Cross-presentation by dendritic cells

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Abstract | The presentation of exogenous antigens on MHC class I molecules, known as cross-presentation, is essential for the initiation of CD8+ T cell responses. *In vivo*, cross-presentation is mainly carried out by specific dendritic cell (DC) subsets through an adaptation of their endocytic and phagocytic pathways. Here, we summarize recent advances in our understanding of the intracellular mechanisms of cross-presentation and discuss its role in immunity and tolerance in the context of specialization between DC subsets. Finally, we review current strategies to use cross-presentation for immunotherapy.

MHC class I-loading complex
A series of endoplasmic reticulum chaperone proteins that stabilize empty MHC class I molecules and control the loading of high-affinity peptides onto MHC class I molecules.

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All nucleated cells express MHC class I molecules, which present peptides derived from endogenous proteins that are degraded in the cytosol by the proteasome. The degradation products of such proteins are transported into the endoplasmic reticulum (ER) to be loaded onto newly formed MHC class I molecules, which are finally exported to the cell surface through the Golgi apparatus. The MHC class I antigen presentation pathway enables the immune system to detect transformed or infected cells displaying peptides from modified-self or foreign proteins, respectively, on their surface MHC class I molecules. Naive antigen-specific CD8+ T cells, however, cannot directly eliminate transformed or infected cells. To become effector cytotoxic T lymphocytes (CTLs), naive CD8+ T cells need first to be activated by 'professional' antigen-presenting cells (APCs). When the APCs are not directly infected, they need to acquire exogenous antigens from the infectious agent and present them on MHC class I molecules, by a mechanism known as cross-presentation.

Although various types of APC can cross-present model antigens *in vitro*, most studies indicate that dendritic cells (DCs) are the main cross-presenting APCs *in vivo*[1]. It is still unclear, however, which DC subtypes can or cannot cross-present antigens, in particular under specific inflammatory situations. It is also unclear so far whether the observed differences in the capacity to cross-present antigens between different DC subtypes are related to their intrinsic adaptation to this function, to their inflammatory environment or to the nature and form of the antigen.

In this Review, we address these questions by summarizing the most recent evidence regarding the intracellular trafficking pathways involved in cross-presentation in DCs. Furthermore, we integrate these pathways into our understanding of the role of cross-presentation in immunity and tolerance (excluding many of the studies in which the cross-presenting cells are not well defined, especially *in vivo*).

Intracellular pathways of cross-presentation

Two main intracellular pathways for the cross-presentation of exogenous antigens have been reported, and these are usually referred to as the 'cytosolic' and 'vacuolar' pathways (FIG. 1). Cross-presentation through the cytosolic pathway is sensitive to proteasome inhibitors[2], which suggests that internalized proteins access the cytosol, where they are degraded by the proteasome. Proteasome-generated peptides can then feed into the classical MHC class I-mediated antigen presentation pathway, which involves the transport of peptides into the ER by transporter associated with antigen processing 1 (TAP1) and TAP2 for loading on newly formed MHC class I molecules. There is, however, no direct evidence that peptide loading on MHC class I molecules occurs in the ER. The recruitment of TAP and the MHC class I-loading complex to phagosomes and endosomes suggests that peptide loading could occur in endocytic compartments[3–5] (see below). Moreover, proteasome-generated polypeptides need to be trimmed by amino-terminal peptidases to be loaded on MHC class I molecules, and both ER-associated aminopeptidase 1 (ERAP1)[6] and endosomal insulin-responsive aminopeptidase (IRAP; also known as cystinyl aminopeptidase)[7] have been shown to be involved in cross-presentation. We therefore refer here to the cytosolic pathway of cross-presentation as a pathway in which antigen processing occurs in the cytosol, regardless of the site of peptide loading.

By contrast, cross-presentation through the vacuolar pathway is resistant to proteasome inhibitors and generally independent of TAP, but is sensitive to inhibitors of lysosomal proteolysis (in particular,
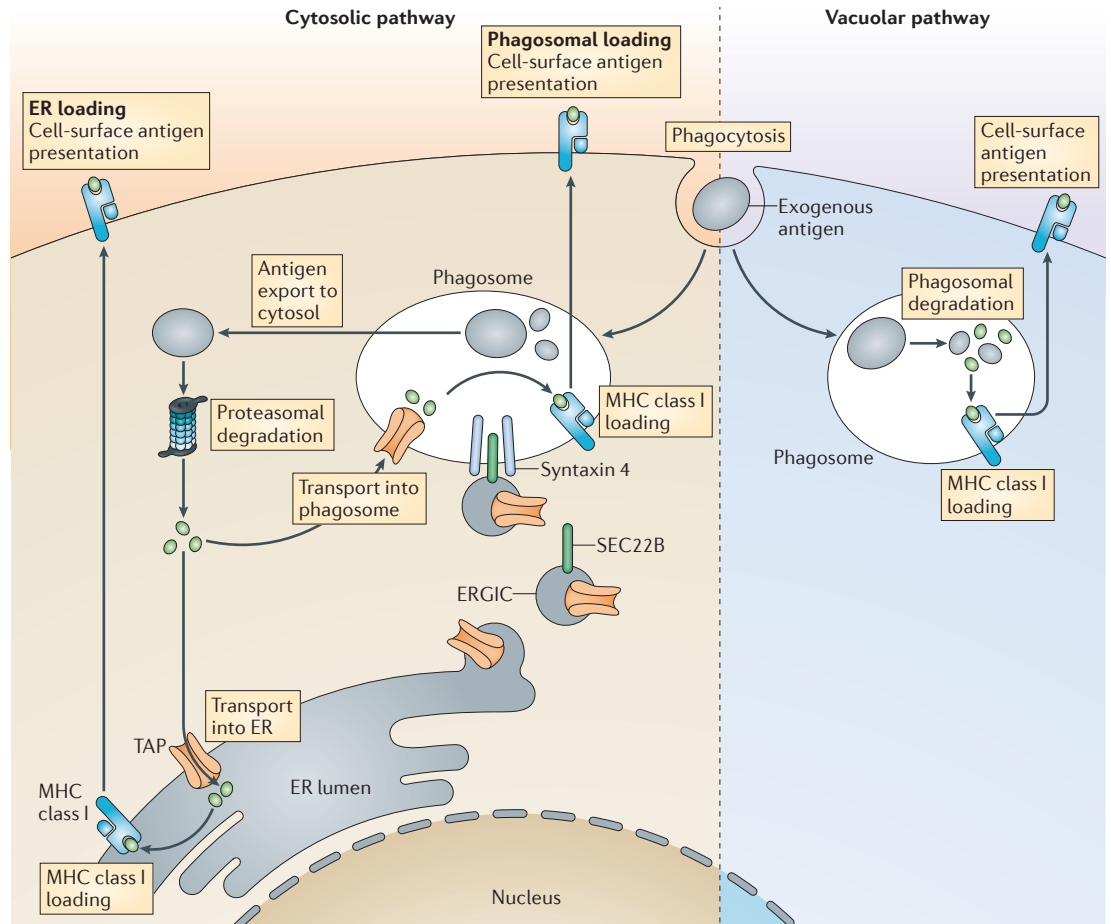
Figure 1 | **Intracellular pathways for cross-presentation in dendritic cells.** After phagocytosis, exogenous antigens can be exported into the cytosol, where they are processed by the proteasome. The processed antigens can then be loaded on MHC class I molecules in the endoplasmic reticulum (ER) (the cytosolic pathway with ER loading) or re-imported into the phagosome to be loaded on MHC class I molecules (the cytosolic pathway with phagosomal loading). The SNARE SEC22B, which localizes in the ER–Golgi intermediate compartment (ERGIC) and interacts with syntaxin 4 on phagosomes, mediates the recruitment of a subset of ER components, including transporter associated with antigen processing (TAP), to phagosomes. Alternatively, exogenous antigens can be degraded into peptides in the phagosome, where they are then loaded on MHC class I molecules (the vacuolar pathway).

cathepsin S inhibitors)[8,9]. This suggests that antigen processing and loading on MHC class I molecules both occur in endocytic compartments in this pathway. Adding to the confusion, it has been shown recently that the cytosolic (proteasome-dependent) pathway can also occur independently of TAP, possibly through another, as yet unidentified, peptide transporter[10].

The relative contributions of the cytosolic and vacuolar pathways to cross-presentation are not easy to determine *in vitro* or *in vivo*, because MHC class I molecules are retained in the ER and are unstable and/or scarce in endosomes in both TAP-deficient cells and proteasome inhibitor-treated cells[11–13]. The best evidence available so far points to the predominant use of the cytosolic (proteasome-dependent) pathway. Indeed, the *in vivo* cross-presentation of an H-Y epitope that is strictly dependent on processing by the immunoproteasome was impaired in mice lacking the inducible proteasome subunit LMP7 (REF. 14).

The origin of the MHC class I molecules involved in cross-presentation is also a matter of debate. Initially, cross-presenting MHC class I molecules were proposed to originate from the plasma membrane and to recycle to endosomes. A conserved tyrosine residue in their cytosolic tail, which is required for internalization from the cell surface, was shown to be crucial for cross-presentation[15]. However it was shown recently that CD74 promotes the trafficking of newly synthesized MHC class I molecules from the ER to endocytic compartments in DCs and that this routing is required for the cross-presentation of cell-associated antigens[16]. Finally, in relation to MHC class I molecule trafficking, RAB3B and RAB3C — two small GTPases that are involved in regulated secretion in other cell types — were identified in a genetic screen as being involved in the cross-presentation of an *Escherichia coli*-associated antigen in DCs[17]. In these studies, however, the pathways (cytosolic versus vacuolar) in which these molecules function were not identified, which complicates the interpretation of the results.

Box 1 | The phagosome pathway in professional phagocytes

In macrophages, the proteolytic activity in phagosomes increases very rapidly after the engulfment of exogenous antigens. Initially, the phagosome is mainly composed of plasma membrane surrounding a large particle (>0.5 μm). Fusion with early endosomes provides the first set of proteolytic enzymes and the acidification machinery. The proteolytic activity in these early phagosomes is limited, and the pH is only slightly acidic (~pH6.5). Fusion with late endosomes and then with lysosomes promotes the maturation of phagosomes into phagolysosomes. After 20–30 minutes, the phagosomal pH drops below 5 as a result of large-scale recruitment from lysosomes and late endosomes of the V-ATPase proton pump. This causes a large increase in the activity of proteases with acidic pH optima.

In neutrophils, the scenario is quite similar, except for a strong and sudden increase in proteolytic activity very early after engulfment. This is due to high-level production of reactive oxygen species by NADPH oxidase 2 (NOX2), causing the alkalinization of the phagosomal lumen (to ~pH7) and the release of proteases with neutral pH optima from the matrix that is present in neutrophil phagosomes.

Overall, the intracellular pathways involved in cross-presentation are still incompletely understood, at both the molecular and cellular levels. In the past 10 years, several groups have started to analyse phagosomal and endosomal functions in DCs, in an attempt to unravel the intracellular transport pathways and compartments involved in cross-presentation.

Regulation of endocytic pH and proteolytic activity. One of the hallmarks of the endocytic pathway of DCs is its decreased proteolytic capacity compared with that of other phagocytes, such as macrophages and neutrophils[18] (BOX 1). Using models of antigen endocytosis, several groups have shown that limited antigen degradation correlates with efficient cross-presentation[18,19]. Moreover, antigens that are artificially delivered to highly degradative, late endocytic compartments by dedicated liposomes are presented on MHC class II molecules more efficiently than on MHC class I molecules[20]. By contrast, antigens are cross-presented more efficiently when the liposomes are targeted to less degradative, early endocytic compartments. Similarly, antigens such as ovalbumin that are internalized by the mannose receptor are targeted to early endosomes, which favours cross-presentation[21]. The nature of the ligand could also influence its endocytic trafficking, as in the case of antigens internalized through DC-specific ICAM3-grabbing non-integrin (DC-SIGN). The extracellular structure of DC-SIGN is composed of two regions: the carbohydrate-recognition domain (CRD) and the neck region. Antigens coupled to antibodies specific for the CRD are internalized and delivered to late degradative compartments, resulting in poor cross-presentation. By contrast, antigens coupled to antibodies specific for the neck region of DC-SIGN are directed to early endosomal compartments, where they are retained, resulting in more efficient cross-presentation[22]. In addition, storage of intact internalized antigens in lysosome-like organelles has been reported, which could favour cross-presentation[23].

The decreased proteolysis that occurs in the endocytic compartments of DCs compared with other phagocytes is mainly due to the low levels of lysosomal proteases in DC endosomes and phagosomes, and to decreased activity of the proteases that are present. The decreased activity of these proteases is caused by a high pH (approximately pH7.5), which is itself a result of both low levels of activity of the V-ATPase (due to its incomplete assembly) and high levels of activity of NADPH oxidase 2 (NOX2) in phagosomal and endosomal compartments[18,24]. In DCs, NOX2 is stored in a population of secretory lysosomes that fuse with incoming phagosomes under the control of RAB27A[25]. In RAB27A-deficient DCs, phagosomal acidification is increased (which decreases the pH), resulting in enhanced proteolysis of phagocytosed antigens and impaired cross-presentation. NOX2 also regulates the phagosomal pH in human monocyte-derived DCs, as DCs from patients with chronic granulomatous disease (which is caused by a genetic defect in *NOX2*) have increased phagosomal acidification and impaired antigen cross-presentation[26].

Recently, it has been proposed that, as in macrophages[27], the phagosomal protease activity in DCs can be regulated by NOX2 through direct oxidation of the proteases, and independently of the pH[28]. It is not clear, however, why the DCs used in this study acidify their phagosomes like macrophages.

The overall picture that emerges from these studies is that cross-presentation requires low levels of protein degradation in phagosomes to preserve potential MHC class I-binding epitopes. However, some degree of degradation favours cross-presentation, probably because small molecules are exported to the cytosol with a higher efficiency than large ones[29]. MHC class II-restricted antigen presentation seems to be more resistant to high levels of proteolysis, perhaps because large polypeptides can bind to MHC class II molecules in endocytic compartments, thereby protecting antigenic epitopes from degradation.

ER–phagosome connections. One of the most intriguing features of DC phagosomes is the presence of ER-resident proteins, in particular those related to MHC class I loading[3,4,30,31]. The possible functions of ER-resident proteins in endocytic compartments and in the membrane transport pathways linking the two compartments have been debated for the past 10 years. The presence of ER-resident proteins in phagosomes was first shown in *Dictyostelium discoideum* and in macrophages[31,32]. Using electron microscopy, ER stacks can be observed in continuity with forming phagosomes at the plasma membrane, indicating that the ER membranes may contribute to the formation of phagosomes. This idea was later challenged by another study that failed to find a major contribution of ER membranes to the formation of phagosomes[33]. The presence in phagosomes or endosomes of proteins that normally reside in the ER has been reported by several groups using biochemical methods, optical and electron microscopy, and functional approaches. In human DCs, bead-bound synthetic peptides bearing an *N*-glycosylation site can be *N*-glycosylated after phagocytosis, when still attached to the beads[34]. As *N*-glycosylation is a feature specific to the ER, these results show that there is direct communication between the two intracellular compartments. We found similar results in mouse DCs[35].

V-ATPase
(Vacuolar ATPase).
A transmembrane protein complex that transports H⁺ ions across intracellular membranes in an ATP-dependent manner.

In addition, we have recently identified a key player in the molecular machinery involved in ER–phagosome interactions. We found that SEC22B, an ER-resident SNARE, is required for the recruitment of a specific cargo of ER proteins to phagosomes[35]. Silencing SEC22B expression impaired cross-presentation but not the presentation of endogenous antigens on MHC class I molecules to CD8+ T cells or antigen presentation on MHC class II molecules to CD4+ T cells. In SEC22B-deficient DCs, antigen transfer from phagosomes to the cytosol was inhibited and antigen degradation in phagosomes was enhanced, suggesting a role for ER proteins in delaying phagosome maturation. It is still unclear whether the inhibition of antigen cross-presentation in SEC22B-deficient cells is a result of decreased antigen export to the cytosol, increased antigen degradation, or other SEC22B-related defects.

Antigen export to the cytosol. The cytosolic pathway of antigen cross-presentation involves antigen export from endocytic compartments to the cytosol. The first indirect evidence for such transport was provided by a study showing that a ribosome-inactivating toxin attached to latex beads inhibits protein synthesis in macrophages. This finding indicates that, after phagocytosis of the latex beads, the toxin was exported to the cytosol, where it could target ribosomes[2]. Morphological evidence was provided some years later using light and electron microscopy[29,36]. In one of these studies[29], subcellular fractionation and western blotting were also used to show antigen delivery to the cytosol in DCs. This export to the cytosol was more efficient in DCs than in macrophages, and low-molecular-mass molecules were exported more efficiently than larger ones. More recently, protein exit from endocytic compartments to the cytosol has been demonstrated using cytochrome *c*-induced apoptosis as a readout: after internalization of exogenous cytochrome *c* by DCs, only cross-presenting DCs transfer cytochrome *c* to their cytosol, triggering caspase-dependent apoptosis[37]. In addition, we have recently reported a new approach to quantify antigen export to the cytosol, based on the cleavage of a fluorescence resonance energy transfer (FRET)-sensitive cytosolic probe by an exogenous enzyme that is transferred to the cytosol after being phagocytosed[35].

The molecular mechanisms that mediate antigen export from endosomes and phagosomes to the cytosol remain poorly understood. The best evidence available indicates that the machinery for the retrotranslocation of misfolded proteins from the ER to the cytosol (the ERAD machinery) might be used for antigen export to the cytosol after the recruitment of ER components to endocytic compartments. Indeed, the ERAD proteins SEC61 and p97 (also known as TER ATPase) are required for antigen export to the cytosol and for cross-presentation[34]. Consistent with this idea, the knockdown of SEC22B expression inhibited both the delivery of ER-resident proteins to phagosomes and the export of exogenous proteins from phagosomes to the cytosol[35]. A role for the mannose receptor in the recruitment of the translocation machinery has been proposed recently[38]; however, the importance of this mechanism remains unclear, as

cross-presentation is not impaired in lymphoid-organ DCs that are deficient for the mannose receptor[39]. Finally, recent work on the role of heat shock protein 90 (HSP90) in cross-presentation presents additional evidence supporting this model. HSP90 is known to associate with the ERAD complex on the cytosolic side of the ER[40], and internalized proteins — which need to be unfolded before their translocation into the cytosol[41,42] — are refolded in the cytosol with the help of HSP90. Moreover, the cytosolic export of internalized antigens is decreased in HSP90-deficient DCs, and the apoptosis of CD8+ DCs after phagocytosis of cytochrome *c* is decreased *in vivo* in *Hsp90*-knockout mice[43].

Although the molecular mechanisms of antigen translocation from endosomes and phagosomes into the cytosol remain poorly understood, it seems that DCs have a specialized endocytic pathway that enables them to limit antigen degradation and to recruit ER components for the transfer of exogenous proteins to the MHC class I-restricted antigen presentation pathway. Our understanding of retrotranslocation from the ER through the ERAD machinery has advanced significantly in recent years[44], for example with regard to the definition of distinct ERAD pathways that use different E3 ubiquitin ligases and the way in which ERAD substrates are recognized. Research on antigen export to the cytosol during cross-presentation should benefit from these advances.

Cross-presentation in DC subpopulations

In recent years, it has become clear that the DC compartment is organized in a complex series of subpopulations with different ontogenies and dedicated functions. In particular, it is now clear that only some DC subsets can cross-present antigens efficiently. The contribution of the different DC subtypes to cross-presentation and cross-priming (the induction of effector CD8+ T cells *in vivo*) varies depending on the experimental setting.

Cross-presentation efficiency of DC subsets. Two main populations of DCs have been described in mice and humans in steady-state conditions: plasmacytoid DCs (pDCs) and conventional DCs[45]. The mouse conventional DC population includes lymphoid organ-resident DCs and migratory DCs. Both of these conventional DC populations can be divided into several subsets on the basis of cell phenotype. Two main types of resident DC are found in the spleen, lymph nodes and thymus: CD8+CD11b− and CD8−CD11b+ DCs[46]. Migratory DCs are present in most non-lymphoid organs and migrate to draining lymph nodes. They comprise two main subpopulations: CD103+CD11b− and CD103−CD11b+ DCs[47–50]. In the skin, there is a third subset of migratory DCs known as Langerhans cells. Finally, an additional subset of DCs, known as inflammatory DCs, differentiates from monocytes during inflammation[51]. These DC subsets express different endocytic receptors and pattern-recognition receptors (PRRs)[52,53] and secrete different cytokines and chemokines following activation. There is accumulating evidence that different DC populations have specialized functions, including in terms of antigen presentation and the regulation of T cell responses.

SNARE

(Soluble NSF attachment protein receptor). A member of a class of proteins that are required for membrane fusion events that occur in the course of vesicle trafficking and secretion.

Fluorescence resonance energy transfer

(FRET). A technique that is used to measure protein–protein interactions either by microscopy or by flow cytometry. Proteins fused to cyan, yellow or red fluorescent proteins are expressed and assessed for interaction by measuring the energy transfer between fluorophores. Such transfer can occur only if the proteins physically interact.

ERAD

(Endoplasmic reticulum-associated protein degradation). A pathway that targets proteins that are misfolded in the ER to the proteasome for degradation.

Pattern-recognition receptors

(PRRs). Host receptors (such as Toll-like receptors and NOD-like receptors) that can sense pathogen-associated molecular patterns. The term PRR is usually restricted to molecules that signal to modify gene expression programmes, leading to the expression and/or secretion of immune modulators (such as pro-inflammatory cytokines) that coordinate innate and adaptive immune responses. PRRs can be localized at the plasma membrane, in endosomal compartments or in the cytosol.

Initially, resident CD8^{+} DCs were shown to be more efficient at cross-presentation than CD8^{−} DCs in the steady state[54], whereas both DC subtypes presented antigens efficiently after receptor-mediated endocytosis (for example, by CD205 or Fc receptors for IgG (FcγRs))[55,56]. Other DC subsets, however, have also been shown to cross-present antigens efficiently. Among migratory DCs, CD103^{+} DCs are the most efficient for the cross-presentation in the lymph nodes of antigens that were captured in the lungs[57–60] or skin[61,62]. Whether Langerhans cells can cross-present antigens has been controversial. Recent studies using *in vivo* ablation of Langerhans cells clearly showed that, in mice, Langerhans cells do not have a major role in the cross-presentation of peripheral antigens[63–65]. Mouse pDCs are generally poor APCs[66]. Although cross-presentation by pDCs can be observed *ex vivo* after Toll-like receptor (TLR) stimulation[67], several studies have failed to demonstrate a role for pDCs in cross-presentation *in vivo*[58,68,69]. Finally, inflammatory DCs also cross-present antigens both *ex vivo* and *in vivo* in different models of inflammation[39,70–73].

The cross-presentation ability of different DC subsets is also developmentally regulated. The immediate precursors of CD8^{+} DCs have limited cross-presentation ability compared with terminally differentiated CD8^{+} DCs[74]. The ability to cross-present antigens is promoted by cytokines that are involved in the development of the CD8^{+} DC lineage, such as granulocyte–macrophage colony-stimulating factor (GM-CSF)[75]. This suggests that the ability for cross-presentation is only acquired at the late stages of CD8^{+} DC development.

Human DCs are also heterogenous. Blood DC antigen 1 (BDCA1)[+] DCs (also known as CD1c^{+} DCs), BDCA3^{+} DCs (also known as CD141^{+} DCs) and pDCs are found in the blood, spleen, tonsils and lymph nodes[76–79]. In the skin, three DC subsets have been described: Langerhans cells, CD1a^{+} dermal DCs and CD14^{+} dermal DCs[80,81], all of which migrate to the skin-draining lymph nodes[79]. Among the skin DCs, Langerhans cells and CD1a^{+} DCs are the most efficient at cross-presentation, whereas CD14^{+} DCs are less efficient[79,80]. BDCA3^{+} DCs were recently proposed to be the homologue of mouse lymphoid organ-resident CD8^{+} DCs[82–85] and therefore to be specialized at cross-presentation. The evidence available so far shows that BDCA3^{+} DCs cross-present antigens from dead cells better than other DC subtypes[84], but it remains to be further investigated whether this corresponds to a specialization of their endocytic pathway compared with that of other DC subtypes (as is the case in mouse CD8^{+} DCs) (BOX 2).

Cross-presentation as a functional specialization. The notion of 'efficiency' is central to what we call 'specialization'. We actually know little about the efficiency of antigen cross-presentation *per se*. In most cases, cross-presentation by mouse DCs is quantified using a biological read-out (in terms of the activation of either T cell receptor (TCR)-transgenic CD8^{+} T cells or CD8^{+} T cell hybridomas expressing an antigen-specific TCR), rather than being measured directly. Indeed, only a few groups have succeeded in detecting cross-presentation using the only available antibody specific for a peptide–MHC complex (the ovalbumin peptide–H-2K[b]-specific monoclonal antibody 25D.1 (REF. 86)), and we have not been able to reproduce this. The outcome that is actually detected using T cell read-outs results from a combination of stimulation by the peptide–MHC class I complex and a series of ill-defined cell-related parameters that enhance or inhibit T cell activation (including co-stimulation, adhesion and cytokines). It is very difficult, under these experimental conditions, to be sure that increased T cell activation is due to increased cross-presentation, rather than to other factors that influence antigen uptake (and intracellular trafficking) or T cell activation itself. In particular, antigen targeting to individual surface receptors influences

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Box 2 | Cross-presentation in human DCs

Several recent studies have established a clear phylogenetic homology between mouse CD8^{+} dendritic cells (DCs) and human blood DC antigen 3 (BDCA3)[+] DCs based on phenotypic and transcriptomic evidence[82–85]. However, the notion that BDCA3^{+} DCs are also the functional counterparts of mouse CD8^{+} DCs in terms of cross-presentation remains, from our point of view, to be definitively established (at least for soluble antigens). Several groups have shown that human BDCA1^{+} DCs also cross-present antigens[79,83,84,178,179]. Most of the studies using *ex vivo*-isolated DCs are based on very low numbers of donors, and variability between human donors should not be underestimated. In one study, non-activated BDCA1^{+} DCs cross-presented a soluble antigen more effectively than BDCA3^{+} DCs, whereas the opposite was seen after activation of the two DC subsets by the Toll-like receptor 3 (TLR3) ligand polyinosinic–polycytidylic acid (polyI:C)[84]. BDCA1^{+} DCs, however, express lower levels of TLR3 than BDCA3^{+} DCs[83,84]. Other modes of activation should therefore be tested before drawing definitive conclusions. The results are more convincing when analysing the cross-presentation of antigens from necrotic cells. In this case, BDCA3^{+} DCs are clearly more efficient than BDCA1^{+} DCs[84], which is consistent with their selective expression of CLEC9A, a receptor for necrotic cells[180]. Moreover, most studies on human DCs use blood DCs, but a recent study suggests that blood DCs have not yet developed their full functional abilities as compared with lymphoid organ-resident DCs[79]. In addition, human plasmacytoid DCs (pDCs) can cross-present soluble or particulate antigens efficiently, in contrast to resting mouse pDCs[178,181]. The antigen form and mode of delivery, as well as the activation signals for the DCs, are probably crucial in determining the efficiency of cross-presentation.

The intracellular pathways involved in cross-presentation by human DCs remain poorly characterized. Cross-presentation by blood BDCA1^{+} DCs[84,178], BDCA3^{+} DCs[84] and pDCs[178] has been shown to be inhibited by proteasome inhibitors, which indicates that exogenous antigens need to access the cytosol for cross-presentation. However, another study in human pDCs showed that cross-presentation was independent of the proteasome and sensitive to inhibitors of endosomal acidification and lysosomal proteases[181], indicating that antigen processing occurred in endosomal compartments.

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both the amount of antigen taken up and the intracellular trafficking route (which can also vary between different cell types, even when the same receptor is used). In addition, many of the conclusions about the 'efficiency' of DC subtypes are limited by the fact that the processes required to isolate the cells from tissue can potentially modify their functions. Finally, we should always keep in mind when saying that a subset is 'specialized' for any function that cell functions can change depending on the environment.

Nevertheless, understanding why steady-state lymphoid organ-resident CD8^{+} DCs seem to be the most efficient at cross-presentation has been the focus of sustained attention in the field. Because cross-presentation was initially evidenced using antigens associated with dead cells, it was first proposed that more efficient cross-presentation was due to the more efficient capture of apoptotic cells by CD8^{+} DCs[87,88]. However, both CD8^{+} and CD8^{−} DCs capture soluble and particulate antigens efficiently and present them on MHC class II molecules, but only CD8^{+} DCs can cross-present internalized antigens on MHC class I molecules, which suggests that CD8^{+} DCs have a specialized intracellular machinery for cross-presentation[89,90]. Similarly, the ability of migratory CD103^{+} DCs to cross-present antigens is not only due to their superior ability to capture antigens[57,58,60,61].

CD8^{+} DCs use only the cytosolic pathway for cross-presentation[39]. This is probably because they have a unique combination of features that favour cross-presentation through this pathway (TABLE 1). After antigen uptake, antigen degradation in endocytic compartments remains limited in CD8^{+} DCs as a result of the maintenance of an alkaline pH in endosomes and phagosomes[91]. This phenomenon is due to the production of reactive oxygen species (ROS) in the endocytic compartments of CD8^{+} DCs following the recruitment of NOX2 by RAC2. In CD8^{−} DCs, RAC1 directs the assembly of NOX2 to the plasma membrane rather than to endosomal membranes, thus limiting the levels of ROS produced in endosomes and phagosomes. The transfer of exogenous antigens to the cytosol is another key step in cross-presentation that is more efficient in

CD8^{+} DCs[37]. It has also been shown that CD8^{+} and CD8^{−} DCs overexpress molecules involved in MHC class I and MHC class II loading, respectively, which could explain their relative preferences for presenting antigens to CD8^{+} versus CD4^{+} T cells[92]. Nevertheless, a functional link between the overexpression of these molecules and the selectivity of antigen presentation in the two subsets is missing. CD8^{+} DCs also express XC-chemokine receptor 1 (XCR1), and its ligand XC-chemokine ligand 1 (XCL1) is produced by CD8^{+} T cells following antigen recognition (as well as by other cells)[93]. The engagement of XCR1 by XCL1 was proposed to facilitate interactions between cross-presenting DCs and CD8^{+} T cells, and this receptor interaction is involved in the development of effector CTLs[83,93].

The biology of cross-presentation in CD103^{+} DCs has not been addressed owing to the paucity of these cells (they account for less than 10% of the total DCs in skin-draining lymph nodes[62]). It was recently shown, however, that CD103^{+} DCs and CD8^{+} DCs are developmentally related[94] and share some functional properties, such as the expression of XCR1 (REF. 95) and the ability to capture apoptotic cells for antigen presentation[60]. It is therefore possible that CD103^{+} DCs share with CD8^{+} DCs some of the mechanisms involved in cross-presentation, but this issue requires further investigation. By contrast, inflammatory DCs and their *in vitro* equivalents (GM-CSF-cultured bone-marrow-derived DCs) have been shown to use both the cytosolic and vacuolar pathways for cross-presentation[7,39]. The functional relevance of this finding is not yet clear. Altogether, most studies indicate a specialization for cross-presentation of lymphoid organ-resident CD8^{+} DCs and migratory CD103^{+} DCs in mice.

Cross-presentation in immunity

Cross-presentation during infections. During infections, antigen-specific CD8^{+} T cells can be primed not only by cross-presentation, but also by direct presentation of pathogen-derived antigens on MHC class I molecules when the DCs are themselves infected. The relative contributions of these two pathways to CD8^{+} T cell activation depend largely on the type and

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Table 1 | **Functional specializations of mouse DC subsets for cross-presentation**

Feature	Inflammatory DCs	pDCs	Lymphoid organ-resident DCs		Migratory DCs		
			CD8^{+} DCs	CD8^{−} DCs	CD103^{+} DCs	CD103^{−} DCs	Langerhans cells
Cross-presentation	+	−	++	−	++	−	−
pH in endocytic compartments	ND	ND	Alkaline	Acidic	ND	ND	ND
Transfer of antigens to the cytosol	ND	ND	+	−	ND	ND	ND
Proteasome-dependent pathway	+	ND	+	ND	ND	ND	ND
Vacuolar pathway	+	ND	−		ND	ND	ND

DC, dendritic cell; ND, not determined; pDC, plasmacytoid DC.

tropism of the pathogen. Dynamic intravital imaging has shown that, during vaccinia virus infection, direct antigen presentation accounts for CD8+ T cell priming[96,97]. By contrast, during *Toxoplasma gondii* infection, CD8+ T cell activation is mainly mediated by uninfected DCs that cross-present parasite-derived antigens captured from neighbouring infected cells[98]. The acquisition by DCs of antigens from infected cells can occur through multiple mechanisms. DCs express a large repertoire of endocytic and phagocytic cell-surface receptors specific for dead cell-associated molecular patterns, and these receptors allow for the recognition and engulfment of infected dead cells. It has also been shown that uninfected APCs can capture and present pathogen-derived antigens through a process known as 'cross-dressing', which involves the acquisition from infected cells of preformed peptide–MHC class I complexes that do not require further processing[99]. In that study, cross-dressing was mediated by trogocytosis, but peptide–MHC complexes might also be transferred through secreted membrane vesicles, such as exosomes[100].

During skin infection with herpes simplex virus 1 (HSV-1), lymph node-resident CD8+ DCs have a dominant role in cross-presentation to CD8+ T cells, whereas migratory dermal DCs are essential for the transport of viral antigens from the site of infection to lymph nodes[101,102]. This result suggested that migratory dermal DCs might transfer antigens to lymph node-resident CD8+ DCs for cross-presentation. One of these studies[101] demonstrated that lymph node-resident CD8+ DCs are not infected by the virus. In addition, a histological analysis showed that HSV-1-infected DCs do not migrate out of the skin[103]. More recently, it was shown that migratory dermal CD103+ DCs are responsible for cross-presentation in secondary HSV-1 skin infection[61]. During *Candida albicans* skin infection, lymph node-resident DCs do not acquire *C. albicans* antigens, and migratory CD103+ DCs are responsible for cross-presentation in draining lymph nodes[65].

In a model of pulmonary infection with influenza virus, several groups found that both migratory pulmonary CD103+ DCs and draining lymph node-resident CD8+ DCs are involved in the cross-presentation of viral antigens[58,104]. Similar observations were made for respiratory syncytial virus infection[105], whereas, in pulmonary infections with poxvirus, migratory CD103+ DCs were the main cross-presenting DCs in lymph nodes[59]. Prolonged tissue infection can also lead to the generation of inflammatory DCs that can migrate to the draining lymph nodes. In the case of influenza virus infection, inflammatory DCs become the main cross-presenting DC subset at late time points after infection[73]. This could be due to the impairment of cross-presentation by conventional DCs, as shown for CD8+ DCs after TLR-mediated systemic activation[106].

In the case of blood-borne pathogens, cross-presentation is carried out exclusively by lymphoid organ-resident CD8+ DCs, as shown for blood-stage *Plasmodium berghei* infection[107] and systemic infection with *P. berghei* sporozoites[108]. Finally, it has been shown

that cross-presentation can occur directly in infected tissues for the stimulation of tissue-resident memory CD8+ T cells during secondary infections[71] or activated CD8+ T cells during primary infections[72]. In both cases, inflammatory DCs are the cross-presenting cells.

The most direct evidence for a crucial role of cross-presentation in antiviral CD8+ T cell responses probably comes from basic leucine zipper transcriptional factor ATF-like 3 (BATF3)-deficient mice, which lack the two main cross-presenting DC subsets (lymphoid organ-resident CD8+ DCs and migratory CD103+ DCs). These mice have impaired CD8+ T cell responses against Sendai virus and West Nile virus[94,109], which indicates that cross-presentation by DCs *in vivo* is essential for the initiation of cytotoxic T cell responses to these viruses. However, a role for direct presentation by infected DCs cannot be completely excluded in these experiments. An analysis of γ-interferon-inducible lysosomal thiol reductase (GILT)-deficient mice provides additional evidence for the crucial role of cross-presentation in promoting cytotoxic T cell responses against viruses. Indeed, although GILT is dispensable for direct presentation to CD8+ T cells by infected cells, GILT-deficient mice infected with HSV-1 or influenza virus have impaired CD8+ T cell responses against viral antigens containing disulphide bonds (which need to be reduced by GILT to allow the antigens to be cross-presented)[42]. Although the contribution of cross-presentation to CD8+ T cell responses during infections has been a matter of debate for many years, concurring evidence is accumulating to support a crucial role for this process, including during certain viral challenges.

Cross-presentation and cancer. The cross-presentation of tumour antigens is less well understood. Although DCs can be found inside tumours, it is not yet clear to which DC subtypes these tumour-infiltrating DCs correspond. A defect of CD8+ DCs and CD103+ DCs severely compromises CD8+ T cell responses to immunogenic syngeneic tumours[109], which shows that cross-presenting DC subsets have a major role in antitumour immune responses. It is unclear, however, whether this phenotype is due to a defect in cross-presentation or to some other function of these DC subsets, such as interleukin-12 production[110]. Recently, it was shown that type I interferons are involved in tumour rejection through their effects on CD8+ DCs in tumour-draining lymph nodes[111,112]. Indeed, type I interferons can enhance cross-presentation by decreasing antigen degradation in endocytic compartments and promoting the survival of CD8+ DCs[113]. However, the way in which tumour antigens reach lymphoid organ-resident CD8+ DCs is still poorly understood. A recent study in which dead tumour cells were injected into the footpads of mice suggested that CD11c+CD169+ macrophages in the lymph nodes cross-present dead tumour cell-associated antigens[114]. Moreover, depletion of CD169+ cells impairs tumour rejection after vaccination with dead tumour cells. However, it is unclear whether the cross-presenting cells in this system are DCs or macrophages, as CD8+ DCs also express CD169 (REF. 53).

Trogocytosis
The transfer of plasma membrane fragments from one cell to another.

Cross-presentation during allospecific responses. The ability of cross-presenting cells to activate allospecific CD8^{+} T cells was highlighted in early studies of cross-priming[115,116], in which immunization with allogeneic cells resulted in the priming of recipient T cells specific for allogeneic peptides and restricted for both donor and host MHC molecules. We now know that cross-presentation of cell-associated antigens depends on host CD8^{+} DCs in that particular model[1,117].

After allogeneic organ transplantation, two recognition pathways account for allospecific T cell activation. In addition to the 'direct' pathway (direct recognition of allogeneic cells by host T cells) (BOX 3), host DCs that migrate into the transplanted tissue can capture, take up and process donor antigens *in situ* and cross-present alloantigen-derived peptides on self MHC molecules after migration to the draining lymphoid organs. The importance of such 'indirect' allorecognition in the shaping of the alloreactive CD8^{+} T cell compartment was demonstrated in a skin allograft model[118], in which CD8^{+} T cells cross-primed through this pathway were the main component of the donor-specific T cell repertoire. Similar results were obtained in a mouse model in which MHC class I-deficient skin allografts were grafted onto severe combined immunodeficiency (SCID) recipients reconstituted with host-type polyclonal CD8^{+} T cells[119]. Recently, the innate and adaptive immune responses against skin allografts were tracked using two-photon microscopy, and it was proposed that host CD11b^{+} DCs (probably inflammatory DCs)

that infiltrate the graft have a key role in the priming process[120]. It is important, however, to consider that cross-priming of host T cells through the indirect pathway can also be mediated by non-haematopoietic cells, including endothelial cells[121,122].

Cross-presentation in tolerance

The cross-presentation of exogenous antigens on MHC class I molecules can also result in CD8^{+} T cell tolerance. Depending on the physiological environment, cross-presentation can induce clonal deletion, functional inactivation (anergy) or programming into a suppressive (regulatory) T cell phenotype. In the steady state, cross-presentation by DCs is involved in both central tolerance and peripheral tolerance of CD8^{+} T cells to self and environmental antigens.

Central tolerance. In the thymus, negative selection eliminates or functionally inactivates developing thymocytes based on their affinity for self-derived peptides. To be completely effective, this process would require the expression of all the immunogenic self antigens in the thymus. The ectopic expression of peripheral tissue-specific antigens (TSAs) by medullary thymic epithelial cells (mTECs) is mediated by the transcription factor autoimmune regulator (AIRE)[123,124], and direct antigen presentation by mTECs has been implicated in the deletion of self-specific CD8^{+} thymocytes[125]. Several groups, however, have suggested that thymic DCs could also have a role in negative selection through the cross-presentation of mTEC-associated TSAs. In support of this hypothesis, AIRE expression is rapidly followed by the proliferative arrest and apoptosis of mTECs[126], which could then be taken up by thymic DCs, resulting in the cross-presentation of TSAs. Indeed, the transfer of membrane-bound or cytosolic material from mTECs to thymic DCs through trogocytosis or the engulfment of dead cells has been reported in several models[127,128], and thymic DCs that do not express AIRE have been implicated in the deletion of TSA-specific thymocytes[125,128]. The relative contributions of the different subpopulations of DCs, including migratory DCs[129], to negative selection remain to be fully established. In the case of central tolerance to circulating antigens, cross-presentation by thymic DCs might have a dominant role, owing to the high efficacy of DCs compared with other populations of stromal cells in the capture of circulating antigens[130].

Peripheral tolerance. T cells expressing TCRs with a low or intermediate affinity for self antigens can escape negative selection in the thymus and access peripheral tissues. These potentially harmful T cells are controlled through a series of mechanisms usually referred to as 'peripheral tolerance'. In the past 15 years, it has become clear that natural regulatory T cells have a major role in controlling self-specific T cells[131–133]. A second important mechanism for silencing self-specific CD8^{+} T cells in the periphery is mediated through the cross-presentation of antigens by resting DCs, a process often referred to as 'peripheral cross-tolerance'.

Box 3 | **Direct allorecognition and cross-priming: the peptide in question**

After transplantation of allogeneic tissue, donor dendritic cells (DCs), which migrate from the graft to the draining lymphoid organs of the recipient, can activate host T cells through direct interactions between donor peptide–MHC complexes and host T cell receptors (TCRs). The contribution of this pathway to allorecognition was evidenced by studies involving the depletion of bone marrow-derived 'passenger' leukocytes from the graft[182]. Interestingly, the immunogenicity of passenger cell-depleted transplants could be restored by injection of donor-strain DCs[183]. For a long time, it was not clear to what extent cross-presentation was necessary for CD8^{+} T cell priming through this direct allorecognition pathway, as the involvement of the peptide in the TCR–peptide–MHC interaction was questioned. To explain the high frequency of allospecific precursors among the recipient T cell repertoire, it was first proposed that allospecific T cells recognize both allogeneic cell-derived peptides and donor MHC molecules. This hypothesis indicated a putative role for cross-presentation of tissue-derived antigens by DCs in allospecific CD8^{+} T cell priming[184]. It was later proposed that alloreactive T cells are activated by direct recognition of donor MHC molecules, regardless of the presented peptides[185]. Some studies have supported this model[186,187], thus implying that cross-presentation of donor antigens has no direct effect on the activation of the allospecific CD8^{+} T cell fraction by donor DCs.

More recently, these two models were reconciled by the demonstration of the importance of self MHC molecules in determining the repertoire of alloreactive T cells[188]. Thus, the peptide would be involved in TCR–peptide–MHC interactions only if donor and host MHC molecules are closely related. However, only a few reports support a peptide-independent activation of allospecific T cells, and several studies analysing the crystal structure of TCRs interacting with allogeneic MHC molecules clearly demonstrate an involvement of the peptide[189,190], which argues in favour of an important, albeit not systematic, involvement of the peptide in allospecific CD8^{+} T cell priming by the direct pathway. To conclude, it is important to note that even if the peptide is involved in allospecific CD8^{+} T cell priming by donor DCs, the relative contribution of endogenous versus exogenous peptides, and so of cross-presentation versus endogenous MHC class I-mediated presentation, has never been formally addressed.

Pioneering work in mice expressing a model antigen in the pancreas showed that peripheral TSAs are constitutively cross-presented to CD8^{+} T cells by haematopoietic cells in the steady state, resulting in limited cell activation and tolerance[134–136]. In this model, cross-presentation and cross-tolerance are mediated by lymph node-resident CD8^{+} DCs[137]. The notion that DCs can directly mediate the clonal deletion of T cells in the periphery was reinforced by experiments targeting model antigens to DCs *in vivo*, either genetically or using DC-specific monoclonal antibodies[138–140]. However, the models used in these studies are based on the adoptive transfer of high-affinity TCR-transgenic T cells. More recently, it was shown that peripheral cross-tolerance is also involved in the control of polyclonal CD8^{+} T cells expressing TCRs with a low or intermediate affinity for TSAs. The authors took advantage of the inability of RAC1-deficient DCs to efficiently take up and cross-present soluble and cell-associated antigens to show that the impaired cross-presentation of dead cell-associated self antigens by RAC1-deficient DCs leads to impaired cross-tolerance and to the accumulation of autoreactive T cells in peripheral lymphoid organs. When appropriately stimulated, these cells had the ability to trigger autoimmune disorders[141].

The types of DC that are involved in cross-tolerance have been investigated in some detail. CD8^{+} DCs were initially thought to have a unique role in cross-tolerance[137], but some studies have also shown involvement of the CD8^{–} DC population[142–144]. Antigen cross-presentation to CD8^{+} T cells in tolerogenic conditions is also not restricted to lymph node- or spleen-resident DCs. Migratory CD103^{+} dermal DCs are highly efficient at cross-presenting keratinocyte-derived antigens to CD8^{+} T cells in the steady state, thus challenging the initial paradigm that cross-tolerance requires the transfer of tissue-derived antigens from migratory DCs to lymph node-resident DCs[61,62]. Similar observations of cross-presentation by CD103^{+} DCs from both lung and small intestine lamina propria were made when an innocuous antigen was administered intratracheally or orally, respectively[57,145]. Interestingly, human lamina propria CD103^{+} DCs with a similar phenotype and function to mouse lamina propria CD103^{+} DCs have been identified[145], but their role in cross-tolerance has not yet been addressed.

So, peripheral tolerance to self and environmental antigens relies, at least in part, on the cross-presentation of exogenous material by both migratory and lymph node-resident DCs. However, recent studies have challenged this model by demonstrating that lymph node stromal cells (LNSCs) can directly present TSAs to self-specific CD8^{+} T cells, thus allowing the deletion of self-reactive T cell precursors[146–150]. Several subpopulations of LNSC seem to be involved in this process, including fibroblastic reticular cells, lymphatic endothelial cells and extrathymic AIRE-expressing cells. Interestingly, these different LNSC populations express distinct self antigens, and the patterns of TSAs expressed by mTECs and extrathymic AIRE-expressing cells have only limited overlap[147]. These observations suggest a qualitative, rather than quantitative, contribution of DCs and LNSCs to cross-tolerance. In support of this idea, DCs do not efficiently cross-present antigens derived from LNSCs[151]. Without dismissing a role for the cross-presentation of self antigens by DCs, these studies call for a more detailed analysis of the relative contributions of the different populations of APCs, haematopoietic or not, in the shaping of the peripheral CD8^{+} T cell compartment.

Towards the clinic

The contribution of cross-presentation to protective immunity after vaccination has been difficult to evaluate. Early vaccines based on synthetic peptides (which were either directly injected or loaded on DCs) were relatively inefficient at inducing consistent clinical benefit, which led the field to use long peptides or intact proteins (both of which require cross-presentation) for vaccination. Enhanced immune protection was obtained[152–154], which could be explained in part by increased endocytosis, processing and presentation of long versus short peptides[155], resulting in long-lasting T cell stimulation[156]. Two main approaches to improve cross-presentation-based vaccination have been envisaged. First, several groups have developed drug-based strategies to directly modulate the process *in vivo*. They hypothesized that a short course of chloroquine could favour cross-presentation by blocking endosomal and phagosomal acidification, thus protecting antigens from degradation and favouring their routing to the MHC class I antigen presentation pathway. In mice, it was shown that cross-priming in response to a soluble nominal antigen could be substantially boosted by a short treatment with chloroquine[157]. Chloroquine administration together with live sporozoites was also efficient at inducing T cell-dependent protection against a subsequent homologous malaria challenge in both mice and humans[158–160]. In those studies, however, it was unclear whether chloroquine boosted cross-priming or was only required to kill the parasites in the asexual blood stage. In humans, hepatitis B virus (HBV) vaccine responders boosted with a dose of HBV envelope protein vaccine in the presence of chloroquine had substantially increased antigen-specific CD8^{+} T cell responses compared with responders who were boosted without chloroquine[19].

An alternative approach is to target antigens *in vivo* to DC surface molecules that can mediate endocytosis[161]. This approach is very versatile, as it can efficiently induce both CD8^{+} T cell cross-priming (to induce a protective immune response against cancer or infectious diseases) and CD8^{+} T cell cross-tolerance (to prevent autoimmunity or allograft rejection). Whether antigen targeting to DCs results in immunity or tolerance depends on multiple parameters, including the nature and pattern of expression of the target molecule[92,161], the vector used (most recently specific antibodies, but toxins or chaperones have also been used), the co-administered adjuvants, the formulation of the vaccine[162], and the region of the targeted molecule bound by the vector[22,163]. In mice, a large panel of receptors has been used in vaccine-targeting studies.

Peripheral tolerance
Peripheral tolerance refers to mechanisms that control the reactivity of self-specific lymphocytes that have escaped central tolerance. These mechanisms include 'active' suppression by cells endowed with immunomodulatory functions (such as regulatory T cells), as well as the induction of anergy or deletion, for example through antigen presentation to T cells in the absence of co-stimulation.

REVIEWS

Most of these molecules are members of the C-type lectin receptor family, such as CD205 (REFS 139,164), CLEC9A (also known as DNGR1)[165,166] and CD207 (also known as langerin)[167]. However, efficient cross-priming has also been reported when targeting antigens to other endocytic cell-surface molecules, including integrins[168,169], HSP receptors[170] and glycolipids (such as globotriaosylceramide (Gb3))[171].

It seems that all of these receptors successfully route antigens to the MHC class I antigen presentation pathway after endocytosis, but their relative efficiency and their potential for clinical intervention remain difficult to estimate, as few studies have systematically compared their efficacies in the same model systems[172–174]. In addition, we still know very little about the subpopulations of human DCs, and almost nothing about their functions. With the exception of CLEC9A — which seems to be expressed specifically by the BDCA3+ DC subset and which allows cargo delivery to both the MHC class I and class II antigen presentation pathways in both mice and humans[165,166,175–177] — specific markers for the different DC populations are still lacking.

Altogether, antigen targeting is an appealing strategy for future vaccination, but there is still a long way to go. The phenotypic and functional analysis of human DC subtypes, together with the identification of endocytic molecules selectively expressed by these cells, will hopefully allow the development in the next few years of rational targeted vaccination strategies for the induction of both immunity and immune tolerance, depending on the pathology.

Conclusion

Cross-priming was described more than 30 years ago in allografts, but its contribution to immune responses — particularly for antiviral and antitumour immune responses — remains uncertain. The finding that certain DC subsets are more efficient at cross-presentation than others, and that their absence in mutant mice prevents immune responses against certain viruses or tumours, indicates that cross-presentation might have a crucial role in all CD8+ T cell responses. One of the main problems for evaluating the actual contribution of cross-presentation *per se* to immune responses has been the lack of genetically modified mice with selective defects in cross-presentation, rather than in cross-presenting cells (which might also have functions other than cross-presentation). Although DCs with genetic defects in cross-presentation were recently reported (owing to mutations in the genes encoding NOX2 or RAC2, for example[24,91]), the defects are not complete and the affected molecules are widely expressed and involved in other functions in different cell types. It is therefore still not possible to analyse the role of cross-presentation *in vivo* using these mice. Providing an accurate evaluation of the role of cross-presentation in tolerance or immunity will be one of the important challenges in the field for the next few years. But, whatever its physiological role turns out to be, the manipulation of cross-presentation emerges as a promising tool for immune intervention. However, the identification of cross-presenting DC subpopulations in humans will be a prerequisite for the development of novel targeted vaccination strategies.

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