Cross-presentation: underlying mechanisms and role in immune surveillance

Summary: It was originally thought that a cell’s major histocompatibility complex (MHC) class I molecules presented peptides derived exclusively from proteins synthesized by the cell itself. However, in some circumstances, antigens from the extracellular environment can be presented on MHC class I molecules and stimulate CD8+ T-cell immunity, a process termed cross-presentation. Cross-presentation was originally discovered as an obscure phenomenon in transplantation immunity. However, it is now clear that it is a major mechanism by which the immune system monitors tissues and phagocytes for the presence of foreign antigen. Cross-presentation is the only pathway by which the immune system can detect and respond to viral infections or mutations that exclusively occur in parenchymal cells rather than in bone marrow-derived antigen-presenting cells (APCs). Professional APCs, such as dendritic cells, are the principal cells endowed with the capacity to cross-present antigens. In this process, the APCs acquire proteins from other tissue cells through endocytic mechanisms, especially phagocytosis or macropinocytosis. The internalized antigen can then be processed through at least two different mechanisms. In one pathway, the antigen is transferred from the phagosome into the cytosol, where it is hydrolyzed by proteasomes into oligopeptides that are then transported by the transporter associated with antigen processing to MHC class I molecules in the endoplasmic reticulum or phagosomes. In a second pathway, the antigen is cleaved into peptides by endosomal proteases, particularly cathepsin S, and bound by class I molecules probably in the endocytic compartment itself. Depending on the nature of the antigen, one or both of these pathways can contribute to cross-presentation in vivo. The outcome of cross-presentation can be either tolerance or immunity. Which of these outcomes occurs is thought to depend on whether antigens are acquired by themselves alone, leading to tolerance, or with immunostimulatory signals, leading to immunity. One source of such signals is from dying cells that release immunostimulatory ‘danger’ signals that promote the generation of immunity to their cellular antigens. In addition to the critical role of cross-presentation in normal immune physiology, this pathway has considerable potential for being exploited for developing subunit vaccines that elicit both CD4+ and CD8+ T-cell immunity.

Introduction

After a virus enters a host and infects cells, the major adaptive immune response that clears the infection is mediated by CD8+ cytotoxic T lymphocytes (CTLs). These cells also
provide the major defense against cancers. The CD8+ lymphocyte recognizes infected or transformed cells that display on their surface major histocompatibility complex (MHC) class I molecules presenting antigenic peptides derived from viral proteins or mutated gene sequences. The CTL then kills the offending cells and thereby eliminates the source of viral replication or the abnormal/cancerous cells.

In the naïve state, CD8+ T cells of any particular specificity are typically present at very low frequencies. These cells are also initially in a quiescent state. In the initiation phase of an immune response, foreign antigens stimulate the resting CD8+ T cells to clonally expand and express effector functions, including cytolytic activity and cytokines. In the effector phase of CD8+ T-cell immune responses, the CTL effector cells seek out the cells synthesizing foreign antigen and kill them. The pathways through which antigen is presented and stimulates CD8+ T cells in the initiation and effector phases of responses can be different. In the initiation phase of responses, naïve CD8+ T cells must be stimulated by antigen presented on the MHC class I molecules of professional antigen-presenting cells (APCs), such as dendritic cells (DCs). If the APC is not itself synthesizing the antigen, then it must acquire the antigen exogenously from the tissues and display it through a process termed cross-presentation. In the effector phase of the response, however, CTLs are stimulated by antigen presented on the MHC class I molecules of the infected or transformed host cells that are synthesizing the antigen. The mechanisms through which cells present their own internally synthesized antigens on MHC class I molecules is referred to as the classical class I pathway. In this article, we review the MHC class I antigen-presenting pathways with a primary focus on the work from our laboratory on cross-presentation.

The classical MHC class I pathway

All cells continually turn over their proteins. The majority of cellular proteins are degraded in the cytoplasm or nucleus by the ubiquitin-proteasome pathway (1, 2). In this process, proteins are conjugated with a chain of ubiquitin molecules that marks them for rapid degradation (3–5). Polyubiquitinated and some unmodified proteins are degraded by proteasomes into oligopeptides ranging in size from about 2–3 residues to >20 amino acids (6, 7). The majority of these peptides are further hydrolyzed by cytosolic peptidases ultimately into amino acids that are re-utilized by the cell for protein synthesis or energy (2). However, a fraction of the peptides >7 residues escapes destruction and is transported into the endoplasmic reticulum (ER) through the transporter associated with antigen processing (TAP). In the ER, long peptides are further trimmed by ER aminopeptidase-1 (ERAP1) to peptides of eight or nine residues (8–11). MHC class I molecules then bind 8-mer or 9-mer peptides with the appropriate sequences and transport them to the cell surface for display.

As a consequence of these mechanisms, MHC class I molecules display peptides derived from the vast majority of proteins that are synthesized by cells. External proteins that were not produced by the cell itself are not processed and presented on MHC class I molecules of most cells, because they cannot pass through the plasma membrane or endosomes to gain access to the cytosolic compartment. However, if such proteins are experimentally injected into the cytosol, then they are degraded by proteasomes and the resultant peptides are presented on MHC class I molecules (1, 12). Thus, in most cells, MHC class I molecules faithfully display peptides only from the expressed genes in cells. This restriction allows the CD8+ T cells of the immune system to identify cells that are synthesizing abnormal genes (viral or mutant) and eliminate them.

The phenomenon of cross-presentation

It has long been known that CTLs were primed when animals were injected with allogenic cells that had genetic differences in their MHC class I molecules or minor (polymorphic cellular) antigens. However, in 1976, Bevan (13, 14) made the remarkable finding that in animals immunized with fully allogenic cells (differing at minor antigens and all MHC genes), some of the CTLs generated were specific for minor antigens from the graft presented on MHC class I molecules of the host. This finding indicated that during the priming process, minor antigens were somehow transferred from the transplanted cells in a way that they were presented by the host. The phenomenon of transferred antigen being presented on host cells was named cross-presentation, and the resulting stimulation of CTL responses in vivo was termed cross-priming.

The phenomenon of cross-presentation was surprising, because cells were only thought to present on MHC class I molecule antigens that they themselves had synthesized. In contrast, protein antigens in the extracellular fluids were not presented on class I molecules of all of the cell types that had been examined at that time. As discussed above, it was subsequently determined that this was because exogenous antigens could not access the appropriate subcellular compartment (the cytosol) wherein the MHC class I pathway was operative. The only situation in which cells were known to acquire external
antigens for class I presentation was when they were pulsed with exogenous peptides (15). However, this mechanism seemed an unlikely one to account for cross-priming, as peptides are not generally immunogenic, probably because in vivo there are few peptide-receptive MHC class I molecules on the surface of cells (16, 17). Therefore, the form of antigen and the mechanism by which it was cross-presented was a mystery.

Nature of the cross-presented antigen

Although the form of antigen that was cross-presented from cells was initially unknown, some subsequent studies raised the possibility that it could be whole cellular proteins. It had long been known that immunization of animals with soluble protein antigens failed to stimulate CTL immunity, a finding that we confirmed with the protein ovalbumin (OVA) (18–20). However, we found that injection of OVA in particulate form (e.g., adsorbed onto a microsphere) primed robust CTL responses (18–20). Similar results were obtained with other antigens, e.g., human immunodeficiency virus gp120 (21). Therefore, whole proteins can be cross-presented in vivo. These findings raised the possibility that proteins might be the form of antigen that was cross-presented from cells in vivo, perhaps upon becoming particulate due to aggregation or association with cellular debris (Fig. 1).

Other studies raised the possibility that the form of cross-presented antigen from cells was small peptides (Fig. 1). Srivastava (22) found that heat-shock proteins (HSPs) isolated from tumors were immunogenic and stimulated tumor-specific CD8+ T-cell immunity. Upon further characterization, it was found that the immunogenic material was actually small antigenic peptides bound to the HSPs (23, 24). Several different HSPs, including HSP70, HSP90, and grp96, were found to have such bound peptides and to elicit CD8+ T-cell immunity (22–28). Therefore, like particulate protein preparations, HSP-peptide complexes could also be cross-presented in vivo (Fig. 1).

While these studies showed the potential for whole proteins or HSP-peptide complexes to be cross-presented, the actual form(s) of antigen that was transferred from one cell and cross-presented by another cell of the host in vivo was unknown. Moreover, other sources of antigen, such as antigen expressed from exogenous RNA or DNA-coding sequences, could have the potential to be cross-presented (29–31) (Fig. 1). To address what form(s) of antigen was cross-presented from cells, we investigated whether the cross-priming activity in cells localized to the site of the mature protein antigen, with the antigen's degraded peptide products, e.g., bound to HSPs, or with its coding sequences. When OVA was targeted to the cytosol of cells by deletion of its signal sequence, the cross-priming activity was found exclusively in the cytosol (32) (Fig. 2A).

However, when this same antigen was targeted to membranes by fusion with a transmembrane domain, cross-priming activity was now found in membrane fractions (Fig. 2B). Therefore, the cross-priming activity in cells was determined by the location of the mature protein and not its degradation products. To further analyze the nature of the antigen, we characterized it immunochemically. The cross-priming antigen could be removed from cell lysates with monoclonal antibodies against the OVA protein (Fig. 2C,D). The antibodies that depleted were specific for native protein and bound to regions that were distinct from the class I-presented sequence (SIINFEKL) (32, 33). Together, these findings indicated that cellular protein was the predominant form of cross-presented antigen in vivo. Therefore, peptides, HSP-peptide complexes, RNA, and DNA appear to play a minor role, if any, in the cross-presentation of antigens from cells in vivo. Most (34–36), but not all (37) other studies came to the same conclusion.

Cellular basis for cross-presentation

In the 1980s, there was no known mechanism by which a cellular protein could be cross-presented. As discussed above, all cells that had been analyzed seemed unable to carry out this
function. Moreover, it seemed inherently dangerous and therefore unlikely that cells would acquire and present environmental antigens on MHC class I, because this presentation would potentially lead to the death of otherwise healthy cells by CTLs. Nevertheless, we hypothesized that cross-presentation would occur by such a mechanism and that this pathway would operate in a unique subset of APCs. In the 1990s, we tested this hypothesis and found that there were indeed cells within lymphoid organs, which when explanted ex vivo could generate class I-presented peptides from antigens in the extracellular medium (20, 38–40). Cells with this ability copurified with DCs and macrophages (39, 40). That DCs and macrophages were actually capable of cross-presenting exogenous protein was confirmed by testing cloned macrophage and DC lines (20, 41, 42).

In contrast, B cells and T lymphocytes were unable to cross-present antigen in these assays (39). These results have been confirmed in human as well as in mouse cells by others (43–45).

The ability of primary macrophages and DCs to cross-present antigen had been missed, because they were not typically used, largely for technical reasons, in the conventional killing assays used to measure MHC class I antigen presentation. Instead, fibroblasts, T lymphoblasts, and lymphoma cells were the ‘target cells’ of choice in chromium-release assays. These cells, as with most other cell types, do not cross-present antigen. As is often the case in science, the advance in our knowledge required the development of new technology, in this case CD8⁺ T-cell hybridomas, that allowed non-standard target cells to be assayed (46). Subsequent studies using these systems have revealed that some other cells can cross-present antigen. Neutrophils (47) and B cells (when binding their specific antigen) (48, 49) have both been reported to cross-present antigen, at least in vitro.

The initial experiments showing that DCs and macrophages could cross-present antigen were all performed in vitro. It was subsequently shown that these same cells could similarly cross-present antigen in vivo. For example, when DCs were isolated from mice injected with protein antigens or viruses, they cross-presented the immunogen on their MHC class I molecules (40). In many but not all of these studies, DCs were the only cells found to be cross-presenting antigens (50–52). Consistent with this finding, Jung et al. (53) generated transgenic mice expressing diphtheria toxin receptor under the control of CD11c promoter, and they found that when CD11c⁺ DCs were ablated by treatment with diphtheria toxin, mice failed to generate a CD8⁺ T-cell response to cell-associated antigen and intracellular pathogens. Therefore, DCs cross-present antigen in vivo and play an essential role in cross-priming CD8⁺ T-cell responses to certain antigens. However, in some other studies, macrophages were also found to cross-present antigens injected into animals (40). Similarly, in mice infected with vaccinia virus, both uninfected CD11c⁺ (DCs) and CD11c⁻ (presumably macrophages) cells appeared to...
cross-present antigen to T cells (54). Therefore, under at least some conditions, macrophages can also cross-present antigen in vivo. The role of these APCs in priming T-cell responses is less clear, but we have recently found that macrophages can stimulate naïve CD8\(^+\) T cells to proliferate, express effector functions, and differentiate into memory cells (55). The contribution of neutrophils and B cells to cross-priming in vivo, if any, is unclear. We found that cross-priming of cell-associated OVA was not impaired in B-cell-deficient mice (\(\mu\)MT\(-/-\) mice), suggesting that the role of B cells in cross-presentation in vivo is minimal or redundant (L Shen, unpublished data).

DCs are heterogeneous and can be broadly divided into plasmacytoid DCs and conventional DCs. The latter subset can be subdivided into ‘lymphoid’ DCs and ‘myeloid’ DCs (50, 52, 56). Which subsets cross-prime naïve CD8\(^+\) T cells and their relative contributions are not fully understood. In experiments in which mice were immunized with cell-associated antigen (57–59) or infected with virus (60–62) and DC subsets isolated, the CD8\(^{\text{dx}}\) DC was identified as the primary APC that stimulated naïve CD8\(^+\) T-cell responses. In viral infection models, however, whether CD8\(^{\text{dx}}\) DCs were solely involved in cross-priming is less clear. The form of antigen also influences what type of DC participates in cross-priming. For example, cell-associated OVA is cross-presented by CD8\(^{\text{dx}}\) DCs, while OVA/IC complexes are cross-presented by both CD8\(^{\text{dx}}\) and CD8\(^{\text{dx}}\) DCs (59). It is generally believed that plasmacytoid DCs do not cross-prime exogenous antigens. However, type I interferon has been suggested to promote cross-priming (63). Since plasmacytoid DCs produce type I interferon upon viral infections, they may enhance the cross-priming process to viral antigen during viral infections (52, 64, 65).

**Mechanism of antigen uptake for cross-presentation**

With the identification of macrophages and DCs as the APCs that could cross-present antigens, it became possible to study how they acquired antigen for this process. These cells cross-presented proteins in soluble form only when these APCs were incubated with very high concentrations of exogenous soluble protein (20, 38, 66). This finding suggested that antigen entering APCs by fluid-phase endocytosis accessed the cross-presentation pathway inefficiently, which may explain why immunization with soluble protein antigens generally fails to stimulate CTL immunity.

We found that when a soluble protein was made particulate, e.g. by adsorption to inert particles of 1–5 \(\mu\)m, cross-presentation occurred at 1000–10 000-fold lower concentrations of antigen (20, 42, 67). Similarly, antigens from another form of particulate antigen, bacteria, were cross-presented efficiently by macrophages (68). Consistent with these observations, when soluble protein is bound to beads and injected into animals, it is cross-presented and stimulates strong CTL responses, as described above (18, 20, 67). This phenomenon may explain, at least in part, why cellular antigens are cross-presented in vivo, because it is likely that many cell-associated antigens are essentially particulate in nature (69, 70).

Particles that are >1 \(\mu\)m in size are internalized by the process of phagocytosis. Macrophages and immature DCs are both highly phagocytic and avidly ingest particulate antigen. When phagocytosis was blocked in these cells with agents like cytochalasins, the cross-presentation of particulate antigen was inhibited (41). Therefore, phagocytosis is the mechanism by which particulate antigens were acquired by APCs for cross-presentation. Interestingly, before the discovery of the cells and their mechanisms that cross-presented antigen, Bevan (71) had speculated that phagocytosis might be involved in the process. In addition to phagocytosis, DCs internalize small particles (<1 \(\mu\)m) and fluid by macropinocytosis, and antigens internalized by this process are also cross-presented (66).

Why is particulate antigen cross-presented much more efficiently than soluble antigen? One reason may simply be that the amount of antigen internalized is much greater when a particle is ingested by phagocytosis than when soluble protein is internalized by fluid-phase pinocytosis. However, this factor is not the only one involved. When the amount of antigen internalized is kept constant, soluble protein that is acquired through phagocytosis is presented more efficiently than when it is acquired through endocytosis (72). Therefore, it seems that the pathways by which antigens are cross-presented are present in or more easily accessed from phagosomes and macropinosomes than from other endocytic compartments, a possibility that is discussed further below.

Once antigen is in phagosomes, how are presented peptides generated and how do they get to MHC class I molecules? There are at least two distinct pathways by which these processes occur: one requires escape of the antigen from the phagosome into the cytosol and the other occurs in the phagosome itself.

**The phagosome-to-cytosol pathway of cross-presentation**

The mechanism by which some antigens are processed and cross-presented on MHC class I molecules was first worked out for the protein antigen OVA bound to beads of iron oxide or polystyrene. Proteasome inhibitors (1, 73) blocked the
cross-presentation of these kinds of particulate antigen in vitro (41, 74). In contrast, proteasome inhibitors did not block the ability of the APCs to present peptides that were produced directly in the cytosol from minigenes (74). This latter control indicated that proteasome inhibitors were blocking the generation of peptides from the particulate antigen rather than inhibiting other steps in the MHC class I pathway.

As described above, proteasomes are also the key proteases involved in producing presented peptides from endogenous antigens. In this situation, the peptides produced by the proteasome must be transported into the ER by TAP in order to bind and be presented by MHC class I molecules (75). We therefore tested whether TAP was also required for the cross-presentation of particulate antigen. TAP-deficient macrophages and DCs were unable to cross-present antigen bound to iron oxide or polystyrene beads (74).

The finding that proteasomes and TAP were required for cross-presentation had important implications for how cross-presented antigen was trafficking in cells. This is because proteasomes are not present in endocytic compartments but are exclusively in the cytosol and nucleus of cells. Similarly, TAP functions to transport peptides in the cytosol across cellular membranes. The finding that proteasomes and TAP were required for the cross-presentation of particulate antigens argued that the cross-presented peptides were generated in the cytosol and therefore that the exogenous antigen must be transferred from the phagosome into the cytosol. That antigen was transferred from phagosomes to the cytosol was shown by other techniques. We showed that protein synthesis was inhibited in macrophages that phagocytized particles containing the toxin gelonin (74). Gelonin inhibits protein synthesis by enzymatically modifying ribosomes and therefore could only have this effect if it were transferred from the phagosome to the cytosol. Similarly, when other groups incubated macrophages or DCs with horseradish peroxidase (HRP), dextran, or OVA, they were able to find the internalized molecules transferred to the cytosol (66, 76, 77) (Fig. 3).

The finding that molecules were transferred from phagosomes into the cytosol was surprising because no mechanism by which this could occur was known. Recently, several ER-resident proteins, including most of the antigen-presenting machinery (TAP, Tapasin, etc.), have been found to be associated with phagosomes (78–80). It has been suggested that this is a consequence of membrane from the ER fusing with the phagosome during phagocytosis (81, 82) (Fig. 3). One of the ER proteins found in phagosomes is Sec61, which is involved in the import and export of proteins from the ER (83). Therefore, Sec61 has been suggested as a possible mechanism by which proteins are transported out of the phagosome (84–86). However, there is as yet no direct evidence that Sec61 plays this role, and the mechanism by which antigens are released from phagosomes remains unknown.

The finding that phagosomes contained most of the same antigen-presenting machinery as the ER raised the possibility that the peptides generated in the cytosol from exported proteins might be re-imported by TAP into the phagosome and load onto class I molecules within the vacuole. In other words, this might be a phagosome-to-cytosol-to-phagosome pathway (Fig. 3). Consistent with this model, TAP associated with phagosomes was shown to be active, and peptide–MHC

![Fig. 3. Phagosome-to-cytosol pathway of cross-presentation.](https://example.com/fig3.png)

In the phagosome-to-cytosol pathway, antigen is internalized into phagosomes or macropinosomes and then transferred into the cytosol. Recently, it was found that a subset of phagosomes acquires transporter associated with antigen processing (TAP), major histocompatibility complex (MHC) class I, Tapasin, and SEC61 from the ER, and it is not presently clear to what extent these vesicles versus standard phagosomes participate in this pathway. The mechanism by which proteins are transferred from phagosomes into the cytosol is not understood, although it has been hypothesized that this export may occur through SEC61. Once in the cytosol, the antigen is hydrolyzed by proteasomes into oligopeptides that are then transported by TAP and loaded onto MHC class I molecules in the endoplasmic reticulum (ER) or the ‘ER – phagosome’ vesicles.
class I complexes could form in phagosomes (78–80). Nevertheless, it is also clear that once proteins are exported into the cytosol, they diffuse widely (66, 76, 87). Moreover, peptides produced in the cytosol will be imported into the ER by TAP (Fig. 3). Therefore, it is likely that cross-presented peptides will find their way into both the ER and the phagosomes (85). What remains unclear is the relative contribution of these two subcellular locations to overall cross-presentation.

Phagosomes also fuse with endolysosomes, a process termed phagosome maturation (88, 89), and proteases within the vacuole will degrade internalized proteins. Does this process contribute to presentation through the phagosome-to-cytosol pathway? As enzymes such as gelonin (74) and HRP (66, 76) remain active upon transfer from phagosome into the cytosol, extensive proteolysis cannot be required for proteins to escape from the vacuole. Moreover, inhibitors of endosomal proteolysis do not block cross-presentation and sometimes actually enhance it (41, 74, 90). Therefore, it appears that proteolysis in phagosomes is not necessary for this pathway of cross-presentation, although it is essential for the vacuolar pathway, described below.

The vacuolar pathway of cross-presentation

Certain antigens, e.g. proteins associated with E. coli (68, 91–93) or poly(lactic-co-glycolic acid) (PLGA) beads (94) or viral proteins/virus-like particles (VLP) (95–97), or even soluble antigen alone (98), were found to be cross-presented at least in part by a distinct pathway. This second mechanism did not require TAP and was insensitive to proteasome inhibitors and therefore was clearly different from the phagosome-to-cytosol pathway (99) (Fig. 4).

For some antigens, both pathways operated and contributed to the generation of a portion of the cross-presented peptides (92, 94). The TAP and proteasome independence of this second pathway indicated that the cross-presented peptides were not generated in the cytosol, but instead in a distinct subcellular compartment. It turned out that these peptides were generated within endocytic vacuoles (see below) and consequently, we will refer to this pathway as the vacuolar pathway of cross-presentation. This pathway is less well studied, and its underlying mechanisms are not completely understood. Recently, however, there have been new insights into the mechanisms that generate the peptides for this form of cross-presentation.

The mechanism by which presented peptides were generated in the vacuolar pathway was investigated for the antigen OVA. We found that the protease inhibitor leupeptin blocked the presentation of OVA by the vacuolar pathway, but not the phagosome-to-cytosol pathway (94) (Fig. 5A).

Therefore, the proteases involved in the two pathways were clearly distinct. Among the proteases resident in the endocytic compartment, several of the cathepsins are sensitive to leupeptin (100, 101). Remarkably, macrophages or DCs that were deficient in cathepsin S were unable to present OVA by the vacuolar pathway (94) (Fig. 5B). In contrast, the loss of cathepsin S did not affect the presentation of OVA by the phagosome-to-cytosol pathway (94). On the other hand, an absence of cathepsin B, L, or D had no effect on cross-
presentation by either the vacuolar or phagosome-to-cytosol pathway (94). Cathepsin S therefore plays an essential role in the vacuolar pathway.

Cysteine proteases, including cathepsin S, have been shown to be able to generate peptides for class II presentation (102–104). To test whether cathepsin S was needed to generate the cross-presented MHC class I-binding peptide in phagosomes, we isolated vacuoles from wildtype and cathepsin S-deficient DCs and incubated them with OVA. The antigenic peptide SIINFEKL was generated by wildtype phagosomes but very poorly by cathepsin S-deficient ones (Fig. 6A), showing that cathepsin S was required in the generation of the presented peptide. Moreover, the demonstration that an endosomal protease was needed and that isolated phagosomes could generate the antigen peptide were formal proof that this pathway of presentation operates in endosomal compartments.

While cathepsin S was necessary, was it also sufficient for generating the presented peptide? To examine this issue, we incubated recombinant cathepsin S with OVA in vitro and found that SIINFEKL was generated (Fig. 6B). Therefore, at least in vitro cathepsin S can by itself generate the presented peptide. This finding was surprising, because antigenic peptides must be of a precise size, typically eight or nine residues, to bind stably to a class I molecule. It was not predicted that cathepsin S would be specialized for generating 8-mers and 9-mers, and it may be that SIINFEKL is fortuitously flanked by cathepsin S cleavage sites. However, the precise specificity of cathepsin S is not fully elucidated.

Is OVA a unique case, or does cathepsin S play an important role in the cross-presentation of other antigens through the vacuolar pathway? We examined this question for two influenza viral proteins. Cathepsin S also plays a role in the generation of TAP-independent responses to immunodominant epitopes in influenza nucleoprotein and polymerase (94). Presumably, cathepsin S is involved in actually generating these presented epitopes, although it has not been formally shown. It is also not known yet whether cathepsin S is sufficient for these responses, as it is for OVA, or whether

**Fig. 5.** A transporter associated with antigen processing (TAP)-independent pathway requires cysteine protease, cathepsin S. (A) Effects of proteasome inhibitor (β-lactone) and cysteine protease inhibitor (leupeptin) on PLGA-ovalbumin (OVA) antigen presentation. TAP-deficient bone marrow (BM) DCs were preincubated with no inhibitors, 100 μg/ml leupeptin, or 2 μM β-lactone for 30 min. PLGA-OVA was then added at a concentration of 5 μg/ml for 5 h in the continuous presence of inhibitors, and the cells were then fixed. They were then titrated and tested for their ability to stimulate RF33.70 T-T hybridomas (specific for Kβ/SIINFEKL) to produce interleukin-2 (IL-2). The content of IL-2 in supernatants was measured using CTLL2 indicator cells. The results are expressed as the mean cpm of 3H-Thymidine incorporation of cultures. (B). Cathepsin S is required for the TAP-independent cross-presentation. BM-DCs from C57BL/6, cathepsin S+/+, TAP−/−, and TAP−/−cathepsin S−/− mice were compared for their ability to cross-presentation PLGA-OVA antigen on class I Kβ. The procedures were essentially identical to that described in (A) (modified from Shen et al. (94) with permission from Elsevier).

**Fig. 6.** Cathepsin S can generate the correct peptide for presentation on major histocompatibility complex class I. (A) Generation of an antigenic peptide by phagosomes requires cathepsin S. Peptides generated from ovalbumin (OVA) incubated with phagosomes from bone marrow (BM) DCs of C57BL/6 and cathepsin S−/− were pulsed onto DC2.4 cells and assayed for their ability to stimulate Kβ/SIINFEKL-specific OT-I-transgenic T cells. (B). Generation of SIINFEKL by cathepsin S. OVA was digested with recombinant cathepsin S and the resulting peptides were separated by reverse phase high-performance liquid chromatography. SIINFEKL was assayed as in (A). Under these conditions, SIINFEKL elutes in fraction 25. These data were originally published in Shen et al. (94).
other proteases contribute to the generation of the presented peptides.

On the basis of these results, we conclude that cathepsin S plays a key and non-redundant role in the vacuolar pathway of cross-presentation, at least for several antigens. One unresolved issue is why other cathepsins are not able to substitute for cathepsin S. Possibly this inability reflects differences in their cleavage specificity. Another possibility is that it reflects the unique ability of cathepsin S to be catalytically active at neutral pH (101, 105). In contrast, all other cathepsins require an acidic environment for activity (100, 101). This requirement may be important, because at low pH, class I molecules may not be able to stably bind peptides. Yet another possibility is that cathepsin S may preferentially sort to this vacuolar compartment and be the predominant protease in this site. In other words, cathepsin S may be the predominant protease and/or the only active protease in the peptide loading vacuolar compartment (106). At present, there is no data to distinguish which, if any of these, possibilities contribute to the unique role of cathepsin S in cross-presentation.

It is presently unknown how broad a repertoire of peptides is produced by the vacuolar pathway, compared to the cytosolic pathway. In some cases, as in OVA SIINFEKL (94) and epitopes from influenza antigens (107), both pathways generate the same epitopes. In these situations, this production should contribute to cross-priming and optimize the CD8\(^+\) T-cell response in vivo. As the vacuolar pathway uses a different set of proteases, it seems likely that the repertoire it produces could be different and potentially smaller than the one produced in the cytosol (108). Would a different repertoire of peptides be useful biologically? On the one hand, CTLs generated to peptides produced exclusively by the vacuolar pathway would not be able to directly kill virally infected or transformed parenchymal cells, because the latter cells would not generate the corresponding presented peptide. In other words, there would be a mismatch between the CTL and the target cell. On the other hand, such CTLs could be useful for clearing phagocytes infected with intracellular organisms (see below) or in responding to cross-presenting APCs in sites of infection or tumors to secrete cytokine and induce inflammation. The extent to which this occurs and its biological importance remain to be determined.

Another unresolved issue is how MHC class I molecules acquire peptides generated in the vacuolar compartment. Harding (68, 91) originally suggested that peptides produced in phagosomes were regurgitated and bound to MHC class I molecules on the cell surface. In Harding’s system, the APCs that were used were cells with very high levels of empty class I molecules due to treatment with paraformaldehyde, which chemically cross-links and stabilizes empty class I molecules (109). However, we find that the amount of peptide that leaks from cells is insufficient to be cross-presented by cells with normal levels of peptide-receptive class I molecules on the cell surface (L Shen, unpublished data). Therefore, it is more likely that class I molecules are binding to peptides within the vacuole itself.

How peptide-receptive MHC class I molecules traffic to the vacuole is unknown, although there are several possible routes of entry. One possible route is internalization from the plasma membrane. Class I molecules are carried into the phagosome from invagination of the plasma membrane during phagocytosis. In addition, they may internalize into endosomes (110, 111) that could then fuse with phagosomes. Consistent with these possibilities, vacuolar cross-presentation was reported to be insensitive to brefeldin A (BFA), which blocks exocytosis of proteins from the ER (68); however, this is a controversial finding. We find that this pathway of presentation is inhibited by BFA (L Shen, unpublished data). As an alternate route of entry, newly synthesized class I molecules might traffic from the ER into endocytic compartments. Although class I is not known to have sorting sequences that would direct these molecules into endosomes, it is possible that specialized mechanisms allow this process to occur in macrophages and DCs. One such mechanism might be by the recently discovered fusion of ER and phagosomes (81) (Fig. 3). Another interesting possibility was that invariant chain, which directs MHC class II molecules to endosomes, might also traffic some newly synthesized class I molecules to the vacuolar compartment (112, 113). However, we found that vacuolar presentation was unchanged in invariant chain-deficient DCs and macrophages (94). It should also be kept in mind that it is possible that the cross-presented peptides are being loaded onto class I molecules in some other location in cells. For example, peptides in the endocytic compartment have been reported to be transported to the ER (114). Therefore conceivably, the peptides generated in phagosomes could traffic to class I molecules in the ER through a retrograde transport mechanism.

Yet another unresolved issue is why certain antigens are presented exclusively by the phagosome-to-cytosol pathway, while others are presented through the vacuolar pathway. Possibly this determination is made by whether or not the proteases in the vacuolar compartment are capable of generating the presented peptide. Presumably, the vacuolar proteases have a more limited capacity to generate the mature peptides than proteasomes together with cytosolic and ER...
aminopeptidases. However, this cannot be the only explanation. When OVA is bound to iron oxide particles, it is presented by the phagosome-to-cytosol pathway, but when in PLGA particles, OVA is also presented by the vacuolar pathway (94). In this situation, what seems to influence which pathway is operative is the physical form of the antigen: iron oxide or polystyrene versus PLGA particles. In another example, presentation of OVA formulated differently can be either TAP-dependent or -independent (115). One possible explanation for this difference is suggested by the observation that after internalization, different kinds of particles localize to different vacuoles (116–119). Therefore, it is possible that the different OVA particles end up in different vacuoles that have different cross-presentation mechanisms.

Endosome to ER pathway, a third pathway?

A third potential pathway was proposed recently for the cross-presentation of soluble proteins. This pathway was based on the discovery that at least some exogenous soluble proteins can be internalized in DCs and transported into the ER (120). As proteins in the ER can be transferred to the cytosol for degradation by the ER-associated degradation pathway (ERAD), it was suggested that this mechanism might be one through which soluble proteins are cross-presented. Another recent report provided evidence that ERAD may contribute to the cross-presentation of a soluble protein, although this study did not resolve whether the ERAD components were operating in the phagosome or the ER (121). At this time, further experimentation is needed to elucidate the role, if any, of this interesting phenomenon.

Contribution of the different cross-presentation mechanisms in vivo

The different mechanisms of cross-presentation have all been elucidated by analyzing cells in vitro. An important question is whether similar mechanisms are operative in vivo. We found that the same particulate antigens that target antigen into the cross-presentation of APCs in vitro are similarly acquired by these cells when injected in vivo and stimulate strong CTL immunity (18, 20). In contrast, injection of soluble antigen fails to prime CD8+ T-cell responses. Therefore, in vivo, phagocytosis and phagosomes almost certainly play important roles in cross-priming.

To address which of the phagosomal pathways of cross-presentation operates in vivo, we and others have constructed chimeric mice whose bone marrow lack essential components of the different cross-presentation pathways. In irradiated wildtype mice reconstituted with TAP-deficient bone marrow, the macrophages and DCs are unable to present antigen by the TAP-dependent (phagosome-to-cytosol) pathway. In these animals, polio virus and tumors are unable to cross-prime CD8+ T-cell responses (122, 123). Moreover, CD8+ T-cell responses to a transplanted cell are markedly reduced (94). Therefore, the TAP-dependent cross-presentation is active in vivo and plays a major role in cross-priming of CD8+ T cells to viruses, tumors, and transplanted cells.

In contrast, in TAP-deficient bone marrow chimeras, CD8+ T-cell responses were primed to PLGA-OVA, high-dose influenza virus, and also weakly to transplanted cells (94, 124). To determine whether these TAP-independent responses required cross-presentation through the vacuolar pathway, we examined irradiated wildtype mice reconstituted with wildtype or cathepsin S-deficient bone marrow. The macrophages and DCs in these animals are unable to present by the cathepsin S-dependent (vacuolar) pathway. These animals were injected with the different immunogens and were also depleted of CD4+ T cells so as to not have confounding effects of cathepsin S deficiency on T-cell help for CTL responses. In these animals, there were small, but detectable, reductions in the priming of CD8 T-cell immunity to PLGA-OVA (Fig. 7A), transplanted cells (Fig. 7B), and influenza virus (Fig. 7C) (94).

In mice lacking both cathepsin S and TAP, responses to all these antigens were essentially abolished (Fig. 7). Therefore, the cathepsin S-dependent pathway is operative in vivo, although its overall contribution is less than the TAP-dependent pathway. It potentially might play a major role in situations where the TAP-dependent pathway is inhibited, e.g. due to infection with viruses encoding immune evasion molecules or with antigens that are unable to access the cytosolic pathway. Also recent reports showed that the TAP-independent pathway can be upregulated during DC maturation, e.g. by lipopolysaccharide (LPS) or CpG, suggesting that under certain conditions, it can play a more significant role (98, 125). Indeed, pathogens and innate signals have been shown to have effects on MHC class I biosynthesis (126) and phagosome maturation (127). In any case, together the cathepsin S and TAP-dependent pathways can account for essentially all cross-presentation in vivo, at least for the antigens thus far examined.

Importance of cross-presentation

Cross-presentation initially appeared to be an obscure phenomenon of unclear significance. It was generally
assumed to be a minor pathway for stimulating CTLs and of little importance. However, it is now clear that cross-presentation is a major mechanism for the immune surveillance of tissues.

As part of the immune surveillance process, the immune system monitors all tissues for the presence of foreign antigens. There are many situations where parenchymal cells in tissues will express foreign antigens, e.g. from mutations or infection with a tissue tropic virus, that are not expressed in any professional bone marrow-derived APCs. In these situations, CD8\(^+\) T-cell immune responses are still generated and play an essential role in eliminating the abnormal cells. Remarkably, however, such CD8\(^+\) T-cell responses are not stimulated in chimeric mice whose bone marrow-derived APCs are unable to present antigen (due to the lack of TAP or the proper MHC class I molecules). Such animals are unable to generate CD8\(^+\) T-cell responses to antigens expressed in the tissues, e.g. from polio virus or tumors (122, 123). In contrast, these animals can generate CTL responses if immunized with competent DCs pulsed with antigen or with viruses expressing antigens that can be presented by the host’s bone marrow-derived APCs (122, 123). Therefore, the T cells in these chimeric animals are fully functional, and their failure to respond to tissue antigens is due to a defect in the ability of the endogenous APCs to cross-present the tissue antigens. Therefore, cross-presentation is the obligatory pathway for

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**Fig. 7.** The transporter associated with antigen processing (TAP)-independent, cathepsin S-dependent vacuolar cross-presentation pathway is operative in vivo. (A and B) OT-I T-cell proliferation in hosts lacking antigen presentation components. Carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled OT-I T cells were injected intravenously into the indicated mice. One day after transfer, 2 \(\mu\)g of PLGA-ovalbumin (OVA) (A) or OVA-transfected allogeneic (H-2\(^b\)) DAP cells (B) were injected subcutaneously on the left flank. Three days (A) and 5 days (B) after immunization, draining inguinal lymph nodes (LNs) were harvested, and LN cells were stained with PerCP-CD8 and APC-Thy1.1 and analyzed by flow cytometry. CD8 and Thy1.1 double-positive T cells were analyzed for proliferation (CFSE dilution) using FLOWJO software. (C). Effects of TAP and cathepsin S on generation of primary CTL responses against influenza virus. The indicated bone marrow chimeras (three to five mice in each group) were depleted of CD4\(^+\) T and injected intraperitoneally with 200 HAU influenza virus. Seven days after infection, B6.SJL (Ly5.1\(^+\)) spleen cells (target cells) were pulsed with NP or PA peptide or left unpulsed, and each target was then labeled with a different concentration of CFSE. The three target cells were mixed and injected intravenously into infected or uninfected mice. Ten hours later, blood was taken; cells were stained with APC-Ly5.1 antibody and analyzed with flow cytometry. The percent in vivo killing is expressed as mean percentage killing ± SD (modified from Shen et al. (94) with permission from Elsevier).
detecting and responding to antigens expressed exclusively in parenchymal cells.

These findings fit with the current concept, which has emerged from many studies, that professional APCs such as DCs are essential for initiating T-cell responses. Professional APCs are believed to be required, because they are the only cells that traffic to lymphoid organs and express all the signals that are needed to activate naïve T cells. DCs are initially present in all tissues as part of a surveillance mechanism. When they acquire antigen, they migrate to draining lymph nodes where they interact with and activate naïve T cells. If the DC is not itself synthesizing the foreign antigen, then it must acquire the antigen exogenously, and this acquisition can only happen through the cross-presentation pathways.

What is presently unclear is how important cross-presentation is in viral infections where the virus can infect both parenchymal cells and professional APCs (50, 128, 129). In this situation, the infected professional APC would be able to present the viral antigens through the classical MHC class I presentation pathway. Nevertheless, even in this situation, limited evidence suggests that cross-presentation may still play an important role. This role is suggested from experiments in chimeric animals whose bone marrow is cathepsin S-deficient and consequently lacks the vacuolar pathway of cross-presentation. When infected with influenza virus, these mice show reduced CTL responses to two different influenza viral antigens (94). Therefore, even though influenza virus can infect DCs, cross-presentation is still contributing significantly to the immune response. Moreover, this is almost certainly an underestimate of the contribution of cross-priming, because the APCs in the cathepsin S-deficient mice are expected to present the same antigens through the more dominant phagosome-to-cytosol pathway of cross-presentation. It is likely that cross-presentation will play a particularly important role for responses to those viruses, e.g. cytopathic strains or ones with immune evasion molecules, that impair the ability of directly infected DCs to function (130, 131). In other words, the predominant APCs that will function in these situations are the ones that are not themselves infected but are cross-presenting the viral antigen.

Another situation where cross-presentation may be important is in immune surveillance of phagocytes infected with intracellular bacteria, protozoa, or parasites. These pathogens are internalized into the phagosomes of macrophages and DCs where they survive and often replicate. MHC class II molecules are one mechanism that monitors the antigens in these compartments and this is important for CD4+ T-cell immunity to these organisms (132). However, the cross-presentation pathways also monitor the phagosome. It is known that antigens from internalized bacteria are cross-presented on MHC class I molecules (133, 134), and CD8+ T-cell responses can contribute to immunity to some of these pathogens (132). Presumably, cross-presentation allows CTLs to recognize the infected phagocyte and produce interferon-γ to help activate the phagocytes and/or may kill the infected cells to eliminate the reservoir of the infection.

**Monitoring cells in vivo: role of cell death**

While it is now clear that the immune system uses cross-presentation to monitor tissues for the presence of foreign antigens in cells, it is not clear exactly how these proteins are released and acquired by the APCs. Several different mechanisms have been proposed for this process including release of cellular proteins by secretion or cell death, or that APCs may ‘nibble’ material from living cells (135). It is possible that several of these mechanisms are operative; however, it seems likely that one of the major mechanisms is the release and acquisition of antigens from dying cells. It is known that dying cells are rapidly ingested by phagocytes, which are precisely the cells in which the cross-presentation pathways are operative. Moreover, the cellular debris will be internalized into phagosomes, which are the entry point for the major cross-presentation pathways. Consistent with this notion, when animals are injected with dead cells or subcellular fractions, CTLs are primed to antigens in these cellular preparations (136–138).

It has become apparent recently that dying cells are not simply functioning in the immune surveillance process as a passive source of antigen. Instead, they also release signals that alert the immune system to potential danger in ways that promote immunity (139–141). Thus, when dead cells are coinjected with particulate antigen, they markedly increase the CTL response to the particulate antigen (137). The dead cells thus function as an adjuvant.

The cellular or ‘endogenous’ adjuvant activities are primarily present in the cytosol (136, 138, 142, 143). The molecular basis of these activities is poorly understood and in some cases has been confusing. HSPs have been shown to induce DC maturation in vitro (144–148) to promote DC migration and to prime CTLs in vivo (28, 149, 150), and they have been considered the major source of endogenous adjuvant activity (151, 152). However, recent reports suggest that some of the observed activity is due to contamination of LPS (153–156). Our biochemical fractionation of cytosol has revealed several distinct peaks of activity (138). One of these activities has been identified as uric acid (138). Another recently reported
endogenous adjuvant is high-mobility group box protein 1 (HMGB1) (157), although in our hands, cell fractions containing HMGB1 have not augmented cross-priming (unpublished data). As these molecules are intracellular, they are normally sequestered from the immune system until cells are injured and die. Thus, their release alerts the immune system to cell death and therefore potential pathology or danger (139, 140).

Adjuvants play an essential role in the generation of immunity. They have long been known to enhance the generation of immune responses (158). Moreover, exposure to antigen in the absence of adjuvant activity can lead to tolerance instead of immunity (158, 159). The best characterized adjuvants are ones of microbial origin. As many components of microbes have adjuvant activity, infections with these agents promote strong immunity. Adjuvants are thought to exert their effects at least in part by stimulating DCs to fully mature and express costimulatory molecules. In the absence of such signals, DCs will present antigen in ways that lead to tolerance instead of immunity (159, 160).

As a consequence of these mechanisms, APCs that acquire tissue antigens without exposure to adjuvants would be predicted to induce tolerance. In fact, in situations where foreign antigen is expressed in normal tissues, e.g. from a transgene, it gets cross-presented in ways that induce tolerance (161); this phenomenon has been termed cross tolerance (160). The release of adjuvants from cells provides a mechanism by which a dying cell’s antigens can stimulate immunity instead of tolerance. Consistent with this notion, in transgenic models of tissue antigens that induce cross-tolerance, experimental injury of the transgenic tissue leads to immunity (162, 163). Moreover, dying cells and their components can stimulate DCs to mature (137, 138).

It is possible that the endogenous cellular adjuvants affect more than just APC-stimulatory activity. An intriguing observation is that coinjection of dead cells boosted immunity to particulate antigen, but not to soluble forms of antigen (21). This is very different from microbial adjuvants, as these agents are known to augment immune responses to soluble proteins. Moreover, when dead cells were coinjected with fluorescent particles, many more particle-containing APCs were found in the draining lymph node (21). In contrast, when coinjected with fluorescent soluble molecules, the dead cells stimulated no increase in labeled APCs in the lymphoid tissue (21). Thus, it appears that endogenous adjuvants may help selectively turn on the immune response to precisely the kinds of antigens that would be released from the dying cells, perhaps by enhancing the cross-presentation process itself.

Potential use of cross-presentation pathways for therapy

One of the most efficacious and cost-effective therapies for the prevention of many infectious diseases, such as smallpox and polio, has been the stimulation of specific immune responses through vaccination. However, there remain a number of infectious diseases for which vaccines are unavailable or only stimulate suboptimal immunity. Moreover, there are non-infectious indications, such as cancer, that could be potentially treated with vaccines.

Most current vaccines consist of non-living components of pathogens. Such killed or subunit vaccines are considered safer than ones using live organisms. However, one of the limitations of subunit vaccines is that they generally fail to elicit CD8+ T-cell immunity. This limitation would preclude the use of subunit vaccines for protection against diseases where immunity is mediated by CTLs, e.g. certain viral infections or cancer.

We now know that subunit vaccines fail to stimulate CTL immunity, because the antigens in these preparations do not get presented on the MHC class I molecules of the professional APCs. If, however, these antigens were introduced in ways that led to their being cross-presented, then CTL immunity could be generated. One way in which this can be achieved is to generate particulate forms of antigen. Such preparations are taken up efficiently by APCs and presented on both MHC class I and class II molecules (18, 164). Moreover, these constructs are avidly taken up by immature DCs, which are precisely the cells that can most effectively stimulate immune responses.

When such particulate preparations are injected into animals, they stimulate both CTL and CD4+ T-cell responses (19). A single injection subcutaneously could stimulate protective immunity (18). These responses are sufficiently strong to protect animals against challenge with aggressive tumors. Moreover, it is possible to make particulate preparations from biocompatible and biodegradable materials such as polylactide–coglycolide (164). Therefore, particulate antigens can be used to target antigens into DCs in vivo in ways that elicit both CD4+ and CD8+ T-cell immunity. It is similarly possible to introduce antigens into the cross-presentation pathways of DCs ex vivo and then inject these APCs back in vivo as a cellular vaccine (165–167).

Conclusion

Over the past 15 years, there have been major advances in our understanding of the role and mechanisms of cross-presentation. This once obscure phenomenon is now recognized...
to be a key mechanism though which the immune system monitors tissues for antigens. There have been major advances in our understanding of the underlying mechanisms of cross-presentation. The cells that cross-present antigens are the bone marrow-derived APCs. The form of antigen they monitor from tissues is predominantly cellular protein. These proteins are acquired through phagocytosis and macropinocytosis and then are presented through two distinct pathways. In one pathway, antigen is transferred into the cytosol where it is degraded by proteasomes, and in the other, the antigen is hydrolyzed in the endocytic compartment by cathepsin S and potentially other peptidases. It is also now recognized that cross-presentation can lead to different outcomes, tolerance, or immunity, depending at least in part on whether the antigens are associated with immunostimulatory adjuvants.

While we now understand much about this important pathway of presentation, there is still much to be learned. We do not fully understand its contributions to immunity or peripheral tolerance in many situations or the factors that influence these very different outcomes. In addition, there are key steps in the antigen-presentation pathways that are not yet understood. Furthermore, the potential for exploiting this pathway for therapy is in its infancy.

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