyrosine residue is part of a known targeting motif YXX∅ (Y = tyrosine, X = any amino acid, ∅ = bulky hydrophobic amino acid) that has been shown to bind directly to adaptor protein (AP)-1, 2, or 3 (Bonifacino and Dell'Angelica, 1999). AP-tyrosine motif interaction results in selective incorporation of motif-containing cargo, such as the transferrin receptor, in clathrin-coated vesicles for uptake (Lizee et al., 2005). Besides endosomal targeting mediated by the tyrosine-based motif, the cytoplasmic domain of class I MHC molecules contains two or three conserved lysine residues (Duncan et al., 2006). Lysines are targets for ubiquitination that can also induce clathrin-mediated endocytosis. Studies on immune evasion strategies employed by Kaposi sarcoma associated herpesvirus (KSHV) identified two viral proteins, K3 and K5, that can downregulate cell surface bound class I MHC molecules via poly-ubiquitination (Lorenzo et al., 2002). Two human homologous proteins of K3 and K5, the membrane-associated RING-CH family MARCH IV and IX, are key regulators in class II MHC surface expression in B cells and DC (Gassart de et al., 2008). Moreover, MARCH IV and IX ubiquitinate class I MHC molecules and induce its internalization in an overexpression system (Bartee et al., 2004). Possibly these proteins can facilitate class I MHC endocytosis under physiological conditions, but this remains to be established. Thus, class I MHC molecules are taken up into the endosomal pathway of DC in a clathrin-dependent manner, enabling for sufficient amounts of endosomal class I MHC molecules to assemble into antigenic peptide/class I MHC complexes. As T cell activation requires presentation of multiple antigen-specific peptide/class I MHC complexes, the efficient transport of peptide/class I MHC complexes from peptide loading compartment to the cell surface is a further cross-presentation requirement that needs to be attained.

RECYCLING OF ENDOSOMAL CLASS I MHC

The intracellular location where peptide/class I MHC complexes are assembled dictates the trafficking route that is taken. Peptide loading within the ER probably results in transport via the biosynthetic pathway to the cell surface. In contrast, endosomal peptide/class I MHC assembly suggests an alternative route of transport.

The endosomal pathway contains both vesicular and tubular structures (Kleijmeer et al., 2001; Boes et al., 2002). During endocytosis, cell surface-derived membrane proteins and lipids are concomitantly taken up with antigen into endosomal vesicles (Scita and Di Fiore, 2010). To ensure steady surface display, most of the proteins and lipids are rapidly returned to the plasma membrane via the endosomal recycling pathway that consists of two main routes. Within minutes, retrograde recycling of membrane proteins from the EE to plasma membrane may occur, whereas a slower recycling route exists via juxtanuclear endosomal recycling

compartments (ERC; Sonnichsen et al., 2000). It was estimated that cells internalize the equivalent of their cell surface one to five times per hour (Steinman et al., 1983), demonstrating the importance of endosomal recycling to normal cellular function.

There is experimental support that the recycling pathway may play a considerable role in antigen cross-presentation. Pharmacological inhibition of the recycling pathway by inclusion of primaquine in murine DC cultures abrogates cross-presentation of exogenous soluble antigen, without affecting class I MHC-mediated presentation of endogenously expressed antigen (Burgdorf et al., 2008). Similarly, Di Pucchio et al. (2008) report cross-presentation of a viral antigen by plasmacytoid DC in a Brefeldin A-resistant, but primaquine-sensitive manner. Furthermore, silencing of the small GTPases Rab3b and 3c, that colocalize with class I MHC molecules in recycling endosomes (RE) of DC2.4 cells, inhibits cross-presentation (Zou et al., 2009). Finally, mouse DC lacking class I MHC in recycling compartments due to expression of class I MHC with an aberrant tyrosine-based motif, are defective in cross-presentation (Lizee et al., 2003). Together, these reports demonstrate that interfering with the recycling pathway of class I MHC can abrogate cross-presentation, but the exact DC-intrinsic mechanisms for class I MHC molecule recycling that are involved in cross-presentation remain elusive.

The endosomal targeting of internalized antigen involves the selective recruitment of signaling molecules [i.e., EHD1 (Jovic et al., 2009) and Rab effector molecules (Hayakawa et al., 2007)]. One factor that regulate selective recruitment of signaling molecules is the small GTPase Arf6 (Brown et al., 2001). GDP/GTP cycling affects Arf6 function in membrane lipid and protein recycling. Active GTP-bound Arf6 localizes to the cytosolic side of the plasma membrane for clathrin-independent endocytosis, whereas GDP-bound Arf6 localized to tubular-like endosomal structures (Caplan et al., 2002).

The Rab family of small GTPases are considered key regulators of endocytic trafficking (Stenmark, 2009; **Figure 1**). Rab22a colocalizes with class I MHC in Arf6-associated tubules (Weigert

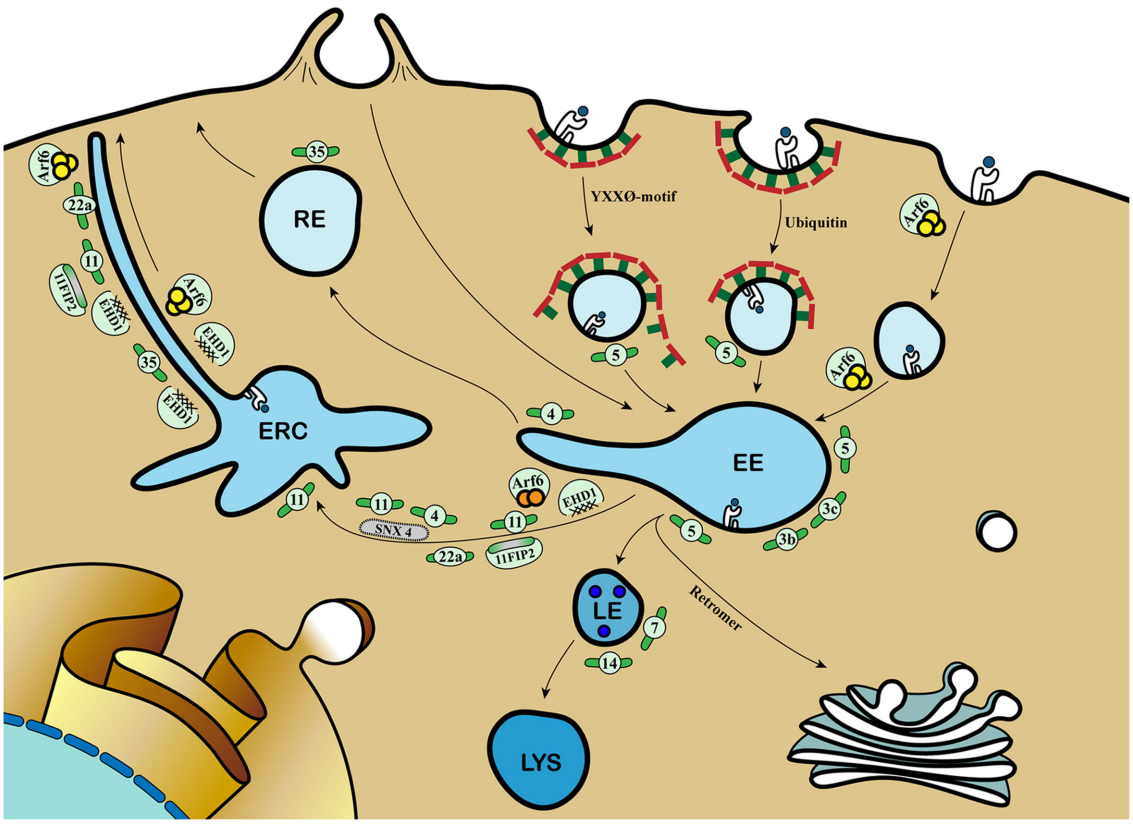


FIGURE 1 | Molecular mechanisms coordinating cargo recycling in the endosomal compartment. Antigens can be internalized by phagocytosis and (receptor-mediated) endocytosis and converge into early endosomes (EE). Class I MHC molecules are taken up by either clathrin-mediated endocytosis dependent on tyrosine-based internalization motif or poly-ubiquitination, or clathrin-independent but GTP-bound Arf6-dependent mechanism. The small GTPase Rab proteins dictate the selection of different effectors and binding partners, thereby directing cargo to distinct endosomal compartments, involving late endosomes (LE), lysosomes (lys), recycling endosomes (RE), and the endosomal recycling compartment (ERC). Rab3b and 3c are involved in rapid recycling of transferrin and are involved in cross-presentation. Rab4 together with Rab11 and Sorting Nexin 4 (SNX4) sort cargo into the ERC. Rab22a regulates class I MHC recycling via Arf6-positive tubules. Rab35 mediates recruitment of EHD1 for class I MHC recycling from early endosomes. EHD1 also colocalizes with Rab11 and its Rab11-FIP2, Arf6, and the Rab4 and Rab5 effector Rabenosyn-5. During Rab5-to-Rab7 transition, the retromer complex directs cargo to the *trans*-Golgi Network (TGN). The increased blue coloration illustrates the drop in endosomal pH. Further information and references are mentioned in the main text. = Class I MHC molecules; = peptide; = Clathrin; = Rab; = GTP-bound Arf6; = GDP-bound Arf6.

et al., 2004), and the expression of dominant active or inactive versions of the Rab22a protein, or depletion of Rab22a, impairs class I MHC recycling to the cell surface (Weigert et al., 2004). Also other members of the Rab family are pivotal to class I MHC recycling. Both Rab35 (Allaire et al., 2010) and Rab11 (Sheff et al., 1999) are implicated in recycling from the ERC to the cell surface. Rab11 in complex with its effector Rab11-FIP2 interacts with one of four known mammalian C-terminal Eps15 homology (EH) domain containing proteins (EHD1; Naslavsky et al., 2006) that all play a role in endosomal trafficking (Naslavsky and Caplan, 2011). EHD1 is essential for recycling of both clathrin-dependent and independent endocytosed molecules, including but not restricted to class I MHC (Caplan et al., 2002) and class II MHC (Walseng et al., 2008).

Next to the recycling pathway via the juxtanuclear ERC, class I MHC molecules may also be directed toward the TGN for entering the biosynthetic pathway. Retrograde transport from the endosomal compartment to the TGN involves a hetero-pentameric complex called the retromer (reviewed in Bonifacino and Hurley, 2008). Thus far, however, interaction between class I MHC molecules and retromer complexes is not reported. However, depletion of the retromer-distinct sorting nexin SNX4 results in disruption of the ERC, and miss-sorting of the transferrin receptor to lysosomes. Therefore, SNX4 appears important for shuttling selective cargo between EE and the ERC (Traer et al., 2007). In conclusion, the ERC is a highly dynamic compartment composed of vesicular and tubular membrane structures, in which proper interplay between molecules including GTPases and Rab proteins contributes to antigen cross-presentation.

DIRECTED MIGRATION ENABLED BY ASSOCIATION OF ENDOSOMAL COMPARTMENTS WITH CYTOSKELETAL ELEMENTS

Membrane-associated cargo, including class I MHC molecules, is selectively transported to distinct endosomal compartments. But what regulates the structural support necessary for endosomal trafficking?

All eukaryotic cells have a filamentous network of cellular proteins, collectively termed the cytoskeleton. It mainly comprises three distinct classes of fibers: microfilaments, microtubules, and intermediate filaments. The cytoskeleton has multiple tasks. It gives the cell its rigidity and strength that helps maintaining cell shape. Moreover, it provides tracks that allow directed movement of organelles and their transport intermediates during intracellular trafficking processes. Microtubules are major components of the cytoskeleton, and are composed of α and β tubulin heterodimers. Disruption of these microtubules perturbs Arf6-associated recycling tubules (Radhakrishna and Donaldson, 1997), and thereby may abrogate endosomal transport of peptide/class I MHC complexes.

The continuous assembly and disassembly of microtubules creates a temporal and spatial dynamic network that allows for long-range endosomal transport (Jiang and Akhmanova, 2011). This network allows directional movements of motor proteins that associate with these microtubule tracks. Kinesins and dyneins are two families of such motor proteins. Most kinesins migrate over the microtubules in plus-end direction toward the cell periphery, whereas dyneins are directed to the minus-end, toward the microtubule-organizing center (MTOC; Wubbolts et al., 1999). Various cargo-selecting molecules or complexes from the endosomal compartment are linked to the motor proteins, allowing separation and movement of endosomal vacuoles over these microtubule tracks. For instance, several Rab proteins are associated directly to motor proteins, such as Rab14 with kinesin (Ueno et al., 2011) and Rab4 with dynein (Bielli et al., 2001). Similar to SNX4, most Rab proteins are indirectly linked to motor proteins via adaptor proteins, allowing separate trafficking processes in distinct responses. For example, Rab6 can interact with Bicaudal-related protein 1 (BICDR-1) or Bicaudal D-2 to associate with kinesin-3 or 1 respectively (Grigoriev et al., 2007; Schlager et al., 2010). Thus, endosomal small GTPase activity of Rab proteins can affect motor–microtubule interaction, thereby altering the segregation or guidance of cargo transport.

Directed assembly of microtubules may also allow for polarized trafficking and delivery of membrane proteins or (soluble mediator) cargo in high concentration to one specific spot. Upon cognate interaction between an antigen presenting cell (APC) and a T cell, the cytoskeleton forms a highly organized structure called the immunological synapse (IS). The IS is a region of spatially and temporally organized, highly concentrated motifs of membrane proteins and cytosolic molecules, formed at the T cell interaction site. The formation of the IS in DC is critical for subsequent T cell activation and depends on cytoskeletal rearrangement (Pulecio et al., 2008). Perturbation of the cytoskeleton abrogates IS formation and subsequent T cell activation (Al-Alwan et al., 2001). Endosomal compartments that transport class II MHC molecules converge at the IS upon cognate DC-T cell interaction (Boes et al., 2002, 2003; Bertho et al., 2003). In addition, a recent study demonstrates that ICAM-1, an adhesion molecule involved in strengthening the DC-T cell interaction, is targeted to the IS. This occurs either via the cell surface by cytoskeleton-dependent active transport, or via RE, where it colocalizes with class II MHC molecules. The latter pathway depends on continuous endocytosis and recycling of ICAM-1. Polarization of the recycling ICAM-1 to the DC-T cell interaction site in its turn depends on the high-affinity state of the ICAM-1 binding partner LFA-1 on T cells (Jo et al., 2010). This was not unexpected, as it was described earlier that blocking LFA-1 with an antibody on antigen-specific $CD4^+$ T cells hampers remodeling of the endosomal class II MHC-containing compartment in murine DC (Bertho et al., 2003). Taken together, these data demonstrate that T cell-directed, cytoskeleton-supported recycling of antigen cargo is crucial for cellular immune responses.

In all cell types mentioned, the small GTPase CDC42 of the Rho family was shown to be responsible for MTOC polarization (Eng et al., 2007; Pulecio et al., 2010; Yuseff et al., 2011). Specifically, Pulecio et al. (2010) show that CDC42-mediated polarization mediates both MTOC polarization and directed transport of the cytokine IL-12 to the DC-T cell interaction site, which was crucial for antigen-specific $CD8^+$ T cell proliferation and IFNγ production. Yuseff et al. (2011) demonstrated that atypical PKC is a downstream target of CDC42, required for MTOC polarization. CDC42 may be responsible for MTOC relocation by a mechanism that recruits the PAR6-atypical PKC complex

to the plasma membrane in an Arf6-dependent manner, as was demonstrated to facilitate the establishment of polarity in migrating astrocytes (Etienne-Manneville and Hall, 2001; Osmani et al., 2010). Moreover, PAR6 overexpression reduced MTOC reorientation in murine macrophages (Eng et al., 2007). Taken these data together, CDC42-based polarity machinery plays an instrumental role in the polarization of the microtubule network and influences the direction of RE and other microtubule-associated trafficking.

IMPLICATIONS TO HUMAN INFECTIOUS DISEASES

Abrogated transport or recycling of class I and class II MHC complexes leads to immune-related disorders, as might be expected considering their importance in the initiation of immune- or tolerogenic responses. Indeed, an inefficient MHC transport leads to severe combined immunodeficiency as in patients with Bare Lymphocyte Syndrome type I. This disease can be caused by mutation in the TAP1, TAP2, or TAPBP genes, all leading to inefficient peptide/class I MHC transport and ultimately decreased cell surface expression (Salle de la et al., 1994). Hampering the transport of peptide/MHC complexes also plays a major role in viral infections. Herpes and Pox viruses can evade immune responses. They do this by several mechanisms including preventing the presentation of newly synthesized class I MHC molecules at the cell surface by blocking peptide translocation via TAP, block of peptide loading, retention of MHC/peptide complexes in the ER or their retrograde translocation into the cytosol for degradation, as illustrated in **Figure 2** (van der Wal et al., 2002; Hansen and Bouvier, 2009; Horst et al., 2011). Increased MHC internalization also limits plasma membrane displayed peptide/class I MHC complexes and subsequent T cell activation (Zuo et al., 2011). Thus far no inborn mutations are known that correlate with mechanisms of MHC recycling or degradation. However, pathogens developed immune evasion strategies that interfere with endosomal transport of MHC or its recycling from the plasma membrane, with possible implications to antigen cross-presentation.

For example, EBV-derived BILF-1 and the previously mentioned K3 and K5 of KSHV decrease class I MHC surface expression by increasing its internalization, aiding the virus in escaping the immune system (Coscoy and Ganem, 2000; Ishido et al., 2000; Lorenzo et al., 2002; Mansouri et al., 2006; Zuo et al., 2011). HIV uses multiple strategies to evade the immune system (Roeth et al., 2004; Dikeakos et al., 2010). HIV-1 expresses

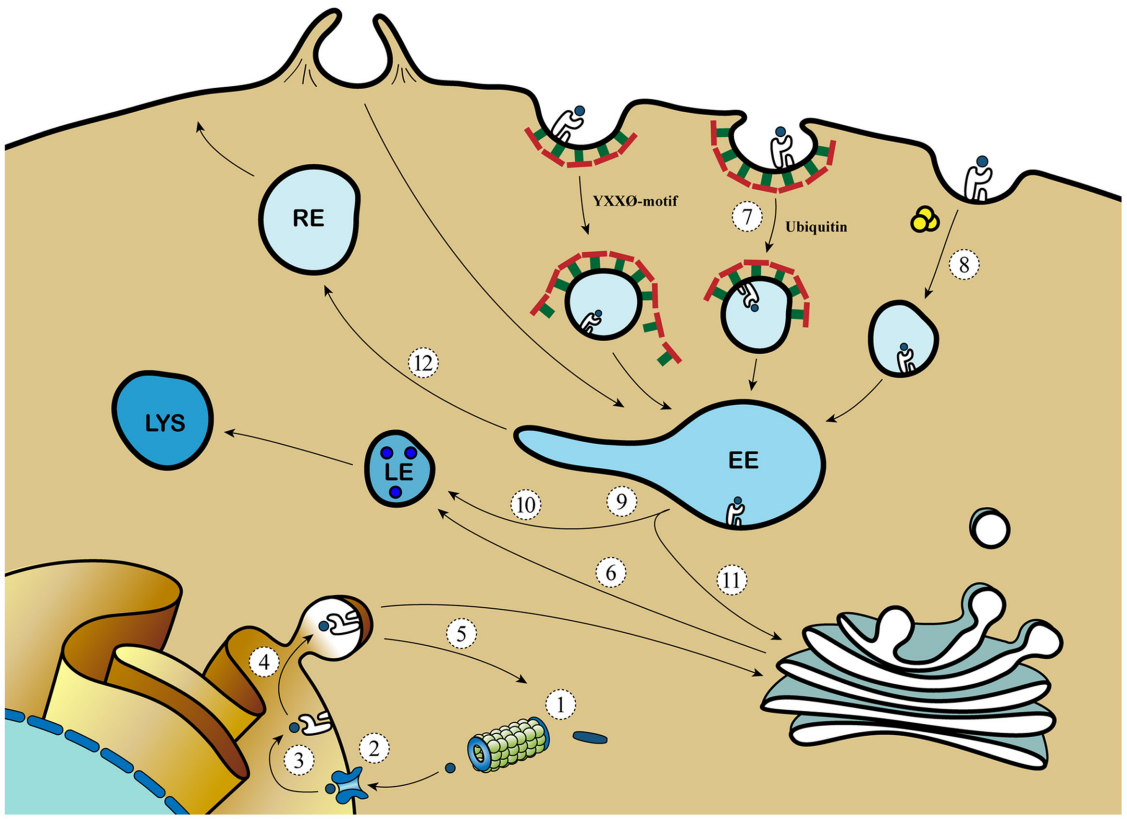


FIGURE 2 | Viral evasion strategies aim at different pathways of MHC transport. 1: Inhibition of proteasomal processing (EBV); 2: Inhibition of TAP (EBV, HSV, CMV); 3: Inhibition of peptide transport (EBV, CMV, adeno); 4: Retaining MHC molecules in the ER (adeno, cowpox); 5: Target MHC for proteasomal degradation (CMV); 6: Target MHC from TGN to endo/lysosomal compartments (HIV); 7: Clathrin-dependent MHC internalization (EBV, KSHV, HIV, CMV); 8: Clathrin independent MHC internalization (HIV); 9: Arresting phagosomal maturation (mycobacteria, *Salmonella*, *Chlamydia*, and *Leishmania*); 10: Targeting MHC to the LE/lysosome (HSV); 11: Inhibition of retromer activity (HVS); 12: Inhibition of progression to the RE (CMV, HSV). = protein; = peptide; =TAP; = Clathrin; = Class I MHC molecules; = proteasome; = GTP-bound ARF6.

the Nef protein that targets newly synthesized class I MHC from the TGN to the destructive lysosomal compartments, thereby preventing cell surface expression of peptide/class I MHC. Moreover, Nef increases the turnover of MHC surface molecules by targeting MHC to lysosomes via the clathrin-dependent retromer-mediated pathway (Peterlin and Trono, 2003). Additionally, Nef and host-derived sorting protein PACS1 interfere with MHC recycling by targeting and retaining MHC from the plasma membrane in the TGN via the previously described clathrin-independent ARF6 endocytic pathway (Blagoveshchenskaya et al., 2002). Recycling of MHC is also abrogated by Murine Cytomegalovirus, which induces an arrest of MHC in EE (Tomas et al., 2010). The retromer is also targeted for immune evasion as was reported for Herpesvirus Saimiri (HVS) infection. HVS-derived tyrosine kinase-interacting protein binds and redistributes the retromer subunit Vps35 from the EE to lysosomes, thereby inhibiting retromer activity. This is physiologically linked to $CD4^+$ T cell downregulation and immortalization (Kingston et al., 2011), but possible retromer-targeted effects by HVS on cross-presentation remain to be shown.

The enormous number of viruses targeting peptide/MHC expression and endosomal trafficking illustrates its crucial role in anti-viral responses. Bacteria can also use these mechanisms to create an environment in which they can thrive (Duclos and Desjardins, 2000). Intracellular pathogens can replicate in vacuoles that retain an elevated pH, show limited hydrolytic activity, and intersect poorly with antigen presentation pathways. To achieve this, pathogens such as Mycobacteria hamper phagosome–lysosome fusion and *Salmonella* delays vacuolar acidification, thereby inducing arrest of phagosomal maturation (Jantsch et al., 2011; Johansen et al., 2011). Bacteria can also use endosomal remodeling and recycling for their own benefit. For example, Rab14 and syntaxin 6, which are together with IRAP involved in cross-presentation (Saveanu et al., 2009; Weimershaus et al., 2012), are recruited to Chlamydial inclusion vacuoles (Capmany et al., 2011; Moore et al., 2011). Also, a recent investigation demonstrates that *Salmonella* induces kinesin activity by the expression of Arl8B, an Arf family member (Kaniuk et al., 2011). Concomitant with increased kinesin activity, endosomal remodeling into tubular-like structures is promoted by Arl8B (Kaniuk et al., 2011), thereby creating an opportunity for *Salmonella* to transfer from cell to cell.

In summary, peptide/MHC surface expression is pivotal in initiating T cell responses and is therefore an important target in pathogen evasion strategies. Pathogens interfere with endosomal transport of MHC molecules to the plasma membrane, internalization of MHC and subsequent recycling or degradation. Knowledge of these processes is important in therapeutic interventions aiming at clearance of infections via appropriately activated MHC-restricted T cell responses. Drugs specifically targeting viral evasion molecules could re-establish proper peptide/MHC presentation, thereby allowing the immune system to clear the virus. Secondly, it is important to clarify evasion strategies employed by prevalent pathogens in future cellular vaccination developments, e.g., DC-based vaccine strategies, since such evasion could impair vaccine effectiveness.

CONCLUDING REMARKS

Efficient cross-presentation of $CD8^+$ T cells that initiates balanced anti-viral and anti-tumor immune responses depends on DC-intrinsic mechanisms that enable the sequential interaction of specific TCR molecules with peptide/MHC complexes in the context of activating or inhibiting (tolerogenic) signals. The molecular mechanisms described in this review all aid to ensure the quantity and quality of this DC-derived signal toward the $CD8^+$ T cells.

Antigen recognition by specific receptors permits the selective and rapid retrieval of antigens present in the extracellular fluid, focusing the antigen pool that is directed toward cross-presentation. Additionally, targeting antigen to specific receptors allow it to target toward superior cross-presenting DC subsets (Sancho et al., 2009), or to overcome prior incapability of antigen cross-presentation (Klechevsky et al., 2010). Not surprisingly, targeting antigen increases antigen cross-presentation *in vivo* (National Library of Medicine US, 2010; Flynn et al., 2011) and is currently used in first phase clinical trials (140, DCVax-001). It now appears that next to efficient antigen uptake, receptor selection is instrumental for antigen delivery to cross-presentation competent compartments. Antigen introduction, as well as co-presence of "danger signals" appears to optimize, at least in some circumstances, the capability of selective endosomal compartments to support antigen cross-presentation, by recruitment of necessary components for cross-presentation (Naslavsky et al., 2006; Jancic et al., 2007; Burgdorf et al., 2008; Kim et al., 2008).

Dendritic cells maturation is accompanied by dramatic changes in cell shape. Since the cytoskeleton is responsible for cell shape, danger signaling is likely involved in cytoskeletal reorganization. Indeed, Toll-like receptor 4 (TLR4) signaling induces actin cytoskeleton remodeling in a MyD88-dependent manner (West et al., 2004). In addition, MTOC reorientation in DC by binding of antigen-specific T cells required TLR signaling (Pulecio et al., 2010). However, this could be an indirect effect due to the fact that mature DC form more stable synapses than immature DC (Benvenuti et al., 2004). Moreover, innate signals via MyD88 are demonstrated in murine DC to remodel the late endosomal compartment in which class II MHC peptide loading occurs (Boes et al., 2002). All together, these data demonstrate a beneficial role for innate signaling in presentation of antigens. However, exact molecular mechanisms that link innate signaling with directed cargo transportation remain elusive.

A large amount of viral immune evasion strategies generated by the evolutionary pressure of the endosomal recycling pathway on anti-viral responses suggests that efficient recycling of class I MHC molecules is essential for an effective $CD8^+$ T cell response. An exon7-deleted variant of class I MHC clearly demonstrates that only a small delay in class I MHC recycling greatly affects $CD8^+$ T cell responses (Rodriguez-Cruz et al., 2011): antigen cross-presentation by exon7-deleted class I MHC molecules-expressing cells results in more robust $CD8^+$ T cell responses.

The quality of the MHC/TCR interaction (e.g., signal 1) affects DC-$CD8^+$ T cell interaction strength, thereby affecting $CD8^+$ T effector function (Bouma et al., 2011), memory differentiation (Teixeiro et al., 2009) and survival (Iezzi et al.,

1999). Hence, primary immune deficiencies with defective quality of signal 1, such as Wiskott-Aldrich syndrome and DOCK8 immunodeficiency patients, share clinical characteristics (e.g., eczema, elevated IgE levels, cutaneous M. *contagiosum* or Papilloma and Herpes viral infections, and increased tumor incidence (Bosticardo et al., 2009; Zhang et al., 2009). Both Dock8 and WAS protein are important for T cell synapse formation (Dupre et al., 2002; Randall et al., 2011), and crucial for interactions between naive $CD8^+$ T cells and DC (Pulecio et al., 2008; Randall et al., 2011). Thus, the endosomal recycling pathway may prove of importance for antigen cross-presentation and prevention of correlated diseases via distinct mechanisms, some of which are outlined above, and likely with more to be uncovered in the years to come.

ACKNOWLEDGMENTS

We acknowledge financial support from the Wilhelmina Research Fund and an NWO/NGI Horizon grant (to Marianne Boes). We thank Michiel de Jong (www.TomatoesandPotatoes.com) for his aid to design the figures. We additionally thank Henk Schipper and Robert van der Burgh for critical reading of the manuscript, the members of the Boes laboratory, and members of the Center for Molecular and Cellular Intervention (CMCI) for helpful discussions.

REFERENCES

Ackerman, A. L., Giodini, A., and Cresswell, P. (2006). A role for the endoplasmic reticulum protein retrotranslocation machinery during crosspresentation by dendritic cells. *Immunity* 25, 607–617.

Ackerman, A. L., Kyritsis, C., Tampe, R., and Cresswell, P. (2003). Early phagosomes in dendritic cells form a cellular compartment sufficient for cross presentation of exogenous antigens. *Proc. Natl. Acad. Sci. U.S.A.* 100, 12889–12894.

Ackerman, A. L., Kyritsis, C., Tampe, R., and Cresswell, P. (2005). Access of soluble antigens to the endoplasmic reticulum can explain cross-presentation by dendritic cells. *Nat. Immunol.* 6, 107–113.

Al-Alwan, M. M., Rowden, G., Lee, T. D., and West, K. A. (2001). The dendritic cell cytoskeleton is critical for the formation of the immunological synapse. *J. Immunol.* 166, 1452–1456.

Albert, M. L., Jegathesan, M., and Darnell, R. B. (2001). Dendritic cell maturation is required for the cross-tolerization of CD8+ T cells. *Nat. Immunol.* 2, 1010–1017.

Allaire, P. D., Marat, A. L., Dall'Armi, C., Di, P. G., McPherson, P. S., and Ritter, B. (2010). The connecdenn DENN domain: a GEF for Rab35 mediating cargo-specific exit from early endosomes. *Mol. Cell* 37, 370–382.

Bachem, A., Guttler, S., Hartung, E., Ebstein, F., Schaefer, M., Tannert, A., Salama, A., Movassaghi, K., Opitz, C., Mages, H. W., Henn, V., Kloetzel, P. M., Gurka, S., and Kroczek, R. A. (2010). Superior antigen cross-presentation and XCR1 expression define human CD11c+ CD141+ cells as homologues of mouse CD8+ dendritic cells. *J. Exp. Med.* 207, 1273–1281.

Bagai, R., Valujskikh, A., Canaday, D. H., Bailey, E., Lalli, P. N., Harding, C. V., and Heeger, P. S. (2005). Mouse endothelial cells cross-present lymphocyte-derived antigen on class I MHC via a. *J. Immunol.* 174, 7711–7715.

Bartee, E., Mansouri, M., Hovey Nerenberg, B. T., Gouveia, K., and Fruh, K. (2004). Downregulation of major histocompatibility complex class I by human ubiquitin ligases related to viral immune evasion proteins. *J. Virol.* 78, 1109–1120.

Basha, G., Lizee, G., Reinicke, A. T., Seipp, R. P., Omilusik, K. D., and Jefferies, W. A. (2008). MHC class I endosomal and lysosomal trafficking coincides with exogenous antigen loading in dendritic cells. *PLoS ONE* 3, e3247. doi:10.1371/journal.pone.0003247

Beauvillain, C., Delneste, Y., Scotet, M., Peres, A., Gascan, H., Guermonprez, P., Barnaba, V., and Jeannin, P. (2007). Neutrophils efficiently cross-prime naive T cells in vivo. *Blood* 110, 2965–2973.

Belizaire, R., and Unanue, E. R. (2009). Targeting proteins to distinct subcellular compartments reveals unique requirements for MHC class I and II presentation. *Proc. Natl. Acad. Sci. U.S.A.* 106, 17463–17468.

Benvenuti, F., Lagaudriere-Gesbert, C., Grandjean, I., Jancic, C., Hivroz, C., Trautmann, A., Lantz, O., and Amigorena, S. (2004). Dendritic cell maturation controls adhesion, synapse formation, and the duration of the interactions with naive T lymphocytes. *J. Immunol.* 172, 292–301.

Bertho, N., Cerny, J., Kim, Y. M., Fiebiger, E., Ploegh, H., and Boes, M. (2003). Requirements for T cell-polarized tubulation of class II+ compartments in dendritic cells. *J. Immunol.* 171, 5689–5696.

Bevan, M. J. (1976). Cross-priming for a secondary cytotoxic response to minor H antigens with H-2 congenic cells which do not cross-react in the cytotoxic assay. *J. Exp. Med.* 143, 1283–1288.

Bielli, A., Thornqvist, P. O., Hendrick, A. G., Finn, R., Fitzgerald, K., and McCaffrey, M. W. (2001). The small GTPase Rab4A interacts with the central region of cytoplasmic dynein light intermediate chain-1. *Biochem. Biophys. Res. Commun.* 281, 1141–1153.

Blagoveshchenskaya, A. D., Thomas, L., Feliciangeli, S. F., Hung, C. H., and Thomas, G. (2002). HIV-1 Nef downregulates MHC-I by a PACS-1- and PI3K-regulated ARF6 endocytic pathway. *Cell* 111, 853–866.

Boes, M., Bertho, N., Cerny, J., Op den, B. M., Kirchhausen, T., and Ploegh, H. (2003). T cells induce extended class II MHC compartments in dendritic cells in a Toll-like receptor-dependent manner. *J. Immunol.* 171, 4081–4088.

Boes, M., Cerny, J., Massol, R., Op den, B. M., Kirchhausen, T., Chen, J., and Ploegh, H. L. (2002). T-cell engagement of dendritic cells rapidly rearranges MHC class II transport. *Nature* 418, 983–988.

Bonifacino, J. S., and Dell'Angelica, E. C. (1999). Molecular bases for the recognition of tyrosine-based sorting signals. *J. Cell Biol.* 145, 923–926.

Bonifacino, J. S., and Hurley, J. H. (2008). Retromer. *Curr. Opin. Cell Biol.* 20, 427–436.

Bonifaz, L., Bonnyay, D., Mahnke, K., Rivera, M., Nussenzweig, M. C., and Steinman, R. M. (2002). Efficient targeting of protein antigen to the dendritic cell receptor DEC-205 in the steady state leads to antigen presentation on major histocompatibility complex class I products and peripheral CD8+ T cell tolerance. *J. Exp. Med.* 196, 1627–1638.

Bosticardo, M., Marangoni, F., Aiuti, A., Villa, A., and Grazia, R. M. (2009). Recent advances in understanding the pathophysiology of Wiskott-Aldrich syndrome. *Blood* 113, 6288–6295.

Bouma, G., Mendoza-Naranjo, A., Blundell, M. P., de, F. E., Parsley, K. L., Burns, S. O., and Thrasher, A. J. (2011). Cytoskeletal remodeling mediated by WASp in dendritic cells is necessary for normal immune synapse formation and T-cell priming. *Blood* 118, 2492–2501.

Bozzacco, L., Trumpfheller, C., Siegal, F. P., Mehandru, S., Markowitz, M., Carrington, M., Nussenzweig, M. C., Piperno, A. G., and Steinman, R. M. (2007). DEC-205 receptor on dendritic cells mediates presentation of HIV gag protein to CD8+ T cells in a spectrum of human MHC I haplotypes. *Proc. Natl. Acad. Sci. U.S.A.* 104, 1289–1294.

Brandes, M., Willimann, K., Bioley, G., Levy, N., Eberl, M., Luo, M., Tampe, R., Levy, F., Romero, P., and Moser, B. (2009). Cross-presenting human gammadelta T cells induce robust CD8+ alphabeta T cell responses. *Proc. Natl. Acad. Sci. U.S.A.* 106, 2307–2312.

Brown, F. D., Rozelle, A. L., Yin, H. L., Balla, T., and Donaldson, J. G. (2001). Phosphatidylinositol 4,5-bisphosphate and Arf6-regulated membrane traffic. *J. Cell Biol.* 154, 1007–1017.

Brown, M. S., Ho, Y. K., and Goldstein, J. L. (1976). The low-density lipoprotein pathway in human fibroblasts: relation between cell surface receptor binding and endocytosis of low-density lipoprotein. *Ann. N. Y. Acad. Sci.* 275, 244–257.

Burgdorf, S., Scholz, C., Kautz, A., Tampe, R., and Kurts, C. (2008). Spatial and mechanistic separation of cross-presentation and endogenous antigen presentation. *Nat. Immunol.* 9, 558–566.

Caplan, S., Naslavsky, N., Hartnell, L. M., Lodge, R., Polishchuk, R. S., Donaldson, J. G., and Bonifacino, J. S. (2002). A tubular EHD1-containing compartment involved in the recycling of major histocompatibility complex class I molecules to the plasma membrane. *EMBO J.* 21, 2557–2567.

Capmany, A., Leiva, N., and Damiani, M. T. (2011). Golgi-associated Rab14, a new regulator for *Chlamydia trachomatis* infection outcome. *Commun. Integr. Biol.* 4, 590–593.

Cebrian, I., Visentin, G., Blanchard, N., Jouve, M., Bobard, A., Moita, C., Enninga, J., Moita, L. F., Amigorena, S., and Savina, A. (2011). Sec22b regulates phagosomal maturation and antigen crosspresentation by dendritic cells. *Cell* 74, 1355–1368.

Coscoy, L., and Ganem, D. (2000). Kaposi's sarcoma-associated herpesvirus encodes two proteins that block cell surface display of MHC class I chains by enhancing their endocytosis. *Proc. Natl. Acad. Sci. U.S.A.* 97, 8051–8056.

Crozat, K., Guiton, R., Contreras, V., Feuillet, V., Dutertre, C. A., Ventre, E., Vu Manh, T. P., Baranek, T., Storset, A. K., Marvel, J., Boudinot, P., Hosmalin, A., Schwartz-Cornil, I., and Dalod, M. (2010). The XC chemokine receptor 1 is a conserved selective marker of mammalian cells homologous to mouse CD8alpha+ dendritic cells. *J. Exp. Med.* 207, 1283–1292.

den Haan, J. M., Lehar, S. M., and Bevan, M. J. (2000). CD8(+) but not CD8(−) dendritic cells cross-prime cytotoxic T cells in vivo. *J. Exp. Med.* 192, 1685–1696.

Di Pucchio, T., Chatterjee, B., Smed-Sorensen, A., Clayton, S., Palazzo, A., Montes, M., Xue, Y., Mellman, I., Banchereau, J., and Connolly, J. E. (2008). Direct proteasome-independent cross-presentation of viral antigen by plasmacytoid dendritic cells on major histocompatibility complex class I. *Nat. Immunol.* 9, 551–557.

Dikeakos, J. D., Atkins, K. M., Thomas, L., Emert-Sedlak, L., Byeon, I. J., Jung, J., Ahn, J., Wortman, M. D., Kukull, B., Saito, M., Koizumi, H., Williamson, D. M., Hiyoshi, M., Barklis, E., Takiguchi, M., Suzu, S., Gronenborn, A. M., Smithgall, T. E., and Thomas, G. (2010). Small molecule inhibition of HIV-1-induced MHC-I down-regulation identifies a temporally regulated switch in Nef action. *Mol. Biol. Cell* 21, 3279–3292.

Doherty, P. C., and Zinkernagel, R. M. (1975). H-2 compatibility is required for T-cell-mediated lysis of target cells infected with lymphocytic choriomeningitis virus. *J. Exp. Med.* 141, 502–507.

Duclos, S., and Desjardins, M. (2000). Subversion of a young phagosome: the survival strategies of intracellular pathogens. *Cell. Microbiol.* 2, 365–377.

Dudziak, D., Kamphorst, A. O., Heidkamp, G. F., Buchholz, V. R., Trumpfheller, C., Yamazaki, S., Cheong, C., Liu, K., Lee, H. W., Park, C. G., Steinman, R. M., and Nussenzweig, M. C. (2007). Differential antigen processing by dendritic cell subsets in vivo. *Science* 315, 107–111.

Duncan, L. M., Piper, S., Dodd, R. B., Saville, M. K., Sanderson, C. M., Luzio, J. P., and Lehner, P. J. (2006). Lysine-63-linked ubiquitination is required for endolysosomal degradation of class I molecules. *EMBO J.* 25, 1635–1645.

Dupre, L., Aiuti, A., Trifari, S., Martino, S., Saracco, P., Bordignon, C., and Roncarolo, M. G. (2002). Wiskott-Aldrich syndrome protein regulates lipid raft dynamics during immunological synapse formation. *Immunity* 17, 157–166.

Eng, E. W., Bettio, A., Ibrahim, J., and Harrison, R. E. (2007). MTOC reorientation occurs during FcgammaR-mediated phagocytosis in macrophages. *Mol. Biol. Cell* 18, 2389–2399.

Etienne-Manneville, S., and Hall, A. (2001). Integrin-mediated activation of Cdc42 controls cell polarity in migrating astrocytes through PKCzeta. *Cell* 106, 489–498.

Flinsenberg, T. W., Compeer, E. B., Boelens, J. J., and Boes, M. (2011). Antigen cross-presentation: extending recent laboratory findings to therapeutic intervention. *Clin. Exp. Immunol.* 165, 8–18.

Flynn, B. J., Kastenmuller, K., Wille-Reece, U., Tomaras, G. D., Alam, M., Lindsay, R. W., Salazar, A. M., Perdiguero, B., Gomez, C. E., Wagner, R., Esteban, M., Park, C. G., Trumpfheller, C., Keler, T., Pantaleo, G., Steinman, R. M., and Seder, R. (2011). Immunization with HIV Gag targeted to dendritic cells followed by recombinant New York vaccinia virus induces robust T-cell immunity in nonhuman primates. *Proc. Natl. Acad. Sci. U.S.A.* 108, 7131–7136.

Fonteneau, J. F., Kavanagh, D. G., Lirvall, M., Sanders, C., Cover, T. L., Bhardwaj, N., and Larsson, M. (2003). Characterization of the MHC class I cross-presentation pathway for cell-associated antigens by human dendritic cells. *Blood* 102, 4448–4455.

Gassart de, A., Camosseto, V., Thibodeau, J., Ceppi, M., Catalan, N., Pierre, P., and Gatti, E. (2008). MHC class II stabilization at the surface of human dendritic cells is the result of maturation-dependent MARCH I down-regulation. *Proc. Natl. Acad. Sci. U.S.A.* 105, 3491–3496.

Graham, D. B., Akilesh, H. M., Gmyrek, G. B., Piccio, L., Gilfillan, S., Sim, J., Belizaire, R., Carrero, J. A., Wang, Y., Blaufuss, G. S., Sandoval, G., Fujikawa, K., Cross, A. H., Russell, J. H., Cella, M., and Swat, W. (2010). ITAM signaling in dendritic cells controls T helper cell priming by regulating MHC class II recycling. *Blood* 116, 3208–3218.

Graham, D. B., Stephenson, L. M., Lam, S. K., Brim, K., Lee, H. M., Bautista, J., Gilfillan, S., Akilesh, S., Fujikawa, K., and Swat, W. (2007). An ITAM-signaling pathway controls cross-presentation of particulate but not soluble antigens in dendritic cells. *J. Exp. Med.* 204, 2889–2897.

Grigoriev, I., Splinter, D., Keijzer, N., Wulf, P. S., Demmers, J., Ohtsuka, T., Modesti, M., Maly, I. V., Grosveld, F., Hoogenraad, C. C., and Akhmanova, A. (2007). Rab6 regulates transport and targeting of exocytotic carriers. *Dev. Cell* 13, 305–314.

Gromme, M., Uytdehaag, F. G., Janssen, H., Calafat, J., van Binnendijk, R. S., Kenter, M. J., Tulp, A., Verwoerd, D., and Neefjes, J. (1999). Recycling MHC class I molecules and endosomal peptide loading. *Proc. Natl. Acad. Sci. U.S.A.* 96, 10326–10331.

Guermonprez, P., Saveanu, L., Kleijmeer, M., Davoust, J., van, E. P., and Amigorena, S. (2003). ER-phagosome fusion defines an MHC class I cross-presentation compartment in dendritic cells. *Nature* 425, 397–402.

Hansen, T. H., and Bouvier, M. (2009). MHC class I antigen presentation: learning from viral evasion strategies. *Nat. Rev. Immunol.* 9, 503–513.

Hayakawa, A., Hayes, S., Leonard, D., Lambright, D., and Corvera, S. (2007). Evolutionarily conserved structural and functional roles of the FYVE domain. *Biochem. Soc. Symp.* 74, 95–105.

Heath, W. R., and Carbone, F. R. (2001). Cross-presentation in viral immunity and self-tolerance. *Nat. Rev. Immunol.* 1, 126–134.

Heit, A., Huster, K. M., Schmitz, F., Schiemann, M., Busch, D. H., and Wagner, H. (2004). CpG-DNA aided cross-priming by cross-presenting B cells. *J. Immunol.* 172, 1501–1507.

Hoeffel, G., Ripoche, A. C., Matheoud, D., Nascimbeni, M., Escriou, N., Lebon, P., Heshmati, F., Guillet, J. G., Gannage, M., Caillat-Zucman, S., Casartelli, N., Schwartz, O., De la, S. H., Hanau, D., Hosmalin, A., and Maranon, C. (2007). Antigen crosspresentation by human plasmacytoid dendritic cells. *Immunity* 27, 481–492.

Horst, D., Verweij, M. C., Davison, A. J., Ressing, M. E., and Wiertz, E. J. (2011). Viral evasion of T cell immunity: ancient mechanisms offering new applications. *Curr. Opin. Immunol.* 23, 96–103.

Houde, M., Bertholet, S., Gagnon, E., Brunet, S., Goyette, G., Laplante, A., Princiotta, M. F., Thibault, P., Sacks, D., and Desjardins, M. (2003). Phagosomes are competent organelles for antigen cross-presentation. *Nature* 425, 402–406.

Iezzi, G., Scotet, E., Scheidegger, D., and Lanzavecchia, A. (1999). The interplay between the duration of TCR and cytokine signaling determines T cell polarization. *Eur. J. Immunol.* 29, 4092–4101.

Ishido, S., Wang, C., Lee, B. S., Cohen, G. B., and Jung, J. U. (2000). Downregulation of major histocompatibility complex class I molecules by Kaposi's sarcoma-associated herpesvirus K3 and K5 proteins. *J. Virol.* 74, 5300–5309.

Jancic, C., Savina, A., Wasmeier, C., Tolmachova, T., El-Benna, J., Dang, P. M., Pascolo, S., Gougerot-Pocidalo, M. A., Raposo, G., Seabra, M. C., and Amigorena, S. (2007). Rab27a regulates phagosomal pH and NADPH oxidase recruitment to dendritic cell phagosomes. *Nat. Cell Biol.* 9, 367–378.

Jantsch, J., Chikkaballi, D., and Hensel, M. (2011). Cellular aspects of immunity to intracellular *Salmonella enterica*. *Immunol. Rev.* 240, 185–195.

Jiang, K., and Akhmanova, A. (2011). Microtubule tip-interacting proteins: a view from both ends. *Curr. Opin. Cell Biol.* 23, 94–101.

Jo, J. H., Kwon, M. S., Choi, H. O., Oh, H. M., Kim, H. J., and Jun, C. D. (2010). Recycling and LFA-1-dependent trafficking of ICAM-1 to the immunological synapse. *J. Cell. Biochem.* 111, 1125–1137.

Johansen, P., Fettelschoss, A., Amstutz, B., Selchow, P., Waeckerle-Men, Y., Keller, P., Deretic, V., Held, L., Kundig, T. M., Bottger, E. C., and Sander, P. (2011). Relief from Zmp1-mediated arrest of phagosome maturation is associated with facilitated presentation and enhanced immunogenicity of mycobacterial antigens. *Clin. Vaccine Immunol.* 18, 907–913.

Jongbloed, S. L., Kassianos, A. J., McDonald, K. J., Clark, G. J., Ju, X., Angel, C. E., Chen, C. J., Dunbar, P. R., Wadley, R. B., Jeet, V., Vulink, A. J., Hart, D. N., and Radford, K. J. (2010). Human CD141+ (BDCA-3)+ dendritic cells (DCs) represent a unique myeloid DC subset that cross-presents necrotic cell antigens. *J. Exp. Med.* 207, 1247–1260.

Jovic, M., Kieken, F., Naslavsky, N., Sorgen, P. L., and Caplan, S. (2009). Eps15 homology domain 1-associated tubules contain phosphatidylinositol-4-phosphate and phosphatidylinositol-(4,5)-bisphosphate and are required for efficient recycling. *Mol. Biol. Cell* 20, 2731–2743.

Jung, S., Unutmaz, D., Wong, P., Sano, G., De los, S. K., Sparwasser, T., Wu, S., Vuthoori, S., Ko, K., Zavala, F., Pamer, E. G., Littman, D. R., and Lang, R. A. (2002). In vivo depletion of CD11c+ dendritic cells abrogates priming of CD8+ T cells by exogenous cell-associated antigens. *Immunity* 17, 211–220.

Kaniuk, N. A., Canadien, V., Bagshaw, R. D., Bakowski, M., Braun, V., Landekic, M., Mitra, S., Huang, J., Heo, W. D., Meyer, T., Pelletier, L., ndrews-Polymenis, H., McClelland, M., Pawson, T., Grinstein, S., and Brumell, J. H. (2011). *Salmonella* exploits Arl8B-directed kinesin activity to promote endosome tubulation and cell-to-cell transfer. *Cell. Microbiol.* 13, 1812–1823.

Kim, S., Shen, T., and Min, B. (2009). Basophils can directly present or cross-present antigen to CD8 lymphocytes and alter CD8 T cell differentiation into IL-10-producing phenotypes. *J. Immunol.* 183, 3033–3039.

Kim, S. H., Visser, A., Cruijsen, C., van, d., and V, Boes, M. (2008). Recruitment of Rab27a to phagosomes controls microbial antigen cross-presentation by dendritic cells. *Infect. Immun.* 76, 5373–5380.

Kingston, D., Chang, H., Ensser, A., Lee, H. R., Lee, J., Lee, S. H., Jung, J. U., and Cho, N. H. (2011). Inhibition of retromer activity by Herpesvirus Saimiri tip leads to CD4 downregulation and efficient T cell transformation. *J. Virol.* 85, 10627–10638.

Klechevsky, E., Flamar, A. L., Cao, Y., Blanck, J. P., Liu, M., O'Bar, A., Gouna-Deciat, O., Klucar, P., Thompson-Snipes, L., Zurawski, S., Reiter, Y., Palucka, A. K., Zurawski, G., and Banchereau, J. (2010). Cross-priming CD8+ T cells by targeting antigens to human dendritic cells through DCIR. *Blood* 116, 1685–1697.

Kleijmeer, M. J., Escola, J. M., Uytdehaag, F. G., Jakobson, E., Griffith, J. M., Osterhaus, A. D., Stoorvogel, W., Melief, C. J., Rabouille, C., and Geuze, H. J. (2001). Antigen loading of MHC class I molecules in the endocytic tract. *Traffic* 2, 124–137.

Kovacsovics-Bankowski, M., Clark, K., Benacerraf, B., and Rock, K. L. (1993). Efficient major histocompatibility complex class I presentation of exogenous antigen upon phagocytosis by macrophages. *Proc. Natl. Acad. Sci. U.S.A.* 90, 4942–4946.

Kovacsovics-Bankowski, M., and Rock, K. L. (1995). A phagosome-to-cytosol pathway for exogenous antigens presented on MHC class I molecules. *Science* 267, 243–246.

Kurts, C., Heath, W. R., Carbone, F. R., Allison, J., Miller, J. F., and Kosaka, H. (1996). Constitutive class I-restricted exogenous presentation of self antigens in vivo. *J. Exp. Med.* 184, 923–930.

Levy, F., Burri, L., Morel, S., Peitrequin, A. L., Levy, N., Bachi, A., Hellman, U., Van den Eynde, B. J., and Servis, C. (2002). The final N-terminal trimming of a subaminoterminal proline-containing HLA class I-restricted antigenic peptide in the cytosol is mediated by two peptidases. *J. Immunol.* 169, 4161–4171.

Limmer, A., Ohl, J., Kurts, C., Ljunggren, H. G., Reiss, Y., Groettrup, M., Momburg, F., Arnold, B., and Knolle, P. A. (2000). Efficient presentation of exogenous antigen by liver endothelial cells to CD8+ T cells results in antigen-specific T-cell tolerance. *Nat. Med.* 6, 1348–1354.

Lin, M. L., Zhan, Y., Villadangos, J. A., and Lew, A. M. (2008). The cell biology of cross-presentation and the role of dendritic cell subsets. *Immunol. Cell Biol.* 86, 353–362.

Lizee, G., Basha, G., and Jefferies, W. A. (2005). Tails of wonder: endocytic-sorting motifs key for exogenous antigen presentation. *Trends Immunol.* 26, 141–149.

Lizee, G., Basha, G., Tiong, J., Julien, J. P., Tian, M., Biron, K. E., and Jefferies, W. A. (2003). Control of dendritic cell cross-presentation by the major histocompatibility complex class I cytoplasmic domain. *Nat. Immunol.* 4, 1065–1073.

Lorenzo, M. E., Jung, J. U., and Ploegh, H. L. (2002). Kaposi's sarcoma-associated herpesvirus K3 utilizes the ubiquitin-proteasome system in routing class major histocompatibility complexes to late endocytic compartments. *J. Virol.* 76, 5522–5531.

Mansouri, M., Douglas, J., Rose, P. P., Gouveia, K., Thomas, G., Means, R. E., Moses, A. V., and Fruh, K. (2006). Kaposi sarcoma herpesvirus K5 removes CD31/PECAM from endothelial cells. *Blood* 108, 1932–1940.

Mant, A., Chinnery, F., Elliott, T., and Williams, A. P. (2012). The pathway of cross-presentation is influenced by the particle size of phagocytosed antigen. *Immunology*. doi: 10.1111/j.1365-2567.2012.03558.x. [Epub ahead of print].

Mayor, S., and Pagano, R. E. (2007). Pathways of clathrin-independent endocytosis. *Nat. Rev. Mol. Cell Biol.* 8, 603–612.

Moore, E. R., Mead, D. J., Dooley, C. A., Sager, J., and Hackstadt, T. (2011). The trans-Golgi SNARE syntaxin 6 is recruited to the chlamydial inclusion membrane. *Microbiology* 157, 830–838.

Naslavsky, N., and Caplan, S. (2011). EHD proteins: key conductors of endocytic transport. *Trends Cell Biol.* 21, 122–131.

Naslavsky, N., Rahajeng, J., Sharma, M., Jovic, M., and Caplan, S. (2006). Interactions between EHD proteins and Rab11-FIP2: a role for EHD3 in early endosomal transport. *Mol. Biol. Cell* 17, 163–177.

National Library of Medicine US. (2010). *A Randomized, Placebo-Controlled, Dose-Escalating, Double-Blinded Phase I Study to Evaluate the Safety and Immunogenicity of Anti-DEC-205 Monoclonal Antibody (Mab) Targeted HIV Gag p24 Vaccine (DCVax-001) with Poly ICLC (Hiltonol) as Adjuvant in HIV-Uninfected Healthy Volunteers.* Bethesda, MD: ClinicalTrials. gov. [May, 19; Identifier: NCT01127464; Internet]. Available at: http://clinicaltrials.gov/ct2/show/NCT01127464

Osmani, N., Peglion, F., Chavrier, P., and Etienne-Manneville, S. (2010). Cdc42 localization and cell polarity depend on membrane traffic. *J. Cell Biol.* 191, 1261–1269.

Pang, B., Neijssen, J., Qiao, X., Janssen, L., Janssen, H., Lippuner, C., and Neefjes, J. (2009). Direct antigen presentation and gap junction mediated cross-presentation during apoptosis. *J. Immunol.* 183, 1083–1090.

Peterlin, B. M., and Trono, D. (2003). Hide, shield and strike back: how HIV-infected cells avoid immune eradication. *Nat. Rev. Immunol.* 3, 97–107.

Pfeifer, J. D., Wick, M. J., Roberts, R. L., Findlay, K., Normark, S. J., and Harding, C. V. (1993). Phagocytic processing of bacterial antigens for class I MHC presentation to T cells. *Nature* 361, 359–362.

Poulin, L. F., Salio, M., Griessinger, E., njos-Afonso, F., Craciun, L., Chen, J. L., Keller, A. M., Joffre, O., Zelenay, S., Nye, E., Le, M. A., Faure, F., Donckier, V., Sancho, D., Cerundolo, V., Bonnet, D., and Reis e Sousa, C. (2010). Characterization of human DNGR-1+ BDCA3+ leukocytes as putative equivalents of mouse CD8alpha+ dendritic cells. *J. Exp. Med.* 207, 1261–1271.

Pratten, M. K., and Lloyd, J. B. (1986). Pinocytosis and phagocytosis: the effect of size of a particulate substrate on its mode of capture by rat peritoneal macrophages cultured in vitro. *Biochim. Biophys. Acta* 881, 307–313.

Pulecio, J., Petrovic, J., Prete, F., Chiaruttini, G., Lennon-Dumenil, A. M., Desdouets, C., Gasman, S., Burrone, O. R., and Benvenuti, F. (2010). Cdc42-mediated MTOC polarization in dendritic cells controls targeted delivery of cytokines at the immune synapse. *J. Exp. Med.* 207, 2719–2732.

Pulecio, J., Tagliani, E., Scholer, A., Prete, F., Fetler, L., Burrone, O. R., and Benvenuti, F. (2008). Expression of Wiskott-Aldrich syndrome protein in dendritic cells regulates synapse formation and activation of naive CD8+ T cells. *J. Immunol.* 181, 1135–1142.

Radhakrishna, H., and Donaldson, J. G. (1997). ADP-ribosylation factor 6 regulates a novel plasma membrane recycling pathway. *J. Cell Biol.* 139, 49–61.

Rammensee, H. G. (1995). Chemistry of peptides associated with MHC class I and class II molecules. *Curr. Opin. Immunol.* 7, 85–96.

Randall, K. L., Chan, S. S., Ma, C. S., Fung, I., Mei, Y., Yabas, M., Tan, A., Arkwright, P. D., Al, S. W., Lugo Reyes, S. O., Yamazaki-Nakashimada, M. A., Garcia-Cruz, L., Smart, J. M., Picard, C., Okada, S., Jouanguy, E., Casanova, J. L., Lambe, T., Cornall, R. J., Russell, S., Oliaro, J., Tangye, S. G., Bertram, E. M., and Goodnow, C. C. (2011). DOCK8 deficiency impairs CD8 T cell survival and function in humans and mice. *J. Exp. Med.* 208, 2305–2320.

Rodriguez, A., Regnault, A., Kleijmeer, M., Ricciardi-Castagnoli, P., and Amigorena, S. (1999). Selective transport of internalized antigens to the cytosol for MHC class I presentation in dendritic cells. *Nat. Cell Biol.* 1, 362–368.

Rodriguez-Cruz, T. G., Liu, S., Khalili, J. S., Whittington, M., Zhang, M., Overwijk, W., and Lizee, G. (2011). Natural splice variant of MHC class I cytoplasmic tail enhances dendritic cell-induced CD8+ T-cell responses and boosts anti-tumor immunity. *PLoS ONE* 6, e22939. doi:10.1371/journal.pone.0022939

Roeth, J. F., Williams, M., Kasper, M. R., Filzen, T. M., and Collins, K. L. (2004). HIV-1 Nef disrupts MHC-I trafficking by recruiting AP-1 to the

MHC-I cytoplasmic tail. *J. Cell Biol.* 167, 903–913.

Rosenberg, S. A., Yang, J. C., and Restifo, N. P. (2004). Cancer immunotherapy: moving beyond current vaccines. *Nat. Med.* 10, 909–915.

Salle de la, H., Hanau, D., Fricker, D., Urlacher, A., Kelly, A., Salamero, J., Powis, S. H., Donato, L., Bausinger, H., and Laforet, M. (1994). Homozygous human TAP peptide transporter mutation in HLA class I deficiency. *Science* 265, 237–241.

Sancho, D., Joffre, O. P., Keller, A. M., Rogers, N. C., Martinez, D., Hernanz-Falcon, P., Rosewell, I., and Reis e Sousa, C. (2009). Identification of a dendritic cell receptor that couples sensing of necrosis to immunity. *Nature* 458, 899–903.

Saveanu, L., Carroll, O., Weimershaus, M., Guermonprez, P., Firat, E., Lindo, V., Greer, F., Davoust, J., Kratzer, R., Keller, S. R., Niedermann, G., and van, E. P. (2009). IRAP identifies an endosomal compartment required for MHC class I cross-presentation. *Science* 325, 213–217.

Savina, A., Jancic, C., Hugues, S., Guermonprez, P., Vargas, P., Moura, I. C., Lennon-Dumenil, A. M., Seabra, M. C., Raposo, G., and Amigorena, S. (2006). NOX2 controls phagosomal pH to regulate antigen processing during crosspresentation by dendritic cells. *Cell* 126, 205–218.

Schlager, M. A., Kapitein, L. C., Grigoriev, I., Burzynski, G. M., Wulf, P. S., Keijzer, N., de, G. E., Fukuda, M., Shepherd, I. T., Akhmanova, A., and Hoogenraad, C. C. (2010). Pericentrosomal targeting of Rab6 secretory vesicles by bicaudal-D-related protein 1 (BICDR-1) regulates neuritogenesis. *EMBO J.* 29, 1637–1651.

Scita, G., and Di Fiore, P. P. (2010). The endocytic matrix. *Nature* 463, 464–473.

Sheff, D. R., Daro, E. A., Hull, M., and Mellman, I. (1999). The receptor recycling pathway contains two distinct populations of early endosomes with different sorting functions. *J. Cell Biol.* 145, 123–139.

Shen, L., Sigal, L. J., Boes, M., and Rock, K. L. (2004). Important role of cathepsin S in generating peptides for TAP-independent MHC class I crosspresentation in vivo. *Immunity* 21, 155–165.

Shen, X. Z., Billet, S., Lin, C., Okwan-Duodu, D., Chen, X., Lukacher, A. E., and Bernstein, K. E. (2011). The carboxypeptidase ACE shapes the MHC class I peptide repertoire. *Nat. Immunol.* 12, 1078–1085.

Simonsen, A., Lippe, R., Christoforidis, S., Gaullier, J. M., Brech, A., Callaghan, J., Toh, B. H., Murphy, C., Zerial, M., and Stenmark, H. (1998). EEA1 links PI(3)K function to Rab5 regulation of endosome fusion. *Nature* 394, 494–498.

Singh, R., and Cresswell, P. (2010). Defective cross-presentation of viral antigens in GILT-free mice. *Science* 328, 1394–1398.

Sonnichsen, B., De, R. S., Nielsen, E., Rietdorf, J., and Zerial, M. (2000). Distinct membrane domains on endosomes in the recycling pathway visualized by multicolor imaging of Rab4, Rab5, and Rab11. *J. Cell Biol.* 149, 901–914.

Steinman, R. M., Mellman, I. S., Muller, W. A., and Cohn, Z. A. (1983). Endocytosis and the recycling of plasma membrane. *J. Cell Biol.* 96, 1–27.

Stelekati, E., Bahri, R., D'Orlando, O., Orinska, Z., Mittrucker, H. W., Langenhaun, R., Glatzel, M., Bollinger, A., Paus, R., and Bulfone-Paus, S. (2009). Mast cell-mediated antigen presentation regulates CD8+ T cell effector functions. *Immunity* 31, 665–676.

Stenmark, H. (2009). Seeing is believing. *Nat. Rev. Mol. Cell Biol.* 10, 582.

Teixeiro, E., Daniels, M. A., Hamilton, S. E., Schrum, A. G., Bragado, R., Jameson, S. C., and Palmer, E. (2009). Different T cell receptor signals determine CD8+ memory versus effector development. *Science* 323, 502–505.

Tomas, M. I., Kucic, N., Mahmutefendic, H., Blagojevic, G., and Lucin, P. (2010). Murine cytomegalovirus perturbs endosomal trafficking of major histocompatibility complex class I molecules in the early phase of infection. *J. Virol.* 84, 11101–11112.

Touret, N., Paroutis, P., Terebiznik, M., Harrison, R. E., Trombetta, S., Pypaert, M., Chow, A., Jiang, A., Shaw, J., Yip, C., Moore, H. P., van der, W. N., Houben, D., Peters, P. J., de, C. C., Mellman, I., and Grinstein, S. (2005). Quantitative and dynamic assessment of the contribution of the ER to phagosome formation. *Cell* 123, 157–170.

Traer, C. J., Rutherford, A. C., Palmer, K. J., Wassmer, T., Oakley, J., Attar, N., Carlton, J. G., Kremerskothen, J., Stephens, D. J., and Cullen, P. J. (2007). SNX4 coordinates endosomal sorting of TfnR with dynein-mediated transport into the endocytic recycling compartment. *Nat. Cell Biol.* 9, 1370–1380.

Trombetta, E. S., Ebersold, M., Garrett, W., Pypaert, M., and Mellman, I. (2003). Activation of lysosomal function during dendritic cell maturation. *Science* 299, 1400–1403.

Ueno, H., Huang, X., Tanaka, Y., and Hirokawa, N. (2011). KIF16B/Rab14 molecular motor complex is critical for early embryonic development by transporting FGF receptor. *Dev. Cell* 20, 60–71.

Urban, R. G., Chicz, R. M., Lane, W. S., Strominger, J. L., Rehm, A., Kenter, M. J., Uytdehaag, F. G., Ploegh, H., Uchanska-Ziegler, B., and Ziegler, A. (1994). A subset of HLA-B27 molecules contains peptides much longer than nonamers. *Proc. Natl. Acad. Sci. U.S.A.* 91, 1534–1538.

van der Wal, F. J., Kikkert, M., and Wiertz, E. (2002). The HCMV gene products US2 and US11 target MHC class I molecules for degradation in the cytosol. *Curr. Top. Microbiol. Immunol.* 269, 37–55.

Vega, M. A., and Strominger, J. L. (1989). Constitutive endocytosis of HLA class I antigens requires a specific portion of the intracytoplasmic tail that shares structural features with other endocytosed molecules. *Proc. Natl. Acad. Sci. U.S.A.* 86, 2688–2692.

Walseng, E., Bakke, O., and Roche, P. A. (2008). Major histocompatibility complex class II-peptide complexes internalize using a clathrin- and dynamin-independent endocytosis pathway. *J. Biol. Chem.* 283, 14717–14727.

Weigert, R., Yeung, A. C., Li, J., and Donaldson, J. G. (2004). Rab22a regulates the recycling of membrane proteins internalized independently of clathrin. *Mol. Biol. Cell* 15, 3758–3770.

Weimershaus, M., Maschalidi, S., Sepulveda, F., Manoury, B., van, E. P., and Saveanu, L. (2012). Conventional dendritic cells require IRAP-Rab14 endosomes for efficient cross-presentation. *J. Immunol.* 188, 1840–1846.

West, M. A., Wallin, R. P., Matthews, S. P., Svensson, H. G., Zaru, R., Ljunggren, H. G., Prescott, A. R., and Watts, C. (2004). Enhanced dendritic cell antigen capture via toll-like receptor-induced actin remodeling. *Science* 305, 1153–1157.

Wubbolts, R., Fernandez-Borja, M., Jordens, I., Reits, E., Dusseljee, S., Echeverri, C., Vallee, R. B., and Neefjes, J. (1999). Opposing motor activities of dynein and kinesin determine retention and transport of MHC class II-containing compartments. *J. Cell Sci.* 112(Pt 6), 785–795.

Yuseff, M. I., Reversat, A., Lankar, D., Diaz, J., Fanget, I., Pierobon, P., Randrian, V., Larochette, N., Vascotto, F., Desdouets, C., Jauffred, B., Bellaiche, Y., Gasman, S., Darchen, F., Desnos, C., and Lennon-Dumenil, A. M. (2011). Polarized secretion of lysosomes at the B cell synapse couples antigen extraction to processing and presentation. *Immunity* 35, 361–374.

Zhang, Q., Davis, J. C., Lamborn, I. T., Freeman, A. F., Jing, H., Favreau, A. J., Matthews, H. F., Davis, J., Turner, M. L., Uzel, G., Holland, S. M., and Su, H. C. (2009). Combined immunodeficiency associated with DOCK8 mutations. *N. Engl. J. Med.* 361, 2046–2055.

Zou, L., Zhou, J., Zhang, J., Li, J., Liu, N., Chai, L., Li, N., Liu, T., Li, L., Xie, Z., Liu, H., Wan, Y., and Wu, Y. (2009). The GTPase Rab3b/3c-positive recycling vesicles are involved in cross-presentation in dendritic cells. *Proc. Natl. Acad. Sci. U.S.A.* 106, 15801–15806.

Zuo, J., Quinn, L. L., Tamblyn, J., Thomas, W. A., Feederle, R., Delecluse, H. J., Hislop, A. D., and Rowe, M. (2011). The Epstein-Barr virus-encoded BILF1 protein modulates immune recognition of endogenously processed antigen by targeting major histocompatibility complex class I molecules trafficking on both the exocytic and endocytic pathways. *J. Virol.* 85, 1604–1614.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 09 December 2011; accepted: 16 February 2012; published online: 07 March 2012.

Citation: Compeer EB, Flinsenberg TWH, van der Grein SG and Boes M (2012) Antigen processing and remodeling of the endosomal pathway: requirements for antigen cross-presentation. Front. Immun. ***3****:37. doi: 10.3389/fimmu.2012.00037*

This article was submitted to Frontiers in Antigen Presenting Cell Biology, a specialty of Frontiers in Immunology.

frontiers in
IMMUNOLOGY

REVIEW ARTICLE
published: 21 March 2012
doi: 10.3389/fimmu.2012.00057

The role of insulin-regulated aminopeptidase in MHC class I antigen presentation

Loredana Saveanu [1,2] **** and Peter van Endert*** [1,2] *******

[1] *Institut National de la Santé et de le Recherche Médicale, Paris, France*
[2] *Faculté de Médecine, Université Paris Descartes, Paris, France*

Edited by:
Christian Kurts, Friedrich Wilhelms-Universität Bonn, Germany

Reviewed by:
Hansjörg Schild, University Medical Center of the Johannes Gutenberg-University, Germany
Sven Burgdorf, Rheinische Friedrich Wilhelms-Universität Bonn, Germany

****Correspondence:***
Loredana Saveanu, Faculté de Médecine, Université Paris Descartes, Sorbonne Paris Cité, 75015 Paris, France.
e-mail: loredana.saveanu@inserm.fr;
Peter van Endert, Institut National de la Santé et de le Recherche Médicale, U1013, Paris 75015, France.
e-mail: peter.van-endert@inserm.fr

Production of MHC-I ligands from antigenic proteins generally requires multiple proteolytic events. While the proteolytic steps required for antigen processing in the endogenous pathway are clearly established, persisting gaps of knowledge regarding putative cross-presentation compartments have made it difficult to map the precise proteolytic events required for generation of cross-presented antigens. It is only in the past decade that the importance of aminoterminal trimming as the final step in the endogenous presentation pathway has been recognized and that the corresponding enzymes have been described. This review focuses on the aminoterminal trimming of exogenous cross-presented peptides, with particular emphasis on the identification of insulin responsive aminopeptidase (IRAP) as the principal trimming aminopeptidase in endosomes and phagosomes.

Keywords: cross-presentation, aminopeptidase, IRAP, ERAP, dendritic cells, MHC class I

HISTORY

Insulin responsive aminopeptidase (IRAP) was initially identified due to its abundance in adipocytes, in specialized endosomes called Glut4 storage vesicles (GSV). The discovery of the glucose transporter Glut4 in 1989 (Birnbaum, 1989; James et al., 1989) was followed by sustained efforts to identify the biochemical composition of GSV, which revealed an abundant protein with a MW of around 160–165 kDa that was constantly associated with isolated GSV and called vp 165. Finally, Keller et al. (1995) identified several tryptic peptides from vp 165, designed oligonucleotide probes based on these peptide sequences and succeeded in cloning full-length IRAP cDNA from an adipose tissue cDNA library. Thus, intracellular localization of IRAP in GSV was the basis for its initial identification. Analysis of IRAP in the context of the regulation of glucose homeostasis has greatly advanced our knowledge about the cellular biology of IRAP endosomes, which are recognized as ubiquitous storage vesicles whose dynamics is regulated by cell-specific stimuli. However, why an aminopeptidase activity is present in GSV remains yet a mystery.

The situation was completely different for the second identification of the aminopeptidase, this time in the context of antigen presentation. In the early 2000s, evidence about the requirement of aminoterminal trimming of MHC class I ligand precursors accumulated (Lauvau et al., 1999; Fruci et al., 2001; Serwold et al., 2001) and prompted several laboratories, including ours, to search for the enzymes performing this final antigen-processing step. Searching for aminopeptidase activities in fractionated protein extracts of crude human B cell microsomes, we identified IRAP (Saveanu et al., 2009) as well as ERAP1–ERAP2 complexes (Saveanu et al., 2005b). The similarity between IRAP and the two ERAP proteins and the co-purification of MHC class I molecules with IRAP suggested that the enzyme was involved in antigen presentation. While the involvement of IRAP in endogenous presentation can not be entirely ruled out, the experimental data available today strongly support the conclusion that IRAP is required for trimming of epitope precursors exclusively in MHC class I antigen cross-presentation.

IRAP AS A MEMBER OF THE M1 METALLOPEPTIDASE FAMILY

The genomic structure of the human IRAP gene (synonyms LNPEP, oxytocinase, P-LAP) is very similar to the ERAP1 and ERAP2 genes (Hattori et al., 2000; Rasmussen et al., 2000; Tanioka et al., 2005). These three genes are located contiguously on the human chromosome 5q15 suggesting the possibility of their divergence from a common ancestral gene. Interestingly, rodents have only two of these enzymes: IRAP (located on murine chromosome 17) and ERAP1 (located on the murine chromosome 13).

While both ER aminopeptidases are strongly induced by cell exposure to interferon gamma (IFN-γ), IRAP protein levels do not change upon IFN-γ stimulation. However, while the ERAP genes have the features of house keeping genes without any TATA or CAAT boxes, the IRAP gene is probably regulated during cell differentiation. Studies performed on the 5′ untranslated region of the IRAP gene in BeWo cells indicated that the transcription factors AP2 and Ikaros cooperatively up-regulate IRAP transcription during differentiation into trophoblastic cells and directly bind to the gene promoter (Iwanaga et al., 2003).

The human IRAP gene codes for a type II transmembrane protein with three domains: a cytoplasmic N-terminal domain of 109-amino-acid, a transmembrane domain of 23-amino-acid, and an intraluminal (or extracellular) domain of 893 amino acids, which include 16 potential N-glycosylation sites (Keller et al., 1995). The long C-terminal, intra-endosomal domain contains a Zn-binding motif HEXXH(X)18E and the exopeptidase motif GAMEN, which are encoded by exons 6 and 7. These two motifs are also found in ERAP1 and ERAP2 and are shared by all members of the M1 family of aminopeptidases (Tsujimoto and Hattori, 2005).

The high percentage of protein sequence identity between IRAP and ERAP1 or ERAP2 (43 or 49% identity, respectively), in correlation with the phylogenetic analysis (Saveanu et al., 2005a) indicates that these enzymes can be classified in a sub-family of M1 aminopeptidases referred to, using an alternative designation of IRAP, "oxytocinase family" (Tsujimoto and Hattori, 2005). The principal difference between the protein sequences of IRAP and the other members of M1 metallopeptidase family, including ERAP1 and 2, is the N-terminal cytoplasmic IRAP domain, which is required for the enzyme localization and its complex intracellular trafficking (see below).

ENZYMATIC ACTIVITY AND NATURAL PEPTIDE SUBSTRATES

SYNTHETIC ANALOGS OF PEPTIDE SUBSTRATES

Several studies analyzed the substrate specificity of IRAP using fluorogenic analogs of peptide substrates (e.g., aminoacyl 7-amino-4-methylcoumarin; Saveanu et al., 2009; Georgiadou et al., 2010; Zervoudi et al., 2011) and demonstrated that the enzyme removes efficiently the following N-terminal aminoacids: Cys, Arg, Lys, Leu, Met, Tyr, Phe, Ala, and Gln. Although IRAP, ERAP1, and ERAP2 display substantial sequence identity and similarity, their substrate specificity as measured using fluorogenic substrates is quite different. Interestingly, the substrate specificity of IRAP is very similar to the specificity of the ERAP1–ERAP2 heterodimer with the difference that IRAP is active in a broader pH range than ERAPs. While 80% of maximal activity of IRAP is conserved at pH 5 or 8, ERAP1 preserved only 30% activity at these pH values (Georgiadou et al., 2010). The functional role of the differences in substrate specificity between IRAP and ERAPs will be discussed later.

HORMONES AND VASOACTIVE PEPTIDES ARE NATURAL IRAP SUBSTRATES

Prior to its characterization as an epitope trimming aminopeptidase, IRAP has been described to inactivate several hormones, vasoactive peptides, and neuropeptides such as oxytocin, somatostatin, cholecystokinin, angiotensin III, Lys-bradikinin, vasopressin, Met-and Leu-enkephalin, neurokinin A, and dynorphin A (Tsujimoto et al., 1992; Herbst et al., 1997; Matsumoto et al., 2001).

A soluble form of human IRAP (starting at residue Ala155) can be detected in human serum during pregnancy. This form is generated by shedding of IRAP from the cell membrane by members of the ADAM (a disintegrin and metalloproteinase) family of endopeptidases (Ito et al., 2004). Increased levels of soluble IRAP in human serum during pregnancy and the ability of IRAP to inactivate the hormone oxytocin by cleavage between the N-terminal cysteine and the adjacent tyrosine residue initially designated IRAP as the enzyme regulating the oxytocin levels to prevent premature delivery. However, it is likely that other serum enzymes can also inactivate oxytocin since murine IRAP does not have the Phe154-Ala155 sequence where cleavage occurs to produce the soluble IRAP enzyme, and since IRAP deficient mice display normal reproductive and maternal behavior (Pham et al., 2009). In support of this conclusion, Keller's group evaluated the oxytocin clearance from the circulation of wt and IRAP deficient mice and showed that oxytocin is inactivated *in vivo* in the absence of IRAP (Wallis et al., 2007). The same authors demonstrated that vasopressin is cleaved *in vivo* exclusively by IRAP and found that IRAP deficient mice have a decreased vasopressin synthesis, probably due to a negative feedback effect. In conclusion, until now, there is only one peptide hormone identified as a specific substrate for IRAP *in vivo*, vasopressin. A genetic study on patients undergoing septic shock further suggested that inactivation of vasopressin by IRAP is physiologically relevant (Nakada et al., 2011). In this study, an increased 28-day mortality in sepsis, which was accompanied by an increased vasopressin clearance, was associated with a SNP (single nucleotide polymorphism: rs4869317) located in a regulatory region of the IRAP gene that may alter the transcription of the gene.

IRAP ACTION ON NEUROPEPTIDES INVOLVED IN LEARNING AND MEMORY

Next to hormones and vasoactive peptides, neuropeptides involved in learning and memory represent an important group of IRAP substrates. Given that memory can be improved by angiotensin IV (AT4) administration (Braszko et al., 1988), the surprising identification of IRAP as a high affinity AT4 receptor in the brain (Albiston et al., 2001) prompted many efforts aiming at characterizing the role of IRAP role in memory and learning. The group of Albiston and Chai showed first that AT4 is a high-affinity inhibitor of IRAP by binding to the active site of the enzyme (Lew et al., 2003) and developed later a new class of IRAP inhibitors which are cognitive enhancers (Albiston et al., 2008). Several hypothetical mechanisms were initially considered for memory enhancement induced by AT4 (Stragier et al., 2008). However, it remains difficult today to draw a conclusion concerning the role of IRAP as an AT4 receptor and in memory mechanisms. Even the analysis of IRAP deficient mice did not shed much light on this issue. A clear result obtained using these mice is that in the absence of IRAP the high affinity-binding site for AT4 is lost in the brain and other tissues, providing strong evidence that IRAP is the AT4 receptor (Albiston et al., 2010). This result is important, considering that the AT4 receptor identity was controversial until recently (De Bundel et al., 2008; Stragier et al., 2008; Wright et al., 2008). Nevertheless, the analysis of the memory phenotype of IRAP deficient mice displayed unexpected results. Despite the fact that acute administration of IRAP inhibitors increases memory, constitutive IRAP deletion in mice does not improve memory (Albiston et al., 2010).

TISSUE DISTRIBUTION AND INTRACELLULAR LOCALIZATION

Even though IRAP was first identified and thus extensively studied in adipocytes, the enzyme is expressed in a very large array of tissues. Initially, IRAP was detected by immunoblot at similarly

high levels in heart, spleen, brain, lung, and adipose tissues and at low level in kidney and muscles. The only tissue in which IRAP amounts were below the limit of immunoblot detection was the liver (Keller et al., 1995). Subsequently a more detailed analysis was performed on brain, placenta, and spleen cells, showing that the enzyme has a cell-specific expression pattern. In the brain its expression is restricted to hippocampus, neocortex, and motor neurons (Fernando et al., 2005). In the placenta, IRAP is expressed mainly in differentiated cells in the trophoblast and the syncytiotrophoblast (Nomura et al., 2005). In the spleen the enzyme is detected in dendritic cells (DCs), B cells, T cells (with highest level of expression observed in conventional CD11c^{high} DCs), and it is absent from granulocytes (Saveanu et al., 2009).

In all the studied cells the enzyme resides in endosomal vesicles. IRAP vesicles recycle slowly in the basal state and can be rapidly and massively translocated to the plasma membrane upon cell-specific stimulation. These vesicles are often called GSV or insulin responsive compartment (IRC) in adipocytes and muscle cells. Since there is no obvious difference between IRAP-containing endosomes in different cell types, we prefer to name the IRAP vesicles "cell-specific storage endosomes." It is now well established that the entire information required for the endosomal localization of IRAP, for its slow recycling and its sensitivity to cell-specific regulation is encoded by the N-terminal cytosolic tail of the protein. For example, a chimera between the N-terminal IRAP domain and the transferrin receptor (TfR) displayed the same intracellular localization and trafficking as the full-length IRAP (Subtil et al., 2000; Hou et al., 2006). Fusion proteins composed by the IRAP cytosolic tail and TfR or EGFP were extensively used as tools to analyze the consequences of different mutations in the targeting motifs that are present in the cytosolic tail of IRAP. The most studied targeting sequences are the two dileucine motifs: LL53,54 and LL76,77. The LL53,54 signal seems to be a form of the (D/E)XXXL(L/I) type signal in which the acidic D or E residue is substituted with a basic R residue (RXXXLL; Bonifacino and Traub, 2003). Interestingly, GLUT4 also contains a RXXXLL sequence, and in this context the RXXXLL signal seems to be important for routing of endocytosed GLUT4 from the plasma membrane to the insulin responsive storage vesicles (Sandoval et al., 2000). However, in the case of IRAP, the substitution of LL53,54 by AA53,54 did not affect the enzyme trafficking in differentiated 3T3-L1 adipocytes (Hou et al., 2006). Unlike this mutation, the substitution of LL76,77 by AA76,77 had a strong impact on IRAP localization and trafficking and resulted in rapid transport of newly synthesized IRAP to the plasma membrane, in a manner indistinguishable from other proteins constitutively directed to the plasma membrane such as vesicular stomatitis virus G coat protein (VSV-G), GLUT1 or syntaxin3 (Watson et al., 2008).

Transient expression of IRAP–GFP demonstrated that newly synthetized IRAP accumulates in storage endosomes as early as 3 h after transfection (Watson et al., 2004). The acquisition of insulin sensitivity starts at 6 h post transfection and is fully accomplished after 9 h. During the first 3 h after enzyme synthesis, IRAP trafficking is brefeldin A sensitive, but once it enters the storage endosomes its translocation to the plasma membrane becomes insensitive to brefeldin A treatment (Watson et al., 2008). In adipocytes, the sorting of newly synthesized IRAP from the trans-Golgi network (TGN) to the storage endosomes requires the clathrin adaptor GGA1, a member of the γ-ear-containing, ADP-ribosylation factor (Arf)-binding (GGA) family of clathrin adaptors (**Figure 1**; Liu et al., 2005; Hou et al., 2006). A chimera between the cytosolic IRAP tail bearing the AA76,77 mutation and the TfR bypasses the GGA1 dependent sorting step in the TGN, but once it arrives at the plasma membrane it can be endocytosed normally, reaches the storage endosomes and is even able to translocate back to the cell surface upon insulin treatment (Watson et al., 2008).

REGULATION OF IRAP TRAFFICKING

SIGNALING PATHWAYS INVOLVED IN IRAP TRAFFICKING REGULATION

The regulation of IRAP trafficking is understood by far in most detail in insulin responsive tissues, mainly in adipocytes, where it has been studied for nearly two decades. As already mentioned before, in these cells, IRAP and GLUT4 are co-localizing in storage endosomes called GSV. Insulin stimulates glucose uptake into adipocytes by stimulating translocation of GLUT4 from the storage endosomes to the plasma membrane (Antonescu et al., 2009). In the absence of insulin, GLUT4 is stored by active mechanisms and sequestered away from the common recycling endosomes (Martin et al., 2006). GLUT4 exocytosis to the plasma membrane from the storage endosomes is very slow compared with general exocytosis from recycling endosomes (Zeigerer et al., 2004). Insulin accelerates GLUT4 and IRAP transport to the cell surface and simultaneously reduces GLUT4 internalization having as a global effect a rapid increase of GLUT4 in the plasma membrane of insulin-stimulated adipocytes. The insulin receptor is a member of the tyrosine kinase receptor family. Binding of insulin

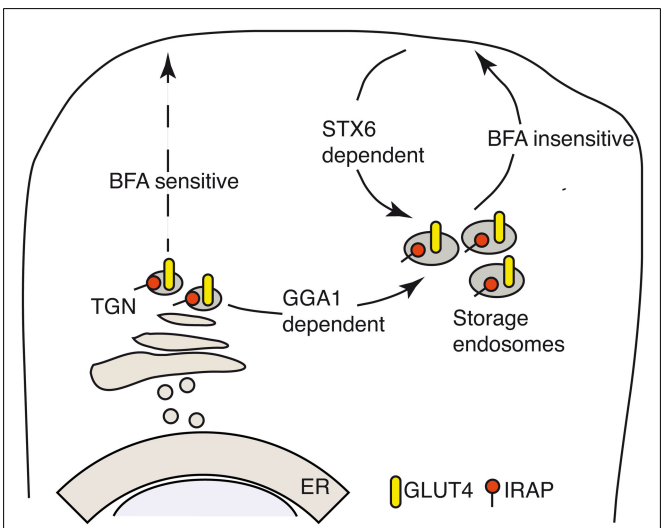


FIGURE 1 | IRAP storage vesicles are slow recycling endosomes. Newly synthetized IRAP accumulates in storage endosomes as early as 3 h after transfection (Watson et al., 2004) and acquires insulin responsiveness after 6–9 h. During the first 3 h after enzyme synthesis, IRAP sorting from the ER is brefeldin A sensitive. Once the enzyme reaches the storage endosomes its translocation to the plasma membrane becomes insensitive to brefeldin A treatment (Watson et al., 2008). Sorting of newly synthetized IRAP from the TGN to the storage endosomes in adipocytes requires the GGA1 clathrin adaptor (Liu et al., 2005; Hou et al., 2006) and the LL76,77 sequence in the cytosolic domain of IRAP (Watson et al., 2008).

to its receptor induces receptor autophosphorylation on several cytoplasmic tyrosines and the rapid recruitment and phosphorylation of the effector proteins insulin receptor substrate 1 and 2 (IRS1, IRS2), and Shc (Goalstone and Draznin, 1997). Tyrosine phosphorylation of IRSs induces the binding of SH2 domains of the regulatory subunits of class I-A PI3Ks, which initiate the subsequent intracellular signaling. Briefly, the PI3K activity produces the lipid PtdIns(3,4,5)P3, which will recruit to membranes the phosphoinositide-dependent kinase-1 (PDK1) and PDK1 substrates, including the kinases of Akt/PKB family (Siddle, 2011). There are three Akt proteins: Akt1, Akt2, and Akt3, which are encoded by separate genes and which have different functional specificities.

Production of different Akt deficient mice demonstrated that Akt1 is important for cell survival, while Akt2 deficient mice have impaired glucose homeostasis and develop type II diabetes and Akt3 deficiency induces abnormal brain development (Gonzalez and McGraw, 2009a). Even though both Akt1 and Akt2 can be recruited to the plasma membrane after PI3K activation, it has been shown by TIRF microscopy that it is mainly Akt2 that is recruited to the plasma membrane upon insulin stimulation. Moreover, siRNA knockdown of Akt2 demonstrated that the protein is required for GLUT4 translocation in response to insulin stimulation (Gonzalez and McGraw, 2009b). These *in vitro* data correlated with the phenotype of Akt2 deficient mice and suggest that Akt2 is the essential Akt isoform controlling trafficking of IRAP–GLUT4 storage endosomes in adipocytes. Nevertheless, a functional overlap among the three Akt isoforms exists. This is suggested by the fact that mice with a single Akt isoform deletion are viable, while mice with double knockouts for Akt are not. The simultaneous deletion of Akt1 and Akt2 leads to death immediately after birth (Peng et al., 2003) and Akt1 and Akt3 double knockout mice are embryonic lethal (Yang et al., 2005). The overlapping roles of Akt1 and Akt2 could also be involved in particular situations in IRAP–GLUT4 trafficking in adipocytes. For example, forced localization of Akt1 to the plasma membrane [via E17K mutation of Akt1 (Gonzalez and McGraw, 2009b) or by addition of a myristoyl group to Akt1 (Kohn et al., 1996)] was demonstrated to induce an Akt2-like signaling and translocation of GLUT4 vesicles (and probably IRAP) to the plasma membrane.

PROTEIN INTERACTIONS OF THE CYTOSOLIC DOMAIN OF IRAP

In adipocytes and muscles PI3K-PDK1-Akt are the protein kinases that are activated in a cascade after insulin receptor stimulation and Rab8, Rab10, and Rab14 the small GTPases that drive GSV translocation to the cell surface. Much of the work leading to the description of this signaling pathway (**Figure 2**) was carried out using GLUT4 or IRAP as markers of GSV, without discriminating between the function of these proteins as simple cargo or active players in GSV trafficking. It is important to note that several findings suggest that both proteins, and especially IRAP, might have more complex roles than simple cargos in vesicular trafficking. First, it was observed that deletion of either of these proteins affects the stability of the other. Mice deficient for IRAP (Keller et al., 2002) displayed a reduced level of GLUT4 protein (50–80% reduction) and mice deficient for GLUT4 had a redistribution of IRAP to the plasma membrane (Jiang et al., 2001). These *in vivo* data, which reflect the situation in primary adipocytes isolated from mice, were only partially confirmed by *in vitro* experiments using siRNA knockdown of GLUT4 and IRAP in 3T3-L1 adipocytes (Jordens et al., 2010). In 3T3-L1 adipocytes, GLUT4 knockdown does not change the distribution of IRAP between the plasma membrane and intracellular vesicles, indicating that IRAP traffic is independent of GLUT4. However, the IRAP knockdown in 3T3-L1 differentiated adipocytes affected GLUT4 trafficking, increasing three times its level at the plasma membrane. At the same time, intracellular GLUT4 was partially rerouted to constitutive endosomes that contain the TfR. When insulin was added to IRAP knockdown adipocytes, the level of GLUT4 at the plasma membrane increased further to a similar extent as in wt adipocytes.

Thus, IRAP is required for intracellular retention of GLUT4 but not for sensitivity of its localization to insulin stimulation. The expression of the cytosolic tail of IRAP was sufficient to recover the normal intracellular distribution of GLUT4 in IRAP knockdown adipocytes. The concomitant analysis of the TfR in IRAP knockdown adipocytes showed that its trafficking was not affected

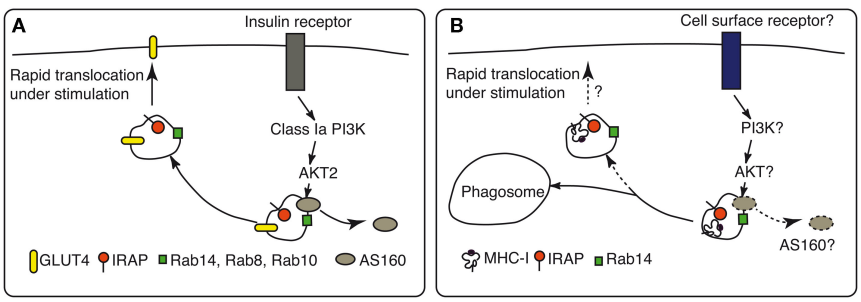


FIGURE 2 | Cell-specific regulation of IRAP storage vesicles. (A) In insulin-responsive tissues, such as adipocytes and muscles, IRAP trafficking is regulated by insulin. Upon insulin binding to its receptor, PI3K-PDK1-Akt protein kinases are activated in a cascade. The most important effector in these cells seems to be the RabGAP AS160, which is phosphorylated by Akt2. Phosphorylation of AS160 leads to its dissociation from the storage endosomes and activation of Rab8, Rab10, and Rab14 that drive the endosome translocation to the cell surface. The biological effect is the increase at cell surface of the principal vesicle cargo, the glucose transporter GLUT4. **(B)** In other cells, the stimuli that regulate IRAP endosome trafficking and the signaling pathways involved in this process are poorly characterized. In DCs, IRAP endosomes are recruited rapidly to the phagosomal membrane. The phagocytic receptors and the signaling molecules responsible for this phenomenon are not yet identified.

by the absence of IRAP, indicating that the role of IRAP is specific to the trafficking of storage endosomes and does not regulate constitutive TfR+ recycling endosomes (Jordens et al., 2010). Thus recruitment of the sorting machinery for GLUT4 targeting to storage endosomes is at least partially accomplished by the cytosolic domain of IRAP. The cytosolic domain of IRAP might recruit sorting machinery components since it interacts with several proteins implicated in protein sorting, vesicle formation, and coupling of the signaling pathways with cytoskeleton remodeling.

Early after its identification, IRAP was found to interact with tankyrase-1 and 2 (Chi and Lodish, 2000), two modular proteins with both poly(ADP)-ribosylation enzymatic activity and scaffolding activities. Tankyrases interact via their ankyrin-repeat domain with diverse partners having multiple biological roles. Among the complex functions attributed to tankyrases, the implication in regulation of Golgi vesicle trafficking and the regulation of protein targeting in response to growth factor signaling are especially relevant in the context of their interaction with IRAP. The identification of the "RXXPDG" sequence as the tankyrase-binding motif of IRAP (Sbodio and Chi, 2002) might allow a better comprehension of the IRAP-tankyrase interaction via mutagenesis experiments.

Another protein involved in the transport between Golgi stacks that was found to interact directly with the cytosolic tail of IRAP is p115 (Hosaka et al., 2005), which assists vesicle fusion by assembling SNARE pin complexes. In addition to these Golgi proteins interacting with IRAP, co-immunoprecipitation experiments and yeast two-hybrid analysis revealed the interaction of the cytosolic domain of IRAP with two cytoskeleton-linked factors: vimentin (Hirata et al., 2011) and FHOS (formin homolog overexpressed in the spleen; Tojo et al., 2003). Vimentin is a part of intermediate filaments in the cytoskeleton, which support and anchor cellular organelles. Formins are multidomain proteins essential for actin polymerization that are involved in the transport of vesicles on actin cables (Goode and Eck, 2007). Since the cytosolic part of IRAP interacts with a protein important in organelle anchoring to the cytoskeleton and another protein involved in guided endosome motility along the cytoskeleton, it is tempting to speculate that the IRAP cytosolic tail regulates GSV trafficking and possibly specialized sorting in several other cell types. It will be of interest to confirm these protein interactions of IRAP in different cell types and assess their function in the regulation of storage endosome trafficking.

Additional indications about a regulatory role of IRAP in GSV trafficking came from two recent studies that reported an interaction between the cytoplasmic domain of IRAP and a well-characterized Akt substrate, the RabGAP AS160, an interaction detected by reciprocal co-immunoprecipitation experiments (Larance et al., 2005; Peck et al., 2006). The IRAP sequence required for the interaction with AS160 was mapped to the region between amino acids 27 and 58 of the IRAP cytosolic domain (Larance et al., 2005). Because the presence of AS160 on GSV is a key player in intracellular retention of GSV in the basal state, the interaction of AS160 with IRAP supports the hypothesis that IRAP is a critical component of GSV retention machinery. However, another recent study concluded that IRAP knockdown in 3T3-L1 adipocytes affect neither AS160 association with GSV nor its regulation by insulin via PI3K-Akt-AS160 (Jordens et al., 2010). It seems therefore that IRAP is not essential for the targeting of AS160 to GSV and even if IRAP binds AS160, additional sites of interaction for AS160 with GSV membranes must exist.

IRAP TRAFFICKING IN IMMUNE CELLS

Among the cell types of the immune system, IRAP was only studied in mast cells (Liao et al., 2006) and DCs (Saveanu et al., 2009; Segura et al., 2009). In bone marrow-derived mast cells and in rat peritoneal mast cells, IRAP is highly expressed and localizes to intracellular vesicles. IRAP vesicles in mastocytes contain VAMP3 and VAMP2 and are different from the secretory granules since there is no overlap between IRAP and histamine or CD63, which are markers of secretory granules. By cell-surface biotinylation experiments, Liao et al. demonstrated that IRAP translocates rapidly to the plasma membrane upon stimulation of mast cells by antigen/immunoglobulin E (IgE) complexes. While exocytosis of mast cell secretory granules following stimulation by antigen-IgE complexes requires PI3K and PKC activities, export of IRAP endosomes to the cell surface is independent of PKC and PI3K activities but depends on intracellular calcium. Thus, interestingly, in mast cells the same extracellular signal, antigen-IgE complexes, induce exocytosis of two intracellular vesicle pools, secretory granules and IRAP vesicles, although the signaling pathways regulating these transport events are different. Contrary to what would be expected by analogy with adipocytes, the activation of PI3K does not induce IRAP translocation to the plasma membrane. These results highlight the cell-specific behavior of IRAP endosomes and show that results obtained in a given cell type cannot readily be extended to others.

We analyzed the intracellular distribution of IRAP in several types of DCs: human monocytes-derived DC, murine bone marrow-derived DC (BMDCs; Saveanu et al., 2009), splenic DC subsets (conventional CD8+ and CD8− DCs and plasmacytoid DCs, pDCs; Weimershaus et al., 2012). In all DC subsets analyzed, the enzyme colocalized at high levels (more than 50% of the total IRAP signal) with Rab14, syntaxin 6 (STX6), and the type1-mannose receptor (MR, alternatively named CD206) and to lesser extent (20 to 30% of total IRAP signal) with EEA1 and MHC class I. The extracellular stimuli and the signaling pathways that regulate IRAP endosomes trafficking in DCs are yet unknown. By several experimental approaches (immunofluorescence microscopy, cell fractionation, flow cytometry analysis of isolated phagosomes), we have demonstrated that IRAP endosomes are recruited to early phagosomes in DCs (Saveanu et al., 2009; Weimershaus et al., 2012), but the phagocytic receptors that induce IRAP vesicle fusion with the phagosome are still unknown.

IMMUNOLOGICAL FUNCTION OF THE ENZYME

Our initial observation that connected IRAP with MHC class I antigen presentation was the co-purification of these two proteins during the screening of crude human microsome lysates for enzymes involved in aminoterminal trimming of MHC class I ligands. IRAP and MHC class I were identified from an IFN-γ-induced peak of aminopeptidase activity isolated by anion exchange chromatography. Surprisingly, subsequent analyses showed that while MHC class I protein synthesis was strongly

increased, IRAP mRNA was not up-regulated upon IFN-γ treatment of HeLa cells. This suggested that either IRAP changed its intracellular localization upon IFN-γ treatment or that increased recovery of IRAP activity was the consequence of its association with IFN-γ-induced MHC class I. While the former hypothesis remains to be studied in detail, association of IRAP with MHC class I molecules was confirmed by reciprocal co-immunoprecipitation experiments in murine BMDCs (Saveanu et al., 2009), as well as in human lymphoblastoid cell lines (our unpublished data).

TRIMMING OF ANTIGENIC PEPTIDES BY IRAP

The ability of IRAP to digest peptide precursors of MHC class I ligands was demonstrated by several teams. Initially we demonstrated that IRAP purified from human microsomes, as well as the recombinant enzyme, efficiently processed two MHC class I ligand precursors: the HIV gag derived peptide, R-SLYNTVATL and the HIV gp160 derived peptide, KIRIQR-GPGRAFVTI (final epitope underlined; Saveanu et al., 2009). Equivalent amounts of isolated ERAP1 or ERAP2 were relatively inefficient in the production of the final epitope from the HIV gp160 precursor, which needs the combined action of ERAP1–ERAP2 (Saveanu et al., 2005b). Notably, IRAP was as efficient as the ERAP1–ERAP2 mixture in the generation of HIV gp160 final epitope, suggesting that the specificity of IRAP resembles that of ERAP1 and ERAP2 combined. The group of Stratikos confirmed this finding in two subsequent reports that analyzed the substrate specificity of ERAP1, ERAP2, and IRAP (Georgiadou et al., 2010; Zervoudi et al., 2011). Using fluorogenic analogs of peptide substrates, they demonstrated that IRAP has broader substrate specificity than isolated ERAP1 or ERAP2 and a broader pH range for optimal activity. However, as it has been demonstrated for ERAP1 (Hearn et al., 2009), the aminopeptidase activity against fluorogenic substrate does not always match the specificity of trimming natural peptides. Therefore, testing IRAP specificity against peptide substrates with systematic variation of the N-terminal residues needs further experimental work. The relative pH independence of IRAP makes the enzyme more appropriate for antigen trimming in endosomes, known for their dynamic pH changes. Taking advantage of the availability of the crystal structure of ERAP1 (Kochan et al., 2011; Nguyen et al., 2011) and the high similarity between these trimming aminopeptidases, the structure of ERAP1 was used to make homology models of the other two aminopeptidases, which allowed the analysis of S1 pocket of all enzymes (Zervoudi et al., 2011). The S1 pocket is defined by 12 amino acids. Six of them are identical for all three enzyme and the other six confer the substrate specificity. In direct correlation with the enzyme ability to cleave basic amino acids, the S1 pocket has different numbers of acidic amino acids: three for ERAP2 (E177, D198, and D888), two for IRAP (E426 and E541), and only one for ERAP1 (E865). Mutagenesis of E541 to R in the S1 pocket of IRAP resulted in a selectivity profile similar to that of ERAP1. The authors concluded that residue E541 is largely responsible for the ability of IRAP to process peptides that are substrates for ERAP2 in the ER.

Among trimming aminopeptidases, only human ERAP1 has been studied extensively with respect to substrate specificity (Chang et al., 2005; Hearn et al., 2009). Using peptide libraries Chang et al. (2005) have demonstrated that human ERAP1 digests optimally peptide with a length of 9–16 amino acids. The transporter associated with antigen presentation (TAP) has an identical length preference for the substrate (van Endert et al., 1994). Given the concordance between ERAP1 and TAP substrate length specificity, Chang et al. proposed the attractive model of "molecular ruler" action for ERAP1. According to this model, ERAP1 is exceptionally adapted to produce MHC class I peptides that have typically 8–10 amino acids length. There are arguments for, and against, the molecular ruler model of ERAP1 mode of action. Several studies *in vitro* showed that ERAP1, as well as its murine homolog ERAAP, could destroy several epitopes, findings in potential conflict with the molecular ruler model (Serwold et al., 2002; York et al., 2002; Georgiadou et al., 2010). On the other hand, the structural analysis of ERAP1 offered a mechanism for the molecular ruler model. According to Nguyen et al. (2011), the enzyme has a regulatory site close to the catalytic site and only long peptides are capable to bind to the regulatory site, inducing the conformational change required for catalytic activity. Next to the structural analysis, one of the strongest arguments for the molecular ruler mode of action of ERAP1 is perhaps the fact that not only the amino terminal residues, but also the carboxy-terminal residues of the peptide substrate control the trimming efficiency, indicating that the enzyme interacts with both peptide ends at the same time (Chang et al., 2005).

The lack of both a systematic analysis of its trimming activity and of a crystal structure does not allow for predicting if IRAP has substrate length or sequence preferences. Georgiadou et al. (2010) performed the so far most detailed analysis of antigenic peptide trimming activity of IRAP. They evaluated the trimming of 14 peptide precursors by IRAP and ERAP1. ERAP1 produced the final epitopes in 13 out of 14 and IRAP in 10 out of 14 cases. The authors concluded that, like ERAP1, IRAP can trim long antigenic peptides efficiently and, in the majority of cases, it accumulates considerable amounts of final antigenic epitope. It is important to mention that both peptidases destroyed some of the final epitopes analyzed. ERAP1 over-digested 6 and IRAP 9 of the 14 precursors. The number of peptides processed in this study is too small to evaluate the substrate length specificity of IRAP. However, a look at the analyzed peptide sequences suggests that IRAP has preferences unrelated to the N-terminal residue, since the same residues are removed with different efficiency depending on the studied peptide. In conclusion, the enzymatic activity data available today indicate that IRAP is capable to trim MHC class I peptide precursors.

THE ROLE OF IRAP IN MHC CLASS I ANTIGEN PROCESSING

Analysis of BMDCs from IRAP deficient mice allowed us to demonstrate that IRAP trimming activity is not required for direct presentation (or endogenous presentation) by MHC class I of two model epitopes: the SIINFEKL peptide, an H-2K^b restricted epitope derived from ovalbumin and the KCSRNRQYL peptide, a D^b restricted epitope derived from the SMCY male antigen (Saveanu et al., 2009). The production of these two epitopes in the endogenous processing pathway requires ERAP and proteasome activity, but not IRAP. These results strongly suggested that IRAP is not involved in the endogenous MHC class I processing pathway and are in concordance with the endosomal localization of IRAP. In steady-state conditions, in human monocyte-derived DCs

(moDCs) and murine BMDCs, we could not detect colocalization of IRAP with ER-resident proteins such as ERAP1, TAP, calnexin or the KDEL receptor. Using ER-targeted variants of IRAP, we verified that the absence of IRAP colocalization with ER markers was not due to the inability of antibodies to recognize ER-resident IRAP. When the KDEL sequence was added to the C-terminal end of the enzyme, the IRAP-specific antibodies detected a strong colocalization of IRAP with two ER markers: TAP and the KDEL receptor. We concluded that the endogenous newly synthesized IRAP molecules exit rapidly from the ER and travel to endosomes containing Rab14, STX6, and MR.

The endosomes containing IRAP are massively recruited to early phagosomes (Saveanu et al., 2009). This was demonstrated by fluorescence microscopy experiments and immunoblot analysis of isolated phagosomes containing latex beads. In the same experimental settings we could not detect the murine ER peptidase (ERAP1/ERAAP) in the phagosomes. These results were in agreement with the proteomic analysis of latex beads phagosomes carried out by Rogers and Foster (2007) who detected IRAP, but not ERAP, in isolated phagosomes. Thus, in our hands, IRAP was the sole member of the oxytocinase family of potential trimming aminopeptidases present in DC phagosomes. The absence of ERAP in phagosomes is of particular interest considering the previous reports suggesting a fusion between ER membranes and newly formed phagosomes (Ackerman et al., 2003; Guermonprez et al., 2003; Houde et al., 2003) and the later controversy concerning the existence and relevance of this phenomenon (Touret et al., 2005). Our results suggest that the ER-phagosome fusion is a selective process, which can provide integral membrane proteins as TAP to the phagosome, but not soluble proteins such as ERAP.

The endosomal and phagosomal localization of IRAP suggested that IRAP might be involved in MHC class I cross-presentation of exogenous antigens. Indeed, IRAP deficiency partially compromised cross-presentation of ovalbumin antigen internalized through phagocytosis both *in vitro* (cross-presentation of ovalbumin-coated latex beads and necrotic cells expressing ovalbumin by BMDCs) and *in vivo* (mice immunized with ovalbumin-polyI:C loaded apoptotic cells). Moreover, IRAP was required for efficient *in vivo* cross-presentation of ovalbumin internalized by receptor-mediated endocytosis (Saveanu et al., 2009; ovalbumin fusion proteins targeted to TLR2 and MR; Kratzer et al., 2010). Importantly, IRAP was involved in a cross-presentation pathway that also requires proteasome activity. While IRAP deficiency decreased the ability of DCs to cross-present ovalbumin by about half, proteasome inhibition (using conditions strictly controlled to avoid toxic side effects) almost abolished cross-presentation completely. These experiments suggested that the initial steps in processing of cross-presented epitopes are almost exclusively performed by the proteasome, while more than one enzyme can perform the last step of antigen processing, the aminoterminal trimming. Analysis of ERAP-deficient mice and BMDCs had previously identified ERAP as another enzyme that can perform the aminoterminal trimming of cross-presented peptides (Yan et al., 2006; Firat et al., 2007). Consistent with this, we found that the absence of either IRAP or ERAP alone reduced cross-presentation of ovalbumin by about half, while the simultaneous deletion of ERAP and IRAP had an additive effect (Saveanu et al., 2009). This functional redundancy, correlated with the absence of ERAP–IRAP colocalization, led us to the hypothesis that the two enzymes act in two independent pathways of proteasome-dependent cross-presentation.

AN UPDATED VIEW OF CROSS-PRESENTATION PATHWAYS

Classically, cross-presentation is divided in two pathways: one that is TAP and proteasome-dependent and one that is TAP and proteasome independent. In the proteasome independent pathway, commonly referred to as vacuolar cross-presentation, internalized exogenous antigens remain in endocytic vesicles where they are processed by acidic lysosomal proteases, with a prominent role for the cathepsin S (Shen et al., 2004) and where loading of the MHC class I molecules occurs. In the proteasome-dependent pathway, which is considered more efficient than the vacuolar pathway (Sigal and Rock, 2000), the antigens are shuttled into the cytosol. Several groups demonstrated that intact, functional proteins such as the toxin gelonin, the enzyme horseradish peroxidase (HRP), or ovalbumin internalized by macrophages and DCs are transferred to the cytosol where they are digested by the proteasome (Kovacsovics-Bankowski and Rock, 1995; Norbury et al., 1995; Rodriguez et al., 1999). One possibility is that the resulting peptides are transported by TAP into the perinuclear ER where they bind to newly synthesized MHC class I molecules (Gromme and Neefjes, 2002; Rock and Shen, 2005). In the context of the latter model, ERAP involvement in proteasome-dependent cross-presentation is an expected finding. However, both experimental observations suggesting fusion between the ER and phagosomes and our discovery that endosomal IRAP plays a role in cross-presentation suggest strongly that MHC class I loading with cross-presented peptides is not limited to the perinuclear ER and can occur in endosomal or phagosomal compartments.

Independent of our work, several recent reports have put forward endosomal subpopulations as active players in cross-presentation (Burgdorf et al., 2007, 2008; Kutomi et al., 2009). It has been demonstrated that ovalbumin endocytosed via the MR reaches an early endosomal compartment (EEA1+, Rab5+) distinct from lysosomes, which enables cross-presentation, while ovalbumin ingested via pinocytosis reaches lysosomes and results in antigen presentation via MHC class II (Burgdorf et al., 2007). Burgdorf proposed that MR+ endosomes are likely to be identical or largely overlap with the early static endosomes described earlier (Lakadamyali et al., 2006). By tracking fluorescent Rabs in live cells, these authors demonstrated that early endosomes consist of two distinct populations: one highly mobile on microtubules and maturing rapidly toward lysosomes and a second "static" maturing much more slowly. Since it is now clearly established that the efficiency of antigen cross-presentation is enhanced by limiting proteolysis and maintaining a close to neutral pH in endolysosomes (Savina et al., 2006; Jancic et al., 2007), it would be interesting to test the overlap of mannose receptor with "static" slowly maturating early endosomes. The substantial colocalization of IRAP with the MR (50–75% depending on the DC type analyzed) and the colocalization of the enzyme with EEA1 and endocytosed soluble ovalbumin (Saveanu et al., 2009; Weimershaus et al., 2012) designate IRAP as an optimal candidate for antigen trimming in these endosomes.

We have found that IRAP was strongly recruited to early phagosomes, where it colocalized with internalized MHC class I and phagocytized antigen. At later time points, when the phagosome was converted to a phagolysosome, the enzyme and MHC class I molecules were detected only in isolated endosomes, often adjacent to the phagosome, but not within. Therefore, IRAP storage endosomes seem to accumulate internalized MHC class I molecules. However, we have not explored whether IRAP vesicles can pinch out internalized antigen and MHC class I during the membrane fusion and fission events occurring during phagosomal maturation.

Our data suggest strongly that MHC class I loading with cross-presented peptides takes place in endocytic vesicles, but it is impossible to ascertain whether these vesicles are endosomes or phagosomes. One argument for loading events in the phagosome derives from the phenomenon of ER-phagosome membrane fusion (Ackerman et al., 2003; Guermonprez et al., 2003; Houde et al., 2003). Several groups demonstrated originally that ER-resident proteins, including key factors of the antigen-presenting machinery (TAP, Tapasin, Calnexin) and Sec61 are associated with the phagosome. Based on these results, it was proposed that the phagosome becomes an autonomous cross-presentation compartment according to the following scenario: phagocytized antigen is transported to cytosol via Sec61, processed by the proteasome and the peptides generated in the cytosol are re-imported by phagosomal TAP complexes and loaded on class I molecules within the phagosome. However, this model was challenged later, when Touret et al. (2005) did not detect any significant contribution of the ER to forming or maturing phagosomes in macrophages or DCs. Although the reasons for this discrepancy remain unclear, different experimental conditions may be the explanation: use of opsonized vs non-opsonized antigen, or possible interference from contaminating TLR ligands that could change phagosomal maturation and membrane fusion events. Molecular characterization of the fusion mechanism and of the proteins controlling the fusion event likely will eventually help to settle this controversy and evaluate the relative contribution of ER-phagosome fusion to overall cross-presentation.

In the light of the experimental data available today, we propose that proteasome-dependent cross-presentation can use at least three intracellular compartments for MHC class I loading with cross-presented peptides (**Figure 3**): the perinuclear ER, phagosomes, and specialized endosomes. MHC class I loading in compartments distinct from the ER in proteasome-dependent cross-presentation is supported also by our recent analysis of TAP deficient BMDCs (Merzougui et al., 2011). We have found that restoration of cell-surface MHC class I molecules on TAP deficient BMDCs by low temperature (26˚C) pre-incubation normalizes cross-presentation of phagocytized ovalbumin, but not cross-presentation of receptor targeted soluble ovalbumin. Surprisingly, restored cross-presentation by TAP deficient cells requires antigen degradation by the proteasome. These findings suggest that the principal role of TAP in proteasome-dependent cross-presentation may be to ensure to provide sufficient levels of cell-surface class I molecules that can be loaded during recycling through phagosomal compartments. Our results also indicate that a TAP-independent mechanism for import of antigenic peptides from the cytosol into phagosomes might exist.

CROSS-PRESENTATION PATHWAYS AND DC SUBSETS

According to our experimental findings, several proteasome-dependent cross-presentation pathways identified by the nature of the trimming peptidase involved can operate simultaneously in DCs (**Figure 3**): (i) a cytosol to ER pathway where the trimming aminopeptidase is ERAP and (ii) a cytosol to endosome pathway dependent on IRAP. For antigens that are shuttled in the cytosol the possibility of cytosolic trimming of peptide precursors also exists. Cytosolic trimming, considered to have a minor impact on overall MHC-I presentation (van Endert, 2011), could be essential for specific epitopes, as for example an epitope derived from the EBNA3C protein of Ebstein Barr virus, which requires nardilysin for amino terminal trimming (Kessler et al., 2011). The relative importance of these pathways in antigen processing may depend on a variety of factors including the nature of the antigen but also the DC subset involved. DCs are a heterogeneous cell population (Shortman and Naik, 2007). Leaving aside tissue resident DCs and migratory DCs, the spleen and the lymph nodes contain at least three main DC subsets in steady-state conditions: pDCs, CD8+, and CD8− conventional DCs (cDCs). Equivalents of these splenic DC subsets can be obtained *in vitro* by FMS-like tyrosine kinase 3 ligand (Flt3L) differentiated cultures of murine BM precursors (Naik et al., 2005). In inflammatory conditions, a new population of DCs, called moDCs arise from blood monocytes. The equivalents of moDCs are obtained *in vitro* by culturing murine BM precursors or human blood monocytes in the presence of granulocyte macrophage colony stimulating factor (GM-CSF) and Interleukin 4 (IL-4; Inaba et al., 1992). pDCs are best known for their ability to secrete high amounts of type I interferons (Reizis et al., 2011), CD8+ DCs for their competence in antigen cross-presentation (Hildner et al., 2008; Lin et al., 2008) and CD8− DCs for their ability to present antigens via MHC class II (Dudziak et al., 2007). moDCs, which proliferate strongly upon inflammatory stimuli, are responsible *in vivo* for highly efficient cross-presentation of gram-negative bacteria and soluble antigens in the presence of TLR ligands (Cheong et al., 2010).

A given DC subset could use preferentially the "cytosol to the ER" or the "cytosol to endosome" pathway of proteasome-dependent cross-presentation. An initial report by Segura et al. (2009) suggested that the cross-presentation pathway involving IRAP and MR is functional only in moDCs. The same authors found that all steady-state DCs have similar amounts of IRAP but did not observe IRAP colocalization with internalized ovalbumin in cell fractionation experiments. We have recently analyzed pDCs and CD8+ and CD8− cDCs (*ex vivo* sorted from the spleens as well as their equivalents obtained from Flt3L cultures) with respect to expression and intracellular localization of IRAP and to the ability to cross-present soluble ovalbumin and yeast cells displaying ovalbumin at cell surface.

Our recent results (Weimershaus et al., 2012) lead to quite different conclusions and indicate that cDCs employ IRAP in cross-presentation. Like Segura and colleagues, we found identical IRAP protein levels in all DC subsets analyzed. Additionally,

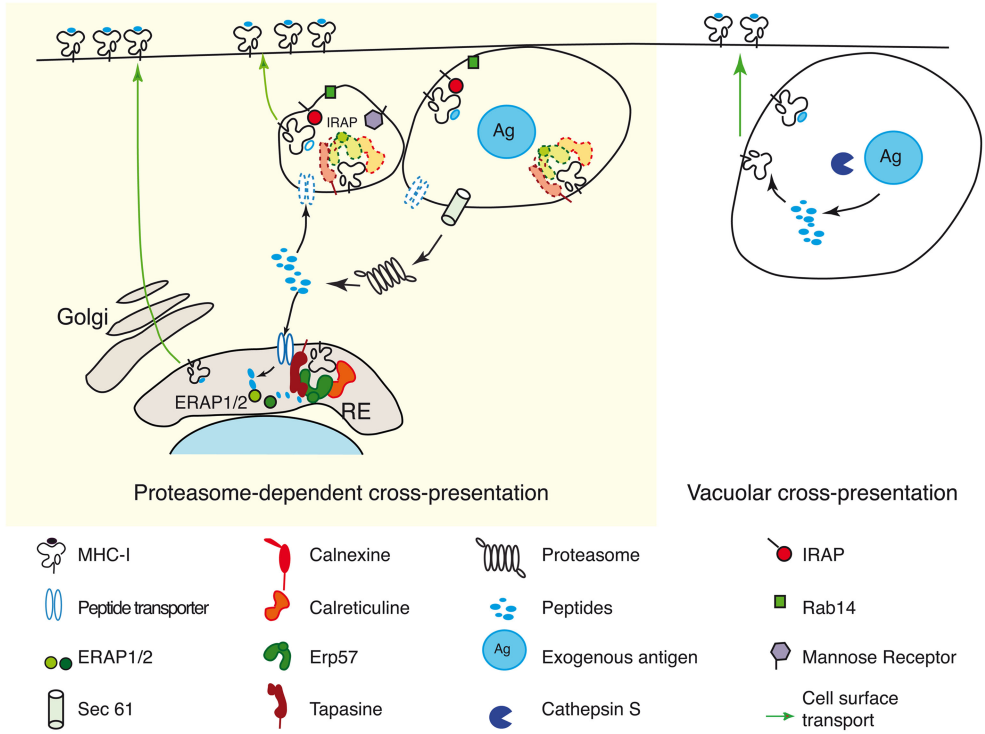


FIGURE 3 | Cross-presentation pathways. According to the involvement of the proteasome in antigen processing cross-presentation is divided in two main pathways: proteasome-dependent cross-presentation (left panel) and vacuolar cross-presentation (right panel). The vacuolar cross-presentation does not require proteasome activity; the entire antigen processing and MHC class I loading with cross-presented peptides occur inside the vacuole. The proteasome-dependent cross-presentation involves the transport of the exogenous antigens (possibly by Sec61) into the DC cytosol of the DC and generation of N-terminal extended precursors of MHC-I ligands in the cytosol. The aminoterminal trimming of these peptide precursors can occur in two different compartments: (i) in a cytosol to ER pathway, epitope precursors will join the endogenous processing pathway after their transport by TAP into the ER (Kovacsovics-Bankowski and Rock, 1995). In this case peptide trimming is carried out by ERAPs; (ii) in a cytosol to endosome pathway, the precursors of class I ligands are retro-transported into specialized endosomes (Burgdorf et al., 2008; Saveanu et al., 2009) or into phagosomes (Guermonprez et al., 2003) and final trimming is performed by IRAP (Saveanu et al., 2009).

we looked at the intracellular distribution of IRAP in DC subsets. In steady-state conditions, the enzyme colocalized with previously known markers of storage endosomes: Rab14 and STX6. However, there was a slight but significant increase in Rab14/IRAP colocalization in CD8+ DCs in comparison with CD8− DCs in steady-state. Considering that Rab14 is one of the small GTPases that drives IRAP translocation upon cell activation, the increased IRAP–Rab14 colocalization in CD8+ DCs may be the reason for higher and prolonged recruitment of IRAP in the phagosomal membrane of these cells. It is conceivable that fusion between Rab14–IRAP endosomes and phagosome contributes to a delay in phagosomal maturation since Rab14 recruitment to the phagosomes prevents phagosome fusion with lysosomes (Kyei et al., 2006; Kuijl et al., 2007). Different from the data obtained by Segura et al. (2009) we also readily detected colocalization of internalized ovalbumin with IRAP using cell fractionation and fluorescence microscopy. Moreover, *in vitro* cross-presentation of soluble ovalbumin and yeasts displaying ovalbumin on the surface was impaired in both CD8+ and CD8− cDCs in the absence of IRAP (Weimershaus et al., 2012).

At first glance, our results on IRAP role in DC subsets are contradictory with those reported by Segura et al. (2009) Examination of the experimental settings, such as for example, the timing of antigen processing, the antigen dose, and the method of DC preparation, can explain some of the observed differences. Multiple cross-presentation pathways can produce the SIINFEKL peptide from internalized ovalbumin. At least four proteolytic enzymes can produce the SIINFEKL epitope from its precursors: cathepsin S in endosomes (Shen et al., 2004), the trimming peptidases ERAP, and IRAP (Serwold et al., 2002; Saveanu et al., 2009) and even cytosolic proteasome complexes (Cascio et al., 2001). It is not surprising in this situation that extended antigen-processing times or high antigen doses overcome the cross-presentation defects observed in the absence of IRAP, ERAP, or even TAP (Weimershaus et al., 2012).

There are several lines of evidence that the cell biology of CD8+ DCs is optimized for cross-presentation. For example, CD8+ DCs have the most efficient transport of internalized antigens to the cytosol (Lin et al., 2008), the closest to neutral phagosomal pH (Savina et al., 2009) and an up-regulated MHC class I loading machinery in comparison with the CD8− cDCs (Dudziak et al., 2007). The maturation state of CD8+ DCs allowing these cells to excel in cross-presentation assays was carefully examined very recently. Using Flt3L BMDCs, Sathe et al. (2011) demonstrated that newly formed CD8+ DC acquire their cross-presentation

capacity in a maturation step (accompanied by CD103 cell-surface expression) triggered by inflammatory cytokines such as GM-CSF, IL3, or TLR ligands (CpG). Interestingly, CD8+ DCs from Flt3L cultures treated with TGF-β up-regulated cell-surface CD103, but did not cross-present. This indicated that CD103 is a marker of the maturation stage but does not correlate with cross-presentation capacity. The work of Sathe et al. highlighted that antigen cross-presentation is not an innate feature of the CD8+ DC lineage, but acquired after cell stimulation by cytokines and TLR ligands. Dresch et al. confirmed the fact that splenic CD8+ DCs require stimulation by GM-CSF or CD40 ligand for acquisition of antigen cross-presentation ability. In contrast, cross-presentation of soluble and cell-associated ovalbumin by thymic CD8+ DCs is very efficient in the absence of licensing factors, such as GM-CSF or CD40 ligand (Dresch et al., 2011).

Thus, peripheral CD8+ DCs, but not their thymic equivalents, need maturation by CD40 ligand, GM-CSF or TLR stimulation to activate the cross-presentation machinery. This recent conclusion perfectly agrees with the longstanding concept that immature DCs are poor antigen-presenting cells and that their activation is crucial for the initiation of immunity (Banchereau and Steinman, 1998). It remains to be seen whether the numerous factors that induce DC activation have an influence on the balance between the cross-presentation pathways depicted in the **Figure 3**.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Current data indicate that the aminopeptidase activity of IRAP is responsible for endosomal aminoterminal trimming of cross-presented peptides. In addition to the peptide trimming activity, the most important IRAP features are its interaction with MHC class I molecules and its presence and role in cell-specific regulation of IRAP endosomes. The substrate specificity of IRAP, as well as the nature of the associated MHC class I molecules will require further investigation. Specifically the IRAP preferences for the length and the internal sequence of peptide substrates are entirely unknown. Another issue of importance is the extent to which the different cross-presentation pathways are used by different DC subsets under various physiologic conditions. Little is known about how the physiological context; e.g., inflammatory cytokines, pathogen-associated danger signals, simultaneous antibody responses, and CD4+ T cell help modulate intracellular cross-presentation pathways.

Another issue that should be addressed in the future is a potential IRAP role in antigen storage by the DCs. van Montfoort et al. (2009) have shown recently that soluble immune complexes can be stored by DCs for several days in lysosome-like organelles thus increasing the potency of cytotoxic T cell priming. Although preliminary results do not suggest a direct presence of IRAP in such compartments, it cannot be ruled out that IRAP plays a role in routing immune complexes and/or phagocytosed antigens to such compartments.

As demonstrated by the study of IRAP trafficking in insulin responsive cells, there are strong indications that the enzyme is not only a cargo of cell-specific storage endosomes but also an active player in the regulation of their trafficking. Our ongoing studies suggest that IRAP is required for normal maturation of phagosomes in BMDCs. We believe that it will be of great interest to characterize the extracellular stimuli and intracellular signaling pathways that regulate the trafficking of IRAP endosomes in DCs. Investigation of the cross-presentation pathways used by thymic DCs may also be of interest. As mentioned above, thymic CD8+ DCs seem to be the only steady-state DC subpopulation with a constitutively activated cross-presentation capacity, a feature that could be important for T cell selection in the thymus and maintenance of immune tolerance in the periphery.

ACKNOWLEDGMENTS

Work on IRAP-related projects by the authors was supported by grant IRAP-DC (NT09_522096) from the Agence Nationale de Recherche.

REFERENCES

Ackerman, A. L., Kyritsis, C., Tampe, R., and Cresswell, P. (2003). Early phagosomes in dendritic cells form a cellular compartment sufficient for cross presentation of exogenous antigens. *Proc. Natl. Acad. Sci. U.S.A.* 100, 12889–12894.

Albiston, A. L., Fernando, R. N., Yeatman, H. R., Burns, P., Ng, L., Daswani, D., Diwakarla, S., Pham, V., and Chai, S. Y. (2010). Gene knockout of insulin-regulated aminopeptidase: loss of the specific binding site for angiotensin IV and age-related deficit in spatial memory. *Neurobiol. Learn. Mem.* 93, 19–30.

Albiston, A. L., Mcdowall, S. G., Matsacos, D., Sim, P., Clune, E., Mustafa, T., Lee, J., Mendelsohn, F. A., Simpson, R. J., Connolly, L. M., and Chai, S. Y. (2001). Evidence that the angiotensin IV (AT(4)) receptor is the enzyme insulin-regulated aminopeptidase. *J. Biol. Chem.* 276, 48623–48626.

Albiston, A. L., Morton, C. J., Ng, H. L., Pham, V., Yeatman, H. R., Ye, S., Fernando, R. N., De Bundel, D., Ascher, D. B., Mendelsohn, F. A., Parker, M. W., and Chai, S. Y. (2008). Identification and characterization of a new cognitive enhancer based on inhibition of insulin-regulated aminopeptidase. *FASEB J.* 22, 4209–4217.

Antonescu, C. N., Foti, M., Sauvonnet, N., and Klip, A. (2009). Ready, set, internalize: mechanisms and regulation of GLUT4 endocytosis. *Biosci. Rep.* 29, 1–11.

Banchereau, J., and Steinman, R. M. (1998). Dendritic cells and the control of immunity. *Nature* 392, 245–252.

Birnbaum, M. J. (1989). Identification of a novel gene encoding an insulin-responsive glucose transporter protein. *Cell* 57, 305–315.

Bonifacino, J. S., and Traub, L. M. (2003). Signals for sorting of transmembrane proteins to endosomes and lysosomes. *Annu. Rev. Biochem.* 72, 395–447.

Braszko, J. J., Kupryszewski, G., Witczuk, B., and Wisniewski, K. (1988). Angiotensin II-(3–8)-hexapeptide affects motor activity, performance of passive avoidance and a conditioned avoidance response in rats. *Neuroscience* 27, 777–783.

Burgdorf, S., Kautz, A., Bohnert, V., Knolle, P. A., and Kurts, C. (2007). Distinct pathways of antigen uptake and intracellular routing in CD4 and CD8 T cell activation. *Science* 316, 612–616.

Burgdorf, S., Scholz, C., Kautz, A., Tampe, R., and Kurts, C. (2008). Spatial and mechanistic separation of cross-presentation and endogenous antigen presentation. *Nat. Immunol.* 9, 558–566.

Cascio, P., Hilton, C., Kisselev, A. F., Rock, K. L., and Goldberg, A. L. (2001). 26S proteasomes and immunoproteasomes produce mainly N-extended versions of an antigenic peptide. *EMBO J.* 20, 2357–2366.

Chang, S. C., Momburg, F., Bhutani, N., and Goldberg, A. L. (2005). The ER aminopeptidase, ERAP1, trims precursors to lengths of MHC class I peptides by a "molecular ruler" mechanism. *Proc. Natl. Acad. Sci. U.S.A.* 102, 17107–17112.

Cheong, C., Matos, I., Choi, J. H., Dandamudi, D. B., Shrestha, E., Longhi, M. P., Jeffrey, K. L., Anthony, R. M., Kluger, C., Nchinda, G., Koh, H., Rodriguez, A., Idoyaga, J., Pack, M., Velinzon, K., Park, C. G., and Steinman, R. M. (2010). Microbial stimulation fully differentiates monocytes to DC-SIGN/CD209(+) dendritic cells for immune T cell areas. *Cell* 143, 416–429.

Chi, N. W., and Lodish, H. F. (2000). Tankyrase is a golgi-associated mitogen-activated protein kinase substrate that interacts with IRAP in GLUT4 vesicles. *J. Biol. Chem.* 275, 38437–38444.

De Bundel, D., Smolders, I., Vanderheyden, P., and Michotte, Y. (2008). Ang II and Ang IV: unraveling the mechanism of action on synaptic plasticity, memory, and epilepsy. *CNS Neurosci. Ther.* 14, 315–339.

Dresch, C., Ackermann, M., Vogt, B., De Andrade Pereira, B., Shortman, K., and Fraefel, C. (2011). Thymic but not splenic CD8 DCs can efficiently cross-prime T cells in the absence of licensing factors. *Eur. J. Immunol.* 41, 2544–2555.

Dudziak, D., Kamphorst, A. O., Heidkamp, G. F., Buchholz, V. R., Trumpfheller, C., Yamazaki, S., Cheong, C., Liu, K., Lee, H. W., Park, C. G., Steinman, R. M., and Nussenzweig, M. C. (2007). Differential antigen processing by dendritic cell subsets in vivo. *Science* 315, 107–111.

Fernando, R. N., Larm, J., Albiston, A. L., and Chai, S. Y. (2005). Distribution and cellular localization of insulin-regulated aminopeptidase in the rat central nervous system. *J. Comp. Neurol.* 487, 372–390.

Firat, E., Saveanu, L., Aichele, P., Staeheli, P., Huai, J., Gaedicke, S., Nil, A., Besin, G., Kanzler, B., Van Endert, P., and Niedermann, G. (2007). The role of endoplasmic reticulum-associated aminopeptidase 1 in immunity to infection and in cross-presentation. *J. Immunol.* 178, 2241–2248.

Fruci, D., Niedermann, G., Butler, R. H., and Van Endert, P. M. (2001). Efficient MHC class I-independent amino-terminal trimming of epitope precursor peptides in the endoplasmic reticulum. *Immunity* 15, 467–476.

Georgiadou, D., Hearn, A., Evnouchidou, I., Chroni, A., Leondiadis, L., York, I. A., Rock, K. L., and Stratikos, E. (2010). Placental leucine aminopeptidase efficiently generates mature antigenic peptides in vitro but in patterns distinct from endoplasmic reticulum aminopeptidase 1. *J. Immunol.* 185, 1584–1592.

Goalstone, M. L., and Draznin, B. (1997). Insulin signaling. *West. J. Med.* 167, 166–173.

Gonzalez, E., and McGraw, T. E. (2009a). The Akt kinases: isoform specificity in metabolism and cancer. *Cell Cycle* 8, 2502–2508.

Gonzalez, E., and McGraw, T. E. (2009b). Insulin-modulated Akt subcellular localization determines Akt isoform-specific signaling. *Proc. Natl. Acad. Sci. U.S.A.* 106, 7004–7009.

Goode, B. L., and Eck, M. J. (2007). Mechanism and function of formins in the control of actin assembly. *Annu. Rev. Biochem.* 76, 593–627.

Gromme, M., and Neefjes, J. (2002). Antigen degradation or presentation by MHC class I molecules via classical and non-classical pathways. *Mol. Immunol.* 39, 181–202.

Guermonprez, P., Saveanu, L., Kleijmeer, M., Davoust, J., Van Endert, P., and Amigorena, S. (2003). ER-phagosome fusion defines an MHC class I cross-presentation compartment in dendritic cells. *Nature* 425, 397–402.

Hattori, A., Kitatani, K., Matsumoto, H., Miyazawa, S., Rogi, T., Tsuruoka, N., Mizutani, S., Natori, Y., and Tsujimoto, M. (2000). Characterization of recombinant human adipocyte-derived leucine aminopeptidase expressed in Chinese hamster ovary cells. *J. Biochem.* 128, 755–762.

Hearn, A., York, I. A., and Rock, K. L. (2009). The specificity of trimming of MHC class I-presented peptides in the endoplasmic reticulum. *J. Immunol.* 183, 5526–5536.

Herbst, J. J., Ross, S. A., Scott, H. M., Bobin, S. A., Morris, N. J., Lienhard, G. E., and Keller, S. R. (1997). Insulin stimulates cell surface aminopeptidase activity toward vasopressin in adipocytes. *Am. J. Physiol.* 272, E600–E606.

Hildner, K., Edelson, B. T., Purtha, W. E., Diamond, M., Matsushita, H., Kohyama, M., Calderon, B., Schraml, B. U., Unanue, E. R., Diamond, M. S., Schreiber, R. D., Murphy, T. L., and Murphy, K. M. (2008). Batf3 deficiency reveals a critical role for CD8alpha+ dendritic cells in cytotoxic T cell immunity. *Science* 322, 1097–1100.

Hirata, Y., Hosaka, T., Iwata, T., Le, C. T., Jambaldorj, B., Teshigawara, K., Harada, N., Sakaue, H., Sakai, T., Yoshimoto, K., and Nakaya, Y. (2011). Vimentin binds IRAP and is involved in GLUT4 vesicle trafficking. *Biochem. Biophys. Res. Commun.* 405, 96–101.

Hosaka, T., Brooks, C. C., Presman, E., Kim, S. K., Zhang, Z., Breen, M., Gross, D. N., Sztul, E., and Pilch, P. F. (2005). p115 Interacts with the GLUT4 vesicle protein, IRAP, and plays a critical role in insulin-stimulated GLUT4 translocation. *Mol. Biol. Cell* 16, 2882–2890.

Hou, J. C., Suzuki, N., Pessin, J. E., and Watson, R. T. (2006). A specific dileucine motif is required for the GGA-dependent entry of newly synthesized insulin-responsive aminopeptidase into the insulin-responsive compartment. *J. Biol. Chem.* 281, 33457–33466.

Houde, M., Bertholet, S., Gagnon, E., Brunet, S., Goyette, G., Laplante, A., Princiotta, M. F., Thibault, P., Sacks, D., and Desjardins, M. (2003). Phagosomes are competent organelles for antigen cross-presentation. *Nature* 425, 402–406.

Inaba, K., Inaba, M., Romani, N., Aya, H., Deguchi, M., Ikehara, S., Muramatsu, S., and Steinman, R. M. (1992). Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J. Exp. Med.* 176, 1693–1702.

Ito, N., Nomura, S., Iwase, A., Ito, T., Kikkawa, F., Tsujimoto, M., Ishiura, S., and Mizutani, S. (2004). ADAMs, a disintegrin and metalloproteinases, mediate shedding of oxytocinase. *Biochem. Biophys. Res. Commun.* 314, 1008–1013.

Iwanaga, K., Nomura, S., Ito, T., Ikoma, Y., Yamamoto, E., Okada, M., Itakura, A., Kikkawa, F., Tsujimoto, M., and Mizutani, S. (2003). Placental leucine aminopeptidase/oxytocinase gene regulation by activator protein-2 in BeWo cell model of human trophoblast differentiation. *FEBS Lett.* 552, 120–124.

James, D. E., Strube, M., and Mueckler, M. (1989). Molecular cloning and characterization of an insulin-regulatable glucose transporter. *Nature* 338, 83–87.

Jancic, C., Savina, A., Wasmeier, C., Tolmachova, T., El-Benna, J., Dang, P. M., Pascolo, S., Gougerot-Pocidalo, M. A., Raposo, G., Seabra, M. C., and Amigorena, S. (2007). Rab27a regulates phagosomal pH and NADPH oxidase recruitment to dendritic cell phagosomes. *Nat. Cell Biol.* 9, 367–378.

Jiang, H., Li, J., Katz, E. B., and Charron, M. J. (2001). GLUT4 ablation in mice results in redistribution of IRAP to the plasma membrane. *Biochem. Biophys. Res. Commun.* 284, 519–525.

Jordens, I., Molle, D., Xiong, W., Keller, S. R., and Mcgraw, T. E. (2010). Insulin-regulated aminopeptidase is a key regulator of GLUT4 trafficking by controlling the sorting of GLUT4 from endosomes to specialized insulin-regulated vesicles. *Mol. Biol. Cell* 21, 2034–2044.

Keller, S. R., Davis, A. C., and Clairmont, K. B. (2002). Mice deficient in the insulin-regulated membrane aminopeptidase show substantial decreases in glucose transporter GLUT4 levels but maintain normal glucose homeostasis. *J. Biol. Chem.* 277, 17677–17686.

Keller, S. R., Scott, H. M., Mastick, C. C., Aebersold, R., and Lienhard, G. E. (1995). Cloning and characterization of a novel insulin-regulated membrane aminopeptidase from Glut4 vesicles. *J. Biol. Chem.* 270, 23612–23618.

Kessler, J. H., Khan, S., Seifert, U., Le Gall, S., Chow, K. M., Paschen, A., Bres-Vloemans, S. A., De Ru, A., Van Montfoort, N., Franken, K. L., Benckhuijsen, W. E., Brooks, J. M., Van Hall, T., Ray, K., Mulder, A., Doxiadis, I. I, Van Swieten, P. F., Overkleeft, H. S., Prat, A., Tomkinson, B., Neefjes, J., Kloetzel, P. M., Rodgers, D. W., Hersh, L. B., Drijfhout, J. W., Van Veelen, P. A., Ossendorp, F., and Melief, C. J. (2011). Antigen processing by nardilysin and thimet oligopeptidase generates cytotoxic T cell epitopes. *Nat. Immunol.* 12, 45–53.

Kochan, G., Krojer, T., Harvey, D., Fischer, R., Chen, L., Vollmar, M., Von Delft, F., Kavanagh, K. L., Brown, M. A., Bowness, P., Wordsworth, P., Kessler, B. M., and Oppermann, U. (2011). Crystal structures of the endoplasmic reticulum aminopeptidase-1 (ERAP1) reveal the molecular basis for N-terminal peptide trimming. *Proc. Natl. Acad. Sci. U.S.A.* 108, 7745–7750.

Kohn, A. D., Summers, S. A., Birnbaum, M. J., and Roth, R. A. (1996). Expression of a constitutively active Akt Ser/Thr kinase in 3T3-L1 adipocytes stimulates glucose uptake and glucose transporter 4 translocation. *J. Biol. Chem.* 271, 31372–31378.

Kovacsovics-Bankowski, M., and Rock, K. L. (1995). A phagosome-to-cytosol pathway for exogenous antigens presented on MHC class I molecules. *Science* 267, 243–246.

Kratzer, R., Mauvais, F. X., Burgevin, A., Barilleau, E., and Van Endert, P. (2010). Fusion proteins for versatile antigen targeting to cell surface receptors reveal differential capacity to prime immune responses. *J. Immunol.* 184, 6855–6864.

Kuijl, C., Savage, N. D., Marsman, M., Tuin, A. W., Janssen, L., Egan, D. A., Ketema, M., Van Den Nieuwendijk, R., Van Den Eeden, S. J., Geluk, A., Poot, A., Van Der Marel, G., Beijersbergen, R. L., Overkleeft, H., Ottenhoff, T. H., and Neefjes, J. (2007).

Intracellular bacterial growth is controlled by a kinase network around PKB/AKT1. *Nature* 450, 725–730.

Kutomi, G., Tamura, Y., Okuya, K., Yamamoto, T., Hirohashi, Y., Kamiguchi, K., Oura, J., Saito, K., Torigoe, T., Ogawa, S., Hirata, K., and Sato, N. (2009). Targeting to static endosome is required for efficient cross-presentation of endoplasmic reticulum-resident oxygen-regulated protein 150-peptide complexes. *J. Immunol.* 183, 5861–5869.

Kyei, G. B., Vergne, I., Chua, J., Roberts, E., Harris, J., Junutula, J. R., and Deretic, V. (2006). Rab14 is critical for maintenance of *Mycobacterium tuberculosis* phagosome maturation arrest. *EMBO J.* 25, 5250–5259.

Lakadamyali, M., Rust, M. J., and Zhuang, X. (2006). Ligands for clathrin-mediated endocytosis are differentially sorted into distinct populations of early endosomes. *Cell* 124, 997–1009.

Larance, M., Ramm, G., Stockli, J., Van Dam, E. M., Winata, S., Wasinger, V., Simpson, F., Graham, M., Junutula, J. R., Guilhaus, M., and James, D. E. (2005). Characterization of the role of the Rab GTPase-activating protein AS160 in insulin-regulated GLUT4 trafficking. *J. Biol. Chem.* 280, 37803–37813.

Lauvau, G., Kakimi, K., Niedermann, G., Ostankovitch, M., Yotnda, P., Firat, H., Chisari, F. V., and Van Endert, P. M. (1999). Human transporters associated with antigen processing (TAPs) select epitope precursor peptides for processing in the endoplasmic reticulum and presentation to T cells. *J. Exp. Med.* 190, 1227–1240.

Lew, R. A., Mustafa, T., Ye, S., Mcdowall, S. G., Chai, S. Y., and Albiston, A. L. (2003). Angiotensin AT4 ligands are potent, competitive inhibitors of insulin regulated aminopeptidase (IRAP). *J. Neurochem.* 86, 344–350.

Liao, H., Keller, S. R., and Castle, J. D. (2006). Insulin-regulated aminopeptidase marks an antigen-stimulated recycling compartment in mast cells. *Traffic* 7, 155–167.

Lin, M. L., Zhan, Y., Proietto, A. I., Prato, S., Wu, L., Heath, W. R., Villadangos, J. A., and Lew, A. M. (2008). Selective suicide of cross-presenting CD8+ dendritic cells by cytochrome c injection shows functional heterogeneity within this subset. *Proc. Natl. Acad. Sci. U.S.A.* 105, 3029–3034.

Liu, G., Hou, J. C., Watson, R. T., and Pessin, J. E. (2005). Initial entry of IRAP into the insulin-responsive storage compartment occurs prior to basal or insulin-stimulated plasma membrane recycling. *Am. J. Physiol. Endocrinol. Metab.* 289, E746–E752.

Martin, O. J., Lee, A., and Mcgraw, T. E. (2006). GLUT4 distribution between the plasma membrane and the intracellular compartments is maintained by an insulin-modulated bipartite dynamic mechanism. *J. Biol. Chem.* 281, 484–490.

Matsumoto, H., Nagasaka, T., Hattori, A., Rogi, T., Tsuruoka, N., Mizutani, S., and Tsujimoto, M. (2001). Expression of placental leucine aminopeptidase/oxytocinase in neuronal cells and its action on neuronal peptides. *Eur. J. Biochem.* 268, 3259–3266.

Merzougui, N., Kratzer, R., Saveanu, L., and Van Endert, P. (2011). A proteasome-dependent, TAP-independent pathway for cross-presentation of phagocytosed antigen. *EMBO Rep.*

Naik, S. H., Proietto, A. I., Wilson, N. S., Dakic, A., Schnorrer, P., Fuchsberger, M., Lahoud, M. H., O'Keeffe, M., Shao, Q. X., Chen, W. F., Villadangos, J. A., Shortman, K., and Wu, L. (2005). Cutting edge: generation of splenic CD8+ and CD8- dendritic cell equivalents in Fms-like tyrosine kinase 3 ligand bone marrow cultures. *J. Immunol.* 174, 6592–6597.

Nakada, T. A., Russell, J. A., Wellman, H., Boyd, J. H., Nakada, E., Thain, K. R., Thair, S. A., Hirasawa, H., Oda, S., and Walley, K. R. (2011). Leucyl/cystinyl aminopeptidase gene variants in septic shock. *Chest* 139, 1042–1049.

Nguyen, T. T., Chang, S. C., Evnouchidou, I., York, I. A., Zikos, C., Rock, K. L., Goldberg, A. L., Stratikos, E., and Stern, L. J. (2011). Structural basis for antigenic peptide precursor processing by the endoplasmic reticulum aminopeptidase ERAP1. *Nat. Struct. Mol. Biol.* 18, 604–613.

Nomura, S., Ito, T., Yamamoto, E., Sumigama, S., Iwase, A., Okada, M., Shibata, K., Ando, H., Ino, K., Kikkawa, F., and Mizutani, S. (2005). Gene regulation and physiological function of placental leucine aminopeptidase/oxytocinase during pregnancy. *Biochim. Biophys. Acta* 1751, 19–25.

Norbury, C. C., Hewlett, L. J., Prescott, A. R., Shastri, N., and Watts, C. (1995). Class I MHC presentation of exogenous soluble antigen via macropinocytosis in bone marrow macrophages. *Immunity* 3, 783–791.

Peck, G. R., Ye, S., Pham, V., Fernando, R. N., Macaulay, S. L., Chai, S. Y., and Albiston, A. L. (2006). Interaction of the Akt substrate, AS160, with the glucose transporter 4 vesicle marker protein, insulin-regulated aminopeptidase. *Mol. Endocrinol.* 20, 2576–2583.

Peng, X. D., Xu, P. Z., Chen, M. L., Hahn-Windgassen, A., Skeen, J., Jacobs, J., Sundararajan, D., Chen, W. S., Crawford, S. E., Coleman, K. G., and Hay, N. (2003). Dwarfism, impaired skin development, skeletal muscle atrophy, delayed bone development, and impeded adipogenesis in mice lacking Akt1 and Akt2. *Genes Dev.* 17, 1352–1365.

Pham, V., Burns, P., Albiston, A. L., Yeatman, H. R., Ng, L., Diwakarla, S., and Chai, S. Y. (2009). Reproduction and maternal behavior in insulin-regulated aminopeptidase (IRAP) knockout mice. *Peptides* 30, 1861–1865.

Rasmussen, T. E., Pedraza-Diaz, S., Hardre, R., Laustsen, P. G., Carrion, A. G., and Kristensen, T. (2000). Structure of the human oxytocinase/insulin-regulated aminopeptidase gene and localization to chromosome 5q21. *Eur. J. Biochem.* 267, 2297–2306.

Reizis, B., Colonna, M., Trinchieri, G., Barrat, F., and Gilliet, M. (2011). Plasmacytoid dendritic cells: one-trick ponies or workhorses of the immune system? *Nat. Rev. Immunol.* 11, 558–565.

Rock, K. L., and Shen, L. (2005). Cross-presentation: underlying mechanisms and role in immune surveillance. *Immunol. Rev.* 207, 166–183.

Rodriguez, A., Regnault, A., Kleijmeer, M., Ricciardi-Castagnoli, P., and Amigorena, S. (1999). Selective transport of internalized antigens to the cytosol for MHC class I presentation in dendritic cells. *Nat. Cell Biol.* 1, 362–368.

Rogers, L. D., and Foster, L. J. (2007). The dynamic phagosomal proteome and the contribution of the endoplasmic reticulum. *Proc. Natl. Acad. Sci. U.S.A.* 104, 18520–18525.

Sandoval, I. V., Martinez-Arca, S., Valdueza, J., Palacios, S., and Holman, G. D. (2000). Distinct reading of different structural determinants modulates the dileucine-mediated transport steps of the lysosomal membrane protein LIMPII and the insulin-sensitive glucose transporter GLUT4. *J. Biol. Chem.* 275, 39874–39885.

Sathe, P., Pooley, J., Vremec, D., Mintern, J., Jin, J. O., Wu, L., Kwak, J. Y., Villadangos, J. A., and Shortman, K. (2011). The acquisition of antigen cross-presentation function by newly formed dendritic cells. *J. Immunol.* 186, 5184–5192.

Saveanu, L., Carroll, O., Hassainya, Y., and Van Endert, P. (2005a). Complexity, contradictions, and conundrums: studying post-proteasomal proteolysis in HLA class I antigen presentation. *Immunol. Rev.* 207, 42–59.

Saveanu, L., Carroll, O., Lindo, V., Del Val, M., Lopez, D., Lepelletier, Y., Greer, F., Schomburg, L., Fruci, D., Niedermann, G., and Van Endert, P. M. (2005b). Concerted peptide trimming by human ERAP1 and ERAP2 aminopeptidase complexes in the endoplasmic reticulum. *Nat. Immunol.* 6, 689–697.

Saveanu, L., Carroll, O., Weimershaus, M., Guermonprez, P., Firat, E., Lindo, V., Greer, F., Davoust, J., Kratzer, R., Keller, S. R., Niedermann, G., and Van Endert, P. (2009). IRAP identifies an endosomal compartment required for MHC class I cross-presentation. *Science* 325, 213–217.

Savina, A., Jancic, C., Hugues, S., Guermonprez, P., Vargas, P., Moura, I. C., Lennon-Dumenil, A. M., Seabra, M. C., Raposo, G., and Amigorena, S. (2006). NOX2 controls phagosomal pH to regulate antigen processing during crosspresentation by dendritic cells. *Cell* 126, 205–218.

Savina, A., Peres, A., Cebrian, I., Carmo, N., Moita, C., Hacohen, N., Moita, L. F., and Amigorena, S. (2009). The small GTPase Rac2 controls phagosomal alkalinization and antigen crosspresentation selectively in CD8(+) dendritic cells. *Immunity* 30, 544–555.

Sbodio, J. I., and Chi, N. W. (2002). Identification of a tankyrase-binding motif shared by IRAP, TAB182, and human TRF1 but not mouse TRF1. NuMA contains this RXXPDG motif and is a novel tankyrase partner. *J. Biol. Chem.* 277, 31887–31892.

Segura, E., Albiston, A. L., Wicks, I. P., Chai, S. Y., and Villadangos, J. A. (2009). Different cross-presentation pathways in steady-state and inflammatory dendritic cells. *Proc. Natl. Acad. Sci. U.S.A.* 106, 20377–20381.

Serwold, T., Gaw, S., and Shastri, N. (2001). ER aminopeptidases generate a unique pool of peptides for MHC class I molecules. *Nat. Immunol.* 2, 644–651.

Serwold, T., Gonzalez, F., Kim, J., Jacob, R., and Shastri, N. (2002). ERAAP customizes peptides for MHC class I molecules in the endoplasmic reticulum. *Nature* 419, 480–483.

Shen, L., Sigal, L. J., Boes, M., and Rock, K. L. (2004). Important role of cathepsin S in generating peptides

for TAP-independent MHC class I crosspresentation in vivo. *Immunity* 21, 155–165.

Shortman, K., and Naik, S. H. (2007). Steady-state and inflammatory dendritic-cell development. *Nat. Rev. Immunol.* 7, 19–30.

Siddle, K. (2011). Signalling by insulin and IGF receptors: supporting acts and new players. *J. Mol. Endocrinol.* 47, R1–R10.

Sigal, L. J., and Rock, K. L. (2000). Bone marrow-derived antigen-presenting cells are required for the generation of cytotoxic T lymphocyte responses to viruses and use transporter associated with antigen presentation (TAP)-dependent and -independent pathways of antigen presentation. *J. Exp. Med.* 192, 1143–1150.

Stragier, B., De Bundel, D., Sarre, S., Smolders, I., Vauquelin, G., Dupont, A., Michotte, Y., and Vanderheyden, P. (2008). Involvement of insulin-regulated aminopeptidase in the effects of the renin-angiotensin fragment angiotensin IV: a review. *Heart Fail. Rev.* 13, 321–337.

Subtil, A., Lampson, M. A., Keller, S. R., and Mcgraw, T. E. (2000). Characterization of the insulin-regulated endocytic recycling mechanism in 3T3-L1 adipocytes using a novel reporter molecule. *J. Biol. Chem.* 275, 4787–4795.

Tanioka, T., Hattori, A., Mizutani, S., and Tsujimoto, M. (2005). Regulation of the human leukocyte-derived arginine aminopeptidase/endoplasmic reticulum-aminopeptidase 2 gene by interferon-gamma. *FEBS J.* 272, 916–928.

Tojo, H., Kaieda, I., Hattori, H., Katayama, N., Yoshimura, K., Kakimoto, S., Fujisawa, Y., Presman, E., Brooks, C. C., and Pilch, P. F. (2003). The Formin family protein, formin homolog overexpressed in spleen, interacts with the insulin-responsive aminopeptidase and profilin IIa. *Mol. Endocrinol.* 17, 1216–1229.

Touret, N., Paroutis, P., Terebiznik, M., Harrison, R. E., Trombetta, S., Pypaert, M., Chow, A., Jiang, A., Shaw, J., Yip, C., Moore, H. P., Van Der Wel, N., Houben, D., Peters, P. J., De Chastellier, C., Mellman, I., and Grinstein, S. (2005). Quantitative and dynamic assessment of the contribution of the ER to phagosome formation. *Cell* 123, 157–170.

Tsujimoto, M., and Hattori, A. (2005). The oxytocinase subfamily of M1 aminopeptidases. *Biochim. Biophys. Acta* 1751, 9–18.

Tsujimoto, M., Mizutani, S., Adachi, H., Kimura, M., Nakazato, H., and Tomoda, Y. (1992). Identification of human placental leucine aminopeptidase as oxytocinase. *Arch. Biochem. Biophys.* 292, 388–392.

van Endert, P. (2011). Post-proteasomal and proteasome-independent generation of MHC class I ligands. *Cell. Mol. Life Sci.* 68, 1553–1567.

van Endert, P. M., Tampe, R., Meyer, T. H., Tisch, R., Bach, J. F., and Mcdevitt, H. O. (1994). A sequential model for peptide binding and transport by the transporters associated with antigen processing. *Immunity* 1, 491–500.

van Montfoort, N., Camps, M. G., Khan, S., Filippov, D. V., Weterings, J. J., Griffith, J. M., Geuze, H. J., Van Hall, T., Verbeek, J. S., Melief, C. J., and Ossendorp, F. (2009). Antigen storage compartments in mature dendritic cells facilitate prolonged cytotoxic T lymphocyte cross-priming capacity. *Proc. Natl. Acad. Sci. U.S.A.* 106, 6730–6735.

Wallis, M. G., Lankford, M. F., and Keller, S. R. (2007). Vasopressin is a physiological substrate for the insulin-regulated aminopeptidase IRAP. *Am. J. Physiol. Endocrinol. Metab.* 293, E1092–E1102.

Watson, R. T., Hou, J. C., and Pessin, J. E. (2008). Recycling of IRAP from the plasma membrane back to the insulin-responsive compartment requires the Q-SNARE syntaxin 6 but not the GGA clathrin adaptors. *J. Cell. Sci.* 121, 1243–1251.

Watson, R. T., Khan, A. H., Furukawa, M., Hou, J. C., Li, L., Kanzaki, M., Okada, S., Kandror, K. V., and Pessin, J. E. (2004). Entry of newly synthesized GLUT4 into the insulin-responsive storage compartment is GGA dependent. *EMBO J.* 23, 2059–2070.

Weimershaus, M., Maschalidi, S., Sepulveda, F., Manoury, B., Van Endert, P., and Saveanu, L. (2012). Conventional dendritic cells require IRAP-Rab14 endosomes for efficient cross-presentation. *J. Immunol.* 188, 1840–1846.

Wright, J. W., Yamamoto, B. J., and Harding, J. W. (2008). Angiotensin receptor subtype mediated physiologies and behaviors: new discoveries and clinical targets. *Prog. Neurobiol.* 84, 157–181.

Yan, J., Parekh, V. V., Mendez-Fernandez, Y., Olivares-Villagomez, D., Dragovic, S., Hill, T., Roopenian, D. C., Joyce, S., and Van Kaer, L. (2006). In vivo role of ER-associated peptidase activity in tailoring peptides for presentation by MHC class Ia and class Ib molecules. *J. Exp. Med.* 203, 647–659.

Yang, Z. Z., Tschopp, O., Di-Poi, N., Bruder, E., Baudry, A., Dummler, B., Wahli, W., and Hemmings, B. A. (2005). Dosage-dependent effects of Akt1/protein kinase Balpha (PKBalpha) and Akt3/PKBgamma on thymus, skin, and cardiovascular and nervous system development in mice. *Mol. Cell. Biol.* 25, 10407–10418.

York, I. A., Chang, S. C., Saric, T., Keys, J. A., Favreau, J. M., Goldberg, A. L., and Rock, K. L. (2002). The ER aminopeptidase ERAP1 enhances or limits antigen presentation by trimming epitopes to 8-9 residues. *Nat. Immunol.* 3, 1177–1184.

Zeigerer, A., Mcbrayer, M. K., and Mcgraw, T. E. (2004). Insulin stimulation of GLUT4 exocytosis, but not its inhibition of endocytosis, is dependent on RabGAP AS160. *Mol. Biol. Cell* 15, 4406–4415.

Zervoudi, E., Papakyriakou, A., Georgiadou, D., Evnouchidou, I., Gajda, A., Poreba, M., Salvesen, G. S., Drag, M., Hattori, A., Swevers, L., Vourloumis, D., and Stratikos, E. (2011). Probing the S1 specificity pocket of the aminopeptidases that generate antigenic peptides. *Biochem. J.* 435, 411–420.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 05 December 2011; accepted: 03 March 2012; published online: 21 March 2012.

Citation: Saveanu L and van Endert P (2012) The role of insulin-regulated aminopeptidase in MHC class I antigen presentation. Front. Immun. ***3****:57. doi: 10.3389/fimmu.2012.00057*

This article was submitted to Frontiers in Antigen Presenting Cell Biology, a specialty of Frontiers in Immunology.

frontiers in
IMMUNOLOGY

REVIEW ARTICLE
published: 05 March 2012
doi: 10.3389/fimmu.2012.00040

Escape from the phagosome: the explanation for MHC-I processing of mycobacterial antigens?

Melanie J. Harriff [1,2]***, Georgiana E. Purdy*** [3] ***and David M. Lewinsohn*** [1,2,3]******

[1] *Portland VA Medical Center, Portland, OR, USA*
[2] *Pulmonary and Critical Care Medicine, Oregon Health & Sciences University, Portland, OR, USA*
[3] *Department of Molecular Microbiology and Immunology, Oregon Health & Sciences University, Portland, OR, USA*

Edited by:
Christian Kurts, Friedrich Wilhelms-Universität Bonn, Germany

Reviewed by:
Kang Liu, Columbia University, USA
Dietmar Zehn, Swiss Vaccine Research Institute, Switzerland

****Correspondence:***
David M. Lewinsohn, Pulmonary and Critical Care Medicine, Oregon Health & Sciences University, 3181 SW Sam Jackson Park Road, Mail Code VA R&D11, Portland, OR, 97239, USA.
e-mail: lewinsod@ohsu.edu

Mycobacterium tuberculosis (Mtb) is thought to live in an altered phagosomal environment. In this setting, the mechanisms by which mycobacterial antigens access the major histocompatibility class I (MHC-I) processing machinery remain incompletely understood. There is evidence that Mtb antigens can be processed in both endocytic and cytosolic environments, with different mechanisms being proposed for how Mtb antigens can access the cytosol. Recently, electron microscopy was used to demonstrate that Mtb has the potential to escape the phagosome and reside in the cytosol. This was postulated as the primary mechanism by which Mtb antigens enter the MHC-I processing and presentation pathway. In this commentary, we will review data on the escape of Mtb from the cytosol and whether this escape is required for antigen presentation to $CD8^+$ T cells.

Keywords: *Mycobacterium tuberculosis*, phagosome, MHC-I antigen processing, $CD8^+$ T cells

INTRODUCTION

Mycobacterium tuberculosis (Mtb) remains a highly prevalent and successful pathogen worldwide, with co-infection with HIV and emergence of multiple drug-resistant strains compounding the impact. Estimates indicate approximately one-third of world population has immunological evidence of infection with Mtb with over 10 million new cases each year (Dye et al., 1999). Mtb is a successful human pathogen because it is able to subvert the host immune response, often choosing an intracellular lifestyle. At the same time, the human immune response is largely successful at containing Mtb-infection, due to an effective Th1 response, reflecting the coordinated action of $CD4^+$ and $CD8^+$ T cell immunity. While $CD4^+$ T cells play an important role in this process, $CD8^+$ T cells are essential because of their unique ability to recognize intracellular infection, particularly in those cells that are major histocompatibility class (MHC)-II negative (Grotzke and Lewinsohn, 2005). One critical question that remains unanswered is how antigens derived from Mtb gain access to the MHC-I antigen processing and presentation pathway.

Following aerosol exposure, Mtb is taken up by lung-resident macrophages and dendritic cells (DC), where it resides in a phagosomal compartment. DCs have been shown to play an essential role in the immune response *in vivo* through cross-presentation and cross-priming functions (Mellman and Steinman, 2001; Segura and Villadangos, 2009; Amigorena and Savina, 2010). Although *in vivo* studies indicate DCs are involved in the immune response following infection with Mtb (Tian et al., 2005; Wolf et al., 2007; Leepiyasakulchai et al., 2012), very little is known about their role in the initiation of $CD8^+$ T cell responses. *In vitro*, many studies have focused on the nature of the Mtb-containing phagosomal compartment. The consensus in the field is that this compartment does not fuse with lysosomes, but intersects with the endosomal pathways, recruiting molecules and necessary nutrients for survival and replication (reviewed in Russell, 2001; Philips, 2008). With regard to MHC-I antigen presentation and initiation of a $CD8^+$ T cell response, studies indicate the presence of processing machinery on phagosomes, including MHC-I and the transporter associated with antigen processing (TAP; Ackerman et al., 2003; Guermonprez et al., 2003; Houde et al., 2003; Grotzke et al., 2009). Work by other groups demonstrates that Mtb antigens can access both cytosolic and vacuolar antigen processing pathways (Mazzaccaro et al., 1996; Neyrolles et al., 2001; Schaible et al., 2003; Lewinsohn et al., 2006). We recently showed that the Mtb compartment is a competent MHC-I antigen processing and presentation organelle. Loaded HLA-E molecules and TAP are present on the phagosome (Grotzke et al., 2009), and peptide import into the lumen of the Mtb phagosome occurs (Harriff, M. J., unpublished data). An alternative model was also proposed that attributes Mtb antigen presentation on MHC-I molecules and subsequent recognition by $CD8^+$ T cells to the ability of Mtb to escape from its phagosomal membrane and reside in the cytoplasm (van der Wel et al., 2007; Weerdenburg et al., 2010).

In this review, we will revisit the literature analyzing the intracellular localization of Mtb and address potential reasons for the discrepancies seen in various *in vitro* and *in vivo* studies. Much of our understanding of the intracellular lifestyle of Mtb is derived from microscopy studies. In particular, electron microscopy (EM) provides sufficient ultrastructural resolution to visualize Mtb within vacuolar structures. In addition, many researchers utilize immuno-fluorescence (IF) microscopy using antibodies against vacuolar membrane proteins to characterize the co-localization of these markers with surface-labeled or fluorescent protein-expressing Mtb. Here, we will focus on studies that document Mtb localization by EM and immuno-EM techniques. We will then look at how these studies correspond to *in vivo* analyses of Mtb localization within cells. Finally, we will assess whether

or not escape from the phagosome is necessary for access of antigen to the MHC-I pathway. If the intracellular lifestyle of Mtb is critical to recognition of infection by $CD8^+$ T cells, it is imperative to characterize Mtb localization in cells for better vaccine design strategies.

INTRACELLULAR LOCALIZATION OF Mtb *IN VITRO*

Intracellular localization of Mtb within phagocytes has been observed since the late 1960s and early 1970s. In their seminal study, Armstrong and Hart analyzed mouse peritoneal macrophages infected with viable or non-viable *M. tuberculosis* H37Rv strain and *Mycobacterium bovis* BCG by EM. They noted that 4 days post infection, "bacteria were never seen to be free in the cytoplasm (i.e., outside phagosomes)," with 23% of these phagosomes exhibiting lysosomal fusion. Macrocyclon was subsequently used to prevent replication of the bacteria. In this case, viable H37Rv remained in membrane-bound phagosomes out to 14 days post infection, with 21% fusing with lysosomes (Armstrong and Hart, 1971).

The first reports of Mtb escape from the phagosome were published in 1980s by the Wright laboratory. Surprisingly, this group reported that at as early as 18–24 h post infection, as many as 60–100% of bacteria had escaped the phagosome and were in the cytosol (Leake et al., 1984; Myrvik et al., 1984). The reasons for this discrepancy remain unclear, however these studies differed dramatically between in the species, source, and cultivation of macrophages, and the preparation of and multiplicity of infection (MOI) of the bacterial inoculum. Specifically, rabbit alveolar macrophages were infected with H37Rv at higher MOI (20–25:1) than was used in the Armstrong and Hart (1971) study (MOI ∼5:1). These studies and others describing the intracellular location of Mtb are summarized in **Table 1**.

A series of papers published in the 1990s evaluated the precise intracellular location of Mtb. McDonough et al. (1993) noted that half of the bacteria infecting J774 mouse macrophages had escaped to the cytosol 4 days following infection. The authors qualified their observations by suggesting "one must exercise caution in distinguishing between a tubercle bacillus free in the cytoplasm and one which is encased in a tightly apposed vacuolar membrane." Subsequent studies by Xu et al. (1994) and Clemens and Horwitz (1995) addressed this concern using H37Rv-infected mouse bone marrow-derived macrophages and primary human monocytes, respectively. Xu et al. observed mycobacteria in a membrane-bound, LAMP1 positive compartment for as long as 14 days post infection. In many cases, the membrane was tightly apposed to the bacteria (Xu et al., 1994). The differences between McDonough et al. and Xu et al. may reflect microscopic technique, since Xu et al. utilized cryopreservation prior to EM, whereas McDonough

Table 1 | *In vitro* ultrastructural analyses of Mtb intracellular localization.

Author and Year	Journal	Cell type	Mycobacterial strain	MOI (if indicated)	Maximum length of infection	% Bacteria free in the cytosol
Armstrong and Hart (1971)	J. Exp. Med.	Mouse peritoneal macrophages	H37Rv	∼5:1	14 d	0
Myrvik et al. (1984)	Am. Rev. Respir. Dis.	Rabbit alveolar macrophages	H37Rv	20–25:1	24 h	60–100
			H37Ra	20–25:1	24 h	<1
Leake et al. (1984)	Infect Immun.	Rabbit alveolar macrophages	H37Rv	20–25:1	18 h	70–99
		Rabbit alveolar macrophages – BCG immunized	H37Rv	20–25:1	18 h	8–28
McDonough et al. (1993)	Infect. Immun.	J774 mouse macrophages	H37Rv	1–10:1	4 d	∼50
Xu et al. (1994)	J. Immunol.	Mouse bone marrow-derived macrophages	H37Rv	10–20:1	14 d	0
Clemens and Horwitz (1995)	J. Exp. Med.	Primary human monocytes	Erdman	0.5:1	5 d	0
Paul et al. (1996)	J. Infect. Dis.	Human monocyte-derived macrophages from PBMC	H37Rv	1:1	6 d	0
Mazzaccaro et al. (1996)	Proc. Natl. Acad. Scei. U.S.A.	Mouse bone marrow-derived macrophages	Erdman	3–10:1	24 h	0
Beatty et al. (2000)	Traffic	Mouse bone marrow-derived macrophages	CD1551 (CSU93)	25:1	16 d	0
Clemens et al. (2002)	Infect. Immun.	Human peripheral blood mononuclear cells, THP-1 monocytes (human)	Erdman	30:1	3 d	0[1]
van der Wel et al. (2007)	Cell	Human monocyte-derived DC	H37Rv	10:1	4 d	32[2]
					7 d	57[2]
Peyron et al. (2008)	PLoS Pathog.	Foamy macrophages (human)	H37Rv	1:100	11 d	0

[1] *Determined by fluorescence microscopy, percentage not given for ultrastructural studies.*

[2] *Percentage of DC containing cytosolic mycobacteria.*

et al. (1993) used organic solvents to dehydrate the sample. The use of organic solvents has the potential to extract the phagosomal membranes and result in the appearance of cytosolic localization. Studies by Clemens and Horwitz also utilized cryopreservation and immuno-EM to delineate the intracellular location of Mtb. In this study, the intracellular compartment of single bacteria versus multiple bacteria was markedly different. Specifically, single bacteria cell were localized to phagosomes that rarely fused with lysosomes and stained positively for multiple markers, including MHC-I, MHC-II, and the transferrin receptor (TfR). These bacteria were not found free in the cytoplasm out to 5 days post infection. In contrast, organelles containing multiple mycobacteria were observed to fuse with lysosomes, and stained positively for CD63 (Clemens and Horwitz, 1995).

Subsequent EM experiments revealed Mtb surrounded by a phagosomal membrane that had not fused with the lysosome. The studies listed in **Table 1** and others have defined the phenotype of this phagosomal membrane. Many proteins are now commonly used markers in the study of host–pathogen interactions of Mtb and other intracellular bacteria (illustrated schematically in **Figure 1**). Identification of the vacuolar trafficking proteins that are acquired and retained or excluded from the Mtb phagosome defined the point of Mtb phagosome maturation arrest as the retention of Rab5 and failure to acquire Rab7 or CD63. While phagosome maturation is arrested, the Mtb-containing vacuole remains dynamic and fuses with other vacuolar compartments. These interactions result in localization of the TfR and MHC-I to the Mtb phagosome (Clemens and Horwitz, 1995, 1996; Sturgill-Koszycki et al., 1996; Via et al., 1997; Kelley and Schorey, 2003). The kinetics and mechanisms of molecule trafficking to and from the phagosome, the bacterial protein and lipid effectors responsible for preventing fusion of Mtb phagosomes with lysosomes have been extensively reviewed elsewhere (Deretic et al., 1997; Deretic and Fratti, 1999; Pieters, 2001; Russell, 2001; Vergne et al., 2004; Brumell and Scidmore, 2007; Philips, 2008).

Recent work by Peter Peters' group challenged this view and suggested that the Mtb phagosome fuses with lysosomes early after infection and that bacteria translocate to the cytosol by 48 h after infection (van der Wel et al., 2007). In contrast to earlier work that had focused on macrophages and monocytes, human monocyte-derived DC that had been differentiated for 5 days prior to bacterial infection were employed. Cryo-immunogold EM was performed at time points from 2 to 96 h following infection. At early time points Mtb was present in a phagosome characterized by the presence of CD63, Lamp1, Lamp2, and Cathepsin D, while MHC-I, TfR, and EEA1 were absent. In spite of the presence of lysosomal markers, these early organelles did not acidify. At later time points, the authors observed the gradual accumulation of Mtb, but not BCG in the cytosol. By 7 days, more than half of the cells contained cytosolic Mtb. Translocation of Mtb to the cytosol was dependent on the ESX-1 Type VII secretion system and EspA. The ESX-1 system is encoded in the region of difference 1 (RD1) region of the Mtb genome that is missing from BCG. Based on their data, van der Wel et al. (2007) proposed that Mtb escape into the cytosol is the primary mechanism by which Mtb antigens

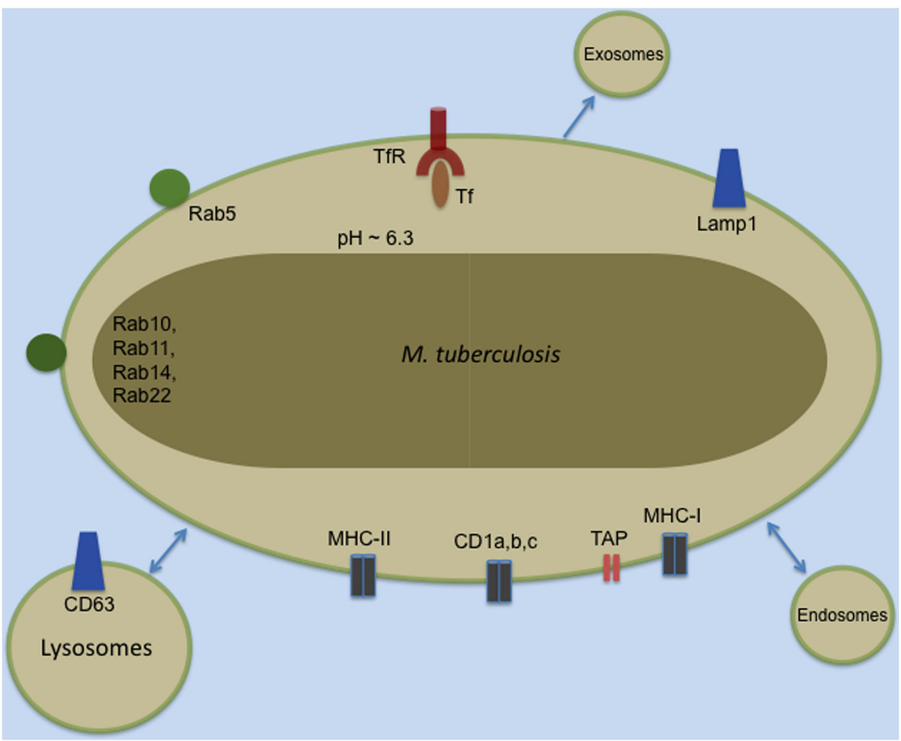


FIGURE 1 | Common markers of the Mtb phagosome.

access the MHC-I processing pathway (van der Wel et al., 2007; Weerdenburg et al., 2010).

Preparation of samples for EM, the type and viability of the host cell, and preparation of the bacterial inoculum may explain the conflicting results regarding phagolysosomal fusion as well as the escape of Mtb to the cytosol. However, van der Wel et al. (2007) used state of the art techniques for EM sample preparation and analysis. Alternatively, it is possible that presence of cytosolic mycobacteria reflected the viability of the DC. First, in our experience, Mtb-infected DC cultured for longer than 7 days have high levels of cell death, and consequently diminished capacity for antigen presentation. Second, it has been reported that Mtb-infection is associated with the death of the host cell. It has been previously demonstrated that virulent mycobacteria inhibit apoptosis. Uptake of Mtb within apoptotic bodies by uninfected cells is a mechanism by which Mtb growth is inhibited (Keane et al., 2000). Conversely, the ability to promote macrophage necrosis is a function of mycobacterial virulence (Hsu et al., 2003; Pan et al., 2005). The induction of necrotic cell death is postulated to promote mycobacterial spread. More recently, it has been demonstrated that high intracellular bacterial burden results in a process termed "atypical cell death." This Mtb-induced cell death is characterized by lysosomal membrane permeabilization followed by degradation of lipid bilayers, and results in morphological characteristics and activation of molecules distinct from apoptosis and necrosis (Lee et al., 2006, 2011; Park et al., 2006). This elevated intracellular mycobacterial burden can result from either a high initial inoculum (Lee et al., 2006), or from ongoing intracellular bacterial replication (Park et al., 2006), and was associated with the disruption of phagosomal membranes and free cytosolic Mtb (Lee et al., 2011). Apoptosis was evaluated by van der Wel et al. (2007) through examination of Caspase 3 levels using fluorescence microscopy in conjunction with analysis of the morphological changes associated with apoptosis by EM. Only 5% of the Mtb-infected DC were apoptotic at 96 h after infection. However, their analysis does not provide an estimate of those cells undergoing atypical and/or necrotic cell death. Although the ability of Mtb to induce atypical cell death is DC is not known, these data suggest that the observation of cytosolic Mtb is a reflection of atypical cell death.

Along with virulence and bacterial burden, the preparation of the inoculum is another possible explanation for discordance between these studies. As discussed previously, both viability and homogeneity of the culture can influence whether or not the Mtb phagosome will undergo lysosomal fusion. For example, it has been previously demonstrated that non-viable mycobacteria are taken up into phagosomes that fuse with lysosomes. As the viability of the inoculum used by van der Wel et al. (2007) was not indicated, this parameter is difficult to evaluate. Furthermore, tight apposition of the phagosomal membrane to the mycobacteria is required to prevent lysosomal fusion (De Chastellier et al., 2009). In this case even modest bacterial clumping will result in lysosomal fusion. The presence of multiple bacteria in a single phagosome at time points shortly after infection in the van der Wel study suggests these mycobacteria-containing phagosomes will likely fuse with lysosomes and may explain the discordant protein co-localization data.

DOES Mtb ESCAPE THE PHAGOSOME *IN VIVO*?

While *in vitro* models are invaluable in the study of host–pathogen interactions, of central importance is the intracellular location of Mtb *in vivo*. In humans, surprisingly little is known regarding the intracellular location of Mtb. Following exposure to Mtb, widely divergent outcomes can occur, ranging from no clinical or immunologic evidence of exposure, to a state of latency defined by immunologic evidence of exposure in the absence of clinical or radiographic manifestations of disease. At present, the means by which aerosolized Mtb enters the lung is poorly understood. Furthermore, it is worth noting that in the limited studies using mycobacterial culture to determine the location of Mtb in those thought latently infected, Mtb can be observed in both granulomatous tissue and areas of normal lung (Opie and Aronson, 1927). Ultrastructural analyses of tissues following natural infection of humans are informative, but limited in scope (**Table 2**). EM of bronchoalveolar lavage samples from infected individuals revealed that Mtb localizes in membrane-bound compartments in infected alveolar macrophages (Russell et al., 2002; Mwandumba et al., 2004). These compartments typically contained a single mycobacterium with the membrane tightly apposed to the organism. In some cases of heavy infection, multiple organisms were observed in a larger compartment. While these studies demonstrate vacuolar Mtb in alveolar macrophages, the intracellular location of Mtb in alternate cells such as DC and epithelial cells *in vivo* is not clear.

The mouse model of TB was designed to cause persistent intracellular infection. Nonetheless, the intracellular localization of Mtb has been examined in lung tissue samples from experimentally infected mice (**Table 2**). While limited in scope, ultrastructural analyses of granulomatous lung lesions and lung homogenates have consistently demonstrated the presence of Mtb and other virulent mycobacteria in a membrane-bound organelle (Merckx et al., 1964; Dumont and Sheldon, 1965; Kondo et al., 1982; Moreira et al., 1997). These studies also revealed heterogeneity in the morphology of the Mtb-containing compartment. In some cases, large membrane-bound vacuoles contained large numbers of bacteria, and in other cases, bacteria were observed singly, in phagosomes with tightly apposed membranes. Moreira et al. (1997) note, however, that multiple mycobacteria per phagosome were only observed in damaged macrophages at inflammatory sites. Within the granuloma, Mtb have been observed in phagosomal compartments in foamy macrophages (Caceres et al., 2009).

IS ESCAPE FROM THE PHAGOSOME REQUIRED FOR IMMUNE RECOGNITION?

It has been postulated that escape of Mtb to the cytosol is the mechanism by which Mtb antigens are processed and presented in the context of MHC-I. Here, we will address the following questions: What is the evidence that MHC-I antigens are exported from the phagosome? Is there enhanced presentation of antigen or recognition by T cells if Mtb escapes to the cytosol?

In contrast to MHC-II, which samples peptide antigens from within the endocytic environment, MHC-I is traditionally considered the primary mechanism by which cytosolically derived antigen can be processed and presented to T cells. The ability of specialized antigen presenting cells such as DC to process

Table 2 | *In vivo* ultrastructural analyses of Mtb intracellular localization.

Author and year	Journal	Tissue type	Bacterial strain	MOI (if indicated)	Days after infection	Observations
Merckx et al. (1964)	Am. Rev. Respir. Dis.	Pulmonary, liver, and spleen tissue (mice)	H37Rv	0.3 mg Mtb/25 g	31 days–5 months 5 months	All bacteria observed in membrane-bound compartments Some bacteria appear free in cytosol
Kondo et al. (1982)	Jpn. J. Med. Sci. Biol.	Granulomatous lung lesions and lung homogenate (mice)	*M. bovis* TCT401	0.5 mg *M. bovis*/mouse	3 weeks	Bacteria in alveolar macrophages are in membrane-bound compartments Bacteria in granulomatous tissue are sometimes observed with disrupted membranes or free in the cytosol
Moreira et al. (1997)	Infect. Immun.	Granulomatous lesions (mice)	*M. tuberculosis* Erdman	200 bacteria/mouse	14–35 days	All bacteria observed in membrane-bound compartments
Russell et al. (2002)	J. Cell Biol.	Alveolar macrophages from bronchoalveolar lavage (BAL; human)	UK[a]	UK[a]	UK[a]	Single and multiple bacteria observed in membrane-bound compartments in heavily infected alveolar macrophages
Mwandumba et al. (2004)	J. Immun.	Alveolar macrophages from bronchoalveolar lavage (BAL; human)	UK[a]	UK[a]	UK[a]	Bacteria observed in membrane-bound compartments Occasionally in cases of heavy infection, multiple bacteria observed in a large vacuole
Caceres et al. (2009)	Tuberculosis	Foamy macrophages (mouse)	H37Rv	20–50 bacteria/mouse	3–9 weeks	Multiple bacteria observed in membrane-bound compartments out to 3 weeks Single bacteria observed in membrane-bound compartments after 3 weeks

[a] *Unknown.*

and present antigens derived from non-cytosolic sources in the context of MHC-I has been termed cross-presentation. Cross-presentation was originally described by Michael Bevan's group (Bevan, 1976; Carbone and Bevan, 1990) and has since been extensively characterized (reviewed in Shen and Rock, 2006; Burgdorf and Kurts, 2008; Lin et al., 2008; Amigorena and Savina, 2010; Van Endert, 2011). Kovacsovics-Bankowski and Rock (1995) originally described the presentation of particulate antigens by demonstrating that bead-associated OVA could stimulate IFNγ production by MHC-I restricted T cells. Many of the details of processing and cross-presentation of antigens have since been elucidated using inert particles such as latex beads, and it is clear the physical nature of the antigen has a profound effect on the mechanisms underlying processing and presentation. Particulate antigens taken up in phagosomes are processed and presented on MHC-I molecules early after uptake, prior to complete acidification of the endocytic compartment (Burgdorf and Kurts, 2008). Soluble antigens, on the other hand, are taken up into distinct endocytic compartments, mediated by binding to cell-surface receptors that determine whether or not the antigen will access MHC-I processing pathways. For example, soluble antigens taken up by the mannose, Langerin, or DEC-205 receptors are targeted to early endosomes and presented on MHC-I molecules (Burgdorf and Kurts, 2008). Once taken up by the cell, both particulate and soluble exogenous antigens access the cytosol by an undefined mechanism, where the antigen undergoes proteasomal degradation, TAP-dependent import into the ER or phagosome, and loading onto MHC-I (Shen and Rock, 2006). In some cases, antigen remains in the phagosome/endosome, where it is proteolytically processed for loading on MHC-I molecules (Lin et al., 2008).

Following infection by intracellular bacterial pathogens, bacterially derived particulate antigens present within the phagosome can also access the MHC-I processing and presentation pathway (Ramachandra et al., 2009; Blanchard and Shastri, 2010). This is in contrast to viral antigens that are present at high levels in the cytosol and presented via classical MHC-I processing and presentation in the endoplasmic reticulum. The mechanisms by which antigens from intracellular bacteria are presented have been the focus of numerous subsequent studies. Although many aspects of these mechanisms are still under debate, it is clear that bacterially derived antigens can gain access to the MHC-I pathway in several distinct, non-mutually exclusive pathways. As noted, these pathways have been reviewed extensively and will be discussed below in more detail as they pertain specifically to the Mtb phagosome and processing and presentation of mycobacterially derived antigens from this phagosome.

The study of antigen processing and presentation in the context of an intracellular infection such as Mtb can provide unique insights into these mechanisms. Broadly, these can be divided into cytosolic and non-cytosolic pathways (**Figure 2**). The cytosolic pathway is defined primarily by a requirement for proteasomal processing, and secondarily for the use of TAP. Direct access of bacterial antigens into the cytosol would allow for proteasomally processed peptides to be transported into the ER by TAP, where they are further processed, loaded onto MHC-I, and subsequently transported to the plasma membrane. Our laboratory and others have used Mtb-specific T cell clones to demonstrate that a number of Mtb antigens access this classical proteasome- and TAP-dependent cytosolic antigen processing pathway *in vitro* (Lewinsohn et al., 1998, 2006; Canaday et al., 1999; Grotzke et al., 2010). Here, proteasomal blockers including lactacystin, LLnL, and epoxomicin, were used to show that certain secreted Mtb proteins, including CFP10, EsxJ, and Ag85B, access the cytosol and that presentation of these antigens by infected DCs requires the proteasome. Furthermore, the use of virally encoded proteins that block the ability of TAP to import peptides into the ER, such as ICP47, indicated that presentation of these same proteins requires TAP. The DCs used in these studies were fixed and used in ELISPOT assays after less than 18 h of infection with Mtb. Mycobacterial escape from the phagosome has not been reported at this time point, indicating that these secreted antigens access the cytosol without a requirement for bacterial escape. *In vivo* data also support a role for TAP in MHC-I presentation of Mtb-derived antigens. For example, Sousa et al. (2000) used TAP1$^{(-/-)}$ mice to show that the protective immunity derived from CD8$^+$ T cells is largely TAP-dependent, although TAP-independent mechanisms also contribute to protection. Taken together, these studies suggest a preferential use of the cytosolic pathway for presentation of Mtb antigens on MHC-I.

The extent to which particulate phagosomal proteins gain access to the cytosol and can compete with abundant self proteins is unclear. As a result, it has been postulated that the phagosome itself participates in antigen processing and presentation, thus enhancing the display of these proteins (Gagnon et al., 2002; Ackerman et al., 2003; Guermonprez et al., 2003; Houde et al., 2003; Burgdorf et al., 2008). In contrast to the traditional cytosolic pathway, antigens transiently access the cytosol and are proteasomally processed. Peptides are then re-imported via TAP into the phagosome where they are loaded onto MHC-I. Proteins and molecules involved in antigen processing and presentation, including TAP, calnexin, tapasin, calreticulin, ERp57, and MHC-I, are present on bead phagosomes as well as on Mtb phagosomes (Grotzke et al., 2009). Providing direct evidence for this alternative phagosome–cytosolic pathway after Mtb infection, isolated Mtb phagosomes can directly stimulate IFN-γ production by Mtb-specific CD8$^+$ T cells, indicating the presence of loaded MHC-I complexes (Grotzke et al., 2009). Additionally, as demonstrated with latex bead phagosomes (Ackerman et al., 2003), TAP in the Mtb phagosome is functional for peptide translocation (Harriff, M. J., unpublished data). Interestingly, ER molecules have also been observed on phagosomes containing other intracellular pathogens. Goldszmid et al. (2009) first demonstrated the presence of ER markers on the *Toxoplasma gondii* parasitophorous vacuole (PV). Further studies have identified a role for Sec22b in the recruitment of these ER proteins to the PV. In the absence of Sec22b, ER-derived proteins do not access the PV, and cross-presentation of *T. gondii* antigens is inhibited (Cebrian et al., 2011). While these studies define a role for the phagosome in cytosolic processing and loading of pathogen-derived antigens, they do not provide an estimate of the extent to which peptides are loaded within the phagosome versus the ER.

What is the direct evidence that mycobacterial antigens can access the cytosol? The Bloom laboratory was among the first to show *in vitro* that cells infected with Mtb could facilitate

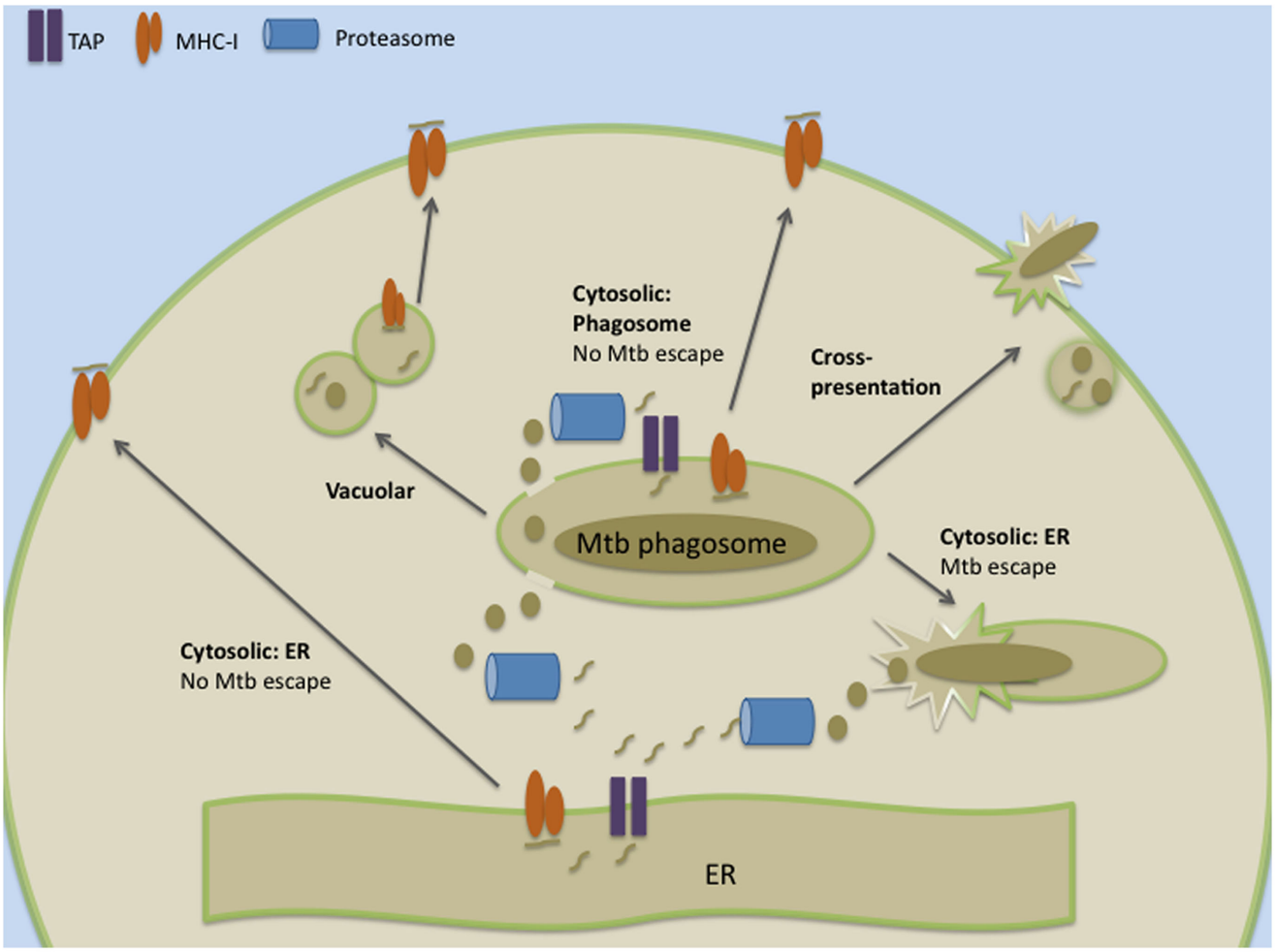


FIGURE 2 | Cytosolic and vacuolar pathways for processing and presenting Mtb antigens in the context of MHC-I.

the presentation of a soluble antigen. Infection of mouse macrophages with live Mtb led to TAP-dependent presentation of co-administered soluble OVA (Mazzaccaro et al., 1996). Similar experiments by this group with wild-type and listeriolysin (LLO)-deficient *Listeria monocytogenes* demonstrated LLO-dependent pore formation was required for the MHC-I presentation of soluble OVA antigen. Taken together, these data led the authors to postulate a similar mechanism for Mtb (Mazzaccaro et al., 1996). Subsequently, fluorescent dextrans, ovalbumin, and polystyrene beads were microinjected into the cytosol of macrophages infected with live or heat killed BCG. The BCG phagosome was found to be permeable to molecules up to 70 kDa in size. Uptake of fluorescent molecules was dependent on viable BCG, suggesting that BCG actively generates pores in the phagosome (Teitelbaum et al., 1999). It is unclear, however, what role these pores play in the access of proteins to the cytosol, as this study demonstrated unidirectional transport of molecules into the phagosome. Furthermore, a similar study employing the electroporation of fluorescently labeled 50 kDa Fab fragments indicated that Mtb phagosomes are impermeable to these molecules (Clemens et al., 2002). To evaluate the transport of Mtb-derived antigens from the phagosome to the cytosol, Schaible et al. co-administered Mtb with membrane impermeable fluorescent molecules (HPTS and a FITC-labeled peptide), and found that these molecules did not access the cytosol. Additionally, after infection with radiolabeled Mtb, minimal amounts of radiolabeled, Mtb-derived proteins (<4%) could be found in the cytosol (Schaible et al., 2003).

However, accumulating evidence suggests that certain proteins can gain access to the cytosol. The well-known antigenic proteins secreted by the ESX-1 Type VII secretion system, such as CFP10 and ESAT-6 can be found in the cytosol (Abdallah et al., 2007). Rv1694 (*tlyA*), a hemolysin and ribosomal RNA methyltransferase, can be exported to the cytosol (Rahman et al., 2010), and epitopes from this protein are recognized by $CD8^+$ T cells in the context of HLA-A∗0201 (Shams et al., 2003). The tyrosine phosphatase PtpA can be exported to the cytosol and phagosome membrane, where it disrupts the trafficking of the V-ATPase complex to the phagosome (Bach et al., 2006, 2008; Wong et al., 2011). Zmp1 is an Mtb- and BCG-encoded Zn^{2+} metalloprotease that interferes with activation of the inflammasome and subsequent maturation of the

phagosome. Zmp1 is required for virulence of Mtb in mice and survival of Mtb and BCG in macrophages and has been observed in the cytosol of infected cells (Master et al., 2008). These results suggest that mycobacterial proteins can access the cytosol without a requirement for Mtb escape from the phagosome

Particulate antigens can also be processed and presented in a manner that does not require access of proteins into the cytosol. This pathway has been termed the vacuolar pathway (**Figure 2**). Here, the generation of antigenic peptides depends on the presence of late endocytic peptidases such as cathepsins normally associated the processing of antigens for MHC-II. It has been postulated that subsequent loading of these peptides occurs through the exchange of peptides with MHC-I molecules found within the relatively acidic endocytic environment (Pfeifer et al., 1993; Song and Harding, 1996; Chefalo and Harding, 2001; Shen et al., 2004). Neyrolles et al. (2001) found that the Mtb 19 kDa lipoprotein is trafficked to distinct compartments from the mycobacteria, and that peptides derived from the 19 kDa lipoprotein are presented to $CD8^+$ T cells by a TAP-independent mechanism. Furthermore, Mtb antigens can also be found in exosomes that are continuously trafficked from the phagosome as well as apoptotic bodies derived from infected cells, allowing for the uptake of antigen by uninfected bystander APCs (Beatty and Russell, 2000; Beatty et al., 2000, 2001; Schaible et al., 2003). The fact that Mtb antigens can be presented to $CD8^+$ T cells without accessing the cytosol suggests that escape to the cytosol is not an absolute requirement.

IS RD1/ESX-1 NECESSARY FOR ANTIGEN PRESENTATION?

It is postulated that the ESX-1 secretion system is central to the escape of Mtb into the cytosol and thereby promotes MHC-I antigen presentation. Currently, there is no experimental evidence comparing the efficiency of $CD8^+$ T cell activation in response to cytosolic versus phagosomal Mtb. However, there are studies both *in vitro* and *in vivo* that have examined the effect of RD1 deletion on the acquisition and maintenance of mycobacterially reactive $CD8^+$ T cells. From these studies it is clear that presence of ESX-1 is not an absolute requirement for access of Mtb antigens into the cytosol. We have demonstrated that infection of human DC with the MtbΔRD1 mutant was as efficient as the complemented Mtb strain in activating $CD8^+$ T cell clones specific for the TB8.4 antigen (Lewinsohn et al., 2006). Billeskov et al. (2007) similarly observed robust induction of TB10.4 specific $CD8^+$ T cells upon infection of mice with either wild-type Mtb or the MtbΔRD1 mutant. The ability of specific mutants of the RD1 region, including the Δ*espA* mutant, to induce antigen-specific $CD8^+$ T cells was also unchanged. Woodworth et al. (2008) concluded that Mtb escape is not required for access of antigen to the MHC-I antigen processing pathway.

For *in vivo* experiments, virulence and the association of Mtb-specific $CD8^+$ T cells with bacterial burden make interpretation of these experiments difficult. It is clear that both the virulence and the induction of $CD8^+$ T cell responses are enhanced when the RD1 region is restored to BCG (Pym et al., 2003; Brodin et al., 2004; Majlessi et al., 2005). A number of experiments have addressed the issue of reduced bacterial burden in BCG-infected animals with regard to $CD8^+$ T cell activation. Russell et al. (2007) used an OVA expressing-BCG strain to show that increasing the dose of BCG leads to a more rapid $CD8^+$ T cell response, due to an increase in the amount of antigen. Ryan et al. (2009) administered greater numbers of BCG than Mtb to mice, such that the lymph node mycobacterial burden was comparable. By establishing equivalent levels of antigen at the site of T cell priming, functionally equivalent, antigen-specific $CD8^+$ T cells were induced (Ryan et al., 2009). Taken together, these data imply that reduced $CD8^+$ T cell frequencies in response to bacteria lacking RD1 reflect decreased availability of antigen due to lower virulence, as opposed to an inability of these bacteria to escape to the cytosol.

CONCLUSION

While Mtb is not an obligate intracellular pathogen, its ability to co-opt the intracellular environment is central to Mtb to persistence in its human host. Maintaining a poorly acidified phagosomal environment provides protection, nutrients, and many other benefits to this pathogenic mycobacteria. Conversely, egress of Mtb into the cytosol with concomitant induction of apoptosis, autophagy, and atypical cell death are likely critical events in the interrelationship of pathogen and host, and may eventually be key to transmission. Mtb is a successful pathogen yet it is also contained by the host to a large extent. Here, it is important for the immune system to recognize and respond to infected cells. Knowing the intracellular niche of Mtb, and understanding how Mtb antigens are processed and presented to T cells within this context is critical to design of better vaccines. In this review, we have examined the evidence suggesting that Mtb escapes the phagosome, and whether or not this is a critical event in the induction and maintenance of MHC-I dependent immunity.

In vitro, it is clear that although escape of Mtb into the cytosol is possible, we have an incomplete understanding of the circumstances necessary for this to occur. While limited, ultrastructural evidence does not support the escape of Mtb from the phagosome *in vivo*. The presence of Mtb in cytosol could have consequences both for the host and the microbe. For example, cytosolic Mtb could lead to stimulation of innate intracellular sensors such as NOD2. While the presence of NOD2 has not been associated with mycobacterial growth control in the mouse, it has recently been shown that NOD2 stimulation can inhibit growth of Mtb in human macrophages (Brooks et al., 2011). It is also likely that presence of mycobacteria in the cytosol host cell leads to the death of this cell. At present, whether this event leads to transmission or enhanced control is not known.

At present, the evidence from both *in vitro* and *in vivo* studies does not support the case that mycobacterial escape to the cytosol is necessary for $CD8^+$ T cell recognition. *In vivo*, while $CD8^+$ T cell responses are observed at a higher frequency in the presence of the RD1-associated proteins, it is likely that these differences are a reflection of enhanced antigen load. (Russell et al., 2007; Woodworth et al., 2008; Ryan et al., 2009) rather than escape to the cytosol. While an *in vivo* system does not exist that models cytosolic Mtb, some insight can be gained from vaccination studies. The laboratory of Stefan Kaufmann has developed a BCG vaccine strain that secretes the LLO protein, a protein whose ability to form pores has been optimized via the deletion of urease leading to decreased phagosomal acidification (Grode et al., 2005). It was postulated that this strain could allow for more

efficient egress of mycobacterial proteins and/or the bacterium itself into the cytosol, and thus improved $CD8^+$ T cell responses. Indeed, vaccination of mice with this recombinant BCG provides increased protection against subsequent infection with Mtb, but the mechanism has not been completely defined. *In vitro*, there is increased cytosolic BCG-derived protein. Furthermore, infection of mouse macrophages with this vaccine strain induces apoptosis, possibly leading to cross-priming by bystander APCs via the uptake of apoptotic vesicles containing mycobacterial antigens (Grode et al., 2005). As such, it remains unclear as to whether or not this vaccine leads to improved frequency and/or quality of the Mtb-specific $CD8^+$ T cell response, and whether or not this is responsible for the improved vaccine efficacy that has been observed.

Based on our understanding of how Mtb antigens are processed and presented in the context of MHC-I to $CD8^+$ T cells *in vitro*, and the differences in antigen load and subsequent protection between Mtb and BCG *in vivo*, it is our opinion that Mtb escape to the cytosol is not critical for induction of $CD8^+$ T cell responses. Rationale vaccine design requires that those $CD8^+$ T cells induced during the course of vaccination can recognize those cells infected with Mtb. In this regard, an enhanced understanding of both the mechanisms of antigen processing and presentation as well as the repertoire of those antigens is of more than semantic importance.

REFERENCES

Abdallah, A. M., Gey Van Pittius, N. C., Champion, P. A., Cox, J., Luirink, J., Vandenbroucke-Grauls, C. M., Appelmelk, B. J., and Bitter, W. (2007). Type VII secretion – mycobacteria show the way. *Nat. Rev. Microbiol.* 5, 883–891.

Ackerman, A. L., Kyritsis, C., Tampe, R., and Cresswell, P. (2003). Early phagosomes in dendritic cells form a cellular compartment sufficient for cross presentation of exogenous antigens. *Proc. Natl. Acad. Sci. U.S.A.* 100, 12889–12894.

Amigorena, S., and Savina, A. (2010). Intracellular mechanisms of antigen cross presentation in dendritic cells. *Curr. Opin. Immunol.* 22, 109–117.

Armstrong, J. A., and Hart, P. D. (1971). Response of cultured macrophages to *Mycobacterium tuberculosis*, with observations on fusion of lysosomes with phagosomes. *J. Exp. Med.* 134, 713–740.

Bach, H., Papavinasasundaram, K. G., Wong, D., Hmama, Z., and Av-Gay, Y. (2008). *Mycobacterium tuberculosis* virulence is mediated by PtpA dephosphorylation of human vacuolar protein sorting 33B. *Cell Host Microbe* 3, 316–322.

Bach, H., Sun, J., Hmama, Z., and Av-Gay, Y. (2006). Mycobacterium avium subsp. paratuberculosis PtpA is an endogenous tyrosine phosphatase secreted during infection. *Infect. Immun.* 74, 6540–6546.

Beatty, W. L., Rhoades, E. R., Ullrich, H. J., Chatterjee, D., Heuser, J. E., and Russell, D. G. (2000). Trafficking and release of mycobacterial lipids from infected macrophages. *Traffic* 1, 235–247.

Beatty, W. L., and Russell, D. G. (2000). Identification of mycobacterial surface proteins released into subcellular compartments of infected macrophages. *Infect. Immun.* 68, 6997–7002.

Beatty, W. L., Ullrich, H. J., and Russell, D. G. (2001). Mycobacterial surface moieties are released from infected macrophages by a constitutive exocytic event. *Eur. J. Cell Biol.* 80, 31–40.

Bevan, M. J. (1976). Cross-priming for a secondary cytotoxic resonse to minor H antigens with H-2 congenic cells which do not cross-react in the cytotoxic assay. *J. Exp. Med.* 143, 1283–1288.

Billeskov, R., Vingsbo-Lundberg, C., Andersen, P., and Dietrich, J. (2007). Induction of CD8 T cells against a novel epitope in TB10.4: correlation with mycobacterial virulence and the presence of a functional region of difference-1. *J. Immunol.* 179, 3973–3981.

Blanchard, N., and Shastri, N. (2010). Cross-presentation of peptides from intracellular pathogens by MHC class I molecules. *Ann. N. Y. Acad. Sci.* 1183, 237–250.

Brodin, P., Majlessi, L., Brosch, R., Smith, D., Bancroft, G., Clark, S., Williams, A., Leclerc, C., and Cole, S. T. (2004). Enhanced protection against tuberculosis by vaccination with recombinant *Mycobacterium microti* vaccine that induces T cell immunity against region of difference 1 antigens. *J. Infect. Dis.* 190, 115–122.

Brooks, M. N., Rajaram, M. V., Azad, A. K., Amer, A. O., Valdivia-Arenas, M. A., Park, J. H., Nunez, G., and Schlesinger, L. S. (2011). NOD2 controls the nature of the inflammatory response and subsequent fate of *Mycobacterium tuberculosis* and *M. bovis* BCG in human macrophages. *Cell. Microbiol.* 13, 402–418.

Brumell, J. H., and Scidmore, M. A. (2007). Manipulation of rab GTPase function by intracellular bacterial pathogens. *Microbiol. Mol. Biol. Rev.* 71, 636–652.

Burgdorf, S., and Kurts, C. (2008). Endocytosis mechanisms and the cell biology of antigen presentation. *Curr. Opin. Immunol.* 20, 89–95.

Burgdorf, S., Scholz, C., Kautz, A., Tampe, R., and Kurts, C. (2008). Spatial and mechanistic separation of cross-presentation and endogenous antigen presentation. *Nat. Immunol.* 9, 558–566.

Caceres, N., Tapia, G., Ojanguren, I., Altare, F., Gil, O., Pinto, S., Vilaplana, C., and Cardona, P. J. (2009). Evolution of foamy macrophages in the pulmonary granulomas of experimental tuberculosis models. *Tuberculosis (Edinb)* 89, 175–182.

Canaday, D. H., Ziebold, C., Noss, E. H., Chervenak, K. A., Harding, C. V., and Boom, W. H. (1999). Activation of human CD8+ alpha beta TCR+ cells by *Mycobacterium tuberculosis* via an alternate class I MHC antigen-processing pathway. *J. Immunol.* 162, 372–379.

Carbone, F. R., and Bevan, M. J. (1990). Class I-restricted processing and presentation of exogenous cell-associated antigen in vivo. *J. Exp. Med.* 171, 377–387.

Cebrian, I., Visentin, G., Blanchard, N., Jouve, M., Bobard, A., Moita, C., Enninga, J., Moita, L. F., Amigorena, S., and Savina, A. (2011). Sec22b regulates phagosomal maturation and antigen crosspresentation by dendritic cells. *Cell* 147, 1355–1368.

Chefalo, P. J., and Harding, C. V. (2001). Processing of exogenous antigens for presentation by class I MHC molecules involves post-Golgi peptide exchange influenced by peptide-MHC complex stability and acidic pH. *J. Immunol.* 167, 1274–1282.

Clemens, D. L., and Horwitz, M. A. (1995). Characterization of the *Mycobacterium tuberculosis* phagosome and evidence that phagosomal maturation is inhibited. *J. Exp. Med.* 181, 257–270.

Clemens, D. L., and Horwitz, M. A. (1996). The *Mycobacterium tuberculosis* phagosome interacts with early endosomes and is accessible to exogenously administered transferrin. *J. Exp. Med.* 184, 1349–1355.

Clemens, D. L., Lee, B. Y., and Horwitz, M. A. (2002). The *Mycobacterium tuberculosis* phagosome in human macrophages is isolated from the host cell cytoplasm. *Infect. Immun.* 70, 5800–5807.

De Chastellier, C., Forquet, F., Gordon, A., and Thilo, L. (2009). Mycobacterium requires an all-around closely apposing phagosome membrane to maintain the maturation block and this apposition is re-established when it rescues itself from phagolysosomes. *Cell. Microbiol.* 11, 1190–1207.

Deretic, V., and Fratti, R. A. (1999). *Mycobacterium tuberculosis* phagosome. *Mol. Microbiol.* 31, 1603–1609.

Deretic, V., Via, L. E., Fratti, R. A., and Deretic, D. (1997). Mycobacterial phagosome maturation, rab proteins, and intracellular trafficking. *Electrophoresis* 18, 2542–2547.

Dumont, A., and Sheldon, H. (1965). Changes in the fine structure of macrophages in experimentally produced tuberculous granulomas in hamsters. *Lab. Invest.* 14, 2034–2055.

Dye, C., Scheele, S., Dolin, P., Pathania, V., and Raviglione, M. C. (1999). Consensus statement: global burden of tuberculosis: estimated incidence, prevalence. and mortality by country. WHO Global Surveillance and Monitoring Project. *JAMA* 282, 677–686.

Gagnon, E., Duclos, S., Rondeau, C., Chevet, E., Cameron, P. H., Steele-Mortimer, O., Paiement, J., Bergeron, J. J., and Desjardins, M. (2002). Endoplasmic reticulum-mediated phagocytosis is a mechanism of entry into macrophages. *Cell* 110, 119–131.

Goldszmid, R. S., Coppens, I., Lev, A., Caspar, P., Mellman, I., and Sher, A. (2009). Host ER-parasitophorous vacuole interaction provides a route of entry for antigen cross-presentation in *Toxoplasma gondii*-infected dendritic cells. *J. Exp. Med.* 206, 399–410.

Grode, L., Seiler, P., Baumann, S., Hess, J., Brinkmann, V., Nasser Eddine, A., Mann, P., Goosmann, C., Bandermann, S., Smith, D., Bancroft, G. J., Reyrat, J. M., Van Soolingen, D., Raupach, B., and Kaufmann, S. H. (2005). Increased vaccine efficacy against tuberculosis of recombinant *Mycobacterium bovis* bacille Calmette-Guerin mutants that secrete listeriolysin. *J. Clin. Invest.* 115, 2472–2479.

Grotzke, J. E., Harriff, M. J., Siler, A. C., Nolt, D., Delepine, J., Lewinsohn, D. A., and Lewinsohn, D. M. (2009). The *Mycobacterium tuberculosis* phagosome is a HLA-I processing competent organelle. *PLoS Pathog.* 5, e1000374. doi:10.1371/journal.ppat.1000374

Grotzke, J. E., and Lewinsohn, D. M. (2005). Role of CD8+ T lymphocytes in control of *Mycobacterium tuberculosis* infection. *Microbes Infect.* 7, 776–788.

Grotzke, J. E., Siler, A. C., Lewinsohn, D. A., and Lewinsohn, D. M. (2010). Secreted immunodominant *Mycobacterium tuberculosis* antigens are processed by the cytosolic pathway. *J. Immunol.* 185, 4336–4343.

Guermonprez, P., Saveanu, L., Kleijmeer, M., Davoust, J., Van Endert, P., and Amigorena, S. (2003). ER-phagosome fusion defines an MHC class I cross-presentation compartment in dendritic cells. *Nature* 425, 397–402.

Houde, M., Bertholet, S., Gagnon, E., Brunet, S., Goyette, G., Laplante, A., Princiotta, M. F., Thibault, P., Sacks, D., and Desjardins, M. (2003). Phagosomes are competent organelles for antigen cross-presentation. *Nature* 425, 402–406.

Hsu, T., Hingley-Wilson, S. M., Chen, B., Chen, M., Dai, A. Z., Morin, P. M., Marks, C. B., Padiyar, J., Goulding, C., Gingery, M., Eisenberg, D., Russell, R. G., Derrick, S. C., Collins, F. M., Morris, S. L., King, C. H., and Jacobs, W. R. Jr. (2003). The primary mechanism of attenuation of bacillus Calmette-Guerin is a loss of secreted lytic function required for invasion of lung interstitial tissue. *Proc. Natl. Acad. Sci. U.S.A.* 100, 12420–12425.

Keane, J., Remold, H. G., and Kornfeld, H. (2000). Virulent *Mycobacterium tuberculosis* strains evade apoptosis of infected alveolar macrophages. *J. Immunol.* 164, 2016–2020.

Kelley, V. A., and Schorey, J. S. (2003). Mycobacterium's arrest of phagosome maturation in macrophages requires Rab5 activity and accessibility to iron. *Mol. Biol. Cell* 14, 3366–3377.

Kondo, E., Yasuda, T., and Kanai, K. (1982). Electron microscopic demonstration of close contact between intracellular mycobacteria and the phagosomal membrane. *Jpn. J. Med. Sci. Biol.* 35, 197–201.

Kovacsovics-Bankowski, M., and Rock, K. L. (1995). A phagosome-to-cytosol pathway for exogenous antigens presented on MHC class I molecules. *Science* 267, 243–246.

Leake, E. S., Myrvik, Q. N., and Wright, M. J. (1984). Phagosomal membranes of *Mycobacterium bovis* BCG-immune alveolar macrophages are resistant to disruption by *Mycobacterium tuberculosis* H37Rv. *Infect. Immun.* 45, 443–446.

Lee, J., Remold, H. G., Ieong, M. H., and Kornfeld, H. (2006). Macrophage apoptosis in response to high intracellular burden of *Mycobacterium tuberculosis* is mediated by a novel caspase-independent pathway. *J. Immunol.* 176, 4267–4274.

Lee, J., Repasy, T., Papavinasasundaram, K., Sassetti, C., and Kornfeld, H. (2011). *Mycobacterium tuberculosis* induces an atypical cell death mode to escape from infected macrophages. *PLoS ONE* 6, e18367. doi:10.1371/journal.pone.0018367

Leepiyasakulchai, C., Ignatowicz, L., Pawlowski, A., Kallenius, G., and Skold, M. (2012). Failure to recruit anti-inflammatory CD103+ dendritic cells and a diminished CD4+ Foxp3+ regulatory T cell pool in mice that display excessive lung inflammation and increased susceptibility to *Mycobacterium tuberculosis*. *Infect. Immun.* 80, 1128–1139.

Lewinsohn, D. M., Alderson, M. R., Briden, A. L., Riddell, S. R., Reed, S. G., and Grabstein, K. H. (1998). Characterization of human CD8+ T cells reactive with *Mycobacterium tuberculosis*-infected antigen-presenting cells. *J. Exp. Med.* 187, 1633–1640.

Lewinsohn, D. M., Grotzke, J. E., Heinzel, A. S., Zhu, L., Ovendale, P. J., Johnson, M., and Alderson, M. R. (2006). Secreted proteins from *Mycobacterium tuberculosis* gain access to the cytosolic MHC class-I antigen-processing pathway. *J. Immunol.* 177, 437–442.

Lin, M. L., Zhan, Y., Villadangos, J. A., and Lew, A. M. (2008). The cell biology of cross-presentation and the role of dendritic cell subsets. *Immunol. Cell Biol.* 86, 353–362.

Majlessi, L., Brodin, P., Brosch, R., Rojas, M. J., Khun, H., Huerre, M., Cole, S. T., and Leclerc, C. (2005). Influence of ESAT-6 secretion system 1 (RD1) of *Mycobacterium tuberculosis* on the interaction between mycobacteria and the host immune system. *J. Immunol.* 174, 3570–3579.

Master, S. S., Rampini, S. K., Davis, A. S., Keller, C., Ehlers, S., Springer, B., Timmins, G. S., Sander, P., and Deretic, V. (2008). *Mycobacterium tuberculosis* prevents inflammasome activation. *Cell Host Microbe* 3, 224–232.

Mazzaccaro, R. J., Gedde, M., Jensen, E. R., Van Santen, H. M., Ploegh, H. L., Rock, K. L., and Bloom, B. R. (1996). Major histocompatibility class I presentation of soluble antigen facilitated by *Mycobacterium tuberculosis* infection. *Proc. Natl. Acad. Sci. U.S.A.* 93, 11786–11791.

McDonough, K. A., Kress, Y., and Bloom, B. R. (1993). Pathogenesis of tuberculosis: interaction of *Mycobacterium tuberculosis* with macrophages. *Infect. Immun.* 61, 2763–2773.

Mellman, I., and Steinman, R. M. (2001). Dendritic cells: specialized and regulated antigen processing machines. *Cell* 106, 255–258.

Merckx, J. J., Brown, A. L. Jr., and Karlson, A. G. (1964). An electron-microscopic study of experimental infections with acid-fast bacilli. *Am. Rev. Respir. Dis.* 89, 485–496.

Moreira, A. L., Wang, J., Tsenova-Berkova, L., Hellmann, W., Freedman, V. H., and Kaplan, G. (1997). Sequestration of *Mycobacterium tuberculosis* in tight vacuoles in vivo in lung macrophages of mice infected by the respiratory route. *Infect. Immun.* 65, 305–308.

Mwandumba, H. C., Russell, D. G., Nyirenda, M. H., Anderson, J., White, S. A., Molyneux, M. E., and Squire, S. B. (2004). *Mycobacterium tuberculosis* resides in nonacidified vacuoles in endocytically competent alveolar macrophages from patients with tuberculosis and HIV infection. *J. Immunol.* 172, 4592–4598.

Myrvik, Q. N., Leake, E. S., and Wright, M. J. (1984). Disruption of phagosomal membranes of normal alveolar macrophages by the H37Rv strain of *Mycobacterium tuberculosis*. A correlate of virulence. *Am. Rev. Respir. Dis.* 129, 322–328.

Neyrolles, O., Gould, K., Gares, M. P., Brett, S., Janssen, R., O'Gaora, P., Herrmann, J. L., Prevost, M. C., Perret, E., Thole, J. E., and Young, D. (2001). Lipoprotein access to MHC class I presentation during infection of murine macrophages with live mycobacteria. *J. Immunol.* 166, 447–457.

Opie, E. L., and Aronson, J. D. (1927). Tubercule bacilli in latent tuberculous lesions and in lung tissue without tuberculous lesions. *Arch. Pathol.* 1–21.

Pan, H., Yan, B. S., Rojas, M., Shebzukhov, Y. V., Zhou, H., Kobzik, L., Higgins, D. E., Daly, M. J., Bloom, B. R., and Kramnik, I. (2005). Ipr1 gene mediates innate immunity to tuberculosis. *Nature* 434,767–772.

Park, J. S., Tamayo, M. H., Gonzalez-Juarrero, M., Orme, I. M., and Ordway, D. J. (2006). Virulent clinical isolates of *Mycobacterium tuberculosis* grow rapidly and induce cellular necrosis but minimal apoptosis in murine macrophages. *J. Leukoc. Biol.* 79, 80–86.

Paul, S., Laochumroovorapong, P., and Kaplan, G. (1996). Comparable growth of virulent and avirulent *Mycobacterium tuberculosis* inhuman macrophages in vitro. *J. Infect. Dis.* 174, 105–112.

Peyron, P., Vaubourgeix, J., Poquet, Y., Levillain, F., Botanch, C., Bardou, F., Daffé, M., Emile, J. F., Marchou, B., Cardona, P. J., de Chastellier, C., and Altare, F. (2008). Foamy macrophages from tuberculous patients' granulomas constitute a nutrient-rich reservoir for *M. tuberculosis* persistence. *PLoS Pathog.* 4, e1000204. doi: 10.1371/journal.ppat.1000204

Pfeifer, J. D., Wick, M. J., Roberts, R. L., Findlay, K., Normark, S. J., and Harding, C. V. (1993). Phagocytic processing of bacterial antigens for class I MHC presentation to T cells. *Nature* 361, 359–362.

Philips, J. A. (2008). Mycobacterial manipulation of vacuolar sorting. *Cell. Microbiol.* 10, 2408–2415.

Pieters, J. (2001). Entry and survival of pathogenic mycobacteria in macrophages. *Microbes Infect.* 3, 249–255.

Pym, A. S., Brodin, P., Majlessi, L., Brosch, R., Demangel, C., Williams, A., Griffiths, K. E., Marchal, G., Leclerc, C., and Cole, S. T. (2003). Recombinant BCG exporting ESAT-6 confers enhanced protection against tuberculosis. *Nat. Med.* 9, 533–539.

Rahman, A., Srivastava, S. S., Sneh, A., Ahmed, N., and Krishnasastry, M. V. (2010). Molecular characterization of tlyA gene product, Rv1694 of *Mycobacterium tuberculosis*: a non-conventional hemolysin and a ribosomal RNA methyl transferase. *BMC Biochem.* 11, 35. doi:10.1186/1471-2091-11-35

Ramachandra, L., Simmons, D., and Harding, C. V. (2009). MHC molecules and microbial antigen processing in phagosomes. *Curr. Opin. Immunol.* 21, 98–104.

Russell, D. G. (2001). *Mycobacterium tuberculosis*: here today, and here tomorrow. *Nat. Rev. Mol. Cell Biol.* 2, 569–577.

Russell, D. G., Mwandumba, H. C., and Rhoades, E. E. (2002). Mycobacterium and the coat of many lipids. *J. Cell Biol.* 158, 421–426.

Russell, M. S., Iskandar, M., Mykytczuk, O. L., Nash, J. H., Krishnan, L., and Sad, S. (2007). A reduced antigen load in vivo, rather than weak inflammation, causes a substantial delay in CD8+ T cell priming against *Mycobacterium bovis* (bacillus Calmette-Guerin). *J. Immunol.* 179, 211–220.

Ryan, A. A., Nambiar, J. K., Wozniak, T. M., Roediger, B., Shklovskaya, E., Britton, W. J., Fazekas De St Groth, B., and Triccas, J. A. (2009). Antigen load governs the differential priming of CD8 T cells in response to the bacille Calmette Guerin vaccine or *Mycobacterium tuberculosis* infection. *J. Immunol.* 182, 7172–7177.

Schaible, U. E., Winau, F., Sieling, P. A., Fischer, K., Collins, H. L., Hagens, K., Modlin, R. L., Brinkmann, V., and Kaufmann, S. H. (2003). Apoptosis facilitates antigen presentation to T lymphocytes through MHC-I and CD1 in tuberculosis. *Nat. Med.* 9, 1039–1046.

Segura, E., and Villadangos, J. A. (2009). Antigen presentation by dendritic cells in vivo. *Curr. Opin. Immunol.* 21, 105–110.

Shams, H., Barnes, P. F., Weis, S. E., Klucar, P., and Wizel, B. (2003). Human CD8+ T cells recognize epitopes of the 28-kDa hemolysin and the 38-kDa antigen of *Mycobacterium tuberculosis. J. Leukoc. Biol.* 74, 1008–1014.

Shen, L., and Rock, K. L. (2006). Priming of T cells by exogenous antigen cross-presented on MHC class I molecules. *Curr. Opin. Immunol.* 18, 85–91.

Shen, L., Sigal, L. J., Boes, M., and Rock, K. L. (2004). Important role of cathepsin S in generating peptides for TAP-independent MHC class I crosspresentation in vivo. *Immunity* 21, 155–165.

Song, R., and Harding, C. V. (1996). Roles of proteasomes, transporter for antigen presentation (TAP), and beta 2-microglobulin in the processing of bacterial or particulate antigens via an alternate class I MHC processing pathway. *J. Immunol.* 156, 4182–4190.

Sousa, A. O., Mazzaccaro, R. J., Russell, R. G., Lee, F. K., Turner, O. C., Hong, S., Van Kaer, L., and Bloom, B. R. (2000). Relative contributions of distinct MHC class I-dependent cell populations in protection to tuberculosis infection in mice. *Proc. Natl. Acad. Sci. U.S.A.* 97, 4204–4208.

Sturgill-Koszycki, S., Schaible, U. E., and Russell, D. G. (1996). Mycobacterium-containing phagosomes are accessible to early endosomes and reflect a transitional state in normal phagosome biogenesis. *EMBO J.* 15, 6960–6968.

Teitelbaum, R., Cammer, M., Maitland, M. L., Freitag, N. E., Condeelis, J., and Bloom, B. R. (1999). Mycobacterial infection of macrophages results in membrane-permeable phagosomes. *Proc. Natl. Acad. Sci. U.S.A.* 96, 15190–15195.

Tian, T., Woodworth, J., Skold, M., and Behar, S. M. (2005). In vivo depletion of CD11c+ cells delays the CD4+ T cell response to *Mycobacterium tuberculosis* and exacerbates the outcome of infection. *J. Immunol.* 175, 3268–3272.

van der Wel, N., Hava, D., Houben, D., Fluitsma, D., Van Zon, M., Pierson, J., Brenner, M., and Peters, P. J. (2007). M. tuberculosis and *M. leprae* translocate from the phagolysosome to the cytosol in myeloid cells. *Cell* 129, 1287–1298.

Van Endert, P. (2011). Post-proteasomal and proteasome-independent generation of MHC class I ligands. *Cell. Mol. Life Sci.* 68, 1553–1567.

Vergne, I., Chua, J., Singh, S. B., and Deretic, V. (2004). Cell biology of *mycobacterium tuberculosis* phagosome. *Annu. Rev. Cell Dev. Biol.* 20, 367–394.

Via, L. E., Deretic, D., Ulmer, R. J., Hibler, N. S., Huber, L. A., and Deretic, V. (1997). Arrest of mycobacterial phagosome maturation is caused by a block in vesicle fusion between stages controlled by rab5 and rab7. *J. Biol. Chem.* 272, 13326–13331.

Weerdenburg, E. M., Peters, P. J., and Van Der Wel, N. N. (2010). How do mycobacteria activate CD8+ T cells? *Trends Microbiol.* 18, 1–10.

Wolf, A. J., Linas, B., Trevejo-Nunez, G. J., Kincaid, E., Tamura, T., Takatsu, K., and Ernst, J. D. (2007). *Mycobacterium tuberculosis* infects dendritic cells with high frequency and impairs their function in vivo. *J. Immunol.* 179, 2509–2519.

Wong, D., Bach, H., Sun, J., Hmama, Z., and Av-Gay, Y. (2011). *Mycobacterium tuberculosis* protein tyrosine phosphatase (PtpA) excludes host vacuolar-H+-ATPase to inhibit phagosome acidification. *Proc. Natl. Acad. Sci. U.S.A.* 108, 19371–19376.

Woodworth, J. S., Fortune, S. M., and Behar, S. M. (2008). Bacterial protein secretion is required for priming of CD8+ T cells specific for the *Mycobacterium tuberculosis* antigen CFP10. *Infect. Immun.* 76, 4199–4205.

Xu, S., Cooper, A., Sturgill-Koszycki, S., Van Heyningen, T., Chatterjee, D., Orme, I., Allen, P., and Russell, D. G. (1994). Intracellular trafficking in *Mycobacterium tuberculosis* and Mycobacterium avium-infected macrophages. *J. Immunol.* 153, 2568–2578.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 23 December 2011; accepted: 17 February 2012; published online: 05 March 2012.

Citation: Harriff MJ, Purdy GE and Lewinsohn DM (2012) Escape from the phagosome: the explanation for MHC-I processing of mycobacterial antigens? Front. Immun. ***3****:40. doi: 10.3389/fimmu.2012.00040*

This article was submitted to Frontiers in Antigen Presenting Cell Biology, a specialty of Frontiers in Immunology.

frontiers in
IMMUNOLOGY

MINI REVIEW ARTICLE
published: 10 February 2012
doi: 10.3389/fimmu.2012.00014

The role of XCR1 and its ligand XCL1 in antigen cross-presentation by murine and human dendritic cells

Richard A. Kroczek* and Volker Henn

Molecular Immunology, Robert Koch-Institute, Berlin, Germany

Edited by:
Christian Kurts, Friedrich-Wilhelms-Universität Bonn, Germany

Reviewed by:
Francesca Granucci, University of Milano-Bicocca, Italy
Thomas Gebhardt, The University of Melbourne, Australia

****Correspondence:***
Richard A. Kroczek, Molecular Immunology, Robert Koch-Institute, Nordufer 20, 13353 Berlin, Germany.
e-mail: kroczek@rki.de

Recently, the chemokine receptor XCR1 has been found to be exclusively expressed on a subset of dendritic cell (DC) known to be involved in antigen cross-presentation. This review aims to summarize the known biology of the XCR1 receptor and its chemokine ligand XCL1, both in the mouse and the human. Further, any involvement of this receptor–ligand pair in antigen uptake, cross-presentation, and induction of innate and adaptive cytotoxic immunity is explored. The concept of antigen delivery to DC via the XCR1 receptor is discussed as a vaccination strategy for selective induction of cytotoxic immunity against certain pathogens or tumors.

Keywords: dendritic cells, XCR1, XCL1, antigen cross-presentation, antigen targeting, vaccination

Recently, the chemokine receptor XCR1 has been found to be exclusively expressed on a subset of dendritic cells (DCs) known to excel in antigen cross-presentation. The impact of this chemokine receptor on DC biology can only be understood through combining the available knowledge on the XCR1 receptor, its chemokine ligand XCL1, and the current information on the function of DCs expressing XCR1.

The chemokine ligand XCL1 has been cloned independently by three groups, by Kelner et al. (1994) as "lymphotactin" from murine immature thymocytes, by Müller et al. (1995) as "ATAC" ("activation-induced, T cell-derived, and chemokine-related molecule") from two-signal activated human T cells, and by Yoshida et al. (1995), who identified "single cysteine molecule-1" ("SCM-1," huXCL1) in PHA-stimulated human PBMC. The latter group later also found a second human gene, termed *SCYC2* (Yoshida et al., 1996), which encodes huXCL2, a protein differing only in amino acids 7 and 8 from the originally identified SCM-1 molecule, redesignated to SCM-1α. Interestingly, a gene corresponding to human *SCYC2/XCL2* has never been identified in the mouse. Dorner et al. (1997) purified human ATAC, determined its mature protein form, and demonstrated that it is secreted as a partially glycosylated 93 aa protein with a calculated M_r of 10.3 kDa. Mature human ATAC corresponds in its sequence to SCM-1α, and is 61.4% identical and 84% similar (NCBI blastp) to murine lymphotactin. Collectively, these three groups thus defined the nature of murine XCL1 and human XCL1 (SCM-1α)/XCL2 (SCM-1β).

The generation of mAb specific for murine ATAC/XCL1 provided the first information on the biological context of XCL1 secretion. *In vitro* activated murine NK cells, Th1-polarized $CD4^+$ T cells, and $CD8^+$ T cells were found to co-secrete ATAC/XCL1 with IFN-γ, MIP-1α, MIP-1β, and RANTES (Dorner et al., 2002), prototypical components of the Th1 immune defense (Moser and Loetscher, 2001). Further, the same co-secretion pattern was observed *in vivo* by NK cells in the early phase, and by antigen-specific $CD8^+$ T cells in the later adaptive phase of murine listeriosis (Dorner et al., 2002), a disease in which the intracellular pathogen is cleared by a Th1-type immune reaction (Pamer, 2004). Together, these expression data strongly indicated the involvement of ATAC/XCL1 in the Th1 immune defense, but did not yet provide a mechanistic model of ATAC/XCL1 action.

The function of XCL1 remained unclear for many years. Kelner et al. (1994) reported in their original publication that their newly identified protein induced chemotaxis of a variety of lymphocytes, hence their designation "lymphotactin." In the years following the cloning of lymphotactin/ATAC/SCM-1, a plethora of reports on the chemotactic action of XCL1 was published, both in the human and the mouse. These, as it later turned out, erroneous reports, claimed chemotaxis on T cells, B cells, NK cells, neutrophils, and other cell types (listed in Supplemental Table S1 of Dorner et al., 2009). Only few groups reported that they failed to observe any chemotaxis using a broad array of cell types (Bleul et al., 1996; Dorner et al., 1997; Johnston et al., 2003).

A major step forward was the identification of the receptor for XCL1 by the group of Yoshie. They matched a previously cloned human orphan G protein-coupled receptor GPR5 (Heiber et al., 1995) with the orphan human chemokines SCM-1α/XCL1 and SCM-1β/XCL2, employing binding and functional assays (Yoshida et al., 1998). Further work also indicated that GPR5, later officially designated XCR1, is the only receptor for XCL1 (Yoshida et al., 1998; Shan et al., 2000).

Usually, the identification of a receptor for a chemokine strongly facilitates the elucidation of the biological role of a given chemokine-receptor system, but not in this case. Since a XCR1-specific mAb was not available at that time, the detection of XCR1 expression in tissues had to be done by RT-PCR. The original description of the murine XCR1 gene assumed the existence of only a single-exon coding for XCR1 (Yoshida et al., 1999), so detection of XCR1 mRNA had to rely on a "single-exon" RT-PCR, a system highly prone to false positive results. As a result, more than

a dozen publications reported the expression of XCR1 in a great variety of cell types, all of which turned out to be incorrect (listed in Supplemental Table S2 of Dorner et al., 2009).

The breakthrough regarding the expression of XCR1 came from studies employing poly (A) RNA for detection of XCR1 by RT-PCR and the recognition that the murine XCR1 gene contains two exons, allowing the use of an "intron-spanning" RT-PCR. This approach revealed that XCR1 mRNA is selectively expressed in "conventional" DCs, and not in resting or activated T cells, B cells, NK cells, or plasmacytoid DCs (pDCs). A more detailed analysis showed that only $CD8^+$ DCs and a small proportion of $CD8^-$ DCs express XCR1 mRNA (Dorner et al., 2009). These expression studies were corroborated by experiments using a reporter mouse, in which *lacZ* is expressed under the control of the XCR1 promoter. Whole-body histological analysis of this reporter mouse yielded signals compatible with the notion that XCR1 is exclusively expressed in DCs and not in other cells. In the spleen, the XCR1 signal was associated with $CD8^+$ DCs in the red pulp and in T cell zones (Dorner et al., 2009; Crozat et al., 2010). In lymph nodes, XCR1 gene expression was identified in paracortical areas and subcapsular sinuses (Dorner et al., 2009) and was found to be highly associated with CD103 expressed by migratory DCs (Crozat et al., 2011).

Functional studies yielded results congruent with the obtained XCR1 receptor expression profile. XCL1 induced strong chemotaxis of murine $CD8^+$ DCs, but not of other DC subtypes, T cells, B cells, or NK cells (Dorner et al., 2009). These experiments thus for the first time clearly defined XCL1 as a chemokine and revealed an unusually restricted target population, the $CD8^+$ DCs. In a series of experiments, the function of XCR1 and its chemokine ligand was then tested *in vivo*. When antigen was targeted to APC via DEC-205 in mice, adoptively transferred $CD8^+$ OT-I T cells became activated by the cross-presented antigen and started to secrete XCL1. Further, studies employing XCL1 gene-deficient mice revealed that XCL1 optimizes the expansion and survival of these OT-I $CD8^+$ T cells and their subsequent differentiation into cytotoxic cells. Collectively, this study demonstrated the involvement of the XCL1–XCR1 axis in the dialog of $CD8^+$ T cells with $CD8^+$ DCs on their way to become cytotoxic T cells (Dorner et al., 2009).

$CD8^+$ DCs in the mouse spleen are part of the "resident" DC population, which make up the large majority of splenic DCs. Resident DCs take up antigen locally and present it to T cells "on site." Resident DCs, which all express the CD11c cell surface protein, have been classically subdivided into $CD4^+$ DCs ($CD11b^{hi}$ $CD4^+$ $CD8^-$ $CD205^{lo}$, around 70% of splenic DCs), $CD8^+$ DCs ($CD11b^{lo}$ $CD8^+$ $CD205^{hi}$, 20%), and DN DCs ($CD11b^{hi}$ $CD4^-CD8^-$ $CD205^{lo}$, 10%; Vremec et al., 2000). In the past, resident $CD8^+$ DCs have been consistently implicated in antigen cross-presentation, in which exogenous antigen is not "classically" presented in the context of MHC-class II, but instead shunted to the MHC-class I pathway (den Haan et al., 2000; Shortman and Heath, 2010).

The correlation of XCR1 expression with $CD8^+$ DCs was not perfect, with only 70–85% of $CD8^+$ DCs showing the XCR1 driven *LacZ*-reporter signal, but also 2–8% of DN DCs (Dorner et al., 2009). These data could be fully reproduced with a mAb to murine XCR1 (unpublished data), raising questions about the functional role of the 20–25% of $CD8^+$ DCs not expressing XCR1, and the 2–8% of DN DCs positive for XCR1, but not expressing CD8. Of interest in this context, CFSE-labeled allogeneic 300–19 pre-B cells injected i.v. were essentially only taken up by $XCR1^+$ DCs, while $CD8^+$ DCs lacking XCR1 and other DCs played a negligible part (unpublished data).

The uptake studies were complemented by tests for antigen cross-presentation. Soluble ovalbumin (OVA) was injected i.v., and 14 h later $CD8^+$ $XCR1^+$, $CD8^-$ $XCR1^+$ (corresponding to the $XCR1^+$ DN DC subset), $CD8^+$ $XCR1^-$, and $CD8^-$ $XCR1^-$ DCs were highly purified from spleens and co-cultured with OT-I *in vitro*. In this classical assay for antigen cross-presentation, $CD8^+$ $XCR1^+$ and $CD8^-$ $XCR1^+$ DCs presented soluble OVA clearly better than $CD8^+$ $XCR1^-$ and $CD8^-$ $XCR1^-$ DCs (unpublished data). When the same experiment was performed with cell-associated antigen (300–19 cells transfected with OVA), splenic $CD8^+$ $XCR1^+$, and $CD8^-$ $XCR1^+$ DCs excelled in antigen cross-presentation, whereas $CD8^+$ $XCR1^-$ and $CD8^-$ $XCR1^-$ DCs fully failed (unpublished data). Both assays with soluble and cell-associated antigen thus demonstrated the superior capacity of the $XCR1^+$ DC populations to cross-present antigen, irrespective of CD8 expression on their surface.

When these functional studies were followed by extensive phenotyping of these four DC populations, the $XCR1^+$ $CD8^+$ DCs appeared homogeneous and very similar to $XCR1^+$ $CD8^-$ DCs. On the other hand, both $XCR1^-$ $CD8^+$ DCs and $XCR1^-$ $CD8^-$ DCs exhibited a quite different surface phenotype (unpublished data). When combining the phenotypic analysis of the various DC subsets with their ability to cross-present antigen, it becomes apparent that the current classification of DCs into $CD8^+$ DCs, $CD4^+$ DCs and DN DCs may no longer be useful. Instead, splenic $XCR1^+$ DCs appear as a rather homogeneous population set apart from the $XCR1^-$ DCs. The $XCR1^-$ DC population, on the other hand, may possibly be further subdivided, e.g., based on the expression of the fractalkine receptor, as suggested recently by Bar-On et al. (2010). More functional data with DCs from other lymphoid organs will be necessary to further characterize the $XCR1^+$ and $XCR1^-$ DC populations. However, what emerges from the current studies is the close correlation of XCR1 expression with the ability of DCs to cross-present antigen. The XCL1–XCR1 axis thus appears as an integral part of the antigen cross-presentation machinery.

How can we imagine the contribution of the XCL1–XCR1 system to the defense of pathogens? From the few *in vivo* studies available it is clear that NK cells, and possibly also other cells of the innate immune system, secrete this chemokine early upon infection with certain pathogens like *Listeria* or MCMV (Dorner et al., 2002, 2004). When NK cells release XCL1, they co-release this chemokine with a set of other cytokines and chemokines, most notably IFN-γ, as part of a Th1-type of defense (Dorner et al., 2002, 2003, 2004). It seems likely that secretion of XCL1 under these circumstances facilitates the communication of NK cells with $XCR1^+$ DCs (and vice versa, **Figure 1**). It is interesting to note that in the adaptive phase of the immune response activated $CD8^+$ T cells communicate with $XCR1^+$ DCs by secreting the same array of XCL1-associated cytokines and chemokines

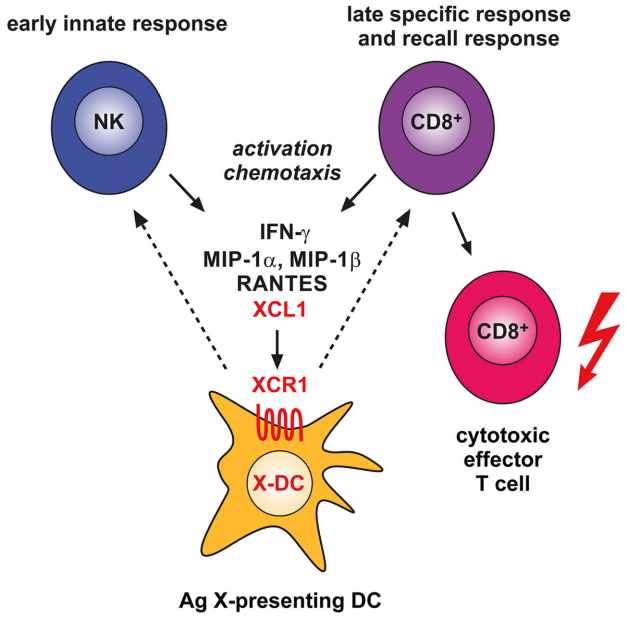


FIGURE 1 | Involvement of the XCL1–XCR1 communication axis in the innate and adaptive cytotoxic responses to cross-presented microbial and tumor antigens. Secretion of the chemokine XCL1 by activated NK cells specifically attracts XCR1-expressing DCs capable of antigen cross-presentation. This ensures an effective communication between these cells in the innate phase of the immune response. In the adaptive phase, secretion of XCL1 by activated $CD8^+$ T cells optimizes the dialog with antigen cross-presenting DCs and facilitates the differentiation of $CD8^+$ T cells to cytotoxic effector cells.

(Dorner et al., 2002). $XCR1^+$ DCs are the ideal communication partners for $CD8^+$ T cells, since they optimally cross-present antigen and integrate any inflammatory signals, so $CD8^+$ T cells can be tolerized or receive a "license to kill."

One additional factor in this complex interplay may be the special capacity of $XCR1^+$ DCs to take up certain forms of antigen. Uptake of *L. monocytogenes* by $CD8^+$ DCs (which now have to be reconsidered as $XCR1^+$ DCs) is critical for infection with *Listeria* (Neuenhahn et al., 2006; Edelson et al., 2011), and possibly also for the development of immunity against this pathogen. Thus, the special ability of $XCR1^+$ DCs to take up certain (intracellular?) pathogens defines them as ideal DCs for instructing cytotoxic effector cells of the innate and adaptive immune systems to eliminate infected cells in the periphery. This mechanism appears important, since pathogens, once "hidden" in infected cells, are otherwise "invisible" to the immune system. A similar function of $XCR1^+$ DCs can be hypothesized in the surveillance of the body for tumor antigens. Live or dead cancerous cells taken up by $XCR1^+$ DCs will be digested and processed, and any tumor antigens will be cross-presented by the MHC-class I molecules to $CD8^+$ T cells. One has to assume, however, that the recognition of "altered self" from cancerous tissue by $CD8^+$ T cells has to be accompanied by some "adjuvant" signal to initiate a cytotoxic response against the tumor. Otherwise, a tolerance reaction to "altered self" may result.

The exquisite specificity of XCR1 expression may in future be exploited for vaccination purposes. Almost a decade ago, the groups of Steinman and Nussenzweig had pioneered the concept of antigen targeting to DCs by employing a mAb directed to CD205 (Hawiger et al., 2001). Such a concept promises to lower the antigen dose required to induce optimal immunity. More importantly, by targeting antigen to functionally different DC subsets, it offers the possibility to elicit *highly specific* immune responses. Based on such a concept, one can envisage future "designer vaccines" which address various components of the immune system to a different extent and thus elicit a protective immune response tailored to given pathogens. Early studies employing antibodies directed to CD205 convincingly demonstrated that potent cytotoxic $CD8^+$ T cell immunity can be induced when the targeted antigen is administered together with agents having adjuvant activity on DCs (Bonifaz et al., 2002). Similar results were obtained later with mAb directed to Clec9A/DNGR-1 (Caminschi et al., 2008; Sancho et al., 2008). None of the mAb used for DC-targeted antigen delivery to date, however, recognizes only one functional DC subset. For example, CD205 in the mouse is expressed on a variety of cells, including B cells, as well as thymic and intestinal epithelia (Witmer-Pack et al., 1995), Clec9A/DNGR-1 on pDCs, and a subset of $CD24^+$ blood DCs (Caminschi et al., 2008; Sancho et al., 2008). This may not be disadvantageous for the induction of the desired immune response, but the lack of "absolute" specificity for a functional DC subset runs against the "designer vaccine" concept, in which only one component of the immune system is specifically addressed. Thus, ideally, antigen could be selectively delivered to B cells, or pDCs, or any of the functionally different DC subsets (or any desired combination thereof). XCR1 as a target molecule appears to fulfill such an ideal. By being expressed only on the subset of DCs preferentially interacting with components of cytotoxic immunity (NK cells and $CD8^+$ T cells), it holds the promise of an entry port for vaccines against intracellular pathogens like *P. falciparum* or *M. tuberculosis*. Initial targeting tests performed with OVA recombinantly engineered to the chemokine ligand XCL1 or with OVA chemically coupled to the XCR1-specific mAb MARX10 gave very promising results. By combining this type of antigen delivery with adjuvants, a potent and specific *in vivo* cytotoxicity could be achieved (own unpublished results). Further tests have to be performed, however, to demonstrate that this response is highly selective and does not address other components of the immune system. Thus, XCR1 appears as an attractive target molecule for the "designer vaccine" concept.

All antigen targeting experiments were performed in the mouse and have to be regarded as model systems only. In the human system, the phenotype and function of DCs is less well understood, since most of the data has been obtained with *in vitro* generated monocyte-derived DCs, which may only partially reflect DC function *in vivo*. In the relatively few studies with primary human DCs, $CD304^+$ pDCs, and conventional DCs encompassing the $CD1c^+$ ($BDCA\text{-}1^+$), $CD16^+$, and $CD141^+$ DC ($BDCA\text{-}3^+$) subsets could be identified in the peripheral blood (Dzionek et al., 2000; MacDonald et al., 2002; Piccioli et al., 2007; for review see Ju et al., 2010). Extensive gene expression comparison suggested that $CD141^+$ DCs may correspond to murine $CD8^+$ DCs (Robbins

et al., 2008). Recent experimental work has confirmed this hypothesis: XCR1 was found to be selectively expressed in CD141$^+$ DCs and not in other cells (Bachem et al., 2010; Crozat et al., 2010), and CD141$^+$ DCs were the only DC subset migrating to XCL1 (Bachem et al., 2010). Further, primary CD141$^+$ DCs were found to be particularly capable of antigen cross-presentation, when tested *in vitro* with soluble or cell-associated antigen (Bachem et al., 2010; Crozat et al., 2010; Jongbloed et al., 2010). These data collectively indicated that CD141$^+$ DCs are the homologs of murine CD8$^+$ DCs and suggest that the lessons learned in the mouse most likely will also hold true in the human. This view is supported by the close structural resemblance of mouse and human XCL1 and mouse and human XCR1. Moreover, the expression pattern of human XCL1 (activated NK cells and CD8$^+$ T cells; Müller et al., 1995; Hedrick et al., 1997; Blaschke et al., 2003) appears very similar to the XCL1 expression profile in the mouse. Thus, the CD141$^+$ DCs are most likely specialized on the surveillance of intracellular pathogens and also aberrant antigens originating from cancerous tissue. Similar to the mouse system, they can be expected to closely cooperate with cells of cytotoxic immunity.

Considering all available functional data, XCR1 also in the human appears to be an ideal target for antigen delivery. Different from CD205, which in the human is expressed on CD11c$^+$ DC, monocytes, pDC, NK cells, and T cells (Kato et al., 2006), and Clec9A/DNGR-1, which is expressed on CD141$^+$ DC, but also found on a subset of B cells and CD14$^+$ CD16$^-$ monocytes (Caminschi et al., 2008; Huysamen et al., 2008), expression of XCR1 is restricted to CD141$^+$ DCs. Given this selectivity and the functional association with antigen cross-presentation, XCR1 emerges as a prime candidate for vaccines designed to induce selective cytotoxic immunity in man.

ACKNOWLEDGMENTS

This work was supported by grants of the Wilhelm-Sander-Foundation and the Deutsche Forschungsgemeinschaft TR52.

REFERENCES

Bachem, A., Güttler, S., Hartung, E., Ebstein, F., Schaefer, M., Tannert, A., Salama, A., Movassaghi, K., Opitz, C., Mages, H. W., Henn, V., Kloetzel, P. M., Gurka, S. and Kroczek, R. A. (2010). Superior antigen cross-presentation and XCR1 expression define human CD11c$^+$ CD141$^+$ cells as homologues of mouse CD8$^+$ dendritic cells. *J. Exp. Med.* 207, 1273–1281.

Bar-On, L., Birnberg, T., Lewis, K. L., Edelson, B. T., Bruder, D., Hildner, K., Buer, J., Murphy, K. M., Reizis, B., and Jung, S. (2010). CX3CR1$^+$ CD8α$^+$ dendritic cells are a steady-state population related to plasmacytoid dendritic cells. *Proc. Natl. Acad. Sci. U.S.A.* 107, 14745–14750.

Blaschke, S., Middel, P., Dorner, B. G., Blaschke, V., Hummel, K. M., Kroczek, R. A., Reich, K., Benoehr, P., Koziolek, M., and Muller, G. A. (2003). Expression of activation-induced, T cell-derived, and chemokine-related cytokine/lymphotactin and its functional role in rheumatoid arthritis. *Arthritis Rheum.* 48, 1858–1872.

Bleul, C. C., Fuhlbrigge, R. C., Casasnovas, J. M., Aiuti, A. and Springer, T. A. (1996). A highly efficacious lymphocyte chemoattractant, stromal cell-derived factor 1 (SDF-1). *J. Exp. Med.* 184, 1101–1109.

Bonifaz, L., Bonnyay, D., Mahnke, K., Rivera, M., Nussenzweig, M. C. and Steinman, R. M. (2002). Efficient targeting of protein antigen to the dendritic cell receptor DEC-205 in the steady state leads to antigen presentation on major histocompatibility complex class I products and peripheral CD8$^+$ T cell tolerance. *J. Exp. Med.* 196, 1627–1638.

Caminschi, I., Proietto, A. I., Ahmet, F., Kitsoulis, S., Shin, T. J., Lo, J. C., Rizzitelli, A., Wu, L., Vremec, D., van Dommelen, S. L., Campbell, I. K., Maraskovsky, E., Braley, H., Davey, G. M., Mottram, P., van, d. V., Jensen, K., Lew, A. M., Wright, M. D., Heath, W. R., Shortman, K., and Lahoud, M. H. (2008). The dendritic cell subtype-restricted C-type lectin Clec9A is a target for vaccine enhancement. *Blood* 112, 3264–3273.

Crozat, K., Guiton, R., Contreras, V., Feuillet, V., Dutertre, C. A., Ventre, E., Vu Manh, T. P., Baranek, T., Storset, A. K., Marvel, J., Boudinot, P., Hosmalin, A., Schwartz-Cornil, I. and Dalod, M. (2010). The XC chemokine receptor 1 is a conserved selective marker of mammalian cells homologous to mouse CD8α$^+$ dendritic cells. *J. Exp. Med.* 207, 1283–1292.

Crozat, K., Tamoutounour, S., Vu Manh, T. P., Fossum, E., Luche, H., Ardouin, L., Guilliams, M., Azukizawa, H., Bogen, B., Malissen, B., and Dalod, M. (2011). Cutting edge: expression of XCR1 defines mouse lymphoid-tissue resident and migratory dendritic cells of the CD8α$^+$ type. *J. Immunol.* 187, 4411–4415.

den Haan, J. M., Lehar, S. M., and Bevan, M. J. (2000). CD8$^+$ but not CD8$^-$ dendritic cells cross-prime cytotoxic T cells in vivo. *J. Exp. Med.* 192, 1685–1696.

Dorner, B., Müller, S., Entschladen, F., Schröder, J. M., Franke, P., Kraft, R., Friedl, P., Clark-Lewis, I., and Kroczek, R. A. (1997). Purification, structural analysis, and function of natural ATAC, a cytokine secreted by CD8$^+$ T cells. *J. Biol. Chem.* 272, 8817–8823.

Dorner, B. G., Dorner, M. B., Zhou, X., Opitz, C., Mora, A., Güttler, S., Hutloff, A., Mages, H. W., Ranke, K., Schaefer, M., Jack, R. S., Henn, V., and Kroczek, R. A. (2009). Selective expression of the chemokine receptor XCR1 on cross-presenting dendritic cells determines cooperation with CD8$^+$ T cells. *Immunity* 31, 823–833.

Dorner, B. G., Scheffold, A., Rolph, M. S., Hüser, M. B., Kaufmann, S. H., Radbruch, A., Flesch, I. E., and Kroczek, R. A. (2002). MIP-1α, MIP-1β, RANTES, and ATAC/lymphotactin function together with IFN-γ as type 1 cytokines. *Proc. Natl. Acad. Sci. U.S.A.* 99, 6181–6186.

Dorner, B. G., Smith, H. R., French, A. R., Kim, S., Poursine-Laurent, J., Beckman, D. L., Pingel, J. T., Kroczek, R. A., and Yokoyama, W. M. (2004). Coordinate expression of cytokines and chemokines by NK cells during murine cytomegalovirus infection. *J. Immunol.* 172, 3119–3131.

Dorner, B. G., Steinbach, S., Hüser, M. B., Kroczek, R. A., and Scheffold, A. (2003). Single-cell analysis of the murine chemokines MIP-1α, MIP-1β, RANTES, and ATAC/lymphotactin by flow cytometry. *J. Immunol. Methods* 274, 83–91.

Dzionek, A., Fuchs, A., Schmidt, P., Cremer, S., Zysk, M., Miltenyi, S., Buck, D. W., and Schmitz, J. (2000). BDCA-2, BDCA-3, and BDCA-4: three markers for distinct subsets of dendritic cells in human peripheral blood. *J. Immunol.* 165, 6037–6046.

Edelson, B. T., Bradstreet, T. R., Hildner, K., Carrero, J. A., Frederick, K. E., Kc, W., Belizaire, R., Aoshi, T., Schreiber, R. D., Miller, M. J., Murphy, T. L., Unanue, E. R., and Murphy, K. M. (2011). CD8α$^+$ dendritic cells are an obligate cellular entry point for productive infection by *Listeria monocytogenes*. *Immunity* 35, 236–248.

Hawiger, D., Inaba, K., Dorsett, Y., Guo, M., Mahnke, K., Rivera, M., Ravetch, J. V., Steinman, R. M. and Nussenzweig, M. C. (2001). Dendritic cells induce peripheral T cell unresponsiveness under steady state conditions in vivo. *J. Exp. Med.* 194, 769–779.

Hedrick, J. A., Saylor, V., Figueroa, D., Mizoue, L., Xu, Y., Menon, S., Abrams, J., Handel, T., and Zlotnik, A. (1997). Lymphotactin is produced by NK cells and attracts both NK cells and T cells in vivo. *J. Immunol.* 158, 1533–1540.

Heiber, M., Docherty, J. M., Shah, G., Nguyen, T., Cheng, R., Heng, H. H., Marchese, A., Tsui, L. C., Shi, X., George, S. R., and O'Dowd, B. F. (1995). Isolation of three novel human genes encoding G protein-coupled receptors. *DNA Cell Biol.* 14, 25–35.

Huysamen, C., Willment, J. A., Dennehy, K. M., and Brown, G. D. (2008). CLEC9A is a novel activation C-type lectin-like receptor expressed on BDCA3$^+$ dendritic cells and a subset of monocytes. *J. Biol. Chem.* 283, 16693–16701.

Johnston, B., Kim, C. H., Soler, D., Emoto, M., and Butcher, E. C. (2003). Differential chemokine responses and homing patterns of murine TCRαβ NKT cell subsets. *J. Immunol.* 171, 2960–2969.

Jongbloed, S. L., Kassianos, A. J., McDonald, K. J., Clark, G. J., Ju, X., Angel, C. E., Chen, C. J., Dunbar, P. R., Wadley, R. B., Jeet, V., Vulink, A. J., Hart, D. N. and Radford, K. J. (2010). Human CD141$^+$ (BDCA-3)$^+$ dendritic cells (DCs) represent a unique myeloid DC subset that cross-presents necrotic cell antigens. *J. Exp. Med.* 207, 1247–1260.

Ju, X., Clark, G., and Hart, D. N. (2010). Review of Human DC Subtypes. *Methods Mol. Biol.* 595, 3–20.

Kato, M., McDonald, K. J., Khan, S., Ross, I. L., Vuckovic, S., Chen, K., Munster, D., MacDonald, K. P., and Hart, D. N. (2006). Expression of human DEC-205 (CD205) multi lectin receptor on leukocytes. *Int. Immunol.* 18, 857–869.

Kelner, G. S., Kennedy, J., Bacon, K. B., Kleyensteuber, S., Largaespada, D. A., Jenkins, N. A., Copeland, N. G., Bazan, J. F., Moore, K. W., Schall, T. J., and Zlotnik, A. (1994). Lymphotactin: a cytokine that represents a new class of chemokine. *Science* 266, 1395–1399.

MacDonald, K. P., Munster, D. J., Clark, G. J., Dzionek, A., Schmitz, J., and Hart, D. N. (2002). Characterization of human blood dendritic cell subsets. *Blood* 100, 4512–4520.

Moser, B., and Loetscher, P. (2001). Lymphocyte traffic control by chemokines. *Nat. Immunol.* 2, 123–128.

Müller, S., Dorner, B., Korthäuer, U., Mages, H. W., D'Apuzzo, M., Senger, G., and Kroczek, R. A. (1995). Cloning of ATAC, an activation-induced, chemokine-related molecule exclusively expressed in CD8$^+$ T lymphocytes. *Eur. J. Immunol.* 25, 1744–1748.

Neuenhahn, M., Kerksiek, K. M., Nauerth, M., Suhre, M. H., Schiemann, M., Gebhardt, F. E., Stemberger, C., Panthel, K., Schroder, S., Chakraborty, T., Jung, S., Hochrein, H., Russmann, H., Brocker, T., and Busch, D. H. (2006). CD8α^+ dendritic cells are required for efficient entry of *Listeria monocytogenes* into the spleen. *Immunity* 25, 619–630.

Pamer, E. G. (2004). Immune responses to *Listeria monocytogenes*. *Nat. Rev. Immunol.* 4, 812–823.

Piccioli, D., Tavarini, S., Borgogni, E., Steri, V., Nuti, S., Sammicheli, C., Bardelli, M., Montagna, D., Locatelli, F., and Wack, A. (2007). Functional specialization of human circulating CD16 and CD1c myeloid dendritic-cell subsets. *Blood* 109, 5371–5379.

Robbins, S. H., Walzer, T., Dembele, D., Thibault, C., Defays, A., Bessou, G., Xu, H., Vivier, E., Sellars, M., Pierre, P., Sharp, F. R., Chan, S., Kastner, P., and Dalod, M. (2008). Novel insights into the relationships between dendritic cell subsets in human and mouse revealed by genome-wide expression profiling. *Genome Biol.* 9, R17.

Sancho, D., Mourao-Sa, D., Joffre, O. P., Schulz, O., Rogers, N. C., Pennington, D. J., Carlyle, J. R., and Reis e Sousa, C. (2008). Tumor therapy in mice via antigen targeting to a novel, DC-restricted C-type lectin. *J. Clin. Invest.* 118, 2098–2110.

Shan, L., Qiao, X., Oldham, E., Catron, D., Kaminski, H., Lundell, D., Zlotnik, A., Gustafson, E., and Hedrick, J. A. (2000). Identification of viral macrophage inflammatory protein (vMIP)-II as a ligand for GPR5/XCR1. *Biochem. Biophys. Res. Commun.* 268, 938–941.

Shortman, K., and Heath, W. R. (2010). The CD8$^+$ dendritic cell subset. *Immunol. Rev.* 234, 18–31.

Vremec, D., Pooley, J., Hochrein, H., Wu, L., and Shortman, K. (2000). CD4, and CD8 expression by dendritic cell subtypes in mouse thymus, and spleen. *J. Immunol.* 164, 2978–2986.

Witmer-Pack, M. D., Swiggard, W. J., Mirza, A., Inaba, K., and Steinman, R. M. (1995). Tissue distribution of the DEC-205 protein that is detected by the monoclonal antibody NLDC-145. II. Expression in situ in lymphoid and nonlymphoid tissues. *Cell. Immunol.* 163, 157–162.

Yoshida, T., Imai, T., Kakizaki, M., Nishimura, M., Takagi, S., and Yoshie, O. (1998). Identification of single C motif-1/lymphotactin receptor XCR1. *J. Biol. Chem.* 273, 16551–16554.

Yoshida, T., Imai, T., Kakizaki, M., Nishimura, M., and Yoshie, O. (1995). Molecular cloning of a novel C or gamma type chemokine, SCM-1. *FEBS Lett.* 360, 155–159.

Yoshida, T., Imai, T., Takagi, S., Nishimura, M., Ishikawa, I., Yaoi, T., and Yoshie, O. (1996). Structure and expression of two highly related genes encoding SCM-1/human lymphotactin. *FEBS Lett.* 395, 82–88.

Yoshida, T., Izawa, D., Nakayama, T., Nakahara, K., Kakizaki, M., Imai, T., Suzuki, R., Miyasaka, M., and Yoshie, O. (1999). Molecular cloning of mXCR1, the murine SCM-1/lymphotactin receptor. *FEBS Lett.* 458, 37–40.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 12 December 2011; accepted: 26 January 2012; published online: 10 February 2012.

*Citation: Kroczek RA and Henn V (2012) The role of XCR1 and its ligand XCL1 in antigen cross-presentation by murine and human dendritic cells. Front. Immun. **3**:14. doi: 10.3389/fimmu.2012.00014*

This article was submitted to Frontiers in Antigen Presenting Cell Biology, a specialty of Frontiers in Immunology.