

Cross presentation of antigen by dendritic cells: mechanisms and implications for immunotherapy

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Dendritic cells (DCs) possess the specialized potential to present exogenously derived antigen to cytotoxic T lymphocytes to elicit an immune response. This process, termed cross presentation, is crucial in the generation of immune response to viruses, tumors and in autoimmune disease. The ability of DCs to cross-present exogenous antigen to cytotoxic T lymphocytes makes them an attractive target for exploitation in immunotherapy. In recent years, significant advances have been made in understanding the mechanism of cross-presentation and the DC subsets involved. The recent discovery of the human cross presenting DC has given this field a new lease of life. In this report, the authors provide an overview of cross-presentation of antigen by DCs, focusing on the current understanding of the molecular mechanisms of the process. The authors also discuss the DC subsets involved in cross presentation and its role in health and disease.

KEYWORDS: antigen presentation • CD141 • CD8 α ⁺ DC • cross presentation • dendritic cells • immunotherapy • XCR1

Dendritic cells (DCs) are sentinels of the immune system, specializing in surveillance of the tissue microenvironment for antigens and their acquisition. They process and present these antigens to T cells, resulting in activation of the appropriate immune response. DCs are the most effective APCs by virtue of high levels of MHC II expression and costimulatory activity. They present antigen either directly via MHC II to naive CD4⁺ T-helper cells (Th), also termed as conventional presentation (FIGURE 1) or via MHC I to naive CD8⁺ or cytotoxic T lymphocytes (CTL). The latter process, when occurring for exogenously derived antigen, is termed cross presentation and was first demonstrated through seminal experiments in 1976 in which a cytotoxic immune response was generated by the administration to recipient mice of allogeneic cells, differing only in their expression of minor histocompatibility antigens [1]. Cross presentation plays an important role in the recognition of tumor cells and tissue-specific viruses [2–7] and has also been implicated in central [8] and peripheral tolerance to self antigens [9], raising the prospect of harnessing its potential in immunotherapy. The recent discovery of the human counterpart of the mouse cross-presenting DCs has once again

put cross presentation in the limelight [10]. This review focuses on the characteristics of the cross presenting DC, both in mice and humans, highlighting the mechanism of cross presentation and its implications in health and disease.

Mechanism of cross presentation

Exogenous antigens may be internalized by macropinocytosis, micropinocytosis, phagocytosis, receptor-mediated endocytosis or via gap junctions [11,12]. They pass through three stages to be cross presented by DCs, namely: the processing of antigen into peptides; loading of peptides onto MHC I molecules; and transport of the MHC I–peptide complex to the cell surface. Two pathways have been proposed for this process, the vacuolar pathway and the cytosolic pathway. In the vacuolar pathway, antigen is processed into peptides by proteases present in the endosome. They are then loaded onto MHC I molecules, which have been recycled from the plasma membrane or obtained from endoplasmic reticulum (ER), and transported to the cell surface. In the case of the cytosolic pathway, internalized antigen is transported into the cytosol, where it is degraded into peptides by conventional

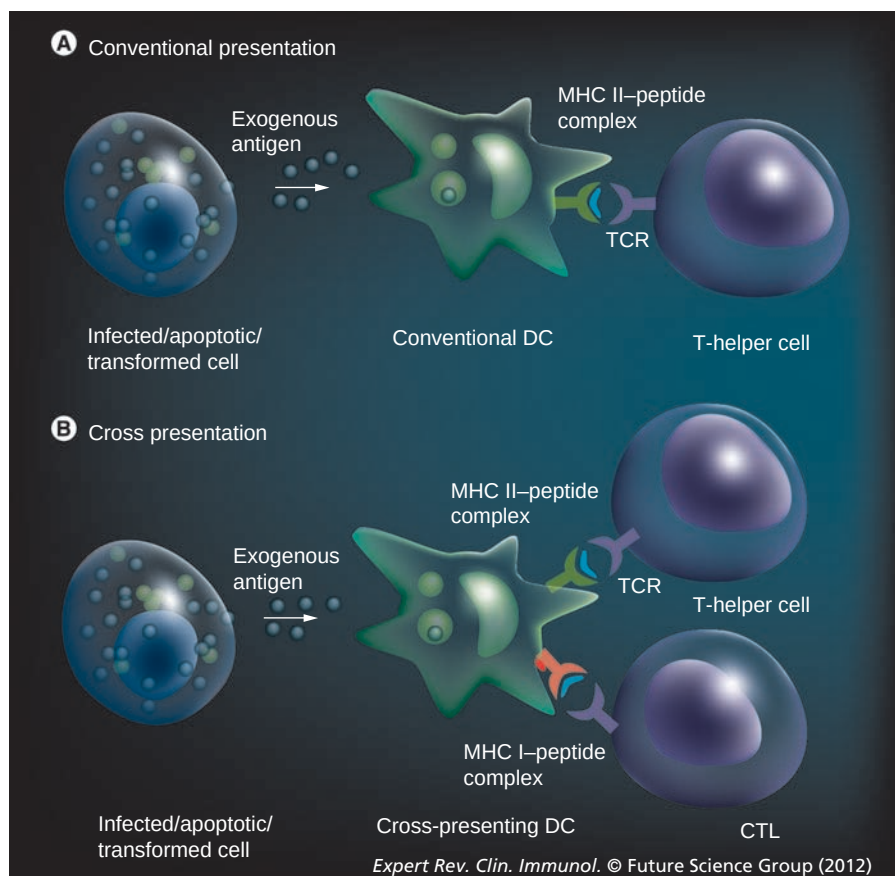


Figure 1. Pathways of exogenous antigen presentation. (A) Conventional presentation – DCs acquire exogenous antigen from apoptotic, infected or transformed cells and display peptides from the processed antigen in complex with MHC II molecules to T-helper cells. **(B)** Cross presentation – DCs acquire exogenous antigen from apoptotic, infected or transformed cells and display the processed antigen in complex with MHC I molecules to CTL.

CTL: Cytotoxic T lymphocyte; DC: Dendritic cell; TCR: T cell receptor.

proteasomes or more specialized immunoproteasomes, upregulated in response to inflammation [13]. These peptides are translocated into the ER via the transporter associated with antigen processing (TAP) where it enters the canonical MHC I presentation pathway. Here, peptides are loaded onto nascent MHC I molecules already present within the ER, aided by calreticulin, calnexin and tapasin, in the antigen-loading pathway [11]. The MHC I–peptide complex is then transported to the cell surface for presentation to CD8⁺ T cells [11,14]. There is strong evidence for both cytosolic and vacuolar pathways for antigen processing. Palmowski *et al.* first demonstrated that cross presentation of certain antigens does not occur in the absence of one of the immunoproteasome subunits, demonstrating a cytosolic contribution to the pathway [13]. However, it has also been shown that cross presentation of some antigens does not require transport via the cytosolic route [15]. The discovery that recruitment of ER-resident proteins to the phagocytic or vacuolar compartments is required for efficient cross presentation also supports the existence of the vacuolar pathway [16].

The segregation of cross presentation into two distinct compartments has been viewed as an oversimplification of the process and

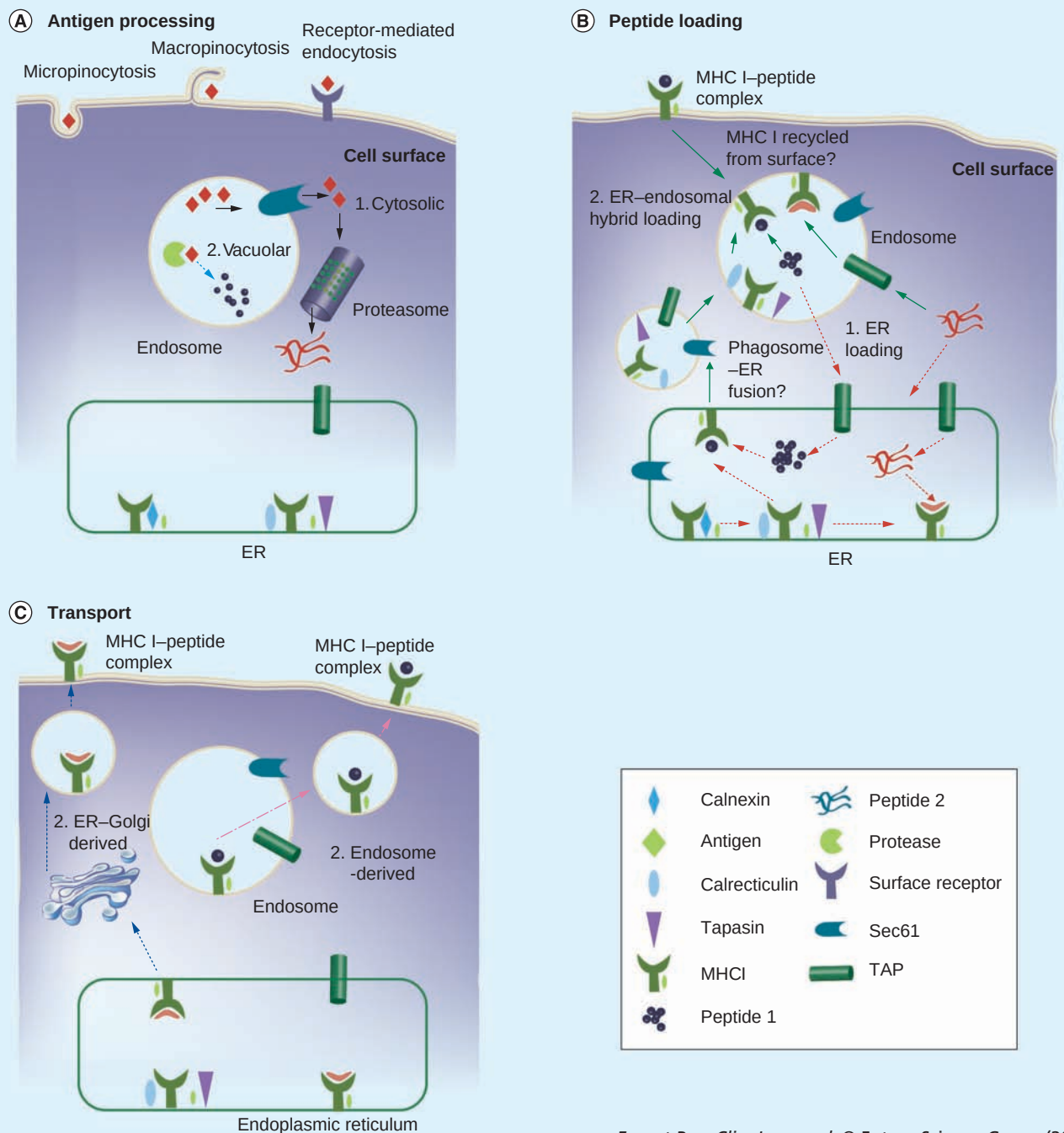
recently, a hybrid or modular view of cross presentation was proposed. In this view, each step or ‘module’ of the cross presentation pathway (antigen processing, loading and transport) can take place either in the vacuolar, cytosolic or even a hybrid of these two compartments. The final pathway used for cross presentation is a combination of each module and is dependent among other factors, on the antigen-binding receptor and the type and size of the antigen [11]. The modular view of cross presentation is depicted in **FIGURE 2**.

Antigen processing

In the cytosolic route, antigen is transported from endosome/phagosome into the cytosol where it is degraded by the immunoproteasome into peptides for loading onto MHC I molecules. This view is supported by early observations of the dependence of cross presentation on the proteasome [16] and later by the direct visualization of internalized antigen in the cytosol [17]. The cytosolic view is appealing as the antigen goes through a similar processing pathway as endogenous presentation. This allows DCs to prime CTLs against the same set of peptides displayed by the infected cell, without themselves being infected. The molecular mechanisms of this process are still unclear, although components of ER-associated degradation machinery, Sec61 complex and the AAA-ATPase p97 [18,19], have been implicated in this process. It is believed that phagosomes acquire Sec61 through fusion

with ER (described later) and, along with AAA-ATPase p97, this is responsible for the transport of antigen from phagosome to cytosol for processing [20,21]. Recently, the cytosolic factor heat shock protein 90 (Hsp90) has been shown to be essential for the process of cross presentation and appears to be involved in the guidance of internalized antigen across the phagosomal membrane into the cytosol [22,23].

In the endosomal route, antigen-containing phagosomes fuse with endosomes and antigen processing takes place in this compartment without transit through the cytoplasm. Compared with other APCs, DCs have been reported to express low levels of lysosomal proteases [24] and maintain a less acidic pH in the endosomes/phagosomes with the help of the NADPH oxidase enzyme NOX2, responsible for the production of reactive oxygen species. This supports the slow degradation of proteins allowing peptides to encounter MHC I molecules for cross presentation. DCs deficient in NOX2, which is the principle source of reactive oxygen species, show rapid phagosomal acidification and decreased efficiency of cross presentation [25]. A few studies have also shown that endosomal antigen processing can take place with the help of the lysosomal protease cathepsin S [26].



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Figure 2. A modular view of cross presentation. (A) Antigen processing – antigen acquired by macropinocytosis, micropinocytosis or receptor-mediated endocytosis can be degraded in the endosome by resident proteases (blue dashed arrows) or in the cytosol by proteasome (black solid arrows). (B) Peptide loading – peptides are either loaded in the ER (red dashed arrows) or in the endosome with the help of molecules derived through fusion with the ER (green solid arrows). (C) Transport – MHC I molecules loaded with peptide in the ER are transported to the cell surface in vesicles formed in the Golgi complex (purple dashed arrows) while those loaded in the endosome are transported directly to the cell surface (pink dashed arrows). ER: Endoplasmic reticulum.

Peptide loading onto MHC I

Initially, peptide loading was thought to occur within the ER with the help of endogenous peptide-loading machinery consisting of molecular chaperones such as calnexin, tapasin and calrecticulin.

ER-associated aminopeptidase-1 is involved in trimming peptides for loading onto MHC I molecules. The dependence of cross presentation on the presence of TAP [27] and decrease in its efficiency in ER-associated aminopeptidase-1-deficient DCs support this view

of antigen loading [28]. However, the presence of TAP and molecules from the ER peptide loading machinery in phagosomes suggests that peptides can be directly loaded onto MHC I molecules in the phagosomal lumen [29–31]. To explain the unusual localization of ER peptide loading machinery in phagosomes, many groups postulated the fusion of the ER and plasma membrane, or with the phagosome at the site of antigen acquisition [31,32]. Although it is recognized that there is an exchange of material between the ER and phagosomes, the fusion theory remains controversial. While certain groups have presented evidence supporting the involvement of the ER in the formation of phagosomes [29–33] and in cross presentation [34], Touret *et al.* have suggested the detection of ER proteins in phagosomes to be more likely the result of contamination of the phagosome preparations by ER [35]. No evidence for a molecular mechanism supporting the ER–phagosome fusion theory has been postulated, although Sec22b, a vesicle trafficking protein thought to play a role in ER–Golgi protein trafficking has been implicated in this process [36]. Until recently, this theory failed to explain how MHC I molecules in the ER could be prevented from binding endogenous peptides via the canonical class I pathway. Although it has been known for some years that invariant chain (Ii or CD74) associates with MHC I molecules in the ER, similar to MHC II [37,38], only recently was its role in cross presentation demonstrated [39]. The same study also showed that MHC I–CD74 complexes are transported to the phagosomal lumen where CD74 dissociates, allowing peptides generated from exogenous antigens to bind [39].

Cross presentation has been shown to occur in TAP-deficient DC, suggesting that peptide loading onto MHC I molecules can occur independently of ER-derived components [40]. This observation was supported by the discovery that insulin-regulated aminopeptidase (IRAP) localizes to the endosome and trims peptides prior to loading onto MHC I molecules [41]. It was also shown that DCs derived from IRAP-knockout mice were impaired in their ability to cross-present antigen to CTL, whereas endogenous presentation was not affected. IRAP deficiency compromised cross presentation both *in vitro* and *in vivo* but did not affect endogenous presentation [41].

Transport of MHC I–peptide complex to cell surface

The transport of peptide-loaded MHC I molecules depends upon the site of peptide loading. If the peptide is loaded onto the MHC molecule within the ER, transport follows the same route used in endogenous presentation, that is, via the Golgi complex. However, if loading occurs within vacuolar compartments, the MHC I molecules required for loading are either diverted from the ER to the endosome or recycled from the plasma membrane (FIGURE 2C). This was supported by studies showing that tyrosine amino acid residues in the cytoplasmic tail of MHC I, required for the internalization of surface MHC I molecules into endosomes, were essential for efficient cross presentation [42,43]. These MHC I molecules, once loaded with peptides, exit the endosomes to the cell surface, bypassing the Golgi complex. This was supported by the observation that MHC I–peptide complexes on the cell surface were not Endo H resistant, a property usually conferred on them within the Golgi [29]. The mechanism involved in the direct transport of MHC I–peptide complexes to the cell surface remains uncertain.

Even though many pathways of antigen processing, loading and transport have been described, many questions remain unanswered. There are various aspects of the molecular mechanism of these pathways that have yet to be elucidated and it remains unclear what determines the final route taken by an antigen during cross presentation *in vivo*. Many factors affecting this decision have been proposed, including the type and size of antigen and the method by which it is acquired. How DCs segregate exogenous antigen for presentation by MHC I and MHC II molecules still remains unresolved. It has been suggested that antigens acquired via the mannose receptor are specifically targeted for cross presentation, whereas those acquired via other receptors undergo presentation by MHC II. Distinct endosomal compartments were proposed to exist for each of these two pathways and the endosomes designated for cross presentation were prevented from fusion with lysosomes, preserving the peptides for cross presentation [44]. However, this observation has not been confirmed by other groups [45]. It is important to note that most cross-presentation experiments were described *in vitro* using the murine model. It still remains unclear what occurs *in vivo*, both in mice and humans.

Characteristics of cross presenting DC

DCs are heterogeneous and consist of discrete subsets, each of which perform different immune functions and express distinct cell surface markers [46]. DCs can be broadly divided into two main subsets: conventional DCs (cDCs) and plasmacytoid DCs (pDCs) [47]. cDCs can be divided into migratory, lymphoid tissue-resident and monocyte-derived DCs [47]. Lymphoid tissue-resident DCs can be further subdivided into three types based on their CD4 and CD8 expression: CD8 α ⁺, CD4⁺ and CD8 α ⁺CD4[−] (double negative) subsets [47]. CD8 α ⁺ DCs, also called CD8 DCs, were found to be the primary cross-presenting DC in mice [48–50]. These cells express markers such as CD103, TLR3, IRF-8 and XCR1 [51,52]. They also express receptors CLEC9A, CLEC7A, DC-SIGN (CD209), DEC205 (CD205) and mannose receptor 1 (CD206), which have been linked to distinct endocytosis mechanisms, possibly explaining their ability to cross-present antigen [4]. These cells have the capacity to cross-present exogenous antigen to CD8⁺ T cells and produce IL-12 on activation [53]. The development of CD8 DCs depends on the transcription factor BATF-3 [54] and it was shown that BATF-3 knockout mice were unable to cross-present antigen to CTLs and had an impaired ability to reject tumors and respond to viral infection [54].

Despite significant effort, the human counterpart to the murine cross-presenting DCs eluded identification until 2010, when CD141⁺ DCs were found to contain the human equivalent of mouse CD8 DC [10,55,56]. CD141⁺ DCs are a subset of conventional DCs present in lymphoid organs and blood. They were found to share expression of many genes with their murine counterpart as determined by transcriptome analysis [57]. Like the murine CD8 DC, CD141⁺ DCs also express CLEC9A, BATF-3, IRF-8, TLR3 and secrete IL-12 when activated. However, they do not express TLR7 and TLR9 [55,56,58]. Bachem *et al.* demonstrated that CD141⁺ cells were the only DC subset in humans to express the chemokine receptor XCR1, also expressed by mouse CD8 DC, which promotes the chemotaxis of DCs towards XCL1-producing CD8⁺ T cells [59]. CD141⁺XCR1⁺ DC were shown to be more efficient in the internalization of exogenous

dead cell material and its cross presentation to CD8⁺ T cells than other human DC subsets from the same donor, namely, CD16⁺ and CD1⁺ cDC [10,55,56]. However, these studies do not rule out the possibility that other DC subsets in humans also possess the ability to cross-present antigen to CTLs, although with less efficiency than the CD141⁺ subset [54, 60]. This is in contrast to mice, where CD8 DCs are the main DC subset specializing in antigen cross presentation [54, 60]. It must be noted that most DCs used in murine cross-presentation experiments were derived from lymphoid tissue whereas human DCs in equivalent experiments were mostly derived from blood or differentiated in culture. It is speculated that blood-derived CD141⁺ DCs may be precursors to the lymphoid CD141⁺ DCs and their cross-presentation efficiency may increase during maturation in lymphoid organs [55]. The similarities and differences of mouse and human cross-presenting DC are outlined in TABLE 1.

Role of cross presentation in immunity & tolerance

To elicit an immune response, CD8⁺ T cells must be activated by DCs, licensing them to recognize antigen presented in the context of MHC I on the surface of the infected cell. This process requires the same antigen to be presented by both the APC and the targeted cell. However, not all pathogens infect APC and some can evade their endogenous presentation pathway. Cross presentation allows DCs to prime CD8⁺ T cells against exogenously acquired antigens, leading to an effective adaptive immune response. This process, termed cross priming, has been shown to be crucial in tumor immune surveillance and in immunity to tissue-specific viruses and viruses that can bypass the antigen presentation pathway [4]. *In vivo*, DCs can take up various types of antigen to cross-prime CD8⁺ T cells including live, infected, transformed, apoptotic or dead cells. Self antigens can also be cross presented, leading to deletion of autoreactive CTLs, a process termed cross tolerance [8, 61]. It is still not clearly understood what regulates the decision of DCs to induce immunity versus tolerance. It has been speculated that DCs induce tolerance under normal steady state conditions and only switch to the immunogenic state on encountering inflammatory stimuli, such as microbial products, IFN α or Hsp, which induce their maturation [62]. To break cross tolerance, DCs are also required to present antigen via MHC II to CD4⁺ T cells, which in turn activate these DC via a CD40–CD40L interaction, allowing them to cross present the antigen to CD8⁺ T cells, a process called ‘DC licensing’ [4]. This limits the occurrence of autoimmunity as it requires the interaction, in a spatially and temporally restricted manner, of three rare cell types. There are cross-presenting DCs as well as antigen-specific CD4⁺ and CD8⁺ T cells, an event that is unlikely to occur serendipitously [63, 64]. It must be noted that these interactions do not have to take place simultaneously as the DC, once primed through interaction with CD4⁺ T cells, is able to cross-present antigen to CTLs upon subsequent encounter [4].

Considering the role of cross presentation in tumor immunity, it is legitimate to speculate why tumors still arise despite their expression of neo-antigens, which lead to continuous cross presentation to CTLs. Several groups that studied cross priming using a tumor model, have shown that tumors usually elicit a tolerogenic or weak immunogenic response due to the lack of inflammatory stimuli required for strong cross priming [4]. Similarly, despite the presence

Table 1. Comparison of the characteristics of the cross-presenting DCs in mice and humans.

Characteristic	Mouse	Human
<i>Surface markers</i>		
CD8 α	+	–
BDCA-3 (CD141)	–	+
Clec9A	+	+
XCR1	+	+
DEC205 (CD205)	+	–
DC-SIGN (CD209)	+	ND
CD11b	+	–
CD103	+	ND
Nec12	+	+
<i>Transcription factors</i>		
BATF3	+	+
IRF-8	+	+
IRF-4	–	–
<i>Pathogen sensors</i>		
TLR3	+	+
TLR9	+	–
TLR7	+	–
IL-12 production	+	+
ND: not determined.		

of effective cross priming against viral antigens, infection can still occur. It has been shown that murine cytomegalovirus-infected cells can escape the cytotoxic activity of CTLs as they present a different viral epitope from the one to which they have been primed [65].

Cross presentation in immunotherapy

The ability of DCs to cross-present antigen to CTLs makes them an attractive target for use in immunotherapy. Cross presentation has been exploited in some forms of chemotherapy that aim to increase presentation of tumor-associated antigens (TAA) to CTLs by inducing apoptosis in tumors. Many agents such as Hsp, uric acid crystals or monoclonal antibodies have been used to improve cross-priming efficiency by costimulating DCs [4]. DCs pulsed with peptide or genetically modified to express TAAs have been exploited in many cellular vaccines to prime an immune response against TAAs and against viruses [66]. Cross presentation of antigen by DCs may also lead to peripheral tolerance, which provides great promise as a strategy to treat autoimmune diseases. However, this potential has not been fully exploited as the mechanism by which DCs induce tolerance rather than immunogenicity remains ambiguous.

Until now, clinical trials involving the use of monocyte-derived DCs for inducing immune response against tumor or viral antigens have proven disappointing. This may be due to the variability between monocyte-derived DCs obtained from different donors and their relative inefficiency in cross presentation. In humans, the recently identified cross-presenting DCs, namely the

CD141⁺ XCR1⁺ subset, make up less than 0.1% of peripheral blood monocyctic cells [56]. Alternative sources of cross-presenting DCs have been reported such as differentiated hematopoietic precursor cells and the use of humanized mouse spleen [67,68]. However, the yield from these methods is still relatively low, hindering the full exploitation of cross-presenting DCs in clinical trials.

Several groups have shown that DCs can be differentiated from murine and human embryonic stem cells [69–71]. However, the ability of these DCs to cross-present antigen has not been demonstrated and the use of animal products in their establishment makes them inappropriate for downstream clinical applications. In 2009, DCs were successfully derived from human embryonic stem cells using an animal product-free approach [72]. These DCs were able to phagocytose, process and present viral antigen to CD8⁺ T cells. Recently, CD141⁺ XCR1⁺ DCs were successfully derived from human induced pluripotent stem (iPS) cells using a similar animal-free culture system [73]. These DCs were able to cross-present TAA to CD8⁺ T cells and prime naive T cells *in vitro*. Importantly, iPS cells have indefinite capacity for self-renewal and therefore provide a potentially unlimited source of autologous cross-presenting DCs for therapy that might bypass the issue of patient-to-patient variability. Furthermore, iPS cells are relatively amenable to genetic modification and their differentiation into DCs provides a relatively straightforward strategy for their genetic manipulation for the purpose of immunotherapy [74]. These developments overcome many of the current limitations surrounding the use of DCs for immunotherapy and may usher in a new era in development of DC-based vaccines.

Expert Commentary & five-year view

Since its first description in 1976, significant progress has been made in understanding the mechanisms involved in cross presentation, the identification of the DC subsets involved in the process and their characterization. The identification of human cross-presenting DCs has rejuvenated interest in their use as vaccines for the induction of immunity against viruses and tumors. The ability of cross-presenting

DCs to induce tolerance also offers the prospect of using these cells to limit neutralizing immune responses to defined therapeutic proteins such as lysosomal enzymes administered during enzyme replacement therapy for the treatment of the lysosomal storage diseases. However, the results of the clinical trials conducted in this field have so far proven disappointing. This may be attributed to the rarity of the cell population and the difficulty in their genetic manipulation. The discovery that cross-presenting DCs can be differentiated from iPS cells using clinically approved methods might help overcome some of the practical issues involved in their use for immunotherapy and may facilitate the development of DC-based vaccines [73]. Nevertheless, many questions still remain unresolved including the differences between cross presentation in mice and humans and the molecular mechanism involved. It is not yet fully understood how DCs determine by which pathway a particular exogenous antigen is presented. Why only certain subsets of DC cross present also remains unknown. Furthermore, a deeper understanding of cross priming and tolerance, the signals required for their induction, and the balance between the two is required. In the next few years, a focus on understanding the mechanism of cross presentation, especially in humans, and research into increasing the efficiency of their derivation from novel sources such as iPS cells will enable us to fully exploit the potential of cross-presenting DCs for immunotherapeutic purposes.

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Key issues

- Cross presentation is a process by which dendritic cells (DCs) present exogenous antigen via MHC I to cytotoxic T lymphocytes.
- The ability of DCs to cross-present exogenous antigen to cytotoxic T lymphocytes makes them attractive targets for use in immunotherapy.
- Classically, cross presentation is believed to occur by two pathways: Vacuolar – in which antigen is processed by proteases in the endosomes and loaded onto the MHC I molecule recycled from the cell surface; and Cytosolic – in which antigen is transported from the endosomes to the cytosol where it is degraded by proteasomes into peptides that, upon translocation to the ER, are loaded onto nascent MHC I molecules by the canonical endogenous pathway.
- Cross presentation takes place in three distinct steps: antigen processing, antigen loading and transport of the MHC I–peptide complex to the cell surface.
- According to the modular view, each step or 'module' of the cross-presentation pathway takes place in either the cytosolic, vacuolar or a hybrid of the two compartments. The final pathway is a combination of each module.
- In mice, CD8 α ⁺ DCs, a subset of lymphoid tissue resident DCs, were found to be the primary cross-presenting DC. These cells express markers such as CD103, TLR3, XCR1 and CD11b.
- Recently, CD141⁺XCR1⁺ DCs were identified as the cross-presenting DC subset in humans. This has rejuvenated interest in the exploitation of cross presentation in immunotherapy.
- Many clinical trials exploiting DCs for immunotherapy against tumors and viruses have been disappointing. This may be due to the low number of cross-presenting DCs that can be extracted from peripheral blood and patient-to-patient variability.
- The discovery that cross-presenting DCs can be differentiated from induced pluripotent stem cells using animal-free culture systems may usher in the next era in the development of DC-based immunotherapy.

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