Mark Howarth
Tim Elliott

The processing of antigens delivered as DNA vaccines

Authors' address
Mark Howarth, Tim Elliott
Cancer Sciences Division, University of Southampton School of Medicine, Southampton General Hospital, Southampton, UK

Correspondence to:
Tim Elliott
Professor of Experimental Oncology
Cancer Sciences Division
University of Southampton School of Medicine
Mailpoint 824, Southampton General Hospital
Southampton SO16 6YD
UK
Tel.: +44 23 8079 6193
Fax: +44 23 8078 3839
E-mail: t.j.elliott@soton.ac.uk

Acknowledgements
We thank Nasia Kontouli for the help in preparing this manuscript. MH was supported by an MRC studentship.

Immunological Reviews 2004
Vol. 199: 27–39
Printed in Denmark. *All rights reserved*

Copyright © Blackwell Munksgaard 2004
Immunological Reviews
0105-2896

Summary: The ability of DNA vaccines to provide effective immunological protection against infection and tumors depends on their ability to generate good CD4$^+$ and CD8$^+$ T-cell responses. Priming of these responses is a property of dendritic cells (DCs), and so the efficacy of DNA-encoded vaccines is likely to depend on the way in which the antigens they encode are processed by DCs. This processing could either be via the synthesis of the vaccine-encoded antigen by the DCs themselves or via its uptake by DCs following its synthesis in bystander cells that are unable to prime T cells. These different sources of antigen are likely to engage different antigen-processing pathways, which are the subject of this review. Understanding how to access different processing pathways in DCs may ultimately aid the rational development of plasmid-based vaccines to pathogens and to cancer.

Introductory remarks

Antigens administered in the form of DNA can stimulate both B- and T-cell responses, and they have been shown to be protective against viral, bacterial, and tumor challenge (1). The most effective vaccines are most likely to be those that stimulate both CD4$^+$ and CD8$^+$ T cells (2), and so it is important to consider antigen-processing of DNA-encoded antigens for presentation by both major histocompatibility complex (MHC) class I and class II molecules. As dendritic cells (DCs) have sole responsibility for priming T-cell responses *in vivo*, it makes sense to focus attention on the antigen-processing properties of this cell type. Here we discuss the four possible antigen-processing 'pathways' identified in DCs separately: (i) processing of endogenous antigen for presentation by MHC class I, (ii) processing of exogenous antigen for presentation by MHC class II, (iii) processing of endogenous antigen for presentation by MHC class II, and (iv) processing of exogenous antigen for presentation by MHC class I (cross-presentation). These pathways are illustrated schematically in *Fig. 1* and are discussed in turn below.

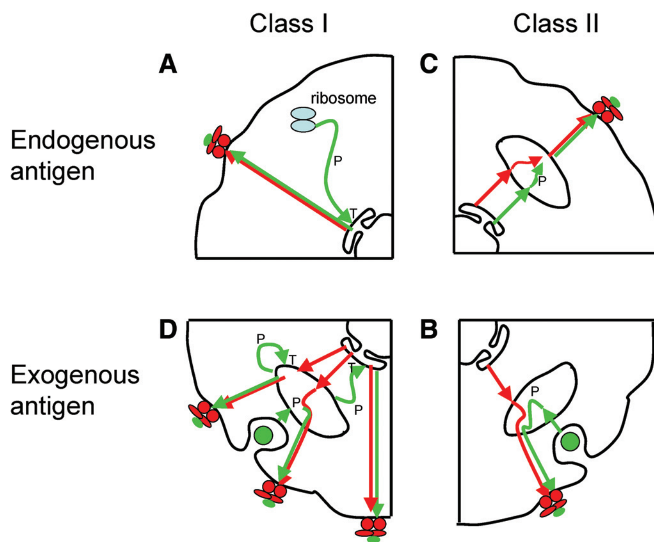

Fig. 1. The four pathways of antigen processing in dendritic cells. The intracellular pathway of antigen is shown in green, and the intracellular pathway of MHC molecules is shown in red. (A) Processing of endogenous antigen for presentation by MHC class I. Endogenously synthesized antigens are degraded in the cytosol by proteases (P), and the peptides generated are transported into the ER by TAP (T), where they are loaded onto class I molecules and migrate to the cell surface. (B) Processing of exogenous antigen for presentation by MHC class II. Antigens taken into phagolysosomes are degraded there by proteases (P) and are then loaded onto class II molecules before migrating to the cell surface. (C) Processing of endogenous antigen for presentation by MHC class II. Antigens synthesized in the cell that contain an endosome/lysosome-targeting signal will be transported there, degraded by proteases (P), and the peptides generated loaded onto class II molecules before the complexes are transported to the cell surface. (D) Processing of exogenous antigen for presentation by MHC class I (cross-presentation). Antigens taken into endosomes or phagolysosomes have three theoretical fates. The first (bottom) is that they are translocated into the cytosol, where they enter the classical endogenous pathway (1) with peptides transported into the ER by tap (T) where they bind newly synthesized class I molecules. The second (middle) is that they are degraded in this low pH compartment, and the peptides generated there bind to class I molecules present as a result of ER/phagosome fusion or direct targeting. It is also possible that peptides generated here are regurgitated and bind to cell surface class I molecules (not shown). The third (top) is that antigens are translocated out of the phagolysosome, degraded by phagolysosome-associated proteases, such as proteasome (P), then transported back into the same phagolysosome by TAP, where they can bind to class I. The presence of TAP and class I in the phagolysosome is a direct result of ER phagolysosome fusion.

Direct transfection of DCs leading to class I presentation

Evidence that direct transfection is important for priming cytotoxic T-lymphocytes

Several groups have shown that DCs can become transfected with vaccine DNA after intramuscular syringe-delivery (3), scarification (4), and gene-gun delivery (5). Although antigen-encoding 'gene' expression by DCs does not tell us whether these cells are primarily responsible for cytotoxic T-lymphocyte (CTL) priming *in vivo*, attempts have been made to show that this is the case. For example, Porgador *et al.* (5) co-expressed a cell-surface marker with the antigen-expressing DNA plasmid and followed immunization with *in vivo* depletion of marker-positive cells. Depletion abrogated priming and, taken together with their observation that $CD8^+$/$DEC205^+$ DCs were the principal cell type involved in CTL priming in this model, these data suggest that directly transfected DCs were the most important source of CTL priming (5).

Processing of antigens following direct transfection

Proteasomal generation of peptide for MHC class I

A key role for the proteasome in cytosolic proteolysis was first suggested by the observation that fusion of influenza nucleoprotein to ubiquitin, a targeting signal for degradation by the proteasome, enhanced presentation of a D^b-restricted nucleoprotein epitope (6). Further, evidence for the importance of the proteasome came from impaired-MHC class I presentation of cytosolic ovalbumin but not a cytosolic peptide minigene in cells with a temperature-sensitive defect in ubiquitin conjugation (7). The use of small-molecule proteasome inhibitors demonstrated the general importance of the proteasome in the generation of ligands for MHC class I (8, 9).

Much attention has been focused on the birth of proteins, but the death and decay of proteins is of equal importance. Protein degradation is a complex highly regulated process. Proteins are targeted for degradation because they have been damaged, because they have misfolded by chance, or because they never folded in the first place. The principal executioner of cytosolic proteins is the proteasome. The proteasome has a 20S catalytic core, consisting of a central barrel formed from $\alpha7\beta7\beta7\alpha7$ subunits into which proteins are threaded (*Fig. 1*) (10). The confinement of the proteolytic activity within this barrel stops uncontrolled degradation of cellular proteins. Regulatory subunits are attached to the catalytic core, principally the 19S regulator, which recognizes and unfolds proteins that have been targeted for degradation by the covalent attachment of ubiquitin. The proteasome has a range of catalytic activities, principally trypsin-like, chymotrypsin-like, and caspase-like. The main products of proteasome cleavage are peptides of 3–24 residues (11, 12). Interferon-γ (IFNγ) induces expression of the catalytic subunits LMP2, LMP7, and MECL-1, which replaces the housekeeping X, Y, and Z subunits of the proteasome. IFNγ also induces the proteasome regulator PA28. These changes produce an immunoproteasome, which cleaves less often after acidic residues, better matching the C-terminal

preference of MHC class-I alleles (13–15). Immature and mature DCs constitutively express immunoproteasomes (16).

Proteasome degradation of ovalbumin *in vitro* only generates the correct C-terminus of the dominant epitope single ovalbumin-derived epitope (SIINFEKL) 8% of the time, and these peptides are principally extended by 1–8 residues at the N-terminus (17). Also, IFNγ increases proteasomal generation of N-terminal extended peptides (17). Thus, a model has arisen whereby the C-terminus of MHC class-I epitopes is principally generated by the proteasome, while the N-terminus can be generated either precisely by the proteasome or by a panel of cytosolic and endoplasmic reticulum (ER) aminopeptidases (18). The ability of the proteasome to generate the correct C-terminus from degradation of the protein is an important predictor of peptide immunogenicity (19).

The source of substrates for the proteasome has recently been revisited. It was first shown by Varshavsky and Turner (20) that 50% of proteins bearing an N-terminal degradation signal could be degraded co-translationally, before the ribosome has finished synthesizing their C-terminus. It was then found that protein synthesis is quite inefficient and a significant source of substrates for the proteasome comes from defective ribosomal products (DRiPs) (21, 22). Thirty percent of proteins are degraded within 10 min of their synthesis (21). Importantly, this degradation allows peptides to be channeled to the MHC class I pathway at the start of their life, rather than at the end. MHC class I can therefore monitor protein synthesis rates and not protein concentrations. This factor could be important when designing DNA vaccines. The proportion of antigenic peptides that derive from folded protein or from DRiPs has been evaluated; DRiPs are only the majority for unstable proteins such as ovalbumin (23). The lack of enrichment of antigenic peptides in the N-terminus of proteins would also suggest that truncated polypeptides are not a dominant source of proteasome substrates (24). Note that proteins in the ER may also be processed to produce peptides for MHC class I, which principally occurs through retrograde transport of misfolded protein from the ER to the cytosol via Sec61p (25–27), but other pathways probably exist (28). High-mannose N-glycans in the cytosol are seen by a ubiquitin ligase (29) causing the protein to be deglycosylated and then degraded by the proteasome.

Non-proteasomal cytosolic generation of peptide for MHC class I

Many MHC class I alleles are resistant to high concentrations of proteasome inhibitors, suggesting that many of their peptide ligands can come from other sources; surface expression of H2-D^k is actually increased by treatment with the proteasome inhibitor lactacystin (30). In the last few years, several other cytosolic proteases have been identified, and their role in generating antigenic peptides has begun to be characterized.

Tripeptidyl peptidase II (TPPII) is a proteolytic complex that is rod-shaped, is larger than the proteasome, and has membrane-bound and cytosolic forms (31). It removes tripeptides from the N-terminus and also has endoproteolytic activity, often after basic residues (32). Like several other proteases, TPPII cannot cut after or before proline. Reports that it could fully substitute in the cell for the proteasome (33, 34) may be an artefact of incomplete proteasomal inhibition (35). TPPII short interfering RNA (siRNA) has been shown to inhibit the processing of a specific epitope from human immunodeficiency virus (HIV) Nef, but the global effects on MHC class I assembly of inactivating TPPII have not been determined (36). This testing of functional importance by siRNA has significant advantages over the use of chemical inhibitors that are rarely, fully specific.

Leucine aminopeptidase is an IFNγ-inducible cytosolic protease that can cleave N-terminally extended SIINFEKL to generate the optimal epitope (37). Overexpression of leucine aminopeptidase greatly reduced MHC class I assembly with peptide, indicating that it degrades many potential MHC class I ligands (38).

Puromycin-sensitive aminopeptidase (PSA) is a 100 kDa zinc metalloprotease that is widely expressed, and it was shown to digest a vesicular stomatitis virus (VSV)-nucleoprotein epitope N-terminally extended by five residues to the optimal epitope (39). PSA prefers N-terminal basic or hydrophobic residues and will not cleave before or after proline or glycine (40). PSA seems to be important for the degradation of peptides poorly cleaved by thimet oligopeptidase (41).

Bleomycin hydrolase is a 50-kDa cysteine protease that is widely expressed. It was also shown to digest a VSV-nucleoprotein epitope N-terminally extended by five residues to the optimal epitope (39). No further functional information is available.

Thimet oligopeptidase is a ubiquitously expressed cytosolic metallo-endopeptidase that cleaves peptides of 6–17 residues (42), and it has been shown by overexpression or siRNA that it mainly acts to degrade potential MHC class I peptides (43). Thimet oligopeptidase siRNA enhanced surface MHC class I expression by ~50%, showing that it has a significant impact on the possible pool of MHC class I peptides.

The combined effect of all these proteolytic activities is that life for a peptide in the cytosol is short and brutal. Free peptides cannot be isolated from the cytosol (44, 45), and

peptides injected into the cytosol seem to have a half-life of a few seconds before they are cleaved and degraded to amino acids (38). The spatial organization of these proteases in the cell has not been established. It has been conjectured many times that there is some system, perhaps involving heat shock proteins (hsps), that protects antigenic peptides in the cytosol and delivers them to the transporter associated with antigen processing (TAP). Peptide-loaded hsp70 or hsp90 introduced into the cytosol caused efficient presentation of the peptide by MHC class I, while the hsp-inhibitor deoxyspergualin blocked antigen presentation (46, 47). Antigenic peptides have been found associated with many proteins: calreticulin (48), protein disulfide isomerase and gp96 (49), or hsp70 (50). However, the rapid diffusion of fluorescein-labeled peptides injected into the cytosol indicates that they are not bound by protein in the cytosol (38), although there may be some association with nuclear proteins. These peptides must move to the cytosol before they can be transported by TAP (38). The efficiency of generation of MHC class I peptide complexes is only 1 in 50 for a minigene (23) and 1 in 4000 for certain protein antigens (51). Thus, chaperoning of antigenic peptides to TAP is not highly efficient, and the peptides may have to take their chances in the proteolytic killing fields.

Transport of peptide into the ER

Peptides are pumped from the cytosol into the ER by TAP (52). TAP is an adenosine triphosphate (ATP)-binding cassette transporter, and low-resolution electron microscopy on immunopurified complexes show that TAP exists as a single heterodimeric complex of TAP1 and TAP2 with a 3 nm pocket visible on the ER luminal side at the interface (53). Assays using microsomes or permeabilized cells have shown ATP-dependent peptide transport by TAP (54, 55). Many MHC class I alleles interact with TAP (56). The co-localization of heavy chain β2m with the principal source of ER peptides, TAP, is thought to promote efficient peptide loading, before the peptides can be degraded in the ER (57, 58) or exported back to the cytosol (59). TAP preferentially transports peptides of 9–15 residues (60) and has some sequence selectivity, most notably that proline is disfavored in the first three residues of the peptide (61).

A small number of peptides can gain access to MHC class I in the ER independent of TAP. A good correlation was shown between presentation of cytosolic Epstein-Barr virus (EBV)-peptide minigenes in TAP-deficient cells and peptide hydrophobicity (62). From a panel of EBV epitopes, all the uncharged peptides were TAP-independent, while all the charged peptides (with D, E, K, or R) were TAP-dependent. Presumably, high hydrophobicity allows the peptide to diffuse straight through the lipid bilayer. Addition of an N-terminal (5′) endoplasmic reticulum-targeting sequence to a minigene encoding a CTL epitope can also bypass the requirement for TAP for its presentation (63).

ER proteolysis of peptide for MHC class I

The length of a peptide may still not be optimal for MHC class I binding when it is transported through TAP. However, proteolysis of antigenic peptides can also occur in the ER. This ER trimming can tailor extended peptides to the optimal size for MHC class I binding. TAP-deficient cells present peptides derived from signal sequences in the ER (64, 65). Also, Snyder *et al.* (66) showed that TAP-negative cells could present the C-terminal but not the N-terminal epitope, when two tandemly linked epitopes were targeted to the ER. This finding was the first indication that the ER contains principally aminopeptidase and not carboxypeptidase activity. However, Elliott *et al.* (63) showed that an ER-targeted 170 residue fragment of influenza nucleoprotein could be processed to give the dominant D^b-restricted epitope in TAP-negative cells, even though this action would require N- and C-terminal trimming.

The ER aminopeptidase was tracked down concurrently by the groups of Shastri and Rock/Goldberg (57, 58, 67). ERAP1 [also called aminopeptidase associated with antigenic processing (ERAAP)] is a 100-kDa soluble zinc metalloprotease, ubiquitously expressed and induced by IFNγ. It does not contain an ER retention motif, but endoglycosidase H digestion and immunofluorescence microscopy showed it to be resident in the ER (58). ERAP1 can cleave all bonds except X-P (68). Many ER proteins have X-P at their N-terminus, such as tapasin, calreticulin, and β2m, and thus may be protected from ERAP1 nibbling (68). Many human MHC class I alleles bind peptides with proline at position 2 (P2) as an anchor residue. Peptides with P2 proline are not transported well by TAP and so would have to be transported as N-terminally extended peptides (69). These peptides could then be trimmed by ERAP1, and the proline would help to prevent excessive trimming and destruction of the epitope (67). *In vitro*, ERAP1 cleaved all peptides of >10 residues, half of those of nine residues, and had little effect on peptides of eight residues or less (57). Degradation of 9-mer peptides would explain why ERAP1 siRNA increased human leukocyte antigen (HLA)-A2 surface expression (67). As ERAP1 siRNA almost completely blocked the presentation of a signal sequence-targeted N-terminally extended SIINFEKL, ERAP1 may well be the principal ER trimmase of short peptides (67). Trimming by

ERAP1 is a rapid process, complete in 2 min in microsomes (70). Peptides not degraded by ERAP1 and not bound to MHC class I probably do not accumulate in the ER but are rapidly retro-translocated to the cytosol (59). Peptide refugees seeking protection from cytosolic proteolysis only find asylum in the ER if they bind a restricting MHC class I molecule.

Interaction of chaperones with MHC class I

Although peptide binds spontaneously to MHC class I *in vitro*, assembly *in vivo* of peptide with MHC class I does not occur in isolation. A number of chaperones have a key role in peptide loading and blocking their action can greatly disrupt antigen presentation. MHC class I heavy chain folds in association with calnexin and then may load with peptide while bound to calreticulin, ERp57, Tapasin, and TAP- the 'peptide-loading complex' (71).

Targeting antigens to the endogenous pathway

Several attempts have been made to target DNA vaccines to the endogenous MHC class I processing pathway in order to maximize the consequence of direct DC transfection. These strategies have included to the following: appending ER targeting sequences to 'pre-processed' immunogenic peptide sequences (72); fusing antigen to substrates that are known to be good for the proteasome (73); as well as using constructs encoding ubiquitin-fusion proteins (74). The latter enhanced CTL production but led to poor B-cell responses. Interestingly, a fusion construct of calreticulin to the human papillomavirus oncoprotein antigen E7 enhanced E7-specific CTL production. This result may have been due to enhanced uptake of the CRT-E7 protein by DCs, but given the intimate association of calreticulin with MHC class I molecules as they assemble with peptides in the ER and the presence of proteases there, it is tempting to speculate that calreticulin may be targeting peptide antigens to newly synthesized class I molecules in the peptide-loading complex. In addition, calreticulin has anti-angiogenic properties, and a significant proportion of the antitumor effect of the CRT-E7 fusion DNA vaccine can attributed to this property (75).

Acquisition of antigen expressed by 'bystander' cells leading to class II presentation

Evidence for priming class II restricted T cells to exogenous antigen following DNA vaccination

Several DNA vaccines currently under evaluation consist of nothing more than a minimal CTL epitope encoded by a plasmid that also incorporates CpG motifs (76). Some of these complexes can generate CTLs indicating that in some instances, T-cell help can be bypassed by the inclusion of sequences that can directly activate APCs. Others have found that CTL priming requires $CD4^+$ T-cell help (77), and the inclusion of helper epitopes in DNA vaccine constructs is now commonplace. MHC class II-restricted antigen processing takes place in the phagolysosome complex, and unless specifically targeted to this system from an endogenous source, antigens gain access to the class II processing compartment from outside the cell via receptor-mediated endocytosis, phagocytosis, or macropinicytosis. It is therefore likely that antigens encoded by DNA vaccines must first be synthesized in bystander cells before uptake by APCs, if they are to stimulate $CD4^+$ T cells.

Processing of exogenous antigens

Antigen uptake

The mode of antigen uptake by DCs can dictate the phagolysosomal compartment that it is first exposed to (78), and hence might influence the pathway by which it is degraded. For example, in B cells, antigens that are taken up by endocytosis via the B-cell receptor can be diverted away from compartments that yield specific-class II-restricted epitopes by introducing a single-point mutation in their transmembrane region (79). Depending on exactly what forms the antigen is encountered by DCs will determine which, if any, receptor system will be used in its uptake. For example, apoptotic cells (which can contain DNA vaccine-encoded antigen) can be taken up by the avb5 integrin/CD36 complex via thrombospondin (80), by the scavenger Receptor LOX-1, by the receptor tyrosine kinase MER (81), and by the $\alpha 1$ macroglobulin receptor CD91, which recognizes surface-bound C1q/mannose-binding lectin via a calreticulin bridge (82). Other pathways for the uptake of soluble proteins include the scavenger-like receptor DEC-205 (83) and FcR, which, by internalizing soluble immune complexes, can take advantage of pre-existing antibodies to an antigen and enhance its processing via the class II-processing pathway. In addition, peptides and possibly even whole antigens bound to stress proteins released from necrotizing cells are taken into DCs by several receptors including LOX-1 (84), CD40 (85, 86), SRA (87), and possibly TLR2 (88) and CD91 (89).

Proteolysis

The proteolysis of antigens in phagolysosomes is likely to be complex, and it will depend on the exact route of uptake and

intracellular routing of the phagocytosed antigen. Garin *et al.* (90) have carried out a proteomic analysis of the contents of phagolysosomes as they mature following the ingestion of latex beads, and they have identified more than 140 novel proteins including over 50 acid hydrolases. The acid proteases were incorporated into phagolysosomes one (or a few) at a time, and so they may be available to act on a substrate antigen in sequence.

The proteases that are likely to be involved in antigen breakdown include broad-specificity aspartate (Cathepsin D and E), cysteine (Cathepsin B, F, H, L, S, and Z) proteases with both endopeptidase (Cathepsin D, E, F, L, and S) and exopeptidase (Cathepsin B, H, and Z) activity, serine proteases, metalloproteases (91), and the asparagine-specific cysteine endopeptidase (AEP) (92). In addition, acid denaturant (pH 5.5–4.0) and an enzyme, GILT (γ interferon-inducible lysosomal thioredoxin), which catalyzes the reduction of intramolecular disulfide bonds, also contribute to antigen breakdown (93). It is unlikely that any single sequence of enzymes is employed for the processing of all antigens, but possibly a limited number of enzymes are important for the generation of any given epitope and will represent a balance between epitope generation and epitope destruction. Thus, the processing of an immunoglobulin Fab′2 fragment internalized by receptor-mediated endocytosis has been shown to have a minimum requirement for Cathepsins B and S (94). The processing of ovalbumin, taken into DCs via macropinocytosis, is blocked by a specific inhibitor of Cathepsin E, and processing can also be generated *in vitro* by Cathepsin D and destroyed *in vitro* by Cathepsins B and L (95, 96).

Because the class II peptide-binding groove is open-ended, it is likely that large polypeptides could bind and that proteolytic processing of the antigen could continue while it is bound to class II and that the class II 'footprint' could protect a continuous stretch of polypeptide from cleavage (97). This so-called MHC-guided antigen processing (98) embodies the idea that peptide selection by MHC class I, and hence immunodominance, could be determined by the first available structure of a protein antigen that becomes available for binding to class II as it starts its journey down the pathway of unfolding, reduction, and hydrolysis. Thus, early acting enzymes in the processing pathway may be crucial for epitope selection. Two candidates for this role have been studied in detail. The first is GILT, whose function may be to expose regions of a protein that are normally protected from endoproteases by virtue of the fact that they remain folded at low pH, thanks to the presence of domain-stabilizing disulfide bonds. Mice lacking this enzyme mount much weaker class II-restricted T-cell responses to the antigens hen egg lysosome (HEL) and RNaseA, which contain intramolecular disulfide bonds, than do normal mice. The response to the non-disulfide-bonded antigens was unaffected. When the specificity of antigen processing was analyzed, it was found that the generation of some HEL epitopes was independent of GILT, perhaps because these are available for further processing in the fully oxidized protein (99). Interestingly, full-length reduced but not oxidized HEL and RNAseA have been shown to bind to class II (100), and so it is possible that GILT-catalyzed reduction of these antigens could represent the first step in MHC-guided processing of the GILT-dependent epitopes.

Another candidate is AEP, which initiates and is the rate-limiting activity for the processing of most antigens (92). In a study of the processing of tetanus toxin C fragment (TTCF), which requires AEP for its processing and presentation by class II, Antoniou *et al.* (101) found that cleavage at a single site was a prerequisite for the generation of three epitopes that are distal to this site in both primary and tertiary structure of TTCF. Interestingly, because of its specificity, cleaving after asparagine residues, this enzyme is inhibited by the presence of N-linked carbohydrate. The glycosylation status of a secreted antigen will influence its pathway of processing, and this effect could have important consequences for antigens with variable glycosylation-site occupancy or in cases where the glycosylation status of the antigen following DNA priming is different to that of the antigen encountered in the secondary challenge.

Loading of MHC class II molecules with peptides

Newly synthesized MHC class II molecules are conducted to a phagolysosome compartment called the MIIC by virtue of its assembly with invariant chain (Ii), which contains an endosome-targeting sequence in its cytoplasmic domain. Here Ii is cleaved by leupeptin-sensitive acid hydrolase leaving an Ii fragment called CLIP bound in the peptide-binding groove. This low-affinity peptide is exchanged for antigenic peptides generated in the phagolysosome complex [exchanged being catalyzed by a MHC II-like molecule called DM (102)].

Direct transfection of DCs leading to class II presentation

Establishing the molecular mechanisms that underpin the MHC class II processing pathway has allowed the rational design of DNA vaccines that directly target antigens to key locations along this pathway from an endogenous source. They are therefore designed to work optimally upon direct transfection of DCs. One appealing strategy is to target the MHC class II-binding site directly by introducing a mutant Ii in which CLIP is replaced by a known class II epitope. This

technology was invented by Wang *et al.* (103) as a way of screening cDNA libraries for class II-restricted tumor antigens. However, Thielemans *et al.* (104) have recently shown that DCs electroporated with mRNA encoding Ii, containing a transplanted MAGE-3 epitope in place of CLIP, showed far greater immunostimulatory capacity than peptide-pulsed DCs for a HLA-DP4-restricted clone and DNA vaccines encoding similar constructs. Fusions of antigen and the phagolysosome-targeted protein LAMP have also been shown to have greater stimulatory capacity for $CD4^+$ T cells when introduced into target cells, and a recombinant vaccinia encoding HIVgp160-LAMP has been shown to elicit enhanced $CD4^+$ T-cell and B-cell induction after infection (105), although whether the same will be true for an analogous DNA vaccine remains to be seen. In another approach, both the immunoprotective antigen from *M. tuberculosis*, ESAT-6, and the human-melanoma antigen MART-1 have been fused to CD1 endosomal targeting sequences and have been shown to stimulate greater numbers of specific $CD4^+$ and $CD8^+$ than non-fused controls in vaccination experiments and in the latter case led to greater protective immunity against a transplantable tumor. The CD1 targeting sequence may turn out to be a useful tool for directing class II antigens to an optimal antigen-processing compartment, because, depending on the CD1 isotype from which the targeting sequence is derived, antigens could be targeted to either late endosomes and lysosomes (CD1b and CD1d) or to early endosomes (CD1c) (106). It is conceivable therefore that by careful intracellular targeting, certain enzymes in the class II processing pathway that can destroy key epitopes might be bypassed.

Relevant to a discussion on presentation of endogenous antigens by class II molecules is the fact that DCs have a capacity for autophagy, the cellular process by which some cellular proteins are normally turned over. Recently, it has been shown that a model cytosolic antigen (the neo resistance gene neomycin phosphotransferase II) can be transported to phagolysosomes for processing, following their uptake into autophagosomes, and presented on MHC class II (107). This is a clear demonstration that inclusion of other encoded DNA (such as an antibiotic resistance marker) in a vaccine plasmid could provide helper epitopes.

Acquisition of antigen expressed by bystander cells leading to class I presentation

Evidence for priming class I-restricted T cells to exogenous antigen following DNA vaccination

The fourth antigen-processing pathway involves the uptake by DCs of soluble or particulate antigens derived from the transfection of neighboring cells for presentation by MHC class I molecules, a process known as 'cross-presentation'. Evidence for the cross-presentation of antigens, derived from infectious agents (which includes DNA vaccines for the sake of discussion), has been reviewed recently (108) and is discussed further in other contributions to this issue. Evidence in favor of cross-presentation as being the primary route for priming class I-restricted CTLs after DNA vaccination rests on experimentally contrived situations in which there is differential expression of the transgene in APCs vs. non-APCs.

An example from the field of infectious immunity is in radiation bone marrow-chimeric mice, where mice transgenic for the human poliovirus receptor (CD155) are reconstituted with wildtype bone marrow (which cannot be infected). Here, CTL priming to recombinant human poliovirus-encoded antigens occurred, even though DCs themselves could not be infected with the virus. Priming was shown to be by DCs and not by infected non-hematopoietic cells by using TAP-knockout donor bone marrow in which the DCs are unable to utilize the class I-processing pathway (109). However, the recent demonstration is that in this model mRNA encoding the antigen can be found in DCs (110), and that they can take up sufficient mRNA from infected neighbors to prime CTLs, prompts a re-evaluation of the experimental evidence for *in vivo* cross-priming. It remains to be seen whether DCs can be 'cross-transfected' in this way following DNA -immunization.

Notwithstanding the latest inquisition from Zurich (110), there are several examples in the DNA vaccine literature where this phenomenon would be expected to be eliminated, or at least minimized. For example, Corr *et al.* (111) immunized $Rag^{-/-}$ mice with a 'tet-off' controlled antigen-encoding DNA plasmid and maintained suppression of transcription in one group of mice by implanting them with slow-release tetracycline pellets. Splenocytes (which contain large numbers of DCs) from these or non-drug-treated animals (in which translation of plasmid-encoded antigen is allowed and therefore available for cross-presentation) were then transferred into mice transgenic for a T-cell receptor recognizing a class I-restricted epitope from the antigen. The recipient mice were not drug treated, and so APCs among the splenocytes from drug-treated donor mice could express their transgene for the first time. T-cell responses stimulated by this group, thought to be the result of direct transfection of APCs only, were weak. However, responses elicited by the non-drug-treated splenocytes, thought to be the combined result of direct transfection and cross-presentation, were strong (111).

Processing of exogenous antigens for cross-presentation

From an antigen-processing perspective, cross-priming raises an interesting question: where are exogenous antigens processed for presentation by class I? This question is pertinent, because it is important that the DCs prime CTLs to the same peptide epitopes that they will encounter in the periphery (on infected cells or tumors). One would assume that the same antigen-processing pathway should be employed in both cells, and that this pathway would have to be the 'endogenous' pathway described earlier. There is, in fact, evidence for both cytosolic and phagolysosome processing of class I antigens by DCs (and macrophages).

Cytosolic processing

The hallmark of non-endosomal-antigen processing (and by implication cytosolic/ER-processing) has usually been that it is not sensitive to chloroquine and that it requires the synthesis of new class I molecules. These parameters have been shown to be the case for phagocytosed antigens in DCs (112). However, a topological paradox results, because antigen and class I are placed on opposite sides of the phagolysosome membrane.

Several mechanisms may come into play to transfer antigens from the phagolysosome lumen into the cytosol. A shuttle system has been described for cytosol to endosome transport of a subset of cytosolic proteins that are degraded rapidly, when cells undergo stress (113). This pathway is dependent on hsps, but it is not known whether it is bi-directional. Antigens can also enter the cytosol from distended-phagolysosomes via leakage or 'indigestion' (114).

Significant progress has been made recently into our understanding of the delivery of exogenous antigens to MHC class I with the discovery that phagosomes in macrophages (initiated by the ingestion of latex beads) fuse with the ER (115). This process is known as 'ER-mediated phagocytosis', because fusion is now thought to be a general requirement for formation of the phagosome, supplying both membrane to the nascent vesicle as well as new receptors to the cell surface such as CD91/calreticulin (116). The same phenomenon has been described in DCs, and its immunological implications have been evaluated. While the delivery of newly synthesized class I molecules, directly to the lumen of the phagolysosome, might be expected to facilitate their loading with peptides generated therein, it has now been demonstrated that these molecules continue to be loaded from the cytosolic pathway. Thus, phagocytosed antigen is translocated to the cytosol for processing via the proteasome, and the resulting peptides are translocated back into the same phagosome by TAP (117, 118). In a subsequent study, Ackerman *et al.* (119) showed that cross-presentation could be blocked by a soluble construct of the TAP inhibitor US6, whose only access to TAP was via phagocytosis (US6, an HCMV protein in its native forms targets TAP only in the ER). This finding indicated that all class I loading with TAP-derived peptides occurred in the phagolysosomes of DCs (119). The same group showed that ER fuses with endosomes formed as a result of receptor-mediated endocytosis.

One very important consequence of ER/phagosome fusion is the delivery to the phagosome of the protein translocon, which has been shown to retrotranslocate proteins from the ER lumen to the cytosol (27) prior to their degradation by proteasome. Assuming that this function is preserved in the ER/phagosome, it provides a means for translocating exogenous antigens out of the phagolysosome and, along with the proteasome (which has been identified on the cytoplasmic face of the ER/phagolysosome) (118), provides a highly efficient means of generating a local supply of peptides that can be pumped back into the ER/phagosome by proximal TAP molecules.

Lizee *et al.* (120) have shown recently that a YXXA motif in the cytoplasmic domain of class I is required for cross-presentation in DCs. This motif targets class I to endolysosomes in these cells, which was confirmed as the primary site for peptide loading (120). Class I molecules lacking this motif did not localize to endolysosomes and were inefficient at cross-presentation, although they were as good as wildtype molecules at presenting antigen from an endogenous source (recombinant vaccinia virus infection). At the time of writing, it is not clear whether cross-presentation in this system is TAP- and proteasome-dependent, and hence whether these data are consistent with data showing delivery of class I molecules to the phagolysosome via ER-fusion. It is possible, for example, that the mechanism of antigen uptake by DCs is a critical factor, such that antigens entering the DCs by means other than ER-mediated phagocytosis (such as macropinocytosis) load class I molecules that are targeted to endolysosomes independently of ER/endosome fusion, or whether the presence of class I in ER/phagolysosome fusion compartments is dependent on their cytoplasmic tail.

It is worth reflecting at this point on the fact that, whatever the mechanism might be for exposing exogenous antigens to the endogenous-processing pathway in DCs, the antigenic substrate is likely to differ from that produced from an endogenous source. Specifically, when endogenously synthesized, antigens enter the endogenous pathway at the beginning of their life as DRiPs, but it is more likely that they are fully

folded and acquire some resistance to proteolysis (i.e. at least middle aged) before they are taken up by DCs and enter the endogenous pathway by way of phagolysosome-to-ER transfer. Indeed, the rapid turnover of ubiquitin-tagged antigens used as DNA vaccines has been cited as the main reason for their inability to generate B-cell responses, simply because they do not live long enough to be taken up by DCs. Conversely, increasing the half-life of a peptide epitope encoded by a DNA vaccine by appending a sequence from *Listeria monocytogenes* (the lem-A7-33 peptide) improves its ability to prime CTLs (121). These antigens are excellent at provoking CTL responses, as long as T-cell help can be bypassed.

Important structural changes are likely to accompany protein maturation to middle age, such as post-translational modifications like glycosylation, sulfation, phosphorylation, and stability, such as secondary and tertiary structure, perhaps stabilized by the formation of intermolecular disulfide bonds or by the binding of cofactors. All of these modifications represent significant differences compared to newborn proteins, when considering them as substrates for the antigen-processing machinery, and may well require the action of different antigen-processing components (such as low pH, GILT, or stress-proteins) before they join the common endogenous pathway for processing. Such differences are likely to underpin the observation that, while the EBV protein EBNA1 cannot be processed by proteasome when it is synthesized endogenously, anti-EBNA1 CTLs can be efficiently primed, presumably as a result of cross-presentation and the action of processing components that neutralize the inhibitory glycine-alanine repeat domain within this protein.

Phagolysosome processing

The processing of some antigens has been shown to be sensitive to lysosomotropic agents including heat-inactivated Sendai virus (122), hepatitis B surface antigen (123), and recombinant *Salmonella* (124). Interestingly, antigens adsorbed onto latex beads, which would enter APCs via ER-mediated phagocytosis, have also been shown to be sensitive to lysosomotrophic drugs (124), although it is not known whether processing of this antigen is also TAP- and proteasome-independent. Very little is known about the mechanism of MHC class I loading with peptides in these examples. One possible mechanism is 'regurgitation', in which peptides generated in (or possibly transported back into) phagosomes are disgorged into the extracellular fluid, where they are captured by peptide-receptive class I molecules at the cell surface (124). Other mechanisms exist, which could include the delivery of class I molecules to phagosomes by ER-fusion, sequon-directed targeting, or recycling from the cell surface (125).

Processing of heat-shock protein-associated peptides

Both cytosolic (hsp90, hsp70) and ER (GRP94, calreticulin) chaperones isolated from tumors elicit strong antitumor CTL responses by virtue of their ability to bind to intracellular peptides, some of which will be immunogenic. Not only do these chaperones act as vehicles for the delivery of partially processed peptides to the class I-processing pathway, but also they activate DCs and so act as biological adjuvants. DCs express several receptors by which they can internalize chaperone-peptide complexes (such as LOX-1, SRA, CD40, CD91, and TLR2), but the route taken by these internalized complexes and the point at which they deliver their bound peptides to the class I-processing pathway are not known. An excellent discussion of this topic can be found in Berwin & Nicchitta (126). A study by Germain and colleagues (127) has shown that, after internalization by macrophages, hsp70 can relinquish its load of antigenic peptide either to the cytosol, which leads to the loading of class I molecule in the ER via the TAP and proteasome-dependent pathway, or to endosomes, which leads to loading of class I molecules in these vesicles. The only factor determining which route was taken by peptide was the orientation of the hsp-binding region of the peptide-cargo. When the antigenic-peptide epitope SIINFEKL was appended at the C terminus with an hsp70-binding peptide, it was delivered to the classical class I pathway. When appended at the N terminus with the same sequence, the peptide was loaded onto class I molecules in endosomes. Additionally, there is indirect evidence that hsp-bound peptides generated in bystander cells following transfection after DNA immunization can be transferred to DCs for cross-presentation (128).

Concluding remarks

Sufficient detail of the molecular mechanisms of antigen processing in professional and non-professional APCs has now accumulated to inform the rational design of DNA vaccines. It is likely that the best DNA vaccines for protection against infections and tumors will be those that stimulate both $CD4^+$ and $CD8^+$ T cells. A complete understanding of the cell types in which the DNA immunogen is transcribed and translated, the form in which it is introduced to DCs, the processing enzymes it is exposed to within DCs, and the intracellular location of MHC class I and class II loading therefore will be

important to evaluate vaccine performance and to provide strategies for improvement. Different antigens are likely to require different treatments in the context of DNA vaccination, and it is worth remarking that most of the model systems used to investigate antigen-processing pathways, and to a lesser extent, DNA vaccination strategies employ the recognition of a single ovalbumin-derived epitope (SIINFEKL) presented by H2-Kb in mice. Improvements may include the specific targeting of antigens to DCs or to antigen-processing pathways within DCs. Given the ease with which DNA vaccines can be constructed and tested in preclinical models, progress in this area should be tremendous over the next five years.

References

1. Ulmer JB, et al. Heterologous protection against influenza by injection of DNA encoding a viral protein. Science 1993;**259**:1745–1749.
2. Bennett SR, Carbone FR, Karamalis F, Miller JF, Heath WR. Induction of a CD8+ cytotoxic T lymphocyte response by cross-priming requires cognate CD4+ T cell help. J Exp Med 1997;**186**:65–70.
3. Bouloc A, Walker P, Grivel JC, Vogel JC, Katz SI. Immunization through dermal delivery of protein-encoding DNA: a role for migratory dendritic cells. Eur J Immunol 1999; **29**:446–454.
4. Akbari O, Panjwani N, Garcia S, Tascon R, Lowrie D, Stockinger B. DNA vaccination: transfection and activation of dendritic cells as key events for immunity. J Exp Med 1999;**189**:169–178.
5. Porgador A, Irvine KR, Iwasaki A, Barber BH, Restifo NP, Germain RN. Predominant role for directly transfected dendritic cells in antigen presentation to CD8+ T cells after gene gun immunization. J Exp Med 1998;**188**:1075–1082.
6. Townsend A, et al. Defective presentation to class I-restricted cytotoxic T lymphocytes in vaccinia-infected cells is overcome by enhanced degradation of antigen. J Exp Med 1988;**168**:1211–1224.
7. Michalek MT, Grant EP, Gramm C, Goldberg AL, Rock KL. A role for the ubiquitin-dependent proteolytic pathway in MHC class I-restricted antigen presentation. Nature 1993;**363**:552–554.
8. Rock KL, et al. Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules. Cell 1994; **78**:761–771.
9. Harding CV, et al. Novel dipeptide aldehydes are proteasome inhibitors and block the MHC-I antigen-processing pathway. J Immunol 1995;**155**:1767–1775.
10. Unno M, et al. The structure of the mammalian 20S proteasome at 2.75 a Resolution. Structure (Camb) 2002; **10**:609–618.
11. Niedermann G, et al. The proteolytic fragments generated by vertebrate proteasomes: structural relationships to major histocompatibility complex class I binding peptides. Proc Natl Acad Sci USA 1996;**93**:8572–8577.
12. Kisselev AF, Akopian TN, Woo KM, Goldberg AL. The sizes of peptides generated from protein by mammalian 26 and 20 S proteasomes. Implications for understanding the degradative mechanism and antigen presentation. J Biol Chem 1999;**274**: 3363–3371.
13. Yang Y, Waters JB, Fruh K, Peterson PA. Proteasomes are regulated by interferon gamma: implications for antigen processing. Proc Natl Acad Sci USA 1992;**89**:4928–4932.
14. Gaczynska M, Rock KL, Goldberg AL. Gamma-interferon and expression of MHC genes regulate peptide hydrolysis by proteasomes. Nature 1993;**365**:264–267.
15. Ustrell V, Pratt G, Rechsteiner M. Effects of interferon gamma and major histocompatibility complex-encoded subunits on peptidase activities of human multicatalytic proteases. Proc Natl Acad Sci USA 1995;**92**:584–588.
16. Morel S, et al. Processing of some antigens by the standard proteasome but not by the immunoproteasome results in poor presentation by dendritic cells. Immunity 2000;**12**:107–117.
17. Cascio P, Call M, Petre BM, Walz T, Goldberg AL. Properties of the hybrid form of the 26S proteasome containing both 19S and PA28 complexes. EMBO J 2002;**21**:2636–2645.
18. Craiu A, Akopian T, Goldberg A, Rock KL. Two distinct proteolytic processes in the generation of a major histocompatibility complex class I-presented peptide. Proc Natl Acad Sci USA 1997;**94**:10850–10855.
19. Kessler JH, et al. Efficient identification of novel HLA-A (*) 0201-presented cytotoxic T lymphocyte epitopes in the widely expressed tumor antigen PRAME by proteasome-mediated digestion analysis. J Exp Med 2001;**193**:73–88.
20. Turner GC, Varshavsky A. Detecting and measuring cotranslational protein degradation in vivo. Science 2000;**289**:2117–2120.
21. Schubert U, Anton LC, Gibbs J, Norbury CC, Yewdell JW, Bennink JR. Rapid degradation of a large fraction of newly synthesized proteins by proteasomes. Nature 2000;**404**:770–774.
22. Reits EA, Vos JC, Gromme M, Neefjes J. The major substrates for TAP in vivo are derived from newly synthesized proteins. Nature 2000;**404**:774–778.
23. Princiotta MF, et al. Quantitating protein synthesis, degradation, and endogenous antigen processing. Immunity 2003;**18**: 343–354.
24. Rammensee HG, Falk K, Rotzschke O. Peptides naturally presented by MHC class I molecules. Annu Rev Immunol 1993;**11**:213–244.
25. Bacik I, Cox JH, Anderson R, Yewdell JW, Bennink JR. TAP (transporter associated with antigen processing)-independent presentation of endogenously synthesized peptides is enhanced by endoplasmic reticulum insertion sequences located at the amino but not carboxyl-terminus of the peptide. J Immunol 1994;**152**:381–387.
26. Plemper RK, Bohmler S, Bordallo J, Sommer T, Wolf DH. Mutant analysis links the translocon and BiP to retrograde protein transport for ER degradation. Nature 1997;**388**:891–895.
27. Wiertz EJ, et al. Sec61-mediated transfer of a membrane protein from the endoplasmic reticulum to the proteasome for destruction. Nature 1996;**384**:432–438.
28. Wood P, Elliott T. Glycan-regulated processing of a protein in the endoplasmic reticulum can uncover cryptic cytotoxic T cell epitopes. J Exp Med 1998;**188**: 773–778.
29. Yoshida Y, et al. E3 ubiquitin ligase that recognizes sugar chains. Nature 2002;**418**:438–442.
30. Vinitsky A, Anton LC, Snyder HL, Orlowski M, Bennink JR, Yewdell JW. The generation of MHC class I-associated peptides is only partially inhibited by proteasome inhibitors: involvement of nonproteasomal cytosolic proteases in antigen processing? J Immunol 1997;**159**:554–564.
31. Rose C, et al. Characterization and inhibition of a cholecystokinin-inactivating serine peptidase. Nature 1996;**380**:403–409.

32. Geier E, et al. A giant protease with potential to substitute for some functions of the proteasome. Science 1999;**283**:978–981.
33. Glas R, Bogyo M, McMaster JS, Gaczynska M, Ploegh HL. A proteolytic system that compensates for loss of proteasome function. Nature 1998;**392**:618–622.
34. Li S, Paulsson KM, Chen S, Sjogren HO, Wang P. Tapasin is required for efficient peptide binding to transporter associated with antigen processing. J Biol Chem 2000;**275**:1581–1586.
35. Princiotta MF, et al. Cells adapted to the proteasome inhibitor 4-hydroxy-5-iodo-3-nitrophenylacetyl-Leu-Leu-leucinal-vinyl sulfone require enzymatically active proteasomes for continued survival. Proc Natl Acad Sci USA 2001;**98**:513–518.
36. Seifert U, et al. An essential role for tripeptidyl peptidase in the generation of an MHC class I epitope. Nat Immunol 2003;**4**:375–379.
37. Beninga J, Rock KL, Goldberg AL. Interferon-gamma can stimulate post-proteasomal trimming of the N terminus of an antigenic peptide by inducing leucine aminopeptidase. J Biol Chem 1998;**273**:18734–18742.
38. Reits E, et al. Peptide diffusion, protection, and degradation in nuclear and cytoplasmic compartments before antigen presentation by MHC class I. Immunolity 2003;**18**:97–108.
39. Stoltze L, et al. Two new proteases in the MHC class I processing pathway. Nat Immunol 2000;**1**:413–418.
40. Levy F, et al. The final N-terminal trimming of a subaminoterminal proline-containing HLA class I-restricted antigenic peptide in the cytosol is mediated by two peptidases. J Immunolol 2002;**169**:4161–4171.
41. Saric T, Beninga J, Graef CI, Akopian TN, Rock KL, Goldberg AL. Major histocompatibility complex class I-presented antigenic peptides are degraded in cytosolic extracts primarily by thimet oligopeptidase. J Biol Chem 2001;**276**:36474–36481.
42. Barrett AJ, et al. Thimet oligopeptidase and oligopeptidase M or neurolysin. Meth Enzymol 1995;**248**:529–556.
43. York IA, et al. The cytosolic endopeptidase, thimet oligopeptidase, destroys antigenic peptides and limits the extent of MHC class I antigen presentation. Immunolity 2003;**18**:429–440.
44. Falk K, Rotzschke O, Stevanovic S, Jung G, Rammensee HG. Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. Nature 1991;**351**:290–296.
45. Paz P, Brouwenstijn N, Perry R, Shastri N. Discrete proteolytic intermediates in the MHC class I antigen processing pathway and MHC I-dependent peptide trimming in the ER. Immunolity 1999;**11**:241–251.
46. Yamano T, et al. Two distinct pathways mediated by PA28 and hsp90 in major histocompatibility complex class I antigen processing. J Exp Med 2002;**196**:185–196.
47. Binder RJ, Blachere NE, Srivastava PK. Heat shock protein-chaperoned peptides but not free peptides introduced into the cytosol are presented efficiently by major histocompatibility complex I molecules. J Biol Chem 2001;**276**:17163–17171.
48. Nair S, Wearsch PA, Mitchell DA, Wassenberg JJ, Gilboa E, Nicchitta CV. Calreticulin displays in vivo peptide-binding activity and can elicit CTL responses against bound peptides. J Immunolol 1999;**162**:6426–6432.
49. Lammert E, Stevanovic S, Brunner J, Rammensee HG, Schild H. Protein disulfide isomerase is the dominant acceptor for peptides translocated into the endoplasmic reticulum. Eur J Immunolol 1997;**27**:1685–1690.
50. Breloer M, Marti T, Fleischer B, von Bonin A. Isolation of processed, H-2Kb-binding ovalbumin-derived peptides associated with the stress proteins HSP70 and gp96. Eur J Immunolol 1998;**28**:1016–1021.
51. Montoya M, Del Val M. Intracellular rate-limiting steps in MHC class I antigen processing. J Immunolol 1999;**163**:1914–1922.
52. Elliott T. Transporter associated with antigen processing. Adv Immunolol 1997;**65**:47–109.
53. Velarde G, Ford RC, Rosenberg MF, Powis SJ. Three-dimensional structure of transporter associated with antigen processing (TAP) obtained by single Particle image analysis. J Biol Chem 2001;**276**:46054–46063.
54. Heemels MT, Schumacher TN, Wonigeit K, Ploegh HL. Peptide translocation by variants of the transporter associated with antigen processing. Science 1993;**262**:2059–2063.
55. Shepherd JC, et al. TAP1-dependent peptide translocation in vitro is ATP dependent and peptide selective. Cell 1993;**74**:577–584.
56. Neisig A, Wubbolts R, Zang X, Melief C, Neefjes J. Allele-specific differences in the interaction of MHC class I molecules with transporters associated with antigen processing. J Immunolol 1996; **156**:3196–3206.
57. Saric T, et al. An IFN-gamma-induced aminopeptidase in the ER, ERAP1, trims precursors to MHC class I-presented peptides. Nat Immunolol 2002;**3**:1169–1176.
58. Serwold T, Gonzalez F, Kim J, Jacob R, Shastri N. ERAAP customizes peptides for MHC class I molecules in the endoplasmic reticulum. Nature 2002;**419**:480–483.
59. Koopmann JO, et al. Export of antigenic peptides from the endoplasmic reticulum intersects with retrograde protein translocation through the Sec61p channel. Immunolity 2000;**13**:117–127.
60. Momburg F, Neefjes JJ, Hammerling GJ. Peptide selection by MHC-encoded TAP transporters. Curr Opin Immunolol 1994;**6**:32–37.
61. Neefjes J, et al. Analysis of the fine specificity of rat, mouse and human TAP peptide transporters. Eur J Immunolol 1995;**25**:1133–1136.
62. Lautscham G, et al. Processing of a multiple membrane spanning Epstein-Barr virus protein for CD8(+) T cell recognition reveals a proteasome-dependent, transporter associated with antigen processing-independent pathway. J Exp Med 2001;**194**:1053–1068.
63. Elliott T, Willis A, Cerundolo V, Townsend A. Processing of major histocompatibility class I-restricted antigens in the endoplasmic reticulum. J Exp Med 1995;**181**:1481–1491.
64. Wei ML, Cresswell P. HLA-A2 molecules in an antigen-processing mutant cell contain signal sequence-derived peptides. Nature 1992;**356**:443–446.
65. Henderson RA, et al. HLA-A2.1-associated peptides from a mutant cell line: a second pathway of antigen presentation. Science 1992;**255**:1264–1266.
66. Snyder HL, Yewdell JW, Bennink JR. Trimming of antigenic peptides in an early secretory compartment. J Exp Med 1994;**180**:2389–2394.
67. York IA, et al. The ER aminopeptidase ERAP1 enhances or limits antigen presentation by trimming epitopes to 8-9 residues. Nat Immunol 2002;**3**:1177–1184.
68. Serwold T, Gaw S, Shastri N. ER aminopeptidases generate a unique pool of peptides for MHC class I molecules. Nat Immunol 2001;**2**:644–651.
69. Lauvau G, et al. Human transporters associated with antigen processing (TAPs) select epitope precursor peptides for processing in the endoplasmic reticulum and presentation to T cells. J Exp Med 1999;**190**:1227–1240.
70. Brouwenstijn N, Serwold T, Shastri N. MHC class I molecules can direct proteolytic cleavage of antigenic precursors in the endoplasmic reticulum. Immunolity 2001;**15**:95–104.
71. Williams A, Peh CA, Elliott T. The cell biology of MHC class I antigen presentation. Tissue Antigens 2002;**59**:3–17.
72. Ciernik IF, Berzofsky JA, Carbone DP. Induction of cytotoxic T lymphocytes and antitumor immunity with DNA vaccines expressing single T cell epitopes. J Immunolol 1996;**156**:2369–2375.
73. Dantuma NP, Lindsten K, Glas R, Jellne M, Masucci MG. Short-lived green fluorescent proteins for quantifying ubiquitin/

proteasome-dependent proteolysis in living cells. Nat Biotechnol 2000;**18**:538–543.
74. Rodriguez F, Zhang J, Whitton JL. DNA immunization: ubiquitination of a viral protein enhances cytotoxic T-lymphocyte induction and antiviral protection but abrogates antibody induction. J Virol 1997;**71**:8497–8503.
75. Cheng WF, et al. Tumor-specific immunity and antiangiogenesis generated by a DNA vaccine encoding calreticulin linked to a tumor antigen. J Clin Invest 2001; **108**:669–678.
76. Jakob T, Walker PS, Krieg AM, Udey MC, Vogel JC. Activation of cutaneous dendritic cells by CpG-containing oligodeoxynucleotides: a role for dendritic cells in the augmentation of Th1 responses by immunostimulatory DNA. J Immunolol 1998;**161**:3042–3049.
77. Maecker HT, Umetsu DT, DeKruyff RH, Levy S. Cytotoxic T cell responses to DNA vaccination: dependence on antigen presentation via class II MHC. J Immunolol 1998;**161**:6532–6536.
78. Watts C. Capture and processing of exogenous antigens for presentation on MHC molecules. Annu Rev Immunolol 1997;**15**:821–850.
79. Mitchell RN, et al. Intracellular targeting of antigens internalized by membrane immunoglobulin in B lymphocytes. J Exp Med 1995;**181**:1705–1714.
80. Albert ML, et al. Immature dendritic cells phagocytose apoptotic cells via alphavbeta5 and CD36, and cross-present antigens to cytotoxic T lymphocytes. J Exp Med 1998;**188**:1359–1368.
81. Scott RS, et al. Phagocytosis and clearance of apoptotic cells is mediated by MER. Nature 2001;**411**:207–211.
82. Ogden CA, et al. C1q and mannose binding lectin engagement of cell surface calreticulin and CD91 initiates macropinocytosis and uptake of apoptotic cells. J Exp Med 2001;**194**:781–795.
83. Jiang W, et al. The receptor DEC-205 expressed by dendritic cells and thymic epithelial cells is involved in antigen processing. Nature 1995;**375**:151–155.
84. Delneste Y, et al. Involvement of LOX-1 in dendritic cell-mediated antigen cross-presentation. Immunolity 2002;**17**:353–362.
85. Becker T, Hartl FU, Wieland F. CD40, an extracellular receptor for binding and uptake of Hsp70-peptide complexes. J Cell Biol 2002;**158**:1277–1285.
86. Wang Y, et al. CD40 is a cellular receptor mediating mycobacterial heat shock protein 70 stimulation of CC-chemokines. Immunolity 2001;**15**:971–983.
87. Berwin B, et al. Scavenger receptor-A mediates gp96/GRP94 and calreticulin internalization by antigen-presenting cells. EMBO J 2003;**22**:6127–6136.
88. Vabulas RM, et al. The endoplasmic reticulum-resident heat shock protein Gp96 activates dendritic cells via the Toll-like receptor 2/4 pathway. J Biol Chem 2002;**277**:20847–20853.
89. Basu S, Binder RJ, Ramalingam T, Srivastava PK. CD91 is a common receptor for heat shock proteins gp96, hsp90, hsp70, and calreticulin. Immunolity 2001;**14**:303–313.
90. Garin J, et al. The phagosome proteome: insight into phagosome functions. J Cell Biol 2001;**152**:165–180.
91. Lennon-Dumenil AM, Bakker AH, Wolf-Bryant P, Ploegh HL, Lagaudriere-Gesbert C. A closer look at proteolysis and MHC-class-II-restricted antigen presentation. Curr Opin Immunolol 2002;**14**:15–21.
92. Manoury B, Hewitt EW, Morrice N, Dando PM, Barrett AJ, Watts C. An asparaginyl endopeptidase processes a microbial antigen for class II MHC presentation. Nature 1998;**396**:695–699.
93. Arunachalam B, Phan UT, Geuze HJ, Cresswell P. Enzymatic reduction of disulfide bonds in lysosomes: characterization of a gamma-interferon-inducible lysosomal thiol reductase (GILT). Proc Natl Acad Sci USA 2000;**97**:745–750.
94. Driessen C, Lennon-Dumenil AM, Ploegh HL. Individual cathepsins degrade immune complexes internalized by antigen-presenting cells via Fcgamma receptors. Eur J Immunolol 2001;**31**:1592–1601.
95. Rodriguez GM, Diment S. Destructive proteolysis by cysteine proteases in antigen presentation of ovalbumin. Eur J Immunolol 1995;**25**:1823–1827.
96. Bennett K, et al. Antigen processing for presentation by class II major histocompatibility complex requires cleavage by cathepsin E. Eur J Immunolol 1992;**22**:1519–1524.
97. Donermeyer DL, Allen PM. Binding to Ia protects an immunogenic peptide from proteolytic degradation. J Immunolol 1989;**142**:1063–1068.
98. Sercarz EE, Maverakis E. Mhc-guided processing: binding of large antigen fragments. Nat Rev Immunol 2003; **3**:621–629.
99. Maric M, et al. Defective antigen processing in GILT-free mice. Science 2001; **294**:1361–1365.
100. Sette A, Adorini L, Colon SM, Buus S, Grey HM. Capacity of intact proteins to bind to MHC class II molecules. J Immunolol 1989;**143**:1265–1267.
101. Antoniou AN, Blackwood SL, Mazzeo D, Watts C. Control of antigen presentation by a single protease cleavage site. Immunolity 2000;**12**:391–398.
102. Kropshofer H, Hammerling GJ, Vogt AB. The impact of the non-classical MHC proteins HLA-DM and HLA-DO on loading of MHC class II molecules. Immunolol Rev 1999;**172**:267–278.
103. Wang RF, Wang X, Atwood AC, Topalian SL, Rosenberg SA. Cloning genes encoding MHC class II-restricted antigens: mutated CDC27 as a tumor antigen. Science 1999;**284**:1351–1354.
104. Bonehill A, et al. Efficient presentation of known HLA class II-restricted MAGE-A3 epitopes by dendritic cells electroporated with messenger RNA encoding an invariant chain with genetic exchange of class II-associated invariant chain peptide. Cancer Res 2003; **63**:5587–5594.
105. Ruff AL, Guarnieri FG, Staveley-O'Carroll K, Siliciano RF, August JT. The enhanced immune response to the HIV gp160/LAMP chimeric gene product targeted to the lysosome membrane protein trafficking pathway. J Biol Chem 1997;**272**:8671–8678.
106. Dutronc Y, Porcelli SA. The CD1 family and T cell recognition of lipid antigens. Tissue Antigens 2002;**60**:337–353.
107. Nimmerjahn F, et al. Major histocompatibility complex class II-restricted presentation of a cytosolic antigen by autophagy. Eur J Immunolol 2003;**33**:1250–1259.
108. den Haan JM, Bevan MJ. Antigen presentation to CD8+ T cells: cross-priming in infectious diseases. Curr Opin Immunolol 2001;**13**:437–441.
109. Sigal LJ, Crotty S, Andino R, Rock KL. Cytotoxic T-cell immunity to virus-infected non-haematopoietic cells requires presentation of exogenous antigen. Nature 1999;**398**:77–80.
110. Freigang S, Egger D, Bienz K, Hengartner H, Zinkernagel RM. Endogenous neosynthesis vs. cross-presentation of viral antigens for cytotoxic T cell priming. Proc Natl Acad Sci USA 2003;**100**:13477–13482.
111. Corr M, von Damm A, Lee DJ, Tighe H. In vivo priming by DNA injection occurs predominantly by antigen transfer. J Immunolol 1999;**163**:4721–4727.
112. Brossart P, Bevan MJ. Presentation of exogenous protein antigens on major histocompatibility complex class I molecules by dendritic cells: pathway of presentation and regulation by cytokines. Blood 1997;**90**:1594–1599.

113. Isenman LD, Dice JF. Selective release of peptides from lysosomes. J Biol Chem 1993;**268**:23856–23859.
114. Reis e Sousa C, Germain RN. Major histocompatibility complex class I presentation of peptides derived from soluble exogenous antigen by a subset of cells engaged in phagocytosis. J Exp Med 1995;**182**:841–851.
115. Gagnon E, et al. Endoplasmic reticulum-mediated phagocytosis is a mechanism of entry into macrophages. Cell 2002;**110**:119–131.
116. Desjardins M. ER-mediated phagocytosis: a new membrane for new functions. Nat Rev Immunolol 2003;**3**:280–291.
117. Guermonprez P, Saveanu L, Kleijmeer M, Davoust J, Van Endert P, Amigorena S. ER-phagosome fusion defines an MHC class I cross-presentation compartment in dendritic cells. Nature 2003;**425**:397–402.
118. Houde M, et al. Phagosomes are competent organelles for antigen cross-presentation. Nature 2003;**425**:402–406.
119. Ackerman AL, Kyritsis C, Tampe R, Cresswell P. Early phagosomes in dendritic cells form a cellular compartment sufficient for cross presentation of exogenous antigens. Proc Natl Acad Sci USA 2003;**100**:12889–12894.
120. Lizee G, et al. Control of dendritic cell cross-presentation by the major histocompatibility complex class I cytoplasmic domain. Nat Immunolol 2003;**4**:1065–1073.
121. Kurlander RJ, Chao E, Fields J, Nataraj C. The adjacent flanking region plays a critical role in facilitating the presentation of the Listeria monocytogenes product lemA to H2 M3wt-restricted, peptide-specific murine CD8 cells. J Immunolol 1999; **163**:6741–6747.
122. Liu T, Chambers B, Diehl AD, Van Kaer L, Jondal M, Ljunggren HG. TAP peptide transporter-independent presentation of heat-killed Sendai virus antigen on MHC class I molecules by splenic antigen-presenting cells. J Immunolol 1997;**159**:5364–5371.
123. Schirmbeck R, Reimann J. Peptide transporter-independent, stress protein-mediated endosomal processing of endogenous protein antigens for major histocompatibility complex class I presentation. Eur J Immunolol 1994;**24**:1478–1486.
124. Harding CV, Song R. Phagocytic processing of exogenous particulate antigens by macrophages for presentation by class I MHC molecules. J Immunolol 1994;**153**:4925–4933.
125. Gromme M, et al. Recycling MHC class I molecules and endosomal peptide loading. Proc Natl Acad Sci USA 1999; **96**:10326–10331.
126. Berwin B, Nicchitta CV. To find the road traveled to tumor immunity: the trafficking itineraries of molecular chaperones in antigen-presenting cells. Traffic 2001;**2**:690–697.
127. Castellino F, et al. Receptor-mediated uptake of antigen/heat shock protein complexes results in major histocompatibility complex class I antigen presentation via two distinct processing pathways. J Exp Med 2000;**191**:1957–1964.
128. Kumaraguru U, Rouse RJ, Nair SK, Bruce BD, Rouse BT. Involvement of an ATP-dependent peptide chaperone in cross-presentation after DNA immunization. J Immunolol 2000;**165**:750–759.