MHC ligand generation in T cell-mediated immunity and MHC multimer technologies for T cell detection

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Chapter 1

The cell biology of antigen presentation

THE CELL BIOLOGY OF ANTIGEN PRESENTATION

Innate and adaptive immunity

The immune system protects the body from pathogenic threats such as viruses, parasites and bacteria. Basal immunity in the first place operates by keeping a potentially dangerous pathogen out. The skin, mucus and nasal hair are all examples of providing a barrier between the body and the outside world. However, once a microbe or virus has entered the body many defense mechanisms work to eliminate this potential threat. The basis of the immune system lies in the recognition of self versus non–self and can be separated into two distinct mechanisms: a general defense mechanism against broad classes of organisms and pathogens called the *innate immune response*, and a more specific response that reacts based on the individual types of invading pathogens called *adaptive immunity*. Both mechanisms operate by recognizing that something does not belong inside the body. However, innate and adaptive immunity have two fundamentally different methods to recognize this: the innate immune system operates by specifically knowing what should be considered 'non–self' and adaptive immunity works by specifically knowing what should be considered 'self'.

In order to detect non–self, cells of the innate immune system contain pathogen recognition receptors that recognize molecular motifs associated with pathogens, the so–called pathogen–associated molecular patterns (PAMPs). These receptors, of which the most well known are the Toll like receptors (TLRs) (1) and a recently discovered set of RIG–like helicases (2,3), exist both on the outside and inside of the cell. Examples of PAMPs that can be recognized are bacterial lipopolysaccharides, viral single–stranded RNA and specific DNA motifs that are more prevalent in bacteria than in mammals. After triggering of these receptors, inflammatory and signal molecules will be generated. Some of these molecules, such as histamine and cytokines produced by mast cells and basophils, can recruit more cells of the immune response while other molecules, such as RNAses and peroxidases produced by eosinophils, will help to destroy microbes and viruses. In addition, microbes can be eliminated by specialized cells such as neutrophils and macrophages after internalization via a process called phagocytosis. All in all the responses attributed to the innate immune system can be described as static; they are based on specific molecular motifs rather than individual pathogens and do not change during the lifetime of an organism.

The adaptive immune system also responds to a pathogen with the main goal to clear it from the system. But it aims for an important additional effect: to improve the host's defense for whenever the same pathogen is encountered again and therefore is more specific in its response than innate immunity. This so–called immunological memory is one of the most important aspects of adaptive immunity. The main cell types of the adaptive immune response are T and B lymphocytes. Both display an antigen receptor on their cell surface that needs to be triggered by recognition of specific pathogen–derived molecules in order to activate the cell. A key aspect of the adaptive immune system is the ability to generate a vast amount of different antigen receptors on the surface of T and B cells (although each individual cell will generally display only a single type

of receptor). This large potential repertoire of antigen receptors (estimated for T cells to be 10^{13}–10^{15} in mouse and 10^{16}–10^{18} in humans (4–6)) aims for the recognition of any potential pathogen that might invade the body. Any molecular structure that is recognized by the immune response is called an antigen (hence the name antigen receptor), while the small fragment of an antigen that actually triggers a T cell receptor (TCR) or B cell receptor (BCR) is called an epitope. Antigens can be many structures such as proteins, lipids, polysaccharides and nucleic acids. This thesis however will exclusively focus on proteinaceous antigens and epitopes. Furthermore, the recognition of pathogens by and activation of B cells via their B cell receptor lies beyond the scope of this thesis, and the focus will mainly lie on the generation of epitopes and processing of antigens in the context of T cells and their T cell receptors.

T cells recognize epitopes that are presented to them by other cells of the body. The epitopes are embedded in a molecular complex on the cell surface called Major Histocompatibility Complex, or MHC. This complex comes in two forms: MHC class I and MHC class II. MHC class I complexes are present on all nucleated cells, while MHC class II complexes primarily exist on specialized cells of the immune system called antigen presenting cells (APCs). In most cells of the body, MHC class I complexes at the cell surface display a sample of peptides that are generated from proteins inside the cell itself. This means that at any given time, epitopes presented by MHC class I on the cell surface represent a blueprint of the internal status of that particular cell. In addition, APCs present peptides on their MHC class II complexes that have been generated from internalized proteins. MHC class II–bound epitopes therefore represent the status of the extracellular environment of the APC. Certain specialized APCs also have the ability to bind epitopes generated from extracellular material to their MHC class I molecules, through a process called cross–presentation.

T cells can be divided in two distinct subtypes based on their MHC recognition, with each type named after a co–receptor involved in MHC–TCR interactions on the cell surface: $CD4^+$ T cells recognize epitopes in the context of MHC class II, while $CD8^+$ T cells interact with MHC class I complexes. After activation, $CD8^+$ T cells (also called cytotoxic T cells) have the ability to directly kill infected cells that present the appropriate epitope on their surface. $CD4^+$ cells (or helper T cells) on the other hand aid the immune response with cytokine production and the transfer of activation signals after they have recognized their cognate epitope.

How are T cells able to distinguish between peptides on the cell surface that are derived from host proteins (self) and those derived from pathogens (non–self)? After all, only the latter should activate the immune response. The basis of this distinction originates from the method by which the T cells are generated in the thymus. During T cell development the T cell receptor locus of both $CD4^+$ and $CD8^+$ T cells undergoes genetic rearrangement. This process of V(D)J recombination results in a wide range of different TCRs on individual T cells with a semi–random specificity. After expression of the rearranged TCR, the cells are tested whether the receptor has high enough affinity to interact with MHC complexes. Only T cells that have the intrinsic ability to recognize MHC complexes will survive this positive selection step. Next, T cells in the thymus that recognize endogenous peptides on MHC complexes will be deleted. This process of negative

selection is facilitated by the fact that thymic epithelial cells display a wide range of pMHC complexes with endogenous peripheral peptides (7). Only T cells that have high enough affinity for pMHC complexes and do not respond to endogenous pMHC complexes will develop into mature T cells. This process of T cell selection thus ensures that T cells in the periphery do not recognize endogenous peptides, but only get activated when unknown foreign material is presented to them. Or in other words, the T cell pool knows what is self and will react to anything that is not part of this self repertoire. Any defects in the thymic T cell selection steps can lead to autoimmune diseases in which T cells attack non–infected tissues. One of the most well known examples of this is type I diabetes mellitus.

This thesis is about the generation and use of epitopes in the context of MHC class I and class II. In the following parts of this introduction the different mechanisms of antigen processing and epitope generation will be discussed as well as the cell types that play a role in generating these antigens.

MHC class I antigen presentation

MHC class I molecules are heterotrimeric complexes consisting of a polymorphic heavy chain (HC), an invariant light chain called ß2–microglobulin (ß2m) and a peptide ligand –the epitope– of approximately 8–11 amino–acid long. These peptide–MHC (pMHC) complexes are recognized by the TCR of $CD8^+$ T cells in a peptide–specific fashion, and this interaction forms the molecular basis of antigen recognition by $CD8^+$ T cells. Three polymorphic genes code for MHC class I heavy chains: HLA–A, –B and –C in humans and H2–K, –D and –L in mice, and consequently each individual has 3–6 different types of MHC class I complexes for peptide presentation to T cells. To date, over 2000 different MHC class I alleles have been identified (http://www.ebi.ac.uk/imgt/hla/stats.html). The HC and ß2m are cotranslationally translocated into the endoplasmic reticulum (ER), where they are incorporated into the peptide–loading complex (PLC). This protein complex is a multi subunit structure containing the proteins HC, ß2m, TAP, calreticulin, ERp57, tapasin (8,9), and, possibly, PDI (10,11). The PLC maintains disulfide bond formation of the MHC complex (11–13) and stabilizes HC and ß2m in the absence of peptide (14). The PLC also facilitates peptide loading by keeping the peptide–binding groove of the MHC complex into a peptide–receptive state, favors binding of high affinity peptides (15–18) and brings HC and ß2m in the vicinity of the peptides that are transported into the ER by TAP. After MHC has associated with a high affinity peptide, the PLC dissociates and the pMHC complex is transported to the cell surface via the golgi apparatus, where it can present the peptide to $CD8^+$ T cells.

Peptides that are loaded onto MHC class I are generated from endogenous proteins (and cross–presented material, see section below) marked for degradation. This includes cytosolic proteins at the end of their functional lifespan, misfolded ribosomal products and ER–resident proteins retrotranslocated back into the cytosol. These products are all targeted to the default ubiquitin–proteasome degradation pathway regulating protein turnover and amino acid recycling. As an intrinsic part of the degradation process, antigenic peptides will be generated for presentation on MHC class I complexes. The key protease involved in cellular protein degradation and peptide generation is the proteasome. This cytosolic complex is capable of degrading large protein structures into fragments of 2–25 amino acids (19,20) and is the only protease in the cytosol able to generate

carboxi–termini of MHC class I binding peptides (21). Recently it was established that fragments longer than 15 amino acids are subsequently processed by the peptidase TPP2 (22,23) and several cytosolic aminopeptidases, such as TOP and LAP, can further degrade the fragments into even smaller products for recycling. Peptides of 8–16 amino acids long that are generated during these degradation steps can be transported into the ER via the transporter TAP (24,25), after which they are available for MHC binding in the PLC. The ER–resident aminopeptidase ERAAP has recently been shown to further trim peptides to 8–10 amino acids (26–28) in agreement with the fact that MHC class I molecules bind peptides of 8–11 amino acids long (29). Only a small portion of total protein content will be turned into antigenic peptides, while the majority of products will be degraded. It is estimated that from all the peptides generated during proteasomal processing, less than 0.1% ultimately bind to MHC class I (30–35).

Since epitopes are generated by proteolysis, two sources of these peptides can be distinguished: native proteins that are degraded at the end of their functional lifespan, and faulty translation products tagged for destruction. This latter group is commonly referred to as defective ribosomal products (DRiPs) (36). While the kinetics with which peptides are derived from DRiPs will be rapid, since destruction of defective proteins occurs within hours after translation, epitopes originating from stable proteins are generated depending on their cellular stability, with half–lives ranging between 10 minutes to 2 weeks and the average being around 1–2 days (37). Considering that the goal of antigen presentation is to display internal pathogenic infections to the outside world, it is important that peptides are generated from pathogenic material rapidly after infection. Indeed, peptides derived from several viral proteins are displayed on the cell surface within hours after infection, despite the long–lived nature of the native source proteins (38–40). In addition, experiments examining the fraction of newly synthesized host proteins that is degraded faster than the half–life of the mature protein indicate that the rapid generation of antigenic peptides is not limited to pathogenic material (41–43). This is in agreement with the 'DRiP hypothesis' postulating that the majority of antigenic epitopes originate from newly synthesized polypeptides that are degraded shortly after their translation rather than from long–lived native proteins (36,43). Recently this hypothesis has been expanded with the suggestion that proteins should be divided in rapidly and slowly degrading polypeptides (RDP and SDP, respectively), regardless whether proteins are folded correctly or not (44). The model proposes that RDPs contribute the bulk of antigenic epitopes. This might be possible due to the presence of currently completely hypothetical immunoribosomes (44) specialized in generating epitopes for MHC binding. Whether these immunoribosomes will explain the observed correlation between protein synthesis and epitope generation remains to be seen and to date no single DRiP has been identified (45), leading to doubts whether this specialized mechanism of epitope generation actually exists (46).

With the display of antigenic peptides on the cell surface that reflect the inner status of that cell, the immune system has developed a way to monitor for potential pathogens infecting cells of the body. Cellular pathogens such as bacteria and viruses make use of the protein synthesis mechanism of the host they have infected, and protein turnover is similarly mediated

by the host's proteasomal degradation pathway described above. Therefore, epitopes presented to $CD8^+$ T cells by an infected cell will also contain peptides derived from a pathogen inside. Since $CD8^+$ T cells that recognize epitopes from self–tissue have been eliminated in the thymus during negative selection, it is exactly these foreign peptides bound to MHC class I that will be recognized by cytotoxic T cells, leading to the destruction of that particular cell, ensuring an effective T cell–mediated adaptive immune response.

MHC class II antigen presentation

MHC class II complexes are heterotrimers structurally similar to class I, with two membrane–anchored heavy chains, the a– and ß–chain. The peptide–binding groove formed by these two chains differs in that fact that the C– and N–termini of peptides are not buried in the groove and therefore allows for longer peptides to bind. Generally, MHC class II–bound antigenic peptides are 15–24 amino acids long. There are 3 different class II alleles in human: HLA–DQ, HLA–DP and HLA–DR, while mouse has two: H2–A and H2–E (or I–A and I–E, respectively). MHC class II complexes are primarily present on specialized cells that have the ability to sample the extracellular environment. These antigen presenting cells (APCs) can take up both soluble and particulate material and include dendritic cells (DCs), macrophages and B cells. The process of taking up extracellular material is called endocytosis, and the cellular route that this material traverses inside a cell is called the endocytic pathway. Binding of antigenic peptides by MHC class II also takes place in compartments of the endocytic pathway, but the alfa and beta chain are assembled in the ER. Therefore, the mechanism of loading MHC class II molecules with antigenic peptides is fundamentally different than that of MHC class I. To prevent ER–resident peptides from binding to MHC class II, the peptide binding groove is occupied by a transmembrane protein called the invariant chain (Ii) during assembly of the two heavy chains. Ii also aids with the folding of the MHC class II molecule, and mediates transport from the ER to the endocytic pathway (47,48). This latter process is initiated after Ii trimerizes, forming nonamers of three MHC class II molecules. This large complex is then transported to the MHC class II compartment (MIIC) (49,50), where Ii is processed by proteases such as cathepsins (Cat) S and L and AEP (51). This results in a small portion of Ii that remains bound to the peptide binding groove of MHC called CLIP. An MHC–like protein called HLA–DM (H–2M in mice) then mediates removal of CLIP and assists in keeping the peptide binding groove open (52). Peptides that have been generated in the endocytic pathway and survived complete degradation can then bind to MHC class II and it is believed that HLA–DM also plays a role in this binding step, ensuring that high affinity peptides bind over low affinity epitopes (53). However, the exact mechanisms of peptide binding to MHC class II complexes and displacement of Ii remain incompletely understood (10).

APC display a wide–range of mechanisms to take up extracellular antigen. Examples are receptor–mediated endocytosis, phagocytosis, and macropinocytosis. All internalized material ends up in the endocytic pathway. This pathway consists of a network of intracellular vesicles that have the ability to fuse with one another. Although the endocytic pathway is not linear and contains several points where vesicles can fuse or branch off, the pathway can be roughly subdivided in 3 stages. Each part is defined by the internal acidity of the vesicles, where the pH progressively

decreases further down the pathway. Early endosomes have a pH of around 6.0, which is lowered via late endosomes (pH 5.0–6.0) to a pH of 4.5–5.0 in lysosomes. Material that ends up in lysosomes is rapidly degraded by the protease content of these vesicles and lysosomes play a large role in degradation and recycling of proteinaceous material. As mentioned, the endocytic pathway is not strictly linear: differential modes of uptake can target material to different parts of the pathway (54–57) and more vesicle types exist than the three mentioned above (50,58), but how this differentiation is regulated remains largely unclear.

A variety of proteases reside in the different vesicles of the endocytic pathway, ranging from aspartic proteases (CatD) to cysteine proteases (CatB, F, L, S, Z and AEP). Most of these are generated in the ER as inactive proenzymes and become active by pH–dependent cleavage of a propiece, ensuring activity only in the appropriate compartments (59,60). Processing of extracellular material in the endocytic pathway into antigenic peptides is dependent on the activity of these different proteases in the different compartments of the endocytic route. One of the first step in antigen processing is the reduction of possible disulfide bonds (61). This is mediated by the enzyme GILT (62), although there are most likely other unidentified factors involved (63). After this step the antigen is structurally more accessible for proteolysis by the different enzymes. In this unfolded state, however, the core of the protein will in most cases not be accessible for cleavage since most proteases in the endocytic route are exoproteases. Indeed, an 'unlocking' step by the endopeptidase AEP has been shown to be important for the generation of several MHC class II epitopes (64,65), although, as with GILT, this protease is not essential for epitope generation (66). Many exoproteases have been identified that play a role in antigen processing, of which the cysteine proteases comprise the largest fraction. The different proteases process internalized antigen while it traverses the endocytic route. Several proteases only act in a strict pH range making them active in specific sections of the endocytic route. Interestingly though, to date none of the proteases that are active in MHC class II related antigen processing have been identified to have a substantial nonredundant function in peptide generation (10). This could mean that the activity of the different proteases in each endocytic compartment overlaps in the generation of antigenic peptides. Or this could mean that the role of a protease in one compartment can be taken over by another in a different section of the endocytic route. Although it has been shown that in the absence of several cathepsins the same MHC class II epitopes can still be generated as in wild type cells, the quantity of these epitopes has not been investigated in great detail. It remains unclear by what mechanism the observed redundancy takes place.

As with MHC class I, there needs to be a balance between destruction of material and generation of antigenic peptides (67). Before antigenic material reaches the lysosomal compartment where final destruction takes place in the highly acidic environment, the endocytic pathway intersects with the transport route of MHC class II molecules and MIIC compartments can be formed. These contain both antigenic material and the MHC molecules and here CLIP is exchanged with epitopes by HLA–DM as described above. Once a stable MHC complex is formed, transport to the cell surface is initiated after which epitopes can be presented to $CD4^+$ T cells.

In recent years it has become apparent that different cell types will process extracellular antigens differently after uptake, which has a direct effect on the generation of MHC class II epitopes. Macrophages, B cells and DCs have long been indicated as the key APCs for triggering CD4 T cell responses. However, these cell types differ in the speed with which material is internalized, degraded and processed ((68) and this thesis) and different modes of uptake will target material to different compartments (54–56). Rather than a model in which several cell types play similar roles in eliciting an immune response, each APC is more and more emerging as a specialized player in immunity. Macrophages seem to be more important for degrading material, removing extracellular debris and encapsulating extracellular bacteria than triggering the adaptive immune system. Additionally, some macrophages are providing antigenic material to B cells rather than T cells (69). In B cells, the circumstances under which the BCR is triggered has direct influence on MHC class II loading (70). And DCs are especially capable of migrating to lymphoid organs, plus the transition from an immature to a mature phenotype is accompanied by strong upregulation of co–stimulatory molecules and MHC class II surface expression (71,72). This makes them quite suited for T cell activation. Finally, the different APCs express a different protease profile. Cat L is absent in B cells and DCs, while Cat S is predominantly expressed in these cells. In macrophages on the other hand both cysteine proteases are present (73). Although the basic mechanism of MHC class II loading and peptide generation is similar in these cells, the fact that individual APC types perform specialized functions is reflected by the fact that they differ in their methods of MHC class II–related antigen processing. What the exact differences between APC types are and how they are unique in the generation of epitopes from extracellular material is something that is currently investigated by many research groups.

Cross–presentation

The generation of epitopes as described thus far, in which endogenous proteins are the sole source of epitopes for MHC class I molecules and MHC class II molecules are occupied with exogenous material, does not hold true for all cells involved in the adaptive immune response. Considering that naïve $CD8^+$ T cells reside in the lymphoid organs while pathogens generally infect cells in the periphery there is a spatial incompatibility for the initiation of the adaptive immune response through antigen presentation. Additionally, in order to activate $CD8^+$ T cells interactions between the MHC class I complex and the TCR alone are not sufficient; interactions between co–stimulatory molecules play a crucial role as well. These co–stimulatory molecules are only present on specific cell subsets in the body and most notably on APCs. Therefore, a primary infection in the periphery in most cases will not directly trigger a $CD8^+$ response, even if pathogen derived epitopes are presented by MHC class I complexes of the infected cells. The T cells in the lymphoid organs with the appropriate TCR first require a signal that they are needed, after which they will get activated and leave the lymphoid organs. In order to achieve this, a mechanism called cross–priming exists, where naïve $CD8^+$ T cells in the lymphoid organs are activated by APCs that are not infected themselves (74).

As mentioned above, dendritic cells are capable of internalizing and processing exogenous material for presentation on MHC class II molecules. These cells are at the same time also capable of processing

extracellular antigen for presentation on MHC class I complexes (75–77). Although other APCs than DCs are able to cross–present antigens (78–82), only DC possess all the mechanisms needed to activate naive $CD8^+$ T cells, such as maturation, co–stimulation and migration after an encounter with pathogens or their associated PAMPs (71,72). Therefore it is generally accepted that DCs are the most relevant cross–priming cell type to activate $CD8^+$ T cells *in vivo*. It is well established that cross–priming plays a key role in activating $CD8^+$ T cells (83–88), although direct priming of T cells also takes places in the initiation of an immune response (89–92). What remains unclear and is currently hotly debated is the mechanism through which external antigens are processed into MHC class I epitopes. Which proteases process external epitopes for presentation on class I? Does a distinct mechanism exist, or are the previously described class I and class II pathways sufficient? And finally, where are the epitopes bound to MHC class I?

It is important to consider in what form cross–presented material enters DCs. Over the years, many forms of antigen have been shown to be cross–presented: soluble proteins (77,93–95) immune complexes (96–98), and cell–associated antigens (82,86,88,99–100) are all examples of material that can be cross–presented by DCs. Cell–associated material was the first form of antigen described for cross–presentation *in vivo* (74) and has also been established as the most efficient form of antigen to be cross–presented. Soluble material can also be cross–presented (77,93–95) but with much lower efficiency than cell–associated material (99). Several publications have proposed that cross–presented material is coupled to chaperones such as heat shock proteins and that by this process oligopeptides are a source for cross–presentation as well (101,102). However, several other groups have not been able to verify and confirm the relevance of this mode of transfer (103). As an alternative mode, this thesis and work done by several other labs provide evidence that proteasomal substrates rather than proteasomal products are the source of cross–presented material (104–108 and this thesis), arguing that stable proteins are the predominant source of antigen for cross–presentation (105,109 and this thesis). At present this is indeed the most commonly endorsed hypothesis. The group of Yewdell recently expanded on this notion and shows that stability is a crucial factor in cross–presentation (110): an exceptionally stable oligopeptide is quite capable of being cross–presented and interestingly, this required the heat shock protein HSP90. Whether this will reignite the debate on the role of chaperones in cross–priming remains to be seen. Another mechanism through which oligopeptides potentially can be transferred between cells for cross–presentation has been put forth by the lab of Neefjes, where connexin–mediated gap–junctions play a role in peptide–transfer between cells (111). It must be said however that to date this mechanism has only been shown in cultured cells (112) and other groups have failed to establish the same pathway in Langerhans cells (113). More work needs to be done to verify the *in vivo* relevance of this mechanism. Despite all these remaining uncertainties and different potential mechanisms, it is clear that protein levels in donor cells are a key factor in cross–presentation and changes in the stability of the cross–presented material directly affect the efficiency of cross–priming.

Another controversy is the mechanism through which cross–presented material is converted to MHC class I epitopes. Since in

general proteins rather than oligopeptides are the transferred material, proteolytic processing needs to take place in the DC after uptake. Regardless whether soluble or cellular antigens are internalized, the material ends up in the endocytic pathway through the uptake process described above, where a multitude of proteases are active. However, the protease that is predominantly responsible for the generation of MHC class I products from endogenous proteins, the proteasome, resides in the cytoplasm. It seems likely that the proteasome is involved in the generation of cross–presented epitopes, since for an effective $CD8^+$ T cell response the epitopes generated by cross–presentation and direct presentation need to be the same. Indeed, several groups have reported a requirement for the proteasome in the generation of cross–presented epitopes (94,114). Similarly, TAP–dependency has also been described (94,96,114,115) although quite a number of reports show TAP independent cross–presentation does exist (116–118). Endocytic protease activity plays a role in generating epitopes via cross–presentation as well. Inhibition of endocytic hydrolases enhances cross–presentation (119,120), while for some epitopes a dependency on CatS has been found (121). It currently remains unclear what the exact roles of the different proteases are in generating epitopes via cross–presentation, but as said, it is generally believed that proteasomal cleavage plays a major role at one point in the process. This raises the next question: how does cross–presented material reach the proteasome and where are the epitopes bound to MHC class I molecules? In 2002 and 2003 several papers shook the field by describing a mechanism in which the ER–membrane and phagocytic vesicles fuse to form an endocytic vesicle equipped with all the components of the previously described peptide–loading complex PLC, including TAP (58,122–124). This ER–phagosome fusion would allow for a self–sufficient mechanism where phagosomes are completely equipped for processing of external antigens and loading of epitopes onto MHC class I molecules. A model emerged where proteasomes were associated to these ER–phagosomes and the transporter Sec61 would transport antigens outside the vesicles, after which proteasomal cleavage could take place. Transport via TAP back into the vesicle would then allow for binding to MHC class I. This is an attractive model, but several issues remain. It has been questioned whether ER–phagosome fusion actually takes place (125) and its critics argue that indeed all groups describing the fusion model have generally used the same technique to analyze the vesicles. On top of that, the size of the proposed channel Sec61 would only allow for small antigens to be transported to the cytosol (126), thereby limiting the range of epitopes that can be generated, which does not agree with the experimentally found range of epitopes. It is of course possible that other transporters are involved, such as those of the Derlin family (8,127,128), or that pre–processing takes place inside the ER–phagosome. Despite the issue of transporter size, the evidence for a role of the retrograde translocation machinery is compelling, especially in the case of soluble antigens. Soluble antigens could directly access the ER and enter the cytosol through the Sec61 channel there (95,129). And it is entirely possible that the specific mode of uptake of the antigen plays a crucial role in this differential processing (130).

Despite the many questions that remain it is quite clear that cross–presentation plays a crucial role in initiating an effective immune response besides the 'more traditional' forms of antigen presentation by MHC class I and class II molecules.

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