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

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Citation

Bakker, A. H. (2009, October 29). *MHC ligand generation in T cell-mediated immunity and MHC multimer technologies for T cell detection*. Retrieved from <https://hdl.handle.net/1887/14268>

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

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

Proefschrift

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van de Rector Magnificus prof. mr. P.F. van der Heijden,
volgens besluit van het College voor Promoties
te verdedigen op donderdag 29 oktober 2009
klokke 16:15

door

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geboren te Haarlem
in 1977

Promotiecommissie

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voor mijn ouders

The research described in this thesis was performed at the Department of Immunology at the Netherlands Cancer Institute (NKI), Amsterdam and the Department of Pathology at Harvard Medical School, Boston.

Part of these studies were financially supported by the Landsteiner Stichting voor Bloedtransfusie Research (LSBR Grant 0522).

The printing of this thesis was financially supported by the NKI, Universiteit Leiden, Sanquin Reagents, and Stichting Melanoom.

Cover image: detail of '*Book of Oblivion 22*' by Ad Arma. Reproduced with permission from the artist.

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SCOPE OF THIS THESIS

This thesis focuses on the generation of MHC ligands and their use in analyzing T cell immunity, both in mouse and man. It is roughly split into two sections: the first part deals specifically with the rules governing the generation of MHC ligands, while the second part describes technological advances in the use of these MHC ligands to analyze T cell immunity.

The first part of this thesis starts with an introduction on antigen presentation, covering both the different mechanisms through which epitopes are generated and the process by which epitopes are presented to T cells. Emphasis lies on the discoveries in this field of the last decade. This introduction is followed by two chapters on the generation of antigenic epitopes. Chapter two describes the analysis of protease activity in the endocytic pathway, while chapter three investigates the antigenic source of epitopes generated during cross-presentation.

The second part of this thesis starts with a review on the status of MHC multimer technology as a tool to analyze antigen specific T cell populations. Chapter five then describes a high-throughput approach to generate a vast array of different peptide-MHC complexes for several human MHC alleles, allowing for faster and more complex applications of MHC multimer technology. In the next chapter, the peptide-exchange technology of chapter 5 is used for the development of another novel strategy: multidimensional encoding of peptide-MHC complexes. This chapter describes the development, validation and use of this encoding technique that allows the parallel detection of antigen-specific T cell responses of up to 25 T cell populations in one single sample.

Finally, this thesis concludes with a summary and discussion chapter, giving a short overview of the presented data and discussing its relevance in the field of antigen presentation.

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 $\beta 2$ -microglobulin ($\beta 2m$) 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 $\beta 2m$ 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, $\beta 2m$, 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 $\beta 2m$ 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 $\beta 2m$ 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 α - and β -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 α and β 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 propeptide, 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 steps 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 MHC 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 place 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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Chapter 2

Analysis of protease activity in live antigen-presenting cells shows regulation of the phagosomal proteolytic contents during dendritic cell activation

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J Exp Med. 2002 Aug 19;196(4):529–40

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Analysis of Protease Activity in Live Antigen-presenting Cells Shows Regulation of the Phagosomal Proteolytic Contents During Dendritic Cell Activation

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Abstract

Here, we describe a new approach designed to monitor the proteolytic activity of maturing phagosomes in live antigen-presenting cells. We find that an ingested particle sequentially encounters distinct protease activities during phagosomal maturation. Incorporation of active proteases into the phagosome of the macrophage cell line J774 indicates that phagosome maturation involves progressive fusion with early and late endocytic compartments. In contrast, phagosome biogenesis in bone marrow–derived dendritic cells (DCs) and macrophages preferentially involves endocytic compartments enriched in cathepsin S. Kinetics of phagosomal maturation is faster in macrophages than in DCs. Furthermore, the delivery of active proteases to the phagosome is significantly reduced after the activation of DCs with lipopolysaccharide. This observation is in agreement with the notion that DCs prevent the premature destruction of antigenic determinants to optimize T cell activation. Phagosomal maturation is therefore a tightly regulated process that varies according to the type and differentiation stage of the phagocyte.

Key words: antigen processing • cathepsin • active site–directed probe • phagocytosis • phagosomal maturation

Introduction

MHC class II molecules expressed on the surface of APCs present antigenic peptides to CD4⁺ T lymphocytes. These peptides are produced mainly from antigens that have been internalized and processed in the endocytic pathway of APCs, where they meet class II molecules en route to the cell surface (1, 2). APCs acquire antigens via endocytosis, either nonspecifically or through receptors expressed on their surface (1, 3). The main function of antigen receptors is to target and concentrate antigen in intracellular compartments competent for processing and for interaction of the resulting digestion products with class II molecules (1, 3).

Endocytic proteases play a key role in two different steps of MHC class II–restricted antigen presentation: invariant chain cleavage and antigen degradation. Indeed, invariant chain, which directs class II molecules to the endocytic pathway and protects them from premature peptide binding, must be proteolyzed in endosomal compartments to

allow its replacement with peptide antigen. The cysteine proteases cathepsin (Cat)*S and CatL are implicated in this process (4–6). Antigen processing involves many hydrolase activities present along the endocytic pathway of APCs (6–8). Among these enzymes are the γ -interferon–inducible lysosomal thiol reductase (9, 10), and several cysteine proteases including CatB, CatS, CatL, and asparaginyl endopeptidase (4–8). Limited proteolysis rather than total breakdown of antigens is the rule, because MHC class II molecules present peptides of 9 to 16 residues to CD4⁺ T cells (2). Therefore, a balanced proteolytic environment is required to ensure adequate processing while preventing complete destruction. The molecular mechanisms that control trafficking and exposure of antigens to endocytic proteases are poorly understood.

Phagocytosis is probably a dominant mode of antigen uptake *in vivo* for professional APCs such as dendritic cells (DCs) and macrophages (11). Phagosomes formed by APCs after uptake of latex beads have been shown to be fully

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*Abbreviations used in this paper: Cat, cathepsin; ConB, concanamycin B; DC, dendritic cell; MACS, magnetic-activated cell sorting.

equipped for antigen processing and peptide loading (12). How do phagosomes acquire the proteolytic activities necessary to degrade antigens? The macrophage cell line J774 is a well-characterized model for analysis of phagosome biogenesis (13, 14). In J774 cells, newly formed phagosomes undergo progressive maturation by fusing sequentially with the early endosomal, late endosomal, and lysosomal compartments (13). A proteomic analysis of latex bead-containing phagosomes in J774 cells identified >140 proteins detected at different stages of phagosomal biogenesis (15). In particular, endocytic proteases from the Cat family are gradually incorporated into the phagosome during its maturation, suggesting that these proteases are found all along the endocytic edge, including early endosomes (15). However, whether these enzymes are active in these nonacidic compartments remains to be addressed.

Endocytic proteases are synthesized as inactive zymogens, including a propeptide that is located at the NH₂ terminus of the protein and occupies their active site, thus preventing premature enzymatic activity (6–8). Once in endosomal compartments, the drop in the pH promotes the removal of the propeptide and conversion to the active, mature protease (6–8). The molecular weight of endosomal proteases can therefore be indicative of their state of activation. However, the activity of these enzymes is also set by the milieu in which they function (pH conditions and the presence of small competitive inhibitors; references 6, 7, and 16). The activity of cysteine proteases can be visualized *ex vivo* in crude cell extracts using fluorescent substrates or active site-directed probes (17). The latter correspond to electrophilic substrate analogs that are subject to nucleophilic attack by the cysteine residue in the protease active site. This reaction modifies the enzyme so that it is now covalently and irreversibly attached to the probe. Because the covalent modification by these probes is mechanism-based, the intensity of labeling is proportional to protease activity (17, 18).

Here, we describe an *in vivo* approach to monitor the proteolytic environment encountered by phagocytosed particles upon internalization into the APC. By loading latex beads with an active site-directed probe, we analyzed the activity of the proteases incorporated into the phagosome during biogenesis. This method was first validated using the monocytic cell line J774, and then applied to peritoneal macrophages, bone marrow-derived macrophages, and DCs. Our results indicate that the proteolytic contents of the phagosome depend on both the differentiation stage of the APC and the extracellular stimuli. This implies that the proteolytic environment to which particulate antigens are exposed in the APC is under tight regulation.

Materials and Methods

Mice. C57BL/6 mice were purchased from The Jackson Laboratory. CatB, CatS, and CatL knockout mice have been previously described (19).

Cells and Culture Conditions. J774, a mouse promonocytic cell line, and RAW264.7, a mouse monocyte/macrophage cell line, were obtained from the American Type Culture Collection

and were cultured in RPMI 1640 supplemented with 2 mM glutamine, 10% FCS, and antibiotics.

For the preparation of bone marrow-derived APCs, bone marrow was obtained from 2–4-mo-old mice and APCs were prepared as previously described (20), by culturing in RPMI 1640 with 10% FCS supplemented with 10 ng/ml recombinant mouse GM-CSF (PeproTech). Culture medium was changed at days 2 and 4, and cells were harvested at day 5 or 6. Magnetic cell sorting with anti-CD11c antibody-coated beads was used to separate bone marrow-derived DCs from macrophages according to the manufacturer's protocol (magnetic-activated cell sorting [MACS]; Miltenyi Biotec). Single cell suspensions were preincubated with goat serum and Fc Block (BD Biosciences) for 20 min at 4°C before magnetic separation. After sorting, cells were incubated with anti-CD11c or anti-I-A^b antibodies (BD Biosciences) and cytofluorometric analysis was performed on a FACScan™ using CELL Quest™ software (Becton Dickinson). Inflammatory peritoneal macrophages were generated as previously described (21).

Active Site Labeling of Cysteine Proteases in Cell Lysates. JPM-565 and DCG-04 were synthesized and purified as previously described (18, 22). Cell lysates were prepared in lysis buffer (50 mM sodium acetate, pH 5, 5 mM MgCl₂, 0.5% NP-40) and protein concentration was measured using the Bi-cinchonic acid protein assay (Pierce Chemical Co.). Lysates (25 µg protein/sample) were incubated with DCG-04 for 60 min at 37°C. After boiling in reducing sample buffer for 10 min, samples were analyzed by 12.5% SDS-PAGE and transferred to a polyvinylidene membrane. After blocking with PBS-10% nonfat milk, the membrane was probed with a 1:1,000 dilution of streptavidin-horseradish peroxidase (Amersham Biosciences) in PBS-0.2% Tween 20 for 60 min followed by five washes with PBS-0.2% Tween 20. Enhanced chemiluminescence was used for visualization. We did not observe any difference in the labeling pattern when including 5 mM dithiothreitol in the lysis buffer (unpublished data).

Immunoprecipitation. Cell lysates (100 µg protein) were incubated with 50 µM DCG-04 in 50 µl lysis buffer, pH 5, for 60 min at 37°C. SDS was added to a final concentration of 1%. Samples were boiled for 5 min and the volume was adjusted to 1.5 ml using pH 7.4 lysis buffer (50 mM Tris-HCl, pH 7.4, 5 mM MgCl₂, 0.5% NP-40). Immunoprecipitations were performed as previously described (23) using 5 µl anti-CatL (provided by A. Erikson, University of North Carolina, Chapel Hill, NC; reference 24), 5 µl anti-CatB serum, or 1 ml of the cell culture supernatant from a hybridoma producing an anti-CatS mAb. Samples were analyzed by 12.5% SDS-PAGE and streptavidin blotting.

Purification and Identification of Cysteine Proteases. J774 cell lysates (8–10 mg total protein) were incubated with 5 µM DCG-04 for 60 min at 37°C. As controls, one sample was preincubated with 25 µM JPM-565 for 60 min before the addition of DCG-04, and for another sample the addition of DCG-04 was omitted. Purification of DCG-04-labeled cysteine proteases was performed with streptavidin agarose as previously described (18), except that 0.5% SDS was added and the samples were boiled before purification over the PD-10 column. Samples were run on a 12.5% SDS-PAGE. 1/25 was used for detection by streptavidin blotting. The remainder was Coomassie stained as follows: the gel was fixed in H₂O/25% isopropanol/10% acetic acid for 45 min, stained with 10% acetic acid/0.006% Coomassie brilliant blue G (Sigma-Aldrich) overnight, and destained with 10% acetic acid for 2 h. Polypeptides retrieved on the DCG-04 matrix were excised, digested with trypsin, and analyzed by mass spectrometry using an ion trap liquid chromatography tandem mass spectrometry.

try system (performed by Steven Gygi, Harvard Medical School, Boston, MA).

Subcellular Fractionation. J774 cells were grown in 20-cm dishes. For each time point, three dishes (10^7 cells/dish) were used. Cells were pulsed with 2 μ m YG fluorescent beads (250 μ l/dish; Polysciences) at 37°C, washed three times for 10 min at 4°C with PBS to remove excess beads, and chased at 37°C. Bead-containing compartments were isolated on a sucrose gradient as previously described (13). After centrifugation, both the fluorescent bead-containing compartments and the membranes free of beads were collected, and reducing SDS sample buffer was added. After boiling, proteins were separated by 12.5% SDS-PAGE and streptavidin blotting.

Active Site Labeling of Cysteine Proteases in Live Cells. Cell lines were plated on 12-well plates (0.5×10^6 cells/well) 1 d before the experiment. Streptavidin-coated carboxylated latex beads (1- or 2- μ m diameter; Polysciences) were incubated with DCG-04 for 60 min at room temperature. Beads were washed twice with PBS and resuspended in complete culture medium. Plated cells were washed and pulsed at 37°C with 500 μ l medium containing DCG-04-coated beads for different times. Cells were then washed three times with medium by agitation to remove excess beads and then incubated in medium for different times at 37°C. Medium was removed and cells were lysed with 100 μ l of 2 $\times$ hot reducing SDS sample buffer, supplemented or not with 100 μ M free JPM-565. Lysates were harvested, boiled, and the DNA was sheared with a syringe or sonication. Samples were analyzed by 12.5% SDS-PAGE and streptavidin blotting.

Bone marrow GM-CSF cultures were harvested after 5 or 6 d and pulsed in suspension with DCG-04-coated beads for 5 min at 37°C. In some experiments, 0.1 μ g/ml LPS was added to the cells during the pulse. After the pulse, excess beads were removed by centrifuging them four times at 500 g for 2 min over an FCS cushion. CD11c⁺ and CD11c⁻ cells were separated by MACS. Equal cell numbers (10%) of both populations were incubated for different times in complete medium at 37°C. Cells were centrifuged in the well for 5 min at 1,000 g and lysed with hot 2 $\times$ SDS reducing sample buffer containing 100 μ M JPM-565. The lysates were harvested, boiled, and the DNA was sheared with a syringe or sonication. Half of the sample was analyzed by 12.5% SDS-PAGE and streptavidin blotting. Identical experiments were performed with murine peritoneal macrophages harvested 4 d after thioglycollate medium injection.

Results

We devised a strategy to sample the proteolytic environment encountered by phagocytosed antigens in professional APCs. For this purpose, we used the biotinylated active site-directed probe, DCG-04, coupled to streptavidin-coated latex beads. DCG-04 is a derivative of the peptide epoxide JPM-565 and specifically targets cysteine proteases (Fig. 1 B; reference 18). Probe-coated beads are internalized by APCs through phagocytosis. Bead-containing phagosomes undergo maturation by fusion with the different endocytic compartments of the APC. Once inside the cell, the active site-directed probe senses its proteolytic environment by reacting with active proteases incorporated into the phagosome (Fig. 1 A). This scenario presupposes that probes immobilized via biotin to the latex beads remain available for interaction with proteases via their COOH-terminal ep-

oxide moiety. Because binding of the probe to the protease active site is covalent and irreversible, proteases labeled *in vivo* can then be visualized after direct lysis of the phagocytes in SDS sample buffer, followed by simple electrophoresis and streptavidin blotting. For a given protease, different labeling intensities correspond directly to differences in activity levels. This approach should therefore allow us to evaluate the activity of individual cysteine proteases delivered to the phagosome of APCs during its maturation.

Cysteine Proteases Recognized by the Active Site-directed Probe DCG-04 in Macrophages. The active site-directed probes JPM-565 and its biotinylated version DCG-04 consist of an epoxide moiety linked to a tyrosine and leucine residue with the biotinylated form containing an additional lysine for the attachment of the biotin moiety (Fig. 1 B; references 17, 18, and 22). In crude cell extracts, JPM-565 is a specific inhibitor of cysteine proteases of the papain family including CatB, CatL, and CatS (17). DCG-04 has a specificity very similar to that of JPM-565 and labels CatB, CatH, CatL, CatZ, and CatC in rat liver cell extracts (18). Neither JPM-565 nor DCG-04 are cell permeable as such.

To establish our *in vivo* protease labeling assay, we used the J774 monocytic cell line, which has been extensively characterized in terms of phagosomal biogenesis using latex beads (13, 15). To identify the targets of DCG-04 in J774 cells, lysates were prepared at pH 5 and incubated with an increasing amount of the DCG-04, followed by SDS-PAGE and streptavidin blotting (Fig. 1 C). At least seven distinct polypeptides were detected in the 20–40-kD range, where most of the known active cysteine proteases are expected to migrate. To identify these proteases, DCG-04-labeled cell lysates were immunoprecipitated with antibodies directed against CatB, CatL, and CatS. This allowed the identification of three major DCG-04-labeled species as being CatB, CatL, and CatS (Fig. 1 D). The identity of these enzymes was additionally confirmed by comparing the labeling pattern of WT and CatB⁻, CatS⁻, or CatL-deficient cells (Fig. 4 B). The polypeptide strongly reactive with DCG-04 and of a mass slightly larger than that of CatB did not react with any of the antibodies tested (Fig. 1 C), including a CatH antiserum (unpublished data). DCG-04-labeled cell lysates were therefore incubated with streptavidin-coated agarose beads on a preparative scale and bound material was resolved by electrophoresis followed by Coomassie staining. The polypeptide of interest was excised, digested with trypsin, and analyzed by microbore and electron spray mass spectrometry, allowing its unambiguous identification as CatZ. The three top bands detected in J774 lysates (Fig. 1, C and D) were not considered in our analysis because they were never detected in *in vivo* labeling assays (see below).

In conclusion, DCG-04 recognizes CatB, CatS, CatL, and CatZ in crude cell extracts from J774 cells. Proteomic analysis of latex bead-containing phagosomes in the J774 cell line has shown that CatB, CatS, CatL, and CatZ are indeed the most abundant cysteine proteases incorporated into the phagosome during maturation (15), although the activity of these proteases could not directly be assessed by this proteomic approach. Therefore, DCG-04 is a probe

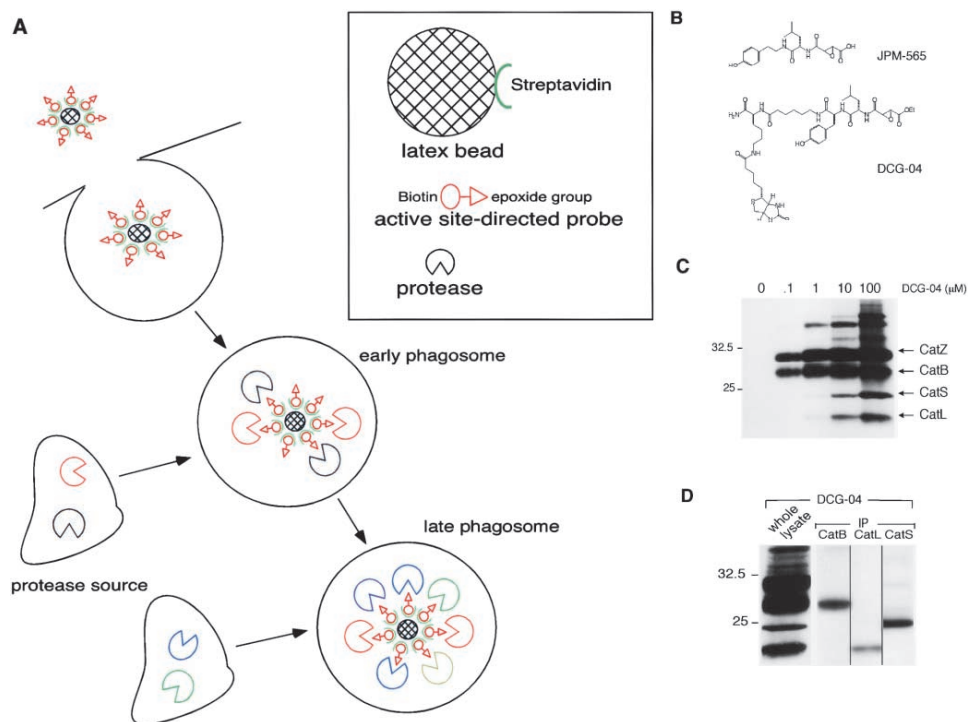


Figure 1. Cysteine proteases recognized by the active site-directed probe DCG-04 in macrophage cell lysates. (A) Schematic overview of the approach designed to examine phagosomal proteolytic contents. To target phagosomal proteases, biotinylated active site-directed probes are coupled to streptavidin-coated latex beads. Upon phagocytosis of the beads, the probe reacts with the active proteases it encounters. Analysis of the modified enzymes at different time points allows a sampling of the proteolytic activities acquired by the phagosome upon fusion with the various endosomal compartments. (B–D) Proteins were separated by SDS-PAGE on a 12.5% gel and reactive proteases were visualized by streptavidin blotting. (B) Structure of the active site-directed probe JPM-565 and its biotinylated derivative DCG-04. (C) Titration of DCG-04 for the labeling of the total cell lysates from J774 cells (pH 5). Lysates were incubated with increasing concentrations of DCG-04. Only the bands in the 15–40-kD range are shown because the larger polypeptides have been shown to be contaminants (reference 18). (D) Identification of individual cysteine proteases labeled by DCG-04. Total extracts from J774 cells (pH 5) were incubated with 5 μ M DCG-04 and subjected to immunoprecipitation using anti-CatB, anti-CatL, or anti-CatS antibodies.

suitable for sampling phagosomal proteolytic activities in live phagocytes.

Visualization of Phagosomal Proteolytic Activity In Vivo. To check whether DCG-04-loaded streptavidin-coated beads can be acquired by phagocytes and target cysteine proteases in endosomal compartments, J774 cells were incubated with DCG-04-coated beads according to the procedure outlined in Fig. 2 A. To ensure that an adequate amount of beads was taken up, cells were pulsed with DCG-04-coated beads for 30 min. Excess beads were removed by washing and cells were chased for 60 min to allow the beads to reach the relevant endocytic compartments. At the end of the chase, cells were lysed in reducing SDS sample buffer immediately followed by heating to prevent postlysis modification of proteases by the probe. Sam-

ples were resolved by electrophoresis and analyzed by streptavidin blotting to visualize the DCG-04-modified polypeptides. DCG-04 coupled to beads labeled CatZ, CatB, CatS, and CatL, demonstrating that the activity of these four enzymes can indeed be visualized in phagosomes of live cells (Fig. 2 B). By loading the latex beads with increasing concentrations of DCG-04, the signal could be enhanced with maximal labeling being achieved at 0.1 μ M DCG-04 (Fig. 2 B).

We performed control experiments to ascertain that the detected proteases were indeed being labeled by the DCG-04 immobilized on the beads. DCG-04 in solution was added to J774 cells to determine if the free compound can be delivered to cysteine proteases by fluid phase endocytosis. We did not detect labeled Cats (Fig. 2 B). To exclude

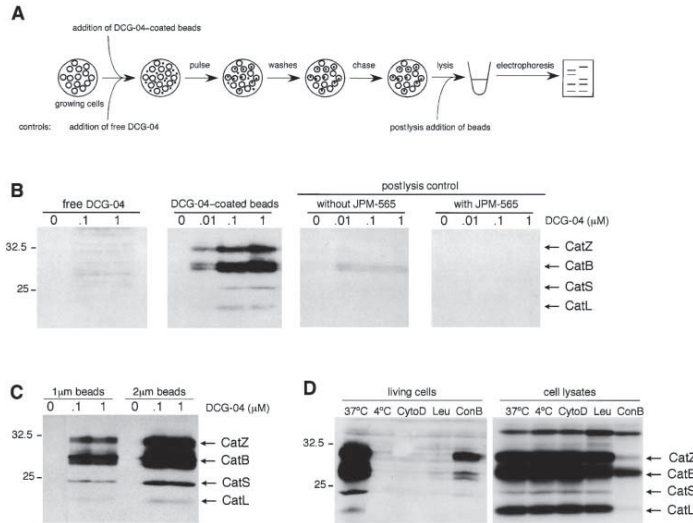


Figure 2. Visualization of phagosomal cysteine protease activity in vivo. (A) Schematic outline of the experimental design. (B–D) Proteins were separated by SDS-PAGE on a 12.5% gel and reactive proteases were visualized by streptavidin blotting. (B) Analysis of phagosomal proteases. J774 cells were incubated for 30 min at 37°C with latex beads previously coated with different concentrations of DCG-04 (pulse). After the removal of excess beads, cells were additionally incubated for 1 h at 37°C (chase). Cells were then directly lysed in 2X concentrated reducing sample buffer. Controls: on the left, cells were treated as in A, but free soluble DCG-04 was added during the pulse instead of DCG-04 coupled to beads, and on the right, cells were treated as in A, but DCG-04-coated beads were added just before lysis in the presence or absence of 100 μ M JPM-565. (C) Analysis of phagosomal proteases comparing beads of 1- and 2- μ m diameter. J774 cells were incubated for 30 min at 37°C with various concentrations of DCG-04 coupled to latex beads. Cells were then washed in PBS, incubated

for an additional 60 min at 37°C, and lysed in sample buffer containing 100 μ M JPM-565. (D) Labeling of phagosomal proteases requires phagocytosis. On the left, J774 cells were pretreated with the indicated inhibitors for 60 min (10 μ g/ml Cytochalasin D, 1 mM Leupeptin, or 20 nM ConB), pulsed with beads coupled to 0.1 μ M DCG-04 for 30 min at 37°C (lanes 1, 3, 4, and 5) or 4°C (lane 2), washed in PBS, and chased for 60 min at 37°C or 4°C before lysis in reducing sample buffer containing 100 μ M JPM-565. On the right, cells were treated as shown on the left except that they were lysed at pH 5. Cell lysates were incubated with 10 μ M DCG-04 for 60 min at 37°C. Note that the two differentially glycosylated forms of CatB appear as a doublet at low expression levels (left).

the possibility that proteases liberated upon cell lysis could bind to the probe despite the fully denaturing conditions of the sample buffer, we added DCG-04-loaded beads to cells just before lysis (Fig. 2 B). A very faint band of CatB was detected under these conditions, suggesting that a small fraction of this protease reacts with the probe at the time of lysis (Fig. 2 B). This postlysis labeling could be suppressed when cells were lysed in SDS sample buffer, to which an excess of nonbiotinylated JPM-565 had been added (Fig. 2 B). For subsequent experiments, an excess of JPM-565 was included in the lysis buffer. Finally, a comparison of 1- and 2- μ m beads showed that 0.1 μ M DCG-04 was saturating for both bead sizes with a stronger signal for 2- μ m beads (Fig. 2 C). We concluded that the amount of protease activities detected is proportional to the amount of probe internalized. Therefore, subsequent experiments were performed with 2- μ m beads incubated with 0.1–1 μ M DCG-04, and cells were lysed in reducing sample buffer containing an excess of JPM-565.

Additional controls exploring pharmacological inhibitors (Cytochalasin D, Concanamycin B [ConB], and leupeptin) or low temperature demonstrated that protease labeling was dependent on phagocytosis of the DCG-04-coated beads. Cells were incubated with DCG-04-coated beads and chased in the presence of inhibitors or at 4°C (Fig. 2 D). Whole cell lysates from each sample were labeled separately with DCG-04 in solution to determine that the inhibitory

effect was due to suppression of phagocytosis rather than a mere reduction in cysteine protease activity (Fig. 2 D). In parallel, fluorescent beads were used to verify whether inhibition of phagocytosis had occurred. Phagocytosis was blocked at 4°C in the presence of Cytochalasin D, an inhibitor of actin polymerization (12), as observed by the absence of colocalization of the fluorescent beads with the lysosomal marker Lamp1 under these conditions (unpublished data). No active cysteine proteases were labeled at 4°C or in Cytochalasin D-treated cells (Fig. 2 D), even though their total activity remained unaffected as shown by labeling of whole cell lysates with DCG-04 in solution (Fig. 2 D). As expected, colocalization of fluorescent beads with Lamp1 was strongly reduced in the presence of ConB (unpublished data), an inhibitor of the H⁺ proton-pumping ATPase that blocks orderly transport along the endocytic pathway, especially at the early to late endosome transition (25, 26). ConB itself did not affect the activity of cysteine proteases in lysates as measured by labeling with DCG-04 (Fig. 2 D). When cells were incubated with DCG-04-coated beads and chased in the presence of ConB, the activity of CatS and CatL was no longer detected in the phagosome, whereas CatB activity was greatly reduced (Fig. 2 D). In contrast, CatZ activity was only slightly reduced by exposure to ConB (Fig. 2 D). This is in agreement with the previous observation that CatZ is incorporated into the phagosome early during maturation

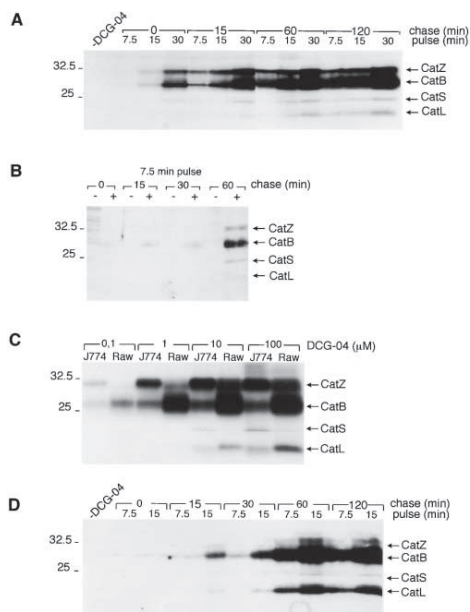


Figure 3. Changes in cysteine protease activity during phagosome biogenesis. (A–D) Proteins were separated by SDS-PAGE on a reducing 12.5% SDS gel and reactive proteins were visualized by streptavidin blotting. (A) Analysis of proteolytic activities incorporated into the phagosome of J774 cells during maturation. Cells were incubated at 37°C with latex beads coupled to DCG-04 as indicated for different periods of time (pulse). After removing excess beads, cells were additionally incubated at 37°C (chase). After each time point, cells were lysed in reducing sample buffer containing 100 μ M JPM-565. (B) Purification of phagosomes and analysis of their proteolytic contents. J774 cells were incubated for 7.5 min at 37°C with DCG-04-coated beads (pulse). After removal of excess beads, cells were incubated for the times indicated at 37°C (chase). Subcellular fractionation was then performed using a sucrose gradient. Both phagosomal (+beads) and nonphagosomal fractions (–beads) were recovered and directly lysed in reducing SDS sample buffer. (C) Comparison of the total contents in cysteine proteases of J774 and RAW264.7 (Raw) cells. Lysates (pH 5) were incubated with various concentrations of DCG-04 for 1 h at 37°C. (D) Analysis of proteolytic activities incorporated into the phagosome of RAW264.7 cells during maturation. Cells were treated as in A.

(15) and would therefore be predicted to be resistant to the effect of such H^+ -ATPase inhibitors. The protease labeling approach used here indicates that the bulk of CatZ is active in early endosomes, even though the pH in these compartments is only mildly acidic. As expected, although leupeptin, a cell-permeable inhibitor of cysteine proteases (27), had no effect on phagocytosis as assessed by the uptake of beads (unpublished data), it completely abolished DCG-04 labeling *in vivo* and partially *in vitro* (Fig. 2 D). We conclude that the labeling of phagosomal proteases by DCG-04-coated beads in living cells is strictly dependent on phagocytosis. Therefore, our approach is suitable to analyze

the proteolytic activities to which particles that have been captured by APCs are exposed.

Monitoring Protease Activity During Phagosome Biogenesis. To monitor the activity of individual cysteine proteases at different stages of phagosome maturation, J774 cells were pulsed and chased with DCG-04-coated beads for different times. We observed a time-dependent increase in active protease labeling (Fig. 3 A). As previously shown by Garin et al. (15), this suggests that active cysteine proteases are not delivered into the phagosome synchronously, but rather incorporated gradually. CatZ and CatB activities were the first detected by DCG-04 labeling (Fig. 3 A), which is consistent with the previous observation that both enzymes are present in early endosomes (15). When cells were chased for longer periods, an increase in total labeling was observed, indicating that the more mature phagosomes were also more proteolytically active. Compared with CatZ, the increase in activity of CatB was more substantial at the later time points, consistent with a continuous delivery of active CatB into the maturing phagosome as previously observed (Fig. 3 A; reference 15). We detected active CatS and CatL only after a 15-min pulse and a 30-min chase, with an additional increase at later chase times (Fig. 3 A). After 2 h of chase, CatB, CatS, CatL, and CatZ activities were all established components of phagosomal proteolytic content (Fig. 3 A). No additional increase in signal was observed between 60 and 120 min of chase (Fig. 3 A). This suggests that DCG-04-coated beads may have reached saturation after these longer chase periods (Fig. 3 A), as it was previously shown that CatS is still delivered 12 h after phagosome formation (15).

Do the observed protease activities reflect phagosomal proteolytic content, rather than diffusion of the probe to acidic endosomal compartments? In other words, does DCG-04 remain attached to the beads during endocytic transport? To address these questions, we isolated the bead-containing compartments by a flotation technique (13). Both the compartments that contain DCG-04-coated beads and the remaining membranes were analyzed for protease content by electrophoresis and streptavidin blotting (Fig. 3 B). Although this method allowed us to visualize active proteases targeted by DCG-04, the procedure was less efficient than the more direct phagocytosis assay (Fig. 3 B). Not until 7.5 min of pulse and 60 min of chase was a signal observed (Fig. 3 B), even though sixfold more cells and beads were used than in the experiment shown in Fig. 3 A. Nonetheless, this experiment showed that DCG-04 remains bound to the beads throughout phagolysosomal maturation, because no signal is observed in fractions that lack beads, even though they contain far more protein (Fig. 3 B). We conclude that the protease activities detected by *in vivo* labeling correspond to active hydrolases incorporated into the phagosome during its maturation.

When we compared lysates prepared from J774 and RAW264.7 cells (a different mouse macrophage cell line) for labeling with increasing amounts of DCG-04, we observed a distinct pattern of labeled proteases. CatB and CatL were more active in RAW264.7 compared with J774 crude cell extracts, whereas there was less CatZ activity

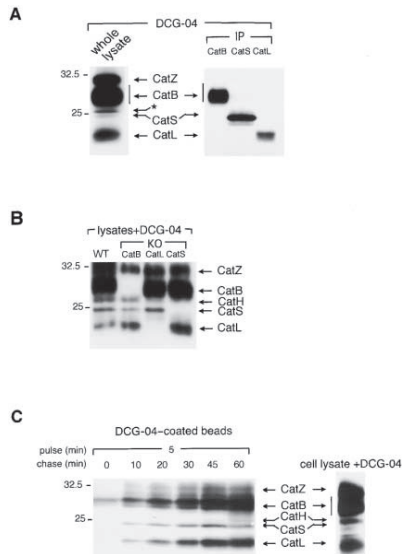


Figure 4. Cysteine protease activity in bone marrow-derived APCs. (A–C) Proteins were separated by SDS-PAGE on a reducing 12.5% SDS gel and reactive proteins were visualized by streptavidin blotting. (A) Analysis of the total contents in cysteine proteases of primary APCs. Total extracts (pH 5) from bone marrow cells cultured in GM-CSF for 6 d were incubated with 0.1 μ M DCG-04 for 1 h at 37°C (left). Total extracts (pH 5) were incubated with 5 μ M DCG-04 and subjected to immunoprecipitation using anti-CatB, anti-CatS, or anti-CatL antibodies (right). *, a labeled protease of unknown identity specific for bone marrow-derived APCs. (B) Analysis of the total contents in cysteine proteases of primary APCs from WT and CatL-deficient mice. Total extracts (pH 5) from bone marrow cells cultured in GM-CSF for 5 d were incubated with 0.1 μ M DCG-04 for 1 h at 37°C. (C) Analysis of proteases incorporated into the phagosome of bone marrow-derived APCs during maturation. Cells cultured in GM-CSF for 6 d were incubated at 37°C with latex beads coupled to DCG-04 for 5 min. After the removal of excess beads, cells were additionally incubated at 37°C (chase). After each time point, cells were lysed in reducing sample buffer containing 100 μ M JPM-565.

(Fig. 3 C). Labeling of CatS in RAW264.7 cells was only barely detectable, even at high concentrations of DCG-04 (100 μ M), indicating that RAW264.7 has a lower level of active CatS than J774 cells (Fig. 3 C). RAW264.7 cells were then incubated with DCG-04-coated beads and chased for different time periods (Fig. 3 D). As for the J774 cell line, the profile of proteases observed in mature phagosome from RAW264.7 cells is comparable to that seen in total cell extracts labeled with DCG-04 (compare the latest chase points from Fig. 3 A and D with Fig. 3 C). This suggests that in these cells, all endosomal compartments containing cysteine proteases fuse with the phagosome during biogenesis. Our results are therefore consistent with progressive phagosomal maturation in these cell lines, involv-

ing distinct endocytic compartments rather than one fusion event with a single target destination, such as the lysosome.

The Phagosome of Primary APCs Selectively Acquires CatS. Do different types of professional APCs use a similar set of endocytic proteases to carry out antigen presentation by class II molecules? Are these proteases accessed in a similar time-dependent manner by internalized particles? How do the distinct APCs modulate the environment of antigens captured by phagocytosis in terms of the proteases they will be exposed to? Having established the methodology for sampling the relevant phagosomal compartments, we applied our protease labeling approach to primary APCs to address these questions.

Mouse bone marrow cells were cultured in GM-CSF, allowing the isolation of two distinct types of professional APCs: DCs (CD11c⁺) and macrophages (CD11c⁻; see Fig. 5). The CD11c⁻ cell population may also contain neutrophils from the granulocyte lineage whose development is equally promoted by GM-CSF. Like macrophages, neutrophils are highly phagocytic cells that are part of the innate immune system but also have the ability to mediate antigen processing and presentation (28). To first analyze the cysteine protease content of bone marrow-derived APCs, day-6 cultures were directly lysed at pH 5 and labeled with soluble DCG-04. Although the activity of CatZ, CatB, CatS, and CatL was detected in these primary APCs, they displayed a more complex pattern of active cysteine proteases than the J774 and RAW264.7 cell lines. In particular, we observed an additional labeled protein migrating at a mass slightly larger than CatS, which was not seen in J774 and RAW264.7 cell lysates (Fig. 4). DCG-04 modification followed by streptavidin-mediated retrieval of the polypeptide and mass spectrometry allowed us to identify it as CatH. The labeling of cells from CatB, CatS, and CatL knockout mice showed that the labeled polypeptides identified as corresponding to these enzymes by immunoprecipitation do not include any other protease (Fig. 4 B). In addition, the presence in bone marrow APC lysates of a labeled polypeptide that comigrates with CatL could be inferred from labeling bone marrow APCs from CatL knockout mice (longer exposed blots; unpublished data). Additional analysis will be needed to establish the identity of this protease.

Next, we performed an *in vivo* analysis of individual protease activities incorporated into the maturing phagosome of bone marrow-derived APCs. As observed for J774 and RAW264.7 cells, the intensity of the signal increases in a time-dependent manner (Fig. 4 B), demonstrating that active proteases are progressively delivered to the phagosomal compartment. However, the relative amount of active CatZ incorporated into the phagosome was considerably lower than the total amount of active CatZ detected in whole cell lysates (Fig. 4, A and B). This indicates that phagosomes of primary APCs show only limited fusion with compartments containing CatZ compared to what we observed in J774 and RAW264.7 cells. Although the same applies to CatH, the opposite is true for the late endocytic protease CatS. Even though little CatS activity is detected in whole cell lysates (Fig. 4 A), relatively large amounts of active CatS are

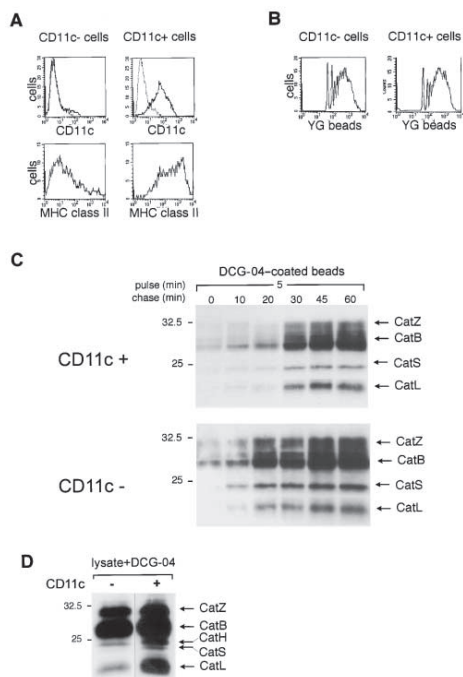


Figure 5. Different rates of protease acquisition by the phagosome of macrophages and DCs. (A) Surface expression of CD11c (top) and MHC class II (bottom) surface in sorted bone marrow-derived APCs. Cells were treated as described in C (see below), incubated with the appropriate antibody, and analyzed by cytofluorometry. Dotted line in top shows isotype control antibody. (B) Uptake of fluorescent latex beads by sorted bone marrow-derived APCs. Cells were treated as described in C. The cells that did not internalize beads (~50%) are not depicted in the histogram because FACS® settings were chosen to visualize the high fluorescence population only. (C–D) Proteins were separated by SDS-PAGE on a 12.5% gel and reactive proteases were visualized by streptavidin blotting. (C) Analysis of proteases incorporated into the phagosome of macrophages (CD11c⁻) and DCs (CD11c⁺). Cells cultured in GM-CSF for 6 d were incubated for 5 min at 37°C with fluorescent yellow beads coupled to DCG-04. Excess beads were removed and cells were separated into CD11c⁺ and CD11c⁻ cells by MACS at 4°C. Equal cell numbers were additionally incubated at 37°C (chase). After the chase, cells were lysed in reducing sample buffer containing 100 μ M JPM-565. (D) Analysis of the total content of cysteine proteases of macrophages (CD11c⁻) and DCs (CD11c⁺). Cells were treated as described in C. CD11c⁻ and CD11c⁺ cells were lysed at pH 5 and incubated for 60 min with 5 μ M DCG-04 at 37°C.

detected in the phagosome (Fig. 4 C). Furthermore, CatS activity is detected earlier during phagosomal biogenesis in primary APCs compared with the macrophage cell lines (compare Fig. 3 and Fig. 4 C). Thus, we conclude that in primary APCs, phagolysosomal fusion preferentially involves endocytic compartments enriched in CatS.

Delivery of Proteases to Phagosomes Is Down Modulated in LPS-activated APCs As mentioned above, mouse bone marrow cells cultured in GM-CSF allow the isolation of two distinct types of professional APCs: DCs (CD11c⁺, MHC class II⁺) and macrophages (CD11c⁻, MHC class II⁺; Fig. 5 A and unpublished data). Expression of MHC class II was induced in the macrophage population by IFN γ (unpublished data).

Do DCs and macrophages display different kinetics of phagolysosomal fusion? To address this question, d6-bone marrow-derived APCs were pulsed with DCG-04-coated beads. After the removal of excess beads by washing, cells were separated by MACS for differential CD11c expression. Separations were performed at 4°C to prevent phagosomal maturation. The CD11c⁻ and CD11c⁺ populations of cells were analyzed by FACS® to verify correct cell separation (Fig. 5 A). A comparison of the DC and macrophage samples (Fig. 5 C) showed that phagosomal maturation occurs more rapidly in macrophages compared with DCs, with maximum levels of protease activity already reached after 20 min of chase (Fig. 5 C). In contrast, the maturation of the DC phagosome progresses more slowly because an increase in CatB and CatL activity is still observed after a chase of 45 min (Fig. 5 C). The difference between macrophages and DCs is not attributable to different amounts of beads having been acquired by these two cell populations, as shown by FACS® analysis of the uptake of fluorescent beads (Fig. 5 B). In fact, when equal amounts of bead-containing cells were lysed at pH 5 and labeled with soluble DCG-04, it was observed that CD11c⁺ cells contain more active cysteine proteases than CD11c⁻ cells (Fig. 5 D). This observation effectively rules out the possibility that the differences observed in the kinetics of phagolysosomal maturation are due to differences in total protease content. Phagolysosomal fusion therefore appears to be a regulated process that varies according to the type of the phagocyte examined.

A remarkable trait of DCs is the phenotypic and functional change evoked by the exposure to inflammatory stimuli, such as bacterial products, e.g., LPS. Indeed, LPS increased B7.2 and MHC class II expression at the surface of our bone marrow-derived DCs (29 and unpublished data). To explore whether phagosome biogenesis in DCs is also modulated by exposure to LPS, day-6 bone marrow cultures were pulsed with DCG-04-coated beads in the presence or absence of LPS for 5 min. After removing excess beads, CD11c⁺ cells were isolated and chased at 37°C. The comparison of untreated and LPS-treated cells revealed drastic differences in the rates of phagosome maturation (Fig. 6 A). Indeed, the delivery of active proteases to the phagosome is considerably delayed in DCs pulsed in the presence of LPS because even after 60 and 120 min of chase, beads have not yet reached saturation (Fig. 6 A and unpublished data). This difference between LPS-treated and control cells does not result from reduced bead uptake by the cells pulsed in the presence of LPS (Fig. 6 C), nor from different amounts of active cysteine proteases (Fig. 6 B).

We were also interested in defining whether the LPS-induced delay in phagosomal maturation is a DC-specific

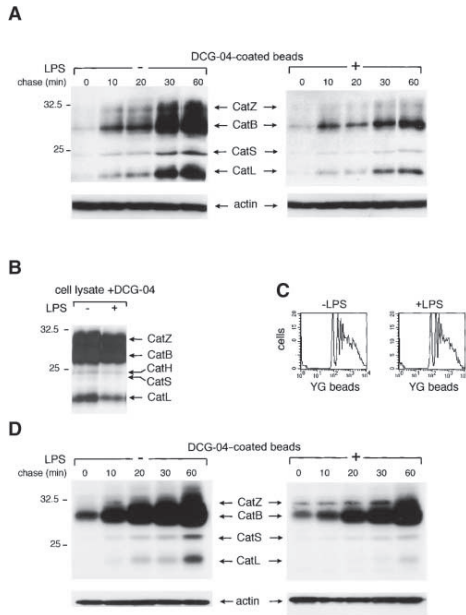


Figure 6. LPS delays phagosome maturation in APCs. (A, B, and D) Labeled proteases were analyzed by SDS-PAGE on 12.5% reducing gel followed by streptavidin blotting. (A) Analysis of proteases incorporated into the phagosome of DCs (CD11c⁺) upon activation. Bone marrow cells cultured in GM-CSF for 6 d were incubated for 5 min at 37°C with fluorescent yellow beads coupled to DCG-04 in the presence or absence of 0.1 µg/ml LPS. Excess beads were removed and CD11c⁺ and CD11c⁻ cells were separated. CD11c⁺ cells were additionally chased at 37°C. After chase, cells were lysed in reducing sample buffer containing 100 µM JPM-565. (B) Analysis of the total contents in cysteine proteases of DCs treated or not with LPS during a 5-min pulse. Cells were treated as described in A. CD11c⁺ cells were lysed at pH 5 and incubated for 60 min with 5 µM DCG-04 at 37°C. (C) Uptake of fluorescent latex beads by bone marrow-derived DCs treated or not with LPS during a 5-min pulse. Cells were treated as described in A. The cells that did not internalize beads (~50%) are not depicted in the histogram because FACS[®] settings were chosen to visualize the high fluorescence population only. (D) Analysis of proteases incorporated into phagosomes of peritoneal macrophages upon LPS activation. Experiments were conducted as described in A.

feature or can be extended to other primary APCs as well. To address this question, we isolated immature peritoneal macrophages and performed uptake experiments with DCG-04-coated beads in the presence or absence of LPS. In line with the results obtained for DCs, LPS treatment of peritoneal macrophages delayed phagosomal maturation when compared with unstimulated cells (Fig. 6 D). The effect was less dramatic in macrophages than in DCs. After a 60-min chase, the proteolytic content of nontreated and LPS-stimulated macrophages were almost equivalent (Fig. 6 D). We conclude that phagolysosomal fusion, and hence exposure of antigen to the proteases in charge of processing

them, is regulated by both the type of APC and the extracellular stimuli they encounter.

Discussion

Here, we describe an approach to directly visualize the activity of proteases that are incorporated into the phagosome at different stages of maturation. Only small numbers of phagocytes are required and there is no need to isolate individual endosomal compartments before analysis. This method is sensitive enough to allow an examination of primary cultures of professional APC, including DCs. Furthermore, the use of covalent active site-directed probes in conjunction with electrophoresis ensures specificity. Methods that employ fluorogenic substrates to detect protease activity suffer from the drawback that more than one enzyme can usually cleave a given peptide substrate. Analysis of the delivery of active hydrolases to the phagosome helped clarify both the distribution of cysteine protease activities among the different endocytic organelles and the dynamics of phagosomal maturation in primary cultures of professional APCs.

We validated our method of *in vivo* labeling of phagosomal proteolytic activities on the mouse monocytic cell line J774, whose phagosomal protein content has been extensively characterized (15). As shown by others, we found that cysteine proteases are acquired by the maturing phagosome of J774 cells sequentially in a time-dependent manner, and not by delivery together (15). We observed a gradual increase in the phagosomal activity of CatZ, CatB, CatS, and CatL, but the rate of increase was different for the four enzymes examined. We detected CatZ activity early during maturation, which is in agreement with the data of Garin et al. (15) who showed that CatZ is one of the first proteases incorporated into the phagosome. Here, we show that CatZ is not only present but also highly active at this early stage of phagosomal biogenesis even though early endosomes are only mildly acidic, an environment not considered optimal for most lysosomal/endosomal hydrolases. By using a fluorogenic substrate, high levels of CatH activity were observed in the early phagosome (30). However, we did not detect any mature CatH in either J774 phagosomes or crude extracts by our method (unpublished data). Moreover, in the proteomic study performed on J774 phagosomes, CatH was not identified as a phagosomal constituent (15). Because CatH and CatZ show strong sequence homology, we think it is possible that both the fluorogenic substrate and the antibody used to identify CatH activity cross reacted with active CatZ. Although CatS is known to be stable and active at neutral pH *in vitro* (31), we did detect CatS activity only at later stages of phagosomal maturation. A similar pattern of activity was displayed by CatL, whereas CatB was continuously incorporated into the phagosome. This suggests that active CatL and CatS are present in late endosomal or lysosomal compartments, whereas CatB is active all along the endocytic pathway. These results are in total agree-

ment with the recently published proteasome analysis of the J774 phagosome (13).

We analyzed bone marrow-derived macrophages and DCs by both in vitro and in vivo active site labeling experiments. In vitro labeling of cell lysates with DCG-04 revealed a more complex pattern of proteases in primary APCs compared with the macrophage cell lines analyzed. In both primary macrophages and DCs, we not only detected the activity of CatZ, CatB, CatS, CatH, and CatL, but also that of several additional species of yet unknown identity. By comparing macrophages and DCs, we found that both APC types display an overall similar pattern of active cysteine hydrolases with certain protease activities being higher in DCs. This applied in particular to CatS, which was barely detectable when labeling macrophage cell lysates. In contrast, the levels of CatZ were only slightly increased in DCs.

Application of our in vivo method showed that in contrast to the macrophage cell lines, there is a selective delivery of proteases to the phagosome of primary APCs. In particular, the relative amount of active CatZ incorporated is considerably lower than the amount of active CatZ detected in whole cell lysates. The opposite was observed for CatS. Even though little CatS activity was detected in whole cell lysates, relatively high levels of CatS activity were detected in the phagosome. Labeling intensities for different proteases cannot be directly related to absolute activities, but changes in the ratios of labeling intensities must correspond to shifts in the protease balance. We estimate that the ratio of CatZ to CatS labeling intensity is >10 in cell lysates, whereas it is <1 when examining phagosomes. Phagosomes from primary APCs must therefore fuse preferentially with endocytic compartments enriched in CatS. This finding raises interesting questions about the relevance of CatZ activity for processing of phagocytosed material. A similar observation was made for CatH, whose labeling ratio to CatS is about >10 in cell lysates, whereas it is <1 in the phagosome. Because in J774 cells most CatZ has been found to reside in early endocytic compartments (15 and this study), phagosomes from primary APCs may undergo only limited fusion with early endosomes. However, we cannot exclude that the subcellular distribution of CatZ in primary APCs is different than in J774 cells. Nonetheless, our data show that the incorporation of active proteases into the phagosome of primary APCs is a selective process that ensures delivery of CatS.

We show that phagosomal maturation occurs more rapidly in macrophages than in DCs. Furthermore, we observed a substantial down-regulation of phagosome maturation in DCs exposed to LPS and to a lesser extent, in LPS-treated macrophages. It is possible that this observation results from the ability of such a stimulus to down-regulate endocytosis in DCs. Indeed, a delay in phagolysosomal fusion could result from a more global decrease in the rates of transport along the endocytic axis. The appearance of class II vesicle-like compartments (32) enriched in MHC class II molecules and early endocytic markers has been reported during DC activation (29). This would allow

DCs to acquire antigen while minimizing the complete destruction of peptide determinants and therefore be beneficial to ensure T cell activation upon arrival in the lymph node (11). In contrast, macrophages are essential players of the innate immune system, can be microbicidal, and constitute an early barrier against infectious agents. Our measurements of protease activity are consistent with these notions.

Based on the usefulness of active site-directed probes for the analysis of protease activity in live cells, we envision the development of a new generation of compounds that can be coupled directly to antigens of immunological relevance, such as intracellular pathogens, apoptotic cells, or single protein antigens. Analysis of the proteolytic environment to which such materials are exposed upon internalization into the APC should help identify the players involved and the sequence in which they act. Do antigens meet proteases immediately after internalization? Does the proteolytic environment to which antigens are targeted differ for distinct APCs and/or modes of internalization? Do features of the antigen itself or extracellular stimuli affect the proteases to which it is exposed in the APC? Certain pathogens not only inhibit phagolysosomal fusion, but may also encode protease inhibitor homologues that could directly affect the activity of surrounding hydrolases (33). These questions may now be addressed by exploring the proteolytic environment encountered by antigen in the course of trafficking in the APC using methodology similar to that reported here.

The authors thank Christine Kocks, José Villadangos, and Guillaume Duménil for their comments on the manuscript, and Steven Gygi and Larry Liekhter for mass spectrometry analysis.

The authors are recipients of fellowships from the Juvenile Diabetes Foundation (A.M. Lennon-Duménil), Boehringer Ingelheim Fonds (R. Maehr), the Max-Kade Foundation (E. Fiebigler), the Netherlands Organization for Scientific Research (H.S. Overkleef), and the American Diabetes Association (Cécile Lagaudrière-Gesbert). This work was supported by grants to H.L. Ploegh from the Juvenile Diabetes Foundation International, through the Juvenile Diabetes Research Foundation Center for Islet Transplantation at Harvard Medical School, from the National Institutes of Health (AI34893 and CA14051), from Boehringer Ingelheim, and Fondecyt (project no. 8000011).

Submitted: 27 February 2002

Revised: 28 June 2002

Accepted: 17 July 2002

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Chapter 3

Antigen bias in T cell cross-priming

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Science 2004 May 28;304(5675):1314–7

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Antigen Bias in T Cell Cross-Priming

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Activated CD8⁺ T cells detect virally infected cells and tumor cells by recognition of major histocompatibility complex class I-bound peptides derived from degraded, endogenously produced proteins. In contrast, CD8⁺ T cell activation often occurs through interaction with specialized antigen-presenting cells displaying peptides acquired from an exogenous cellular source, a process termed cross-priming. Here, we observed a marked inefficiency in exogenous presentation of epitopes derived from signal sequences in mouse models. These data indicate that certain virus- and tumor-associated antigens may not be detected by CD8⁺ T cells because of impaired cross-priming. Such differences in the ability to cross-present antigens should form important considerations in vaccine design.

Cytotoxic CD8⁺ T cells recognize peptides that are presented on the cell surface by major histocompatibility complex (MHC) class I molecules. Two cellular

pathways lead to this form of presentation. In classical antigen presentation, MHC class I molecules bind peptides that are generated via degradation of endogenous

proteins (1). In an alternative pathway, professional antigen-presenting cells (APCs) internalize material derived from virally infected or transformed cells and then present the exogenously derived antigens to naïve CD8⁺ T cells, a process referred to as cross-presentation (2). T cell activation through cross-presentation (cross-priming), can be readily demonstrated for a number of antigens (3–5) but has not been observed for all antigens, including those that are efficiently presented through the endogenous pathway (6, 7).

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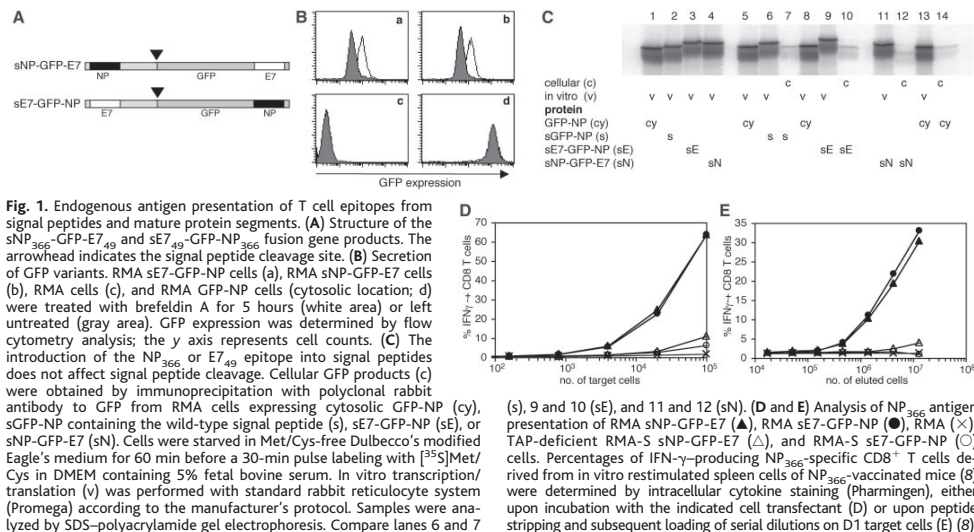
A correction has been published for this article in *Science*. 2004 Sep 24;305(5692):1912: "Antigen bias in T cell cross-priming" by M. C. Wolkers *et al.* (28 May, p. 1314). Two of the symbols in Fig. 3B were denoted incorrectly. The closed triangles represent RMA-S e7-GFP-NP cells, and the closed circles represent RMA-S sNP-GFP-E7 cells.

Whether the parameters governing efficient exogenous and endogenous antigen presentation are equivalent is not clear. To investigate this issue, we generated two green fluorescent protein (GFP) fusion gene constructs that both encode two MHC class I D^b-restricted epitopes. In the first construct (sNP₃₆₆-GFP-E7₄₉), the epitope NP₃₆₆ (derived from influenza A) was inserted into the N terminus of the signal peptide of a secreted GFP molecule. The second epitope, the antigen E7₄₉ (derived from human papilloma virus 16), was inserted close to the C terminus of GFP (Fig. 1A) (8). In the second construct, the same epitopes were introduced but in reverse order (sE7₄₉-GFP-NP₃₆₆) (Fig. 1A). The introduction of each CTL (cytotoxic T lymphocyte) epitope into the hydrophilic fragment of the signal peptide did not affect signal peptide cleavage or intracellular transport of GFP when compared with a GFP molecule harboring an uninterrupted wild-type signal peptide (sGFP-NP) (Fig. 1, B and C) (figs. S1 and S2). This introduction of NP₃₆₆ and E7₄₉ epitopes into the GFP fusion genes allowed us to monitor antigen presentation of two epitopes present in the same protein synthesis product but located in two different protein fragments. Assessing the presentation of signal peptide–encoded epitopes seemed particularly useful because signal peptides represent an important source of endogenously produced MHC class I-binding peptides (9–12).

Presentation of the NP₃₆₆ and E7₄₉ epitopes via the endogenous pathway was examined by introducing the GFP fusion genes into the RMA tumor cell line (13). Cells containing GFP with the NP₃₆₆ epitope located in either the signal peptide or the mature protein were recognized equally well by NP₃₆₆-specific CD8⁺ T cells (Fig. 1D) (8). Similarly, the E7₄₉ epitope was presented efficiently to antigen-specific T cells, whether located in the signal peptide or the mature protein (14). Because saturation of T cell recognition at high antigen densities could potentially mask differences in presentation efficiency, a more quantitative measure of the antigen processing of T cell epitopes was required. To achieve this, we isolated peptides from each transfected cell line by acid elution and used them to sensitize target cells for CTL recognition. In this system, the epitopes contained in the mature protein fragment or in the signal peptide were again presented with comparable efficiency (Fig. 1E). Hence, for this antigen, location within the signal peptide or GFP moiety apparently does not affect the efficiency of endogenous presentation. Endogenous presentation of the NP₃₆₆ epitope present within the hydrophilic segment of the signal peptide was in large part dependent on the transporter associated with antigen processing (TAP), because presentation of NP₃₆₆ by TAP-deficient RMA-S cells containing sNP₃₆₆-GFP-E7₄₉ was inefficient (Fig. 1, D and E) (9, 15). Similarly, endogenous pre-

sensation of the NP₃₆₆ epitope contained within the mature protein required TAP function (Fig. 1, D and E).

To investigate whether antigen location might influence T cell priming by cross-presentation, we challenged naïve mice with RMA-S cells that contain the sNP₃₆₆-GFP-E7₄₉ gene construct. Efficient induction of T cell immunity against the E7₄₉ epitope located within the mature protein was made evident by staining of peripheral blood cells with MHC tetramers containing E7₄₉ (Fig. 2A). In contrast, the NP₃₆₆ epitope contained in the signal peptide of the same fusion gene induced only low numbers of antigen-specific T cells. To determine whether this difference was a direct result of the epitope location within the protein or was due to other factors, such as a difference in T cell precursor frequency, we challenged mice with RMA-S cells containing the fusion gene with the two epitopes in reverse order (sE7₄₉-GFP-NP₃₆₆). In this setting, mice developed a pronounced NP₃₆₆-specific T cell response, whereas the E7₄₉ epitope located within the signal peptide failed to induce efficient T cell immunity, as judged by MHC tetramer staining and by ex vivo interferon- γ (IFN- γ) production (Fig. 2, A and B). In all mice examined ($n = 40$; $P < 0.0001$), T cells specific for the epitope located in the mature protein outnumbered those specific for the signal peptide–encoded epitope. Antigen-specific T cell responses to the C-terminal antigens E7₄₉ and NP₃₆₆ were stronger, on



average by factors of 30 and 16, respectively, than T cell responses against the same epitope when present in the signal peptide. Collectively, these findings indicate that T cell priming by cross-presentation is considerably more efficient for epitopes derived from mature proteins than for epitopes contained within signal peptides.

We wanted to ascertain that the T cell responses induced by tumor inoculation were a result of cross-presentation and were not due to a putative residual capacity of RMA-S cells to present endogenous antigen *in vivo*. Therefore, we sought to establish whether the observed antigen bias would also be evident in a system in which tumor cells lacked the relevant MHC allele (2). Thus, we introduced the two GFP fusion gene constructs into p815 cells (H-2^d background and therefore lacking the required H-2^b-presenting allele) and used them to challenge C57/Bl6 $\times$ Balb/c F₁ (H-2^b $\times$ H-2^d) mice. Efficient antigen-specific T cell induction to E7₄₉ occurred when this epitope was located within the mature protein but, again, not when located within the signal sequence (Fig. 2C). *Ex vivo* T cell responses to NP₃₆₆ were undetectable in this setting (14), potentially because of strain-dependent differences in NP₃₆₆-specific T cell precursor frequency (16). However, when spleen cells of the challenged mice were analyzed for NP₃₆₆-specific T cell responses upon *in vitro* restimulation, a similar bias toward the NP₃₆₆ epitope encoded within the mature protein was apparent (Fig. 2D).

The selective priming of T cell responses against epitopes contained within mature antigens could potentially be a consequence of immunoglobulin (Ig)-protein complex formation. Specifically, Igs recognizing the (secreted) mature protein might form Ig-protein complexes that could be taken up by dendritic cells via Fc receptor-mediated endocytosis (17, 18). To determine whether Ig-protein complex formation is required for the observed bias in antigen-specific T cell immunity, we challenged μ -deficient mice, which lack B cells (19), with RMA-S cells containing either GFP fusion gene construct. Again, T cell responses to the mature antigens were efficiently induced, whereas T cell immunity induced by signal peptide-encoded epitopes was marginal (Fig. 3A). Thus, Ig-protein complex formation does not measurably influence the antigen bias observed in this system.

In an alternative scenario, the difference in the capacity of the two protein fragments to induce T cell immunity by cross-priming might result from a difference in the ability of APCs to extract and present the two epitopes from cells or cellular remnants. To test this idea, we incubated immature murine D1 dendritic cells (20) with irradi-

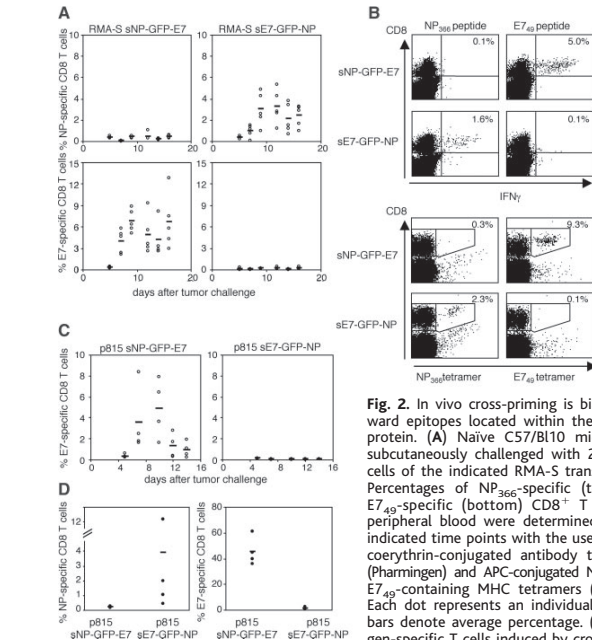


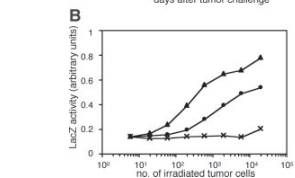
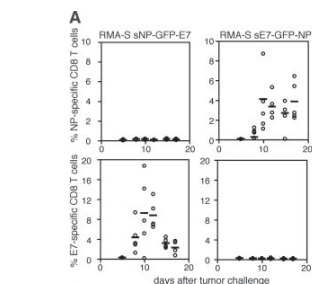
Fig. 2. In vivo cross-priming is biased toward epitopes located within the mature protein. (A) Naive C57/Bl10 mice were subcutaneously challenged with 2×10^6 cells of the indicated RMA-S transfectant. Percentages of NP₃₆₆-specific (top) and E7₄₉-specific (bottom) CD8⁺ T cells in peripheral blood were determined at the indicated time points with the use of phycoerythrin-conjugated antibody to CD8 β (PharMingen) and APC-conjugated NP₃₆₆ or E7₄₉-containing MHC tetramers (27, 28). Each dot represents an individual mouse; bars denote average percentage. (B) Antigen-specific T cells induced by cross-priming are functional. Twelve days after tumor inoculation, IFN- γ production of CD8⁺ T cells was determined directly *ex vivo* upon a 4-hour stimulation with 0.1 μ M NP₃₆₆ or E7₄₉ peptide in the presence of brefeldin A (top) and interleukin-2, and was compared with MHC tetramer staining (bottom). (C and D) MHC tetramer staining of peripheral blood cells (C) or splenocytes (D) from H-2^d $\times$ H-2^b F₁ mice that were challenged with 10^7 cells of the indicated p815 transfectant, measured directly *ex vivo* (C) or after 7 days *in vitro* restimulation with the appropriate peptide (D). Identical results were obtained with H-2^d-restricted mouse embryonic cells that contained either construct (14).

ated RMA-S cells containing either GFP fusion gene construct. Subsequent cross-presentation of the NP₃₆₆ epitope was more efficient by a factor of 10 when derived from the mature protein than when located within the signal peptide (Fig. 3B). Thus, the superior level of T cell induction observed toward epitopes within mature proteins can, at least in part, be attributed to differences in the ability of APCs to cross-present the two classes of antigens.

The observed antigen bias could be a consequence of a specific inability to cross-present T cell epitopes present in signal peptides, or it could reflect a negative effect of N-terminal location of the epitope. As a direct test, we generated a fusion gene construct in which the signal peptide was rendered dysfunctional by removal of the hydrophobic segment that is bound by the signal recognition particle (s*E7₄₉-GFP-NP₃₆₆, Figs. S3 and S4) (8). Mice that were challenged with RMA-S cells transduced with the s*E7₄₉-GFP-NP₃₆₆ construct developed an E7₄₉-

specific T cell response whose magnitude was equal to or greater than that of the NP₃₆₆-specific T cell response (Fig. 3C). These data indicate that poor cross-priming is a direct consequence of location of the epitope within a functional signal peptide.

Prior experiments have sought to determine the contribution of direct priming and cross-priming in the induction of tumor- and virus-specific T cell responses by disrupting either endogenous or exogenous antigen presentation (3–7). Our data show that epitopes derived from signal peptides are efficiently presented through the endogenous pathway but not through the exogenous pathway. Hence, we can analyze the contribution of these two pathways in T cell induction in a setting where both are operational. We challenged naive mice with RMA cells that contained either GFP fusion gene construct. Despite the clear efficiency in endogenous presentation of both epitopes seen in *in vitro* assays (Fig. 1, D and E), antigen-specific T cell responses induced *in vivo* were skewed



epitopes located within a signal peptide. Irradiated RMA-S sNP-GFP-E7 cells ($\blacktriangle$), RMA-S sE7-GFP-NP cells ($\times$), and RMA-S cells ($\times$) were fed to D1 cells, NP₃₆₆-specific fluZ hybridoma cells were added, and T cell activation was determined (8). (C) Antigen bias is dependent on signal peptide location. C57/Bl6 mice were inoculated with 5×10^6 RMA-S sE7₄₉-GFP-NP₃₆₆ cells (left) or RMA-S sE7₄₉-GFP-NP₃₆₆ cells (right). Peripheral blood was analyzed at the indicated time points for NP₃₆₆-specific (top) and E7₄₉-specific (bottom) CD8⁺ T cells by MHC tetramer flow cytometry.

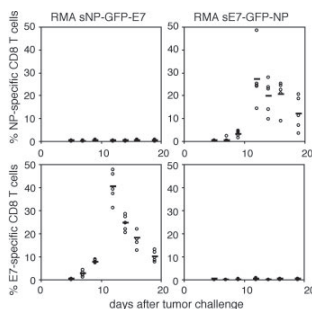


Fig. 4. T cell responses induced after challenge with MHC-proficient RMA cells are biased toward epitopes contained within mature antigens. C57/Bl6 mice were subcutaneously challenged with 5×10^5 RMA sNP₃₆₆-GFP-E7₄₉ cells (left) or RMA sE7₄₉-GFP-NP₃₆₆ cells (right). Peripheral blood was analyzed at the indicated time points for NP₃₆₆-specific (top) and E7₄₉-specific (bottom) CD8⁺ T cells by MHC tetramer flow cytometry.

toward the epitopes contained in the mature antigen, whether NP₃₆₆ or E7₄₉ (Fig. 4). Thus, exogenous antigen presentation proved to be the dominant mechanism for T cell induction, at least in this model system.

Our data show a marked divergence in the efficiency of endogenous and exogenous antigen presentation. The fact that epitopes that are efficiently presented

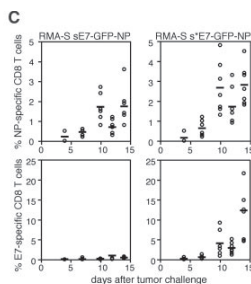


Fig. 3. Differential exogenous presentation of epitopes from signal peptides and mature proteins by dendritic cells. (A) In vivo T cell induction through cross-priming is independent of Igs. NP₃₆₆-specific (top) and E7₄₉-specific (bottom) CD8⁺ T cell responses induced in μ -deficient mice upon tumor inoculation with 2×10^6 RMA-S sNP₃₆₆-GFP-E7₄₉ cells (left) or with RMA-S sE7₄₉-GFP-NP₃₆₆ cells (right). (B) Epitopes derived from mature antigens are more efficiently cross-presented than epitopes located within a signal peptide. Irradiated RMA-S sNP-GFP-E7 cells ($\blacktriangle$), RMA-S sE7-GFP-NP cells ($\times$), and RMA-S cells ($\times$) were fed to D1 cells, NP₃₆₆-specific fluZ hybridoma cells were added, and T cell activation was determined (8). (C) Antigen bias is dependent on signal peptide location. C57/Bl6 mice were inoculated with 5×10^6 RMA-S sE7₄₉-GFP-NP₃₆₆ cells (left) or RMA-S sE7₄₉-GFP-NP₃₆₆ cells (right). Peripheral blood was analyzed at the indicated time points for NP₃₆₆-specific (top) and E7₄₉-specific (bottom) CD8⁺ T cells by MHC tetramer flow cytometry.

through the endogenous pathway are not in all cases efficiently presented upon exogenous presentation may help to explain the lack of cross-priming previously observed in several model systems (6, 7). In this regard, it is useful to note that the lymphocytic choriomeningitis virus (LCMV)-derived GP₃₃ epitope, which has been shown to fail in inducing T cell immunity via cross-priming, is derived from the LCMV GP signal peptide (9). Another interesting issue to address will be whether these findings extend to the efficiency of tolerance induction against peripheral antigens that are not expressed within the thymus. Thus, epitopes derived from peripheral antigens that fail to efficiently enter the cross-presentation pathway may be less likely to be exposed to naïve T cells and may therefore not induce peripheral tolerance (21, 22). Such "ignored" peripheral antigens may prove particularly interesting targets for the induction of tumor-specific T cell immunity, as the T cell repertoire against these antigens may not have been affected by cross-tolerance (23).

How might the bias against signal peptide epitopes be explained at the molecular level? Potentially, signal peptides assemble with cellular factors that reduce their accessibility to the exogenous presentation pathway. Alternatively, and perhaps more likely, if exogenous presentation involves transfer of unprocessed antigens (24), this is likely to be inefficient for molecules that are degraded

rapidly after synthesis (25). In this latter model, exogenous presentation would depend on the steady-state antigen levels in the donor cell, as opposed to the protein synthesis rate for endogenous antigen presentation (26).

The current data suggest a fundamental difference between endogenous presentation and exogenous presentation, a divergence that is likely to influence immunogenicity and immunodominance in virus- and tumor-induced T cell responses. In addition, because vaccine-induced T cell responses often rely on cross-presentation, understanding the rules that determine the efficiency of exogenous presentation should be important in helping to optimize vaccine design.

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29. We thank E. Swart for technical assistance, A. Pfauth and F. van Diepen for flow cytometry assistance, R. Offringa for mouse embryonic cells, P. Ricciardi-Castagnoli for the D1 cells, and J. Neefjes for antibody to GFP. Supported by Netherlands Organization of Scientific Research grant NWO 901-07-233 (N.B.) and by Dutch Cancer Society grants NKI 99-2036 and NKI 2001-2563.

Supporting Online Material
www.sciencemag.org/cgi/content/full/304/5675/1314/DC1

Materials and Methods
Figs. S1 to S4
References

30 January 2004; accepted 13 April 2004

SUPPORTING MATERIAL

Materials and Methods

DNA constructs and cell lines

The gene construct sNP₃₆₆-GFP-E7₄₉ was generated by adding the amino acids **MGVQIASNENMDAMVPCTLLLLLAAALAPTQ** TRAV to the NH₂-terminus of the enhanced Green Fluorescent Protein (GFP). This sequence encodes the NP₃₆₆ epitope (bold) preceded by four NH₂-terminal influenza A NP derived amino acids and followed by the H-2K^b-derived signal peptide (italic). The amino acids **GVQIRAHYNI**VTF SELEKD were introduced at the COOH-terminus of GFP. This sequence encodes the E7₄₉ epitope (bold) preceded by four NH₂-terminal influenza A NP-derived amino acids and followed by a short spacer sequence (underlined) to ensure that antigen presentation required cytosolic processing. In the gene construct sE7₄₉-GFP-NP₃₆₆, the two epitopes were introduced in reverse order, while the sequence of the flanking residues was maintained. The hydrophobic segment of the signal peptide was removed to generate a mutant signal peptide (s*) with the following sequence **MGVQIRAHYNI**VTFVPCTL. The NH₂- and COOH-flanking amino acids were conserved to maintain comparable antigen processing conditions. The gene constructs were inserted into the plasmid pcDNA3.1 for *in vitro* transcription/translation, and into the retroviral vector pMX for introduction into the indicated cell lines as described previously (1). Single cell clones were selected on the basis of matched GFP expression by flow cytometry.

In vitro direct antigen presentation assay

Spleen cells from mice that were immunized with NP₃₆₆-containing RMA tumor cells were restimulated *in vitro* for 14 days in IMDM medium containing 10% fetal bovine

serum, 5x10⁻⁴ μM NP₃₆₆ peptide, and 10 CU human recombinant IL-2/ml (Chiron). NP₃₆₆-specific T cells were incubated with the indicated number of tumor cells, and after 4hr incubation in the presence of Brefeldin A (PharMingen) and IL-2, the percentage of IFN γ -producing CD8⁺ T cells was determined. For peptide elution, peptides were stripped from tumor cells by boiling in 10% acetic acid that contained 10μM of an irrelevant peptide, and passed through a 10 kDa cut-off filter. Serial dilutions were loaded on 5x10⁴ D1 cells, and activation of NP₃₆₆-specific T cell cultures was determined by intracellular IFN γ -staining.

In vitro cross-presentation assay

The indicated RMA-S tumor cells were irradiated with 80 Gray and incubated at 37° C for 24 hrs. Subsequently, 1x10⁵ D1 cells were incubated for 48 hrs with the indicated amount of tumor cells. 1x10⁵ NP₃₆₆-specific fluZ hybridoma cells were added for 16 hrs and T cell activation was determined by monitoring the conversion of chlorophenol red-galactopyranoside to chlorophenol red at 595 nm (2). In this assay, irradiation of tumor cells is required to avoid overgrowth of the cell cultures by the tumor cells but is not essential for antigen uptake by D1 cells. Addition of tumor cell supernatant did not induce detectable cross-presentation by D1 cells, indicating that the source of the material that is cross-presented is cell-associated (3).

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Figures

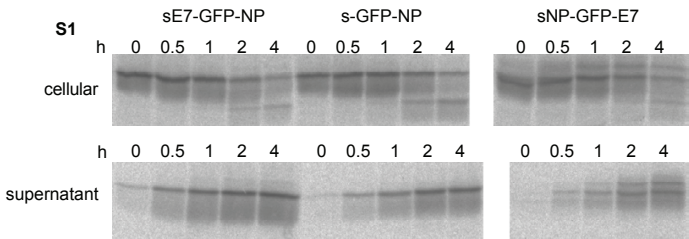


Fig. S1

The introduction of the NP₃₆₆ or E7₄₉ epitope into signal peptides does not affect secretion of GFP variants. Indicated RMA cell transfectants were starved in Met/Cys-free DMEM medium for 60 min prior to a 30 min pulse labeling with ³⁵S-labeled Met/Cys in DMEM medium containing 5% fetal bovine serum. Labeled cells were washed with cold medium, and samples of cells and supernatant were harvested at indicated time points. After cell lysis, GFP variants were immunoprecipitated with polyclonal rabbit anti-GFP. Samples were analyzed by SDS-PAGE. Approximately 50% of the sNP₃₆₆-GFP-E7₄₉ migrates at a higher molecular weight at later chase time points. This material is also secreted and may represent a glycosylated form of this protein.

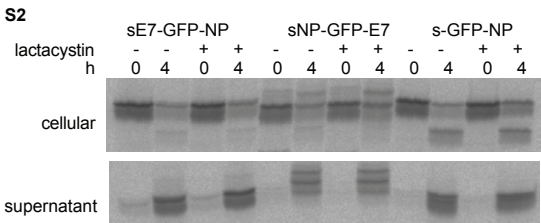


Fig. S2

The introduction of the NP₃₆₆ or E7₄₉ epitope into signal peptides does not affect targeting of nascent proteins to the ER, and does not prevent secretion. Indicated cell lines were starved in Met/Cys-free DMEM medium for 60 min prior to a 30 min pulse labeling with ³⁵S-labeled Met/Cys in DMEM medium containing 5% fetal bovine serum in the presence or absence of proteasome inhibitor lactacystin (Calbiochem) at a final concentration of 10μM. Labeled cells were washed with cold medium, and cultured in the continued presence or absence of lactacystin. Cells were harvested at the indicated time points. GFP variants were immunoprecipitated with polyclonal rabbit anti-GFP, and samples were analyzed by SDS-PAGE.

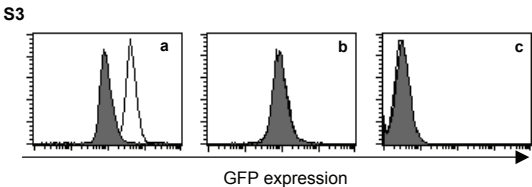


Fig. S3
Deletion of the hydrophobic signal peptide segment yields a cell-associated GFP variant. RMA sE7-GFP-NP cells (a), RMA s*E7-GFP-NP cells (b), and RMA cells (c), were treated with Brefeldin A for 5hrs (white area) or left untreated (gray area). GFP expression was determined by flow cytometry analysis.

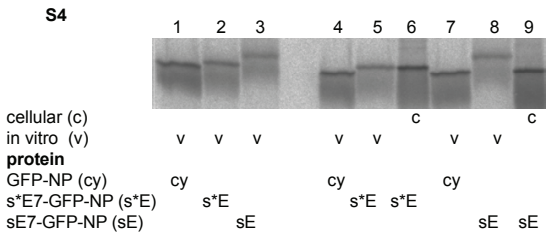


Fig. S4
Deletion of the hydrophobic signal peptide segment leads to loss of signal peptide cleavage. Cellular GFP products (c) were obtained by immunoprecipitation with polyclonal rabbit anti-GFP from RMA cells that expressed cytosolic GFP-NP (cy), sE7-GFP-NP containing a functional signal peptide (sE), or s*E7-GFP-NP containing a disrupted signal peptide (s*E). *In vitro* transcription/translation (v) was performed with standard rabbit reticulocyte system (Promega) according to the manufacturer's protocol using pcDNA3.1 plasmids that encode the indicated GFP variant. Samples were analyzed by SDS-PAGE.

Chapter 4

MHC multimer technology: current status and future prospects

Arnold H. Bakker and Ton N. M. Schumacher

Curr Opin Immunol. 2005 Aug;17(4):428–33

MHC multimer technology: current status and future prospects

Arnold H Bakker and Ton NM Schumacher

The detection of antigen-specific T cell responses by MHC multimer staining is rapidly becoming one of the core immunological techniques, and the technology to produce MHC multimers has been optimized substantially in recent years. Furthermore, recent work demonstrates the potential of high-throughput detection of T cell responses and suggests that manipulation of T cell responses through the use of multimeric MHC reagents is also feasible.

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Current Opinion in Immunology 2005, 17:428–433

This review comes from a themed issue on
Immunological techniques
Edited by Daniel Speiser

0952-7915/\$ – see front matter
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DOI 10.1016/j.coi.2005.06.008

Introduction

T cell receptors (TCRs) are capable of singling out specific peptide–MHC (pMHC) complexes on target cells amidst a wide variety of other pMHC complexes. By exploiting the specificity of this interaction, multimeric forms of MHC molecules (MHC multimers) have been designed with the aim of detecting antigen-specific T cells amidst a multitude of unrelated T cells. The first MHC multimer used for specific T cell analysis, a human MHC class I tetramer, was described in 1996 by John Altman [1]. Today, MHC multimers can range from dimers to octamers, consisting of either MHC class I, MHC class II or nonclassical MHC molecules, from species including mouse, monkey and man.

In this review we discuss the value of currently available MHC class I and class II multimer technologies in terms of valency, expression system and peptide loading strategy. In addition, we provide a roadmap for the future development of multimeric MHC technology, and high-throughput multimer systems in particular, as well as potential clinical applications.

MHC multimer technologies

A great number of strategies has been developed for the production of MHC multimers. As illustrated in Table 1,

the major differences between MHC multimers are found in just three parameters: the valency of the multimeric complex, the expression system through which the molecules are produced, and the peptide-loading strategy used to achieve occupancy of the peptide-binding groove with the desired antigenic peptide.

Valency

TCRs have a low affinity for their cognate pMHC counterparts, with an off-rate of only a few seconds [2]. Monomeric pMHC–TCR interactions are therefore too unstable to be exploited as an effective labelling technique, but — as for any multivalent interaction — combining multiple MHC molecules into one complex greatly increases binding stability [3,4]. In their landmark paper, Davis and colleagues [1] approached this need for multivalency by designing tetrameric forms of MHC molecules. In this strategy, soluble MHC monomers are biotinylated and converted to tetravalent structures by binding to (fluorochrome-conjugated) streptavidin or avidin, which both have four biotin binding sites. The resulting MHC tetramers remain by far the most popular reagents for the detection of antigen-specific CD4⁺ and CD8⁺ T cells by flow cytometry.

What's in a name?

In spite of their name, however, it is quite unclear whether binding of MHC tetramers to T cells occurs in a tetravalent fashion. First, due to the rigid tetrahedral configuration of these complexes only three out of the four available MHC molecules are likely to bind simultaneously to the T cell surface [5]. Second, the conjugates of (strept)avidin with the proteinaceous fluorochromes phycoerythrin (PE) and allophycocyanin (APC) that are used for MHC tetramer production are prepared by chemical crosslinking and therefore also contain multimers of (strept)avidin [6]. These higher order oligomers appear to make an important contribution to T cell binding, as evidenced by the fact that streptavidin–PE ‘tetramers’ show increased binding over Cy5-labeled (true) tetramers, when tested for CD8-independent binding to human cytotoxic T lymphocytes (CTLs) [6]. These data not only suggest that the valency of the standard PE conjugates exceeds four, but also that — at least for (strept)avidin-based multimers — a valency greater than four might be preferred for optimal binding. Other and arguably better defined multimers with valencies greater than four exist, such as the commercially available MHC pentamers, where five pMHC complexes face the same direction through the use of a five-stranded coiled coil as oligomerization domain. On the other side of the spectrum, MHC multimers with a valency of two (i.e. MHC

Table 1										
Distinct MHC multimer formats.										
Peptide-binding strategy	Dimer ^a		Tetramer ^a		Pentamer ^a		Octamer ^a		Polymer/aAPC ^a	
	Euk. ^b	Bact. ^b	Euk. ^b	Bact. ^b	Euk. ^b	Bact. ^b	Euk. ^b	Bact. ^b	Euk. ^b	Bact. ^b
Exchange	[8]		[18 ,24]						[10,11]	
Bound during refolding				[1^c,4^d,21]		^e		[6]		[9,43]
Linked peptide	[16,44]		[17 ,42]						[12]	
Only selected references are cited. MHC class I multimers are shown in bold; MHC class II multimers are shown in italics. ^a Valency. ^b Expression system. ^c Most widely used formats. ^d Monomers and tetramers have been produced, but higher order oligomers are also possible. ^e Limited peer-reviewed data available at present. Euk., Eukaryotic; Bact., bacterial.										

dimers) were designed in the laboratory of Jonathan Schneck [7]. In these molecules, the extracellular domain of MHC molecules is expressed as a genetic fusion with an immunoglobulin scaffold, resulting in an MHC–Ig dimer [7,8]. Although these MHC dimers were originally used for T cell activation, these dimeric MHC molecules are now also used as staining reagents in flow cytometry [8]. Finally, several approaches have been developed for the generation of MHC-coated artificial antigen-presenting cells (aAPCs). In these systems, multivalency is achieved by coupling MHC monomers to magnetic beads [9,10], or by incorporating MHC monomers into liposomal vesicles [11,12].

What’s your favourite number?

Faced with this multitude of multimers, which format should one choose to detect antigen-specific T cells by flow cytometry? The available data suggest that a valency of four or possibly even greater is optimal for the detection of high-avidity CD8⁺ T cells with (strept)avidin-based MHC multimers. It is noted, however, that the scaffold used for multimerization is likely to influence the valency that is required, by affecting the conformational freedom of the attached MHC monomers. MHC dimers are used infrequently in comparison to MHC tetramers, although this could also be due to the more complicated production process (see below). MHC pentamers have the advantage of being more molecularly defined. At present, not many peer-reviewed data on these new molecules are available, but it seems likely that their binding strength will not differ substantially from the standard MHC tetramers.

A higher valency of the complex may become more important when aiming to detect antigen-specific CD4⁺ T cells, as standard MHC class II tetramers appear to miss lower avidity cells present in the antigen-specific CD4⁺ T cell repertoire [12,13]. Low-avidity CD8⁺ T cells, such as T cells specific for self-antigens, can be detected by conventional MHC class I tetramers in at least some cases [14,15] (and one should therefore be aware that the detection of antigen-specific CD8⁺ T cells by MHC tetramer staining is not necessarily indicative of a high-avidity interaction). It seems plausible, however, that the detection of low-avidity self-specific CD8⁺ T cells will

also be facilitated by the use of higher order MHC oligomers.

Expression system

Recombinant MHC molecules used for the production of multimeric MHC reagents have been produced in either bacterial cells or eukaryotic cells, such as insect cells and mammalian cells. A clear advantage of the bacterial expression systems is the ease with which large quantities of proteins can be generated. MHC molecules that are produced in *Escherichia coli* cells generally need to be refolded *in vitro*. This process is straightforward for the majority of MHC class I alleles and, with few exceptions [8,10,16], bacterial expression has indeed been the preferred system for MHC class I multimer production. By contrast, the efficiency with which MHC class II molecules can be refolded *in vitro* is notoriously low, and the majority of MHC class II production systems are therefore based on eukaryotic expression, such as baculovirus-infected insect cells [17] or stable *Drosophila* cell transfectants [18,19]. Although the yield of MHC class II molecules obtained in such eukaryotic expression systems can be optimized [20**], the development of *E. coli* expression strategies that provide higher yields of refolded MHC class II molecules than the current strategies [3,12,21] remains a laudable goal.

Peptide-loading strategy

The third — and rather important — parameter in the generation of MHC multimers is the method by which the MHC molecules are loaded with peptide. Three fundamentally different approaches have been used to date:

1. Antigenic peptides can be included during the *in vivo* process, through genetic linkage to one of the MHC chains.
2. Peptides can be included during the *in vitro* production process.
3. Peptides can be bound after MHC monomer, or even MHC multimer, production.

Low-throughput strategies

From a structural point of view, genetic fusion of the desired antigenic peptide with one of the MHC chains makes sense for MHC class II molecules, but less so for

MHC class I molecules. In the case of MHC class I molecules, the terminal NH_3^+ and COO^- groups are normally buried in the MHC structure and contribute to peptide binding [22,23]; genetic fusion of MHC to one of the peptide termini is therefore bound to result in a local change in the structure of the pMHC complex. Genetic peptide fusions to MHC molecules have been used fairly extensively to produce human and murine MHC class II multimers [17,19]. High throughput synthesis of multimeric MHC molecules by this strategy, however, is precluded by the fact that a different construct and producer cell line is required for each peptide antigen. Production of MHC multimers with several different peptide antigens is more straightforward with the commonly used strategy for MHC class I multimer production, in which a synthetic peptide antigen is included during the *in vitro* refolding process. Because separate refolding and purification is still required for each single MHC class I multimer, the production of large collections of pMHC reagents remains a challenging task.

High-throughput strategies

In cases in which a multitude of MHC multimers with different antigens bound to the same MHC allele is required, the preferred strategy is to bind peptide ligands to preformed MHC monomers or multimers. For MHC class II molecules, such 'exchange' strategies have been developed, using either presumed peptide-free [18] or class II-associated invariant chain peptide (CLIP)-bound [24] MHC class II molecules as starting material. Although the CLIP-based strategy is conceptually somewhat more appealing, as it mimics the *in vivo* binding of ligands to MHC class II, no comparison between the two strategies has been made.

Because of the greater number of identified MHC-class-I-associated epitopes, a similar type of exchange strategy would be of considerable use for the generation of MHC class I multimers [8,10,25]. Because of the instability of peptide-free MHC class I molecules [26,27], however, the conditions that can be used to promote the release of bound ligands, such as low pH, also affect the MHC structure itself, and this type of exchange technology has not gained widespread use for MHC class I. To circumvent this issue, we have recently developed so-called 'conditional' MHC class I ligands. These ligands can be made to dissociate from MHC class I upon exposure to defined triggers, such as ultraviolet light, that do not by themselves destabilize MHC class I molecules (M Toebes *et al.*, personal communication). Using such conditional ligands, a large array of MHC multimers can be generated from preformed MHC complexes in one or two hours, and the resulting complexes appear to bind to antigen-specific T cells with comparable avidity and specificity as conventional MHC class I tetramers. This technique might prove to be of substantial use for the

generation of large collections of pMHC multimers, for conventional MHC multimer flow cytometry, and for high-throughput systems for T cell analysis (see below).

Future strategies and challenges

High throughput analysis with MHC multimers

At present, the detection of antigen-specific T cells by MHC tetramer staining is a technology with a highly limited throughput, as only a single T cell specificity is analyzed per sample. For the definition of novel pathogen- or tumour-associated epitopes, or for the comprehensive screening of T cell responses in blood samples, the simultaneous monitoring of a large number of T cell specificities in a single sample would be highly desirable.

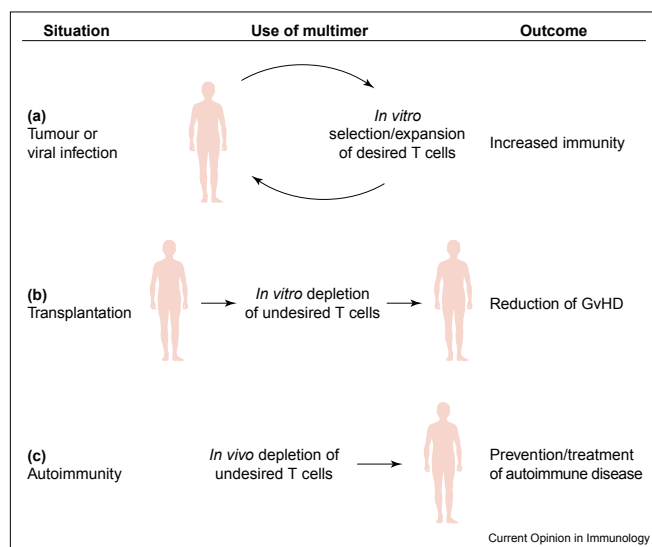
Multiparameter flow cytometry

If MHC multimers with multiple specificities are to be used in a single flow cytometric analysis, this requires that MHC multimers of each separate specificity carry an individual label [28]. The low diversity of fluorochromes routinely used in conjunction with MHC multimers (i.e. PE and APC) has severely limited the potential for multiparameter screening. Although the non-proteinaceous fluorochromes have not worked well with the classical MHC tetramer format, this problem can be overcome by the use of octameric MHC reagents [6]. Perhaps more important for the long-term development of multiparameter MHC multimer analysis is the recent interest in fluorescent nanocrystals called quantum dots (qdots) [29**]. Qdots are more stable than organic fluorochromes, and — importantly for their potential use with MHC multimers — quantum dots are available in a wide fluorescent spectrum and exhibit very narrow emission spectra. On the basis of these properties, we consider it plausible that MHC multimers, built on qdot-coupled streptavidin, for example [30], will enable the simultaneous measurement of 10 or more T cell responses in a single sample in the not-too-distant future. Furthermore, if qdot-coupled MHC multimers can be made to contain defined combinations of qdots, the combinatorial power of the method could increase even further [31].

MHC-microarrays

As an alternative to flow cytometric analysis of multiple T cell responses by MHC multimers, Soen and colleagues [32] have developed an MHC-microarray-based approach for T cell detection. In these arrays, each array spot contains MHC molecules complexed with a specific peptide, and T cell responses are measured by quantifying either T cell binding or T cell activity in distinct spots. Although only a modest number of pMHC specificities were spotted in the first arrays, the use of peptide-exchange systems for MHC class II [18,24] and MHC class I molecules (M Toebes *et al.*, personal communication), in which exchange reactions are performed on the slide, has the promise to produce microarrays with a

Figure 1



Potential clinical applications for MHC multimers. **(a)** MHC multimers can be used for the *ex vivo* selection and expansion of desired antigen-specific T cells. These T cells can then be (re-)infused to enhance reactivity against defined tumour- or virus-associated antigens. **(b)** MHC multimers can be used for the *ex vivo* depletion of undesired (e.g. graft-versus-host disease [GvHD]-associated) T cells from transplant material before transfer to the recipient. **(c)** MHC multimers can be used for the *in vivo* inactivation of undesired (e.g. autoimmune-associated) T cells for the prevention/treatment of autoimmune disease.

substantially larger number of specificities. In comparison to multiparameter analysis of T cell responses by MHC multimer flow cytometry, MHC microarrays are likely to have a somewhat lower sensitivity, but such arrays should be capable of visualizing T cell populations specific for a vast number of (possible) antigens.

Manipulating T cell responses with MHC multimers

A series of studies has provided proof-of-principle for the use of MHC multimers as reagents to boost desirable or suppress unwanted T cell responses (Figure 1). MHC multimers have been used for rapid and efficient *ex vivo* isolation and expansion of specific T cells [11,33,34], which should be of use for adoptive therapy following allogeneic stem cell transplantation, for example [35]. MHC multimer-TCR interactions during such enrichments could affect T cell viability, so to reduce the effects of these interactions, Knabel and colleagues [36] have devised a strategy for reversible multimer staining. This technique forms an elegant addition to the standard MHC multimer approach, but whether such reversible binding improves the *in vivo* activity of adoptively transferred T cells in human trials remains to be determined.

MHC multimers could also conceivably be used for the *ex vivo* removal of unwanted T cells, such as alloreactive T cells contained in peripheral stem cell transplants. Furthermore, several studies provide support for the use of MHC dimers for the *in vivo* inactivation of auto-reactive T cells, thereby preventing type I diabetes and arthritis [37–39]. In addition to these non-conjugated MHC multimers that rely on TCR signalling for their biological effects, isotope-coupled MHC multimers have been generated [40*]. These ^{225}Ac -labeled MHC tetramers are promising reagents to induce killing of specific T cell populations. Although the *in vivo* activity of this type of 'suicide tetramer' remains to be established, the isotope does not seem to be overly toxic *in vivo* [41].

Because the quantity of MHC multimers required for *in vitro* enrichment or depletion is likely to be lower than the amount required for *in vivo* use, these *in vitro* technologies could perhaps be implemented more readily. Before MHC multimers can be used in any of these *ex vivo* or *in vivo* clinical settings, however, it will obviously be essential to develop procedures for the Good Manufacturing Practice production of these reagents, which will prove a challenge for translational researchers in the coming years.

Conclusions

The dissection of T cell responses by MHC multimer staining has become an established technique in preclinical research, and is becoming increasingly important for clinical trial monitoring. The parallel development of a variety of multimeric MHC strategies in the past decade has been important to discovering which formats work best and we expect that in the coming decade a small number of these formats will become standard for MHC multimer flow cytometry. More importantly, we speculate that the near future will bring the arrival of technologies that will enable high-throughput analysis of T cell responses, either by flow cytometry or on solid surfaces. In addition, MHC multimers might find increasing use as therapeutic agents, be it either for the enrichment of desired T cells, or the removal of rogue T cell populations.

Update

Elegant work has recently been published by the Stern laboratory on the generation of MHC microarrays [45**]. In this study, T cell responses against a number of pMHC complexes are analyzed in parallel by cytokine capture.

Cebecauer and colleagues [46*] have recently demonstrated that the distance between individual pMHC molecules of an MHC multimer affects the cytotoxic effect of such multimers on antigen-specific T cells: MHC molecules connected by short linkers induce rapid cell death, whereas MHC molecules connected by long linkers do not. This is valuable information for the development of MHC multimers for clinical use.

Finally, the first papers have been published that make use of the MHC class I pentamer technology to detect antigen-specific T cells by flow cytometry [47,48].

Acknowledgements

We wish to apologize to those colleagues whose work could not be discussed owing to space limitations. We would like to thank our colleagues at the Division of Immunology, and in particular Moniek de Witte, Koen Schepers and John Haanen for critical reading and helpful suggestions.

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Chapter 5

Conditional MHC class I ligands and peptide exchange technology for the human MHC gene products HLA-A1, -A3, -A11, and -B7

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Proc Natl Acad Sci U S A. 2008 Mar 11;105(10):3825–30

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Conditional MHC class I ligands and peptide exchange technology for the human MHC gene products HLA-A1, -A3, -A11, and -B7

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Edited by Jack L. Strominger, Harvard University, Cambridge, MA, and approved January 3, 2008 (received for review October 12, 2007)

Major histocompatibility complex (MHC) class I multimer technology has become an indispensable immunological assay system to dissect antigen-specific cytotoxic CD8⁺ T cell responses by flow cytometry. However, the development of high-throughput assay systems, in which T cell responses against a multitude of epitopes are analyzed, has been precluded by the fact that for each T cell epitope, a separate *in vitro* MHC refolding reaction is required. We have recently demonstrated that conditional ligands that disintegrate upon exposure to long-wavelength UV light can be designed for the human MHC molecule HLA-A2. To determine whether this peptide-exchange technology can be developed into a generally applicable approach for high throughput MHC based applications we set out to design conditional ligands for the human MHC gene products HLA-A1, -A3, -A11, and -B7. Here, we describe the development and characterization of conditional ligands for this set of human MHC molecules and apply the peptide-exchange technology to identify melanoma-associated peptides that bind to HLA-A3 with high affinity. The conditional ligand technology developed here will allow high-throughput MHC-based analysis of cytotoxic T cell immunity in the vast majority of Western European individuals.

epitope | T cell | CD8

MHC Class I molecules are heterotrimeric complexes consisting of an invariant light chain called β 2-microglobulin (β 2m), a polymorphic heavy chain (HC) and an $\approx$ 8- to 11-aa peptide ligand. These peptide-MHC (pMHC) complexes are recognized by the T cell receptor (TCR) of CD8⁺ T cells in a peptide-specific fashion, and this interaction forms the molecular basis of antigen recognition by CD8⁺ T cells. In the past decade, the mapping of pathogen-specific and autoimmune- or cancer-associated T cell epitopes has been a major driving force in the development of assay systems for immunomonitoring. In addition, knowledge of such T cell epitopes forms a cornerstone in the development of vaccine-based or adoptive T cell therapies. As a first step in the mapping of disease-associated T cell epitopes, peptide fragments of disease-associated proteomes may be analyzed for binding to MHC molecules of interest, and subsequent assays can then be used to determine whether T cell reactivity against such pMHC complexes does occur. As demonstrated in a landmark study by Altman and colleagues (1), such antigen-specific T cell reactivity can efficiently be detected by the staining of T cell populations with recombinant fluorescent multimeric MHC molecules.

There is an increasing interest in the development of assay systems, such as MHC-based microarrays, that can monitor a multitude of T cell responses in parallel (2–4). Unfortunately, current technology does not allow for the high-throughput generation of different pMHC complexes, thereby limiting the utility of these techniques. Specifically, because MHC class I complexes that are devoid of peptide are markedly unstable (5, 6), current production processes for recombinant MHC com-

plexes require inclusion of a specific T cell epitope during the initial refolding step (7), and this precludes the production of the large collections of pMHC multimers that would be needed to analyze antigen-specific T cell responses in a comprehensive manner.

Based on these considerations, it seemed valuable to devise technologies that allow the high-throughput parallel generation of peptide-MHC class I complexes. As a step toward this goal, we recently designed an HLA-A2-specific peptide that contains a photocleavable moiety (8). When refolding reactions of HLA-A2 heavy chain and β 2m are performed with this ligand, stable HLA-A2 complexes are formed. However, upon irradiation with long-wavelength UV, the ligand is cleaved and dissociates from the HLA-A2 complex. The resulting empty HLA-A2 complexes disintegrate rapidly, unless UV exposure is performed in the presence of a “rescue peptide.” In this case, the peptide-binding groove that has been vacated by UV exposure will be occupied by the rescue peptide, resulting in the formation of stable pMHC complexes with a distinct T cell specificity. The utility of this approach has been demonstrated by the identification of an HLA-A2-restricted CTL epitope from an H5N1 influenza strain isolated from a lethal case of avian influenza infection in humans (8).

To determine whether this technology can be developed into a broadly applicable high-throughput system for the dissection of human CTL responses, we set out to design and test a panel of UV-sensitive ligands for the human MHC gene products HLA-A1, -A3, -A11, and -B7.

Results

Design of the Conditional Ligands. In an effort to test the feasibility of developing a broadly applicable high-throughput platform for MHC-based detection, we focused on a set of four gene products (HLA-A1, -A3, -A11, and -B7) with a high prevalence in the Western European population. By using the SYFPEITHI database (9), a set of three to six high-affinity 9-mer peptides was designed for each molecule, in which the UV-sensitive β -amino

Author contributions: R.H. and C.L. contributed equally to this work; A.H.B., R.H., C.L., M.T., B.R., C.R.B., H.O., and T.N.M.S. designed research; A.H.B., R.H., C.L., M.T., and C.R.B. performed research; B.R., S.R.H., W.J.E.v.E., M.H.M.H., and H.O. contributed new reagents/analytic tools; A.H.B., R.H., B.R., C.R.B., and T.N.M.S. analyzed data; and A.H.B. and T.N.M.S. wrote the paper.

Conflict of interest statement: The MHC exchange technology described in this manuscript is the subject of a patent application. Based on Netherlands Cancer Institute policy on management of intellectual property, M.T., H.O. and T.N.M.S. would be entitled to a portion of received royalty income in case of future licensing.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0709171105/DC1.

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acid (+/-)-3-amino-3-(2-nitro)phenyl-propionic acid was incorporated at different positions. Amino acid sequences were based on known peptide motifs for each gene product and had a predicted SYFPEITHI peptide-binding score of >25 (excluding a potential detrimental effect of the β -amino acid). In all peptides, the photolabile building block was incorporated at positions predicted to result in solvent exposure of the 2-nitro-phenyl side chain, as based on structural data (10, 11), or the lack of amino acid selectivity at this position (9). Although solvent exposure of the 2-nitrophenyl side-chain is not required for the UV-mediated cleavage step, this strategy was used to reduce the likelihood that incorporation of the photolabile amino acid would affect MHC binding capacity.

First, a series of small-scale refolding reactions was performed with each of the 19 starting ligands [supporting information (SI) Table 2]. The resulting pMHC complexes were subsequently analyzed by gel-filtration HPLC and MHC ELISA to determine three parameters: the efficiency of MHC refolding, the stability of the pMHC complex in the absence of UV exposure, and the UV-sensitivity of this complex. A description of the starting set of conditional ligands and a summary of the outcome of these assays in terms of stability and UV-sensitivity of the different pMHC complexes is given in SI Table 2.

Of the 19 ligands tested, 3 either showed no or very poor refolding with the corresponding MHC molecule or the pMHC complex displayed a substantial instability upon storage or brief 37°C exposure. The remaining 16 pMHC complexes were exposed to UV light and analyzed for unfolding of the pMHC complex: 9 of these pMHC complexes displayed no or low UV-induced unfolding, whereas the remaining 7 displayed efficient UV-induced degradation. From this set, the ligands that yielded the highest efficiency of refolding were selected for further optimization. Specifically, to facilitate rapid release of the resulting peptide fragments upon UV exposure while maximizing complex stability under normal conditions, the anchor residues of the selected ligands were altered while keeping the UV-sensitive amino acid at the same position. After analyzing this pool of second candidate ligands for the same three parameters, an optimal conditional ligand was selected for each HLA gene product, termed p*[allele]: p*A1, STAPGJLEY; p*A3, RYRIGATR; p*A11, RVFAJSPFIK; p*B7, AARGJTJLAM; where J is 3-amino-3-(2-nitro)phenyl-propionic acid.

Analysis of the Conditional pMHC Complexes. Large-scale refolding reactions were performed to enable more detailed analysis of the selected p* ligands for the four different molecules. In parallel, refolding reactions were performed with a set of known epitopes (termed pA1, pA3, pA11, and pB7) lacking a photolabile residue, for use as controls in these experiments. For all four HLA molecules, the efficiency of refolding of the different p*MHC complexes was comparable to that of the corresponding control pMHC (HLA-A1 p*A1, 21%; HLA-A3 p*A3, 20%; HLA-A11 p*A11, 23%; HLA-B7 p*B7, 12%).

To set up a more rapid assay system for the measurement of UV-induced MHC unfolding for the different complexes, pMHC and p*MHC preparations were either exposed to UV or left untreated, and the amount of remaining folded MHC was then analyzed by ELISA (12, 13) of serial dilutions (shown for HLA-A1 in SI Fig. 5). Having established a suitable pMHC concentration to visualize the effect of UV exposure on p*MHC stability via ELISA (between 10 and 20 nM, SI Fig. 5), a kinetic analysis of UV-mediated degradation was performed for each MHC product. UV exposure of control MHC complexes refolded with UV-insensitive peptides had no effect on their stability, independent of the time of exposure. In contrast, each of the p*MHC complexes showed substantial degradation after a 1-min UV treatment, and the effect of UV exposure was close to complete after a 5-min exposure (Fig. 1A).

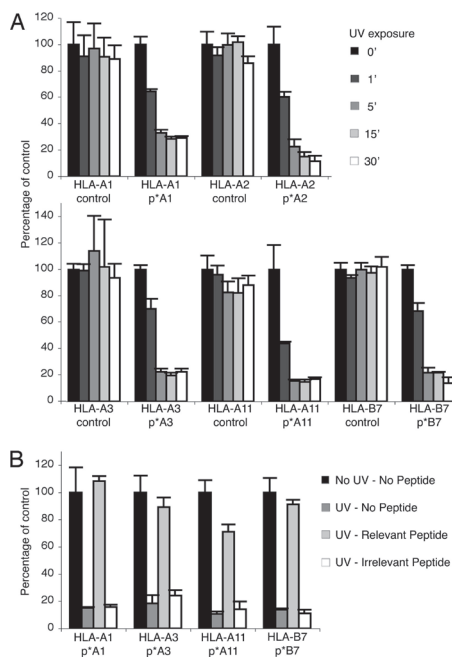


Fig. 1. Characterization of conditional ligands for HLA-A1, -A3, -A11, and -B7. (A) Control and p*-HLA complexes for each complex were exposed to UV for the indicated times, and UV-induced MHC unfolding was measured by ELISA. Control peptides: pA1 VTEHDTLLY, pA2 FLWGPRLV, pA3 RLRAEAQVK, pA11 IVTDFSVIK, and pB7 RPHERNGTFTL. (B) The indicated p*-HLA complexes were exposed to UV light for 0 or 60 min in the presence of no peptide, the HLA-A2 restricted CMV-pp65 epitope NLVPMVATV (irrelevant peptide), or their respective specific ligands, pA1, pA3, pA11, and pB7 (sequences under A) and analyzed by MHC-ELISA. Values indicate means $\pm$ SD of triplicates.

To allow the use of conditional MHC complexes for various high-throughput applications, it is essential that the unstable MHC molecules that are formed upon UV exposure can bind a newly added ligand and thereby be stabilized. To test whether this is the case for the four HLA gene products under study, exchange reactions were performed in the absence of peptide, in the presence of an HLA-A2-restricted control peptide, or in the presence of a known peptide ligand for each complex. For all four HLA gene products, addition of the relevant ligand resulted in a substantial rescue (71–108% of starting material, Fig. 1B), whereas addition of the HLA-A2-restricted control peptide was without effect. Combined, these experiments show that (i) all four conditional ligands refold efficiently into stable MHC complexes, (ii) the resulting complexes display a similar UV-sensitivity, and (iii) the peptide-free MHC molecules generated upon triggering can be charged with newly added allele-specific ligands.

Peptide Cleavage and Exchange Kinetics. A caveat of the MHC ELISA data in Fig. 1 is that the observed unfolding of MHC upon UV exposure is an indirect measure of peptide dissociation and may be influenced by the fact that the stability of the peptide-free state can vary between different MHC molecules

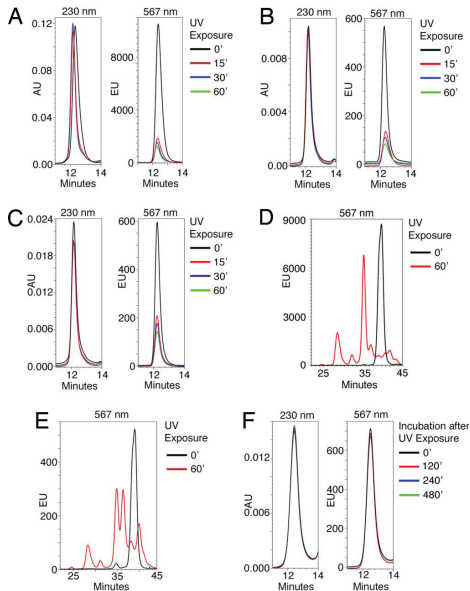


Fig. 2. UV-induced peptide cleavage and exchange kinetics. (A) HLA-A2-monomers refolded with the fluorescent UV-sensitive peptide ^{125} I-p*A2 were treated with UV in the presence of the HLA-A2 ligand NLVPMVATV for different time periods and analyzed by gel-filtration HPLC. Absorbance at 230 nm (Left) and fluorescence at 567 nm (Right) was measured. (B and C) HLA-B7-monomers refolded with either the fluorescent UV-sensitive peptides AARCF(I)JTLAM (B) and AARGJTLCF(I)M (C) were treated with UV in the presence of the HLA-B7 ligand TPRVTGGGAM for different time periods and analyzed as in A. (D) HLA-A2-monomers refolded with the fluorescent UV-sensitive peptide ^{125} I-p*A2 were either left untreated or exposed to UV for 60 min. Extracted peptides were analyzed by reverse-phase HPLC. Black line, untreated; red line, UV-treated. (E) As in D except that before peptide extraction, elution material with the retention time of pMHC molecules was isolated by gel-filtration HPLC. Black line, untreated; red line, UV-treated. (F) p*A2-monomers were treated with UV for 30 min in the presence of 0.5 μ M fluorescent FLPSDC(FI)FPSV and 49.5 μ M FLPSDCFPSV peptide and kept at temperature for the indicated periods before analysis as in A.

(14). To allow a more direct measurement of the dissociation of peptide fragments upon UV exposure, a fluorescently labeled variant of p*A2 was produced: KILGC(FI)VFJV (^{125} I-p*A2). HLA-A2 complexes occupied with this fluorescent ligand were then used in exchange reactions in the presence of an unlabeled CMV pp65-derived HLA-A2 ligand. When aliquots of HLA-A2 ^{125} I-p*A2 complexes are either left untreated or exposed to UV for different time periods and analyzed by gel-filtration HPLC, recovery of total MHC, as reflected by absorbance at 230 nm, is identical for all samples. In contrast, when the amount of remaining MHC-associated ^{125} I-p* ligand is monitored by fluorescence analysis, a clear reduction is observed (Fig. 2A, 88% reduction after 60-min UV exposure). Thus, the instability of MHC upon UV exposure of p*MHC complexes is accompanied by a parallel release of the fluorescent peptide fragment.

In the p*A2 ligand, the J residue is incorporated at p8 of the nonameric peptide. Because the side chain of p9 is buried in the F pocket of the peptide-binding groove, fluorescent labeling at a COOH-terminal position relative to the cleavage site was

precluded for this peptide. To assess whether both peptide fragments that are formed upon cleavage of a UV-sensitive peptide dissociate with similar kinetics from the peptide binding groove, subsequent analyses were performed with variants of the p*B7 ligand, in which the photolabile residue is incorporated at a more central position (p5). Two variants of the p*B7 ligand were produced with a fluorescently labeled cysteine residue at either an NH₂- or COOH-terminal position relative to J (AARCF(I)JTLAM and AARGJTLCF(I)M). HLA-B7 complexes refolded with these peptides were then exposed to UV in the presence of an unlabeled HLA-B7 ligand and analyzed by gel-filtration HPLC. No substantial difference was observed between the rate of dissociation of fluorescently NH₂- and COOH-terminal cleavage products (Fig. 2B and C) and a 60-min UV-exposure led to an 84% and 75% reduction of the fluorescent signal, respectively.

For both the fluorescently labeled NH₂-terminal fragment of p*A2 and the fluorescently labeled NH₂- and COOH-terminal fragments of p*B7 a small amount of fluorescent signal that comigrated with the MHC complex was consistently observed after UV exposure (Fig. 2A–C). Because prolonged UV exposure does not result in a substantial further decrease in this signal (data not shown), it seemed unlikely that this signal reflected the presence of uncleaved MHC-associated ligand. To test this directly, peptides were extracted from UV-exposed or untreated HLA-A2 ^{125} I-p*A2 complexes by acid elution and analyzed by reverse-phase HPLC. This analysis demonstrated that UV exposure leads to a near-complete cleavage of the starting MHC-associated material (Fig. 2D). Next, the fluorescent material that remained MHC-associated upon UV exposure was isolated by gel-filtration HPLC, followed by peptide elution. Subsequent analysis of this material by reverse-phase HPLC demonstrates that also the fluorescent material that remains MHC-associated upon UV exposure does not contain substantial amounts of the starting ^{125} I-p*A2 ligand. The identity of the formed cleavage products was not investigated (Fig. 2E).

To directly visualize the kinetics of binding of newly added ligands to UV-exposed p*MHC complexes, HLA-A2 p*A2 complexes were exposed to UV light for 30 min in the presence of the fluorescent A2 ligand FLPSDC(FI)FPSV (15) and then incubated at room temperature for different periods. Subsequently, the amount of newly bound MHC ligand was determined by gel-filtration HPLC. A strong fluorescent signal was seen when MHC complexes were analyzed directly after UV exposure, and this signal did not increase measurably upon further incubation. This indicates that binding of new ligands to the MHC complex is essentially complete during the time of UV exposure (Fig. 2F).

Detection of Antigen-Specific T Cell Responses with HLA-A1, -A3, -A11, and -B7 Exchange Tetramers. As a stringent test of the value of peptide-exchanged MHC complexes of the different gene products for the detection of antigen-specific T cell responses, exchange reactions were performed with a series of pathogen-derived epitopes and used for multimerization without further purification. Subsequently, T cell staining of these MHC exchange tetramers was compared with that of MHC tetramers generated in classical individual refolding reactions (1). In a first set of experiments, the intensity of staining of epitope specific T cell clones restricted by HLA-A1 and -A3 was analyzed, upon incubation with different concentrations of classical MHC tetramers or MHC exchange tetramers (SI Fig. 6). For both HLA-A1 and -A3 complexes, MHC exchange tetramers and classical MHC tetramers are indistinguishable in their capacity to stain a relevant T cell clone (Fig. 3). Furthermore, in both cases, background staining as revealed by incubation of an irrelevant CTL clone with a high concentration of MHC tetramer is negligible (<0.05%, Fig. 3, SI Fig. 6). To extend this

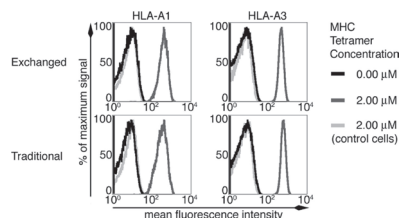


Fig. 3. CD8⁺ T cell clones specific for HLA-A1 CMV-pp65 (Left) and HLA-A3 EBV-EBNA-3a (Right) were stained with equal amounts of HLA-A1 CMV-pp65 or HLA-A3 EBV-EBNA-3a tetramers, generated via peptide exchange (Exchanged, Upper) or classical refolding (Traditional, Lower). Control cells represent staining with the matched MHC tetramer and cross-sample controls.

analysis to clinically more relevant samples, MHC-exchange tetramers and classical MHC tetramers were compared with respect to the ability to detect low-frequency T cell responses in peripheral blood samples. Tetrameric forms of p*MHC complexes that had not been exposed to UV and that are therefore uniformly occupied with the conditional ligand were included as controls. In all cases tested, MHC exchange tetramers stained the relevant T cell populations, and both the percentage and fluorescence intensity were directly comparable to that observed upon staining with MHC tetramers produced by individual refolding reactions (Fig. 4).

Identification of Melanoma-Associated HLA-A3 Ligands. To test the feasibility of the peptide-exchange technology to rapidly screen large panels of peptides for MHC binding, we set out to identify HLA-A3 ligands in melanoma-associated proteins. To date, only four HLA-A3-restricted T cell epitopes have been identified in melanoma-associated proteins, all of them derived from the gp100 antigen (16–18). We designed a library of peptides derived from the melanocyte differentiation antigens (gp100, mart-1, tyrosinase, and tyrosine-related-protein-1 and -2), plus the melanoma-associated protein Nodal (19) by using three binding prediction algorithms for HLA-A3 (9, 20). The resulting 203 peptides (SI Table 3) were then analyzed for HLA-A3 binding in a fluorescence polarization assay. Twenty-two peptides that showed a high inhibition of binding of the tracer peptide were selected for determination of IC₅₀ values (SI Table 3). Importantly, this set of 22 identified HLA-A3 ligands included the four known gp100 epitopes. Table 1 and SI Fig. 7 show that all 22 peptides form high-affinity ligands of HLA-A3, with IC₅₀ values ranging between 73 and 857 nM.

Discussion

The use of multimeric forms of pMHC complexes has become a core immunological technique to visualize antigen-specific CD8⁺ T cell populations (7). Fluorescently labeled MHC multimers are commonly used for the detection of antigen-specific T cells by flow cytometry. In addition, there is an increasing interest in the development of high-throughput assay systems, such as MHC microarrays or combinatorial coding schemes, to visualize pathogen-specific or other disease-associated immune responses in a more comprehensive manner (2, 3, 21). A major obstacle in the development of these high-throughput approaches for the dissection of antigen-specific CD8⁺ T cell immunity has been the fact that for each specific peptide-MHC class I complex, a separate production run is required (1, 22), limiting the practical use of MHC multimer-based T cell detection to a few T cell specificities.

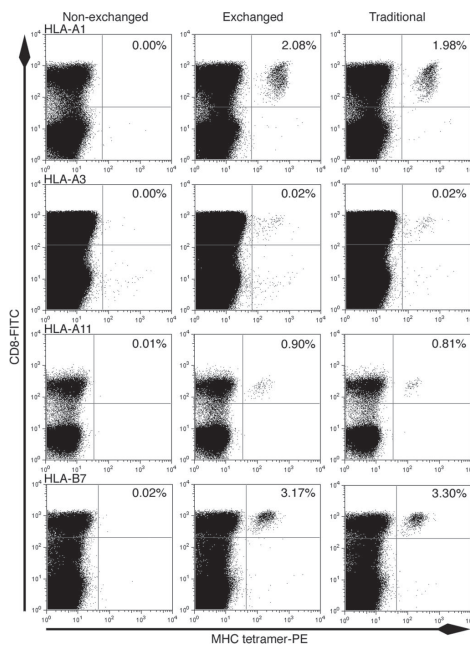


Fig. 4. Indicated pMHC complexes were prepared by classical refolding reactions or by 1-h exchange reactions and converted to tetramers. As a negative control, streptavidin-conjugated nonexchanged p*MHC complexes were used. HLA-typed peripheral blood mononuclear cells were stained with the indicated pMHC tetramers and analyzed by flow cytometry: from top to bottom: HLA-A1 CMV-pp65 (VTEHDTLLY); HLA-A3 EBV-EBNA-3a (RLRAE-AQVK); HLA-A11 EBV-EBNA-3b (IVTDFSVIK); HLA-B7 CMV-pp65 (TPRVTTGG-GAM) tetramers, respectively. For all complexes, stainings were performed at equal concentrations for all three columns. Numbers indicate the percentage of MHC tetramer⁺ cells of CD8⁺ cells.

We have set out to develop technologies that enable the rapid generation of large collections of defined pMHC complexes in parallel reactions. Here, we aimed to determine whether MHC-based peptide-exchange technology can be developed into a broadly applicable platform for the screening of human cytotoxic T cell responses. To this purpose, we defined conditional ligands for four different human MHC products: HLA-A1, -A3, -A11, and -B7 (with the conditional ligands STAPGJLEY, RIYRJ-GATR, RVFAJSFIK, and AARGJTLM, respectively).

Refolding reactions with these conditional ligands are efficient and result in thermostable p*HLA complexes that rapidly degrade upon UV exposure. The presence of a cognate peptide ligand during UV treatment leads to the replacement of the cleaved conditional ligand in the peptide-binding groove of the MHC molecule and thereby results in the generation of pMHC complexes of a desired specificity. In line with this, fluorescently labeled MHC tetramers generated in such exchange reactions stain antigen-specific CD8⁺ T cell populations with equal specificity and sensitivity as MHC tetramers prepared by individual refolding reactions. Dissociation of cleaved peptide fragments displays nonlinear kinetics, with release of 75–95% within 15 min but only limited further release upon prolonged incubation.

Table 1. IC₅₀ values of selected peptides

Protein	Peptide	Position	IC ₅₀ , nM	SEM
Gp100	I ALN F PGSQK	86–95	127	7
	LI YRRRLMK	614–622	159	18
	GTATLRLVK	460–468	204	7
	A LLAVGATK	17–25	212	5
	AL N F PGSQK	87–95	247	12
	GVSRLQRTK	34–42	343	23
	QLVLHQILK	551–559	363	22
	QLRALDGGNK	221–230	415	17
	SLYRDPLPR	46–54	73	2
	HAYIQSLLK	293–301	97	4
Nodal	RTKPLSMILY	317–325	277	6
	RVAGECWPR	175–183	735	5
	YIMVPIPLYR	425–434	384	26
	SLLCRHKK	497–505	498	52
Tyr	VSSKNLMEK	25–33	552	14
	GLVSLLCRHK	494–503	857	42
Tyrp1	SLPYWNFATR	245–254	149	5
	ASYLIRARR	497–505	169	18
Tyrp2	TLLGPRPRYR	196–205	120	1
	GTYEGLLR	301–309	194	14
	RMYNMVPFF	461–469	310	19
	VLLAFQYR	521–529	578	50
Influenza NP	IL RGSVAHK	265–273	181	7
CMVpp65 (HLA-A2)	N LVPVMVATV	495–503	>50,000	ND

IC₅₀ values of selected peptides determined in a competitive binding FP assay after 45 h of incubation. Bold type indicates peptides previously described as HLA-A3 epitopes. Values were determined in three independent experiments. ND, not determined.

Based on reverse-phase analysis of fluorescent peptide that remains MHC-associated upon UV exposure, it is apparent that the residual MHC-bound product primarily consists of reaction products, indicating that lack of dissociation is not due to incomplete cleavage. Prior data have shown that the dissociation of full-length peptides from MHC class I molecules also occurs with biphasic kinetics (23) (although, as expected with half-lives that are orders of magnitude greater). This has been interpreted as evidence for the existence of a “closed” and “open” conformational state of peptide-charged MHC molecules, and it is possible that the same applies to MHC complexes analyzed here.

Of more importance for the practical use of MHC reagents, the presence of a fragment of the conditional ligand in a fraction of UV-exposed MHC molecules has no measurable effect on the ability to detect antigen-specific CD8⁺ T cell responses. Specifically, the amount of MHC exchange multimer required to stain HLA-A1 and -A3 restricted T cells does not substantially deviate from that required when using conventional MHC tetramer reagents (a factor of 0.8 and 1.5 for HLA-A1-CMV-pp65 and HLA-A3-EBV-EBNA-3a). This may be explained by the fact that of the four MHC complexes in a tetrameric MHC molecule a maximum of three is likely to be simultaneously available for binding and the identity of the fourth pMHC complex will in this case be irrelevant (24). In addition, it is noted that also in classical MHC refolding reactions, it is rather unclear to what extent MHC occupancy is homogeneous.

To demonstrate the application of peptide exchange for high-throughput screening of potential T cell epitopes, a screen of melanoma-associated peptides was performed in the context of HLA-A3. The time between obtaining the peptide library and completing the final binding assay was a mere 3 weeks, including quality controls and data analysis. Twenty-two peptides were identified with high affinity for HLA-A3, and this included the four previously described gp100-derived epitopes. These epitopes could be used to monitor naturally occurring T cell

responses in melanoma patients. More intriguingly, this collection of epitopes may be used to isolate TCRs from vaccinated HLA-transgenic mice (25, 26). For those epitopes that are presented at the cell surface of melanoma cells, this would provide a strategy for the targeting of melanomas by TCR gene therapy with an expanding collection of TCRs (27).

With the collection of conditional ligands for five HLA gene products that is now available, coverage of the Western European population has become substantial. Specifically, with this set of molecules, high-throughput analysis is feasible for at least one HLA-A or -B complex for >90% of individuals. However, coverage of human populations in other areas is lower, with 58%, 65%, and 75% for populations in SubSaharan Africa, North America, and Southeast Asia, respectively. Definition of conditional ligands for the HLA-A24, -B15, and -B58 gene products that are prevalent in these areas would be useful to increase coverage in these areas to the same level.

MHC peptide exchange-based strategies may be used for both large-scale T cell epitope discovery, as demonstrated here for HLA-A3, and for T cell screening. The latter large-scale MHC-based analyses of T cell responses will generally not be feasible by standard MHC tetramer flow cytometry approaches, because the amount of patient material that is required would be prohibitive. Rather, it seems essential to develop robust platforms that can be used to analyze large series of antigen-specific cytotoxic T cell responses in parallel. Two conceptually different approaches may possibly be used for this purpose. In a first approach, parallel analysis of a large number of T cell specificities is achieved by spatial encoding schemes, in which T cells with a given specificity are selectively retained or active at defined sites. The MHC microarray platforms as developed by the Davis and Stern groups (2–4), have provided evidence for the feasibility of this approach. As an alternative approach, parallel analysis of antigen-specific T cell responses may potentially be achieved by combinatorial coding schemes, in which T cells specific for a given pMHC complex are defined by the binding of combinations of differentially labeled MHC tetramers. Dual T cell staining by using MHC tetramers conjugated to PE and APC has proven feasible (28) and efforts to test the feasibility of large scale combinatorial coding remain to be completed (A.H.B., unpublished observations).

In addition to the use of peptide exchange strategies for large-scale T cell monitoring, it seems likely that this technology will also be valuable for the development of protocols for antigen-specific adoptive T cell therapy. The infusion of antigen-specific T cell populations is considered valuable to restore antiviral immunity in transplant recipients and other immunocompromised patients (29), and a study in which the feasibility of infusion of CMV-specific T cells obtained by MHC-tetramer-assisted enrichment has been reported (30). In addition, selection of defined T cells may be used to enhance the antitumor effect of allogeneic hematopoietic stem cell transplantation (31) and TCR gene therapy protocols (27, 32, 33).

There is no conceptual difficulty in the production of MHC tetramers or reversible MHC tetramers (34) under GMP conditions. However, the production of the collection of GMP-grade pMHC tetramers required for these various applications may be cost-prohibitive, because for most of these applications, small series of pMHC reagents rather than single pMHC products would be preferred. For example, CMV-specific CD8⁺ T cell responses in healthy individuals are directed toward, on average, eight different ORFs (35). Extrapolating this to the other human Herpesviridae that are a cause of morbidity and mortality in transplant recipients, a cell product intended to prevent activation of herpes simplex virus, varicella zoster virus, cytomegalovirus, and EBV would perhaps ideally include reactivity against one or two dozen distinct CD8⁺ T cell epitopes. Based on these considerations, it seems attractive to develop GMP production

processes for p*HLA complexes, and the use of such GMP-grade p*MHC complexes in simple exchange reactions may facilitate the clinical development of oligoclonal adoptive T cell therapy.

Materials and Methods

Generation of Peptide–MHC Complexes. All peptides were synthesized by standard Fmoc synthesis. (+/–)-3-amino-3-(2-nitro)phenyl-propionic acid was generated as described (12). Fluorescent labeling of peptides was performed as described in *SI Text* and confirmed by LC-MS. Labeled peptides were purified by reverse-phase HPLC.

Recombinant HLA-A1, -A2, -A3, A11, and -B7 heavy chains were produced in *Escherichia coli*. MHC Class I monomer refolding reactions with *E. coli*-derived β 2M were performed as described (22) and purified by gel-filtration HPLC in PBS (pH 7.4). Biotinylation and MHC tetramer formation were performed as described (12). pMHC complexes were stored at -20°C in PBS/16% glycerol, MHC tetramers were stored at -20°C in PBS/16% glycerol/0.5% BSA.

Analysis of Peptide Exchange. Exchange reactions were performed by exposure of pMHC complexes (25 $\mu\text{g}/\text{ml}$ in PBS) to long-wavelength UV, by using a 366-nm UV lamp (Camag) in the presence or absence of 50 μM exchange peptide. After UV-exposure, pMHC complexes intended for subsequent analysis by ELISA were incubated at 37°C for 60 min to promote unfolding of peptide-free MHC molecules (6). For gel-filtration HPLC, incubations after UV exposure were performed at room temperature. pMHC complexes intended for use in flow cytometry were multimerized by the stepwise addition of streptavidin-PE (Invitrogen). For gel-filtration HPLC, 300×21 and 300×7 mm Biosep SEC S3000 columns (Phenomenex) were used for protein isolation and analysis, respectively. Absorbance was monitored at 230 nm, and fluorescence was monitored with excitation at 550 nm and emission at 567 nm. Peptide elution and subsequent reverse-phase chromatography was performed as described in *SI Text*. Sandwich ELISAs were performed as described (12).

Cells and Flow Cytometry. Frozen peripheral blood mononuclear cells from individuals undergoing an HLA-matched allogeneic bone marrow transplan-

tation were obtained after informed consent and with approval from the Leiden University Medical Center Institutional Review Board. For flow-cytometric analysis, cells were stained with PE-labeled MHC tetramers for 5 min, followed by FITC-labeled anti-CD8 (BD Biosciences) staining for 15 min at room temperature. Data acquisition was carried out on a FACSCalibur (Becton Dickinson). Analysis was performed by using FlowJo (Tree Star).

Peptide Library and Binding Studies. Protein sequences for Nodal (NP.060525), Mart-1/Melan-a (NP.005502) Tyrosinase (AAB60319), Tyrosinase-related protein 1 (CAG28611), Tyrosinase-related protein 2 (ABI73976), and GP100/PMEL17 (NP.008859) were analyzed for potential HLA-A3 ligands by using SYFPEITHI (9), and the artificial neural network (ANN) and stabilized matrix method (SMM) algorithms from IEDB (version prior to December 2007) (20). Peptides were selected with a predicted binding value of either >21 for SYFPEITHY (nona- and decamers), <6000 for ANN (nonamers only), or <600 for SMM (decamers only), resulting in 203 peptides. Synthesized peptides (Pepscan Lelystad), were checked by LC-MS. HLA-A3 binding assays were performed by using a fluorescence polarization (FP) assay. For this purpose, a FP assay reported for HLA A2.1 (36) was modified for application with UV-mediated peptide exchange, using fluorescently labeled A3-specific KVP-CALINK (37) as tracer peptide (see *SI Text*). To determine the binding capacity of peptides for HLA-A3, percentage inhibition relative to controls was determined at 5 μM in an FP competition assay with conditional p*A3. For peptides displaying $>63\%$ inhibition at 5 μM , IC_{50} values were determined by generating dose-response curves of serial peptide dilutions from 50 μM to 50 nM.

ACKNOWLEDGMENTS. We thank Drs. Per Thor Straten and Mads Hald Andersen (both of Herlev University, Herlev, Denmark) for HLA-A11* samples, Henk Hilkmann for peptide synthesis, and Anna Keller for critical reading of the manuscript. This work was supported by Landsteiner Foundation of Blood Transfusion Research Grant 0522 (to T.N.M.S.), Dutch Cancer Society Grant UL 2007-3825 (to T.N.M.S. and M.H.M.H.), and Nederlandse Organisatie voor Wetenschappelijk Onderzoek Grant 700.55.422 (to H.O.).

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SUPPORTING INFORMATION

Labeling of Peptides.

Fluorescent labeling of peptides was performed as follows: 6 mg of peptide (50 mg/ml in DMSO) was diluted in 1.5 ml 50 mM Hepes buffer and low amounts of *N,N*-dimethylformamide (DMF; Biosolve) were added whenever precipitates formed. 2.5 mg of TMR-5-maleimide (Tebu-Bio) from a 50 mg/ml DMF stock solution was added, and the reaction mixture was incubated at room temperature for 36 h on an orbital shaker. Five microliters of the reaction was analyzed by LC-MS, and the reaction was terminated by the addition of DTT to a final concentration of 5 mM. Before purification, 1.5 ml of 10% trifluoroacetic acid (TFA; Biosolve)/5 mM DTT/50 mM Hepes was added. Labeled peptides were purified by reverse-phase HPLC (1525EF; Waters) with H₂O/0.05%TFA as solvent A and acetonitrile/0.05% TFA as solvent B at a flow rate of 18 ml/min on a 10- μ m 30 $\times$ 250-mm dc18 Atlantis column (Waters). A linear gradient of 10–95% solvent B over 14 min was used for a first elution. The fractions were then analyzed by LC-MS, and fractions containing fluorescently labeled peptide were further purified by using a similar protocol with a linear gradient of 25–80% solvent B over 19 min for elution. Fractions were then lyophilized, dissolved in DMSO, and stored at –20°C.

Peptide Elution.

HLA-A2 Flp*A2 complexes were either left untreated or exposed to UV light for 60 min. For examination of the effect of UV treatment on the total pool of MHC associated plus released fluorescent ligand, peptides were extracted with 10 μ l of 10% TFA (Biosolve) for 60 min at room temperature. The material was then analyzed by reverse-phase chromatogra-

phy with H₂O/0.05%TFA as solvent A and acetonitrile/0.05% TFA as solvent B at a flow rate of 1 ml/min on a 300 $\times$ 3.9 Delta Pak 15 μ C18 column (Waters). A linear gradient of 10–60% solvent B over 60 min was used for elution. To analyze the composition of material remaining MHC-associated after UV treatment, reactions were separated by gelfiltration chromatography after UV exposure and material eluting with the retention time of pMHC complexes was collected. Subsequently, samples were adjusted to contain an equal amount of fluorescent signal and concentrated to 100 μ l. Ten microliters of 10% TFA was added, and samples were incubated for 60 min at room temperature. Nine hundred microliters of H₂O was added, and samples were analyzed by reverse-phase chromatography as described above.

Fluorescence Polarization Assay.

For all fluorescence polarization binding assays peptides were diluted in bovine γ -globulin in PBS (0.5 mg/ml; Sigma). The HLA-A3-binding peptide KVPCALINK was labeled with TMR-5-maleimide (Anaspec) and purified by reverse-phase HPLC as described above. This peptide (pTAMRA) was used as a tracer for fluorescence polarization (FP) experiments. Labeled peptide was standardized against a fluorescence intensity curve by using free TMR-5-maleimide as a standard. FP measurements were performed on a PerkinElmer Wallac EnVision 2101 Multilabel Reader. Samples were measured by using a 531-nm excitation filter, 579-nm S-channel emission filter, a 579-nm P-channel emission filter, and a Bodipy-TMR FP Dual mirror (all filters and mirrors obtained from PerkinElmer). FP values are given as mP (millipolarization) and calculated by using

the following formula: polarization (mP) = $1,000 \times (S-GP)/(S+GP)$, where S and P are the fluorescence intensities measured in the S (parallel to polarization plane) and P (perpendicular to polarization plane) directions and G is the grating factor. The G factor is a correction factor for instrument (filters, mirror)-dependent variations in sensitivity for measurements in the S and P directions and is determined according to the instrument manufacturer's instructions by measuring a 1 nM sample of free TAMRA. The G factor is then calculated using the following formula: $G = (S/P) \times (1-L/1,000)/(1+L/1,000)$, where L is the theoretical or literature polarization value for the fluorophore, here TAMRA (L = 50 mP). Typically, G factors of 1–1.1 were obtained.

Epitope Screening.

To determine the binding capacity of the predicted ligands for HLA-A3, percentage inhibition of binding of tracer peptide was determined at 5 μ M in an FP competition assay with conditional p*A3. For this purpose, an FP assay was modified for application with UV-mediated peptide exchange for HLA-A3. In a 384 well black nonbinding surface assay plate (Corning) each well was loaded with 10 μ l of a 2.5 $\times$ peptide solution (12.5 μ M), 5 μ l of a pTAMRA solution (5 nM) and 10 μ l of a 2.5 $\times$ HLA-A3 p*A3 solution (1.25 μ M). For all preparations 0.25 mg/ml bovine γ -globulin in PBS was used as a buffer. The plate was spun for 1 min at 1,000 $\times g$ at room temperature. To start UV-mediated peptide exchange, the plate was placed 10 cm under a 365-nm UV lamp (2 $\times$ 15 W blacklight blue tubes, LxWxH 505 $\times$ 140 $\times$ 117 mm; Uvitec) located in a cold room (4°C). After 30 min irradiation, the plate was sealed with thermowell sealing tape (Corning) and incubated at room temperature for 45 h, when periodic readings showed no further increase in polarization, indicating

the establishment of equilibrium. Controls included peptide free samples (1% DMSO) for 0% inhibition of tracer binding and 200 μ M ILRGSVAHK (A3-Flu epitope) samples for 100% inhibition of tracer binding. All data points were determined in triplicate.

Competition Assay.

For peptides displaying >63% inhibition at 5 μ M IC₅₀ values were determined by generating dose-response curves of serial peptide dilutions covering a range of 50 μ M to 50 nM using the UV-mediated FP competition assay described above. The binding affinity (IC₅₀ value) of each competitor peptide was defined as the concentration that inhibits 50% binding of pTAMRA tracer peptide. Data were analyzed by using GraphPad Prism software (GraphPad). Fifty percent inhibitory concentrations were determined in at least three independent experiments for each peptide. Peptide concentrations used for determination of IC₅₀ values were calculated by assuming maximum synthetic yield (2 μ mol). For 13 peptides containing tyrosine or tryptophan residues, concentrations were verified by measuring absorption at 280 nm (for calculations of extinction coefficients see: www.expasy.org/tools/protparam.html). Peptide concentrations as determined by absorption at 280 nm were in between 0.47 and 1.05 of the predicted values.

Table 2. Conditional ligands tested

Allele	Sequence	Refolding efficiency Thermostability UV-sensitivity	Allele	Sequence	Refolding efficiency Thermostability UV-sensitivity
HLA-A1	STDJGVLEY	+	HLA-A11	AIFQJSM TK	+
	STDPJVLEY	- (LD)		AIFQSSM JK	--- (TI)
	STDPGJLEY	++		ATFQJSM TK	--- (LR)
	STDPGVJEY	+		ATFQSSM JK	-- (ND)
	STDPGVLJY	- (LD)		RVFAFSF JK	-- (ND)
				RVFAJSFIK	Candidate
	STAPGJLEY	Candidate	HLA-B7	APRJPTLAL	- (LD)
	STDPGJLEK	--- (LR)		APRGJTLAL	++
	STDPGJLEF	--- (TI)		APRGPJLAL	- (LD)
HLA-A3	RLYJGGATK	- (LD)		APRGPTJAL	-- (ND)
	RLYRJGATK	++		APRGPTLJL	--- (LR)
	RLYRGGAJK	-LD			
				AARGJTLAM	Candidate
	RIYRJGATR	Candidate		AARGJTLAL	++
	RGYRJGATY	--- (TI)		APRGJTLAM	++
	RLYRJGATL	--- (LR)			

Overview of the peptides tested for each of the four HLA molecules. **J** indicated 3-amino-3-(2-nitro)phenyl-propionic acid. Sequences in the top part of each panel reflect the peptides used in a first screen. Arrows indicate which peptides were chosen as leads for a second screen (bottom parts). For HLA-A11, a panel of high and low affinity binders was tested in a single screen with **J** at two different positions. The four conditional ligands selected for further analysis are depicted in bold. LR: low refolding efficiency, TI: thermo-instable, ND: no UV-mediated degradation, LD: low UV-mediated degradation.

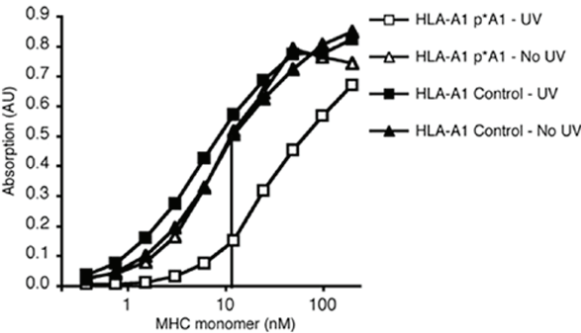


Fig. 5.

Establishment of an optimal MHC con-centration for ELISA analysis. HLA-A1 complexes containing a control HLA-A1 ligand pA1 or the UV-sensitive ligand p*A1 were exposed to 366 nm of UV for 0 or 60 min, and serial dilutions were analyzed by ELISA. Vertical line indicates the MHC concentration used for subsequent analyses. MHC concentrations as established for the other MHC gene products were also in the 10–20 nM range.

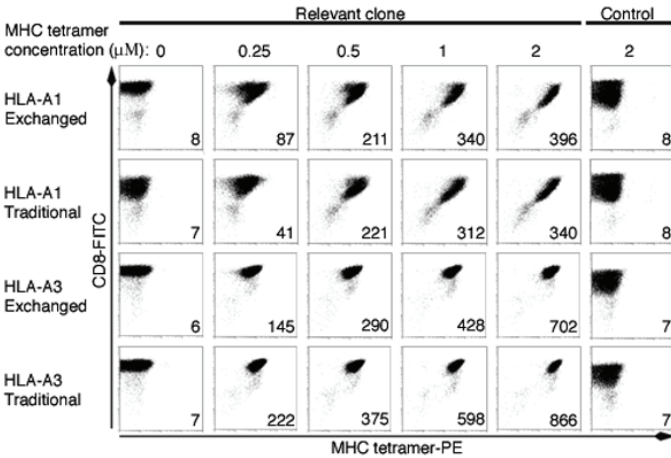


Fig. 6. Tetramer titration on T cell clones. CD8+ T cell clones specific for HLA-A1 CMV-pp₆₅ and HLA-A3 EBV-EBNA-3a were stained with the indicated amounts of HLA-A1 CMV-pp₆₅ (Top) and HLA-A3 EBV-EBNA-3a (Bottom) tetramers. “Exchanged” indicates MHC tetramers generated via peptide exchange, whereas “Traditional” indicates MHC tetramers generated via classical MHC refolding with the specific peptide. Images designated “Relevant Clone” and “Control” represent staining with the matched MHC tetramer and crosssample controls, respectively. Numbers indicate MFI of CD8+ cells.

classical MHC refolding with the specific peptide. Images designated “Relevant Clone” and “Control” represent staining with the matched MHC tetramer and crosssample controls, respectively. Numbers indicate MFI of CD8+ cells.

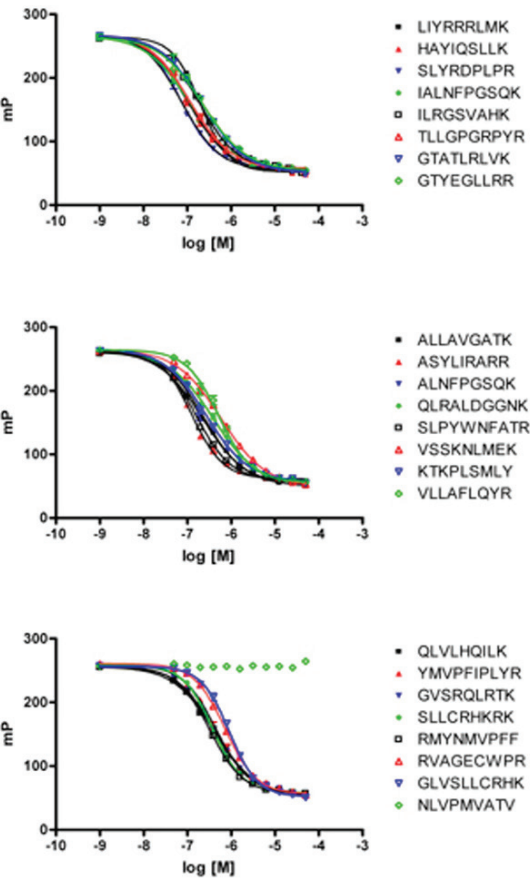


Fig. 7. Dose-response curves of a competitive binding FP assay after 30-min UV irradiation and 45-h incubation using HLA-A3 p*A3 and the indicated peptides. IC₅₀ values were determined from three independent experiments. A representative experiment is shown. mP indicates millipolarization.

Table 3. Peptide library scanned for HLA-A3 binding

Protein	Sequence	Inhibition	SD	Protein	Sequence	Inhibition	SD	Protein	Sequence	Inhibition	SD
Tyrosinase-related protein 2	TLLGPRPYR	84	1	Tyrosinase	VSSKNLMEK	74	3	GP100	<u>LIYRRRLMK</u>	90	2
	GTYEGLRR	83	2		YMPVPIPLYR	72	2		<u>IAINFPGSOK</u>	86	1
	VLLAFLOYR	74	11		SLLCRHKR	70	2		<u>GTATRLVK</u>	84	2
	RMVNMVPPF	69	6		GLVSLCRHK	64	5		<u>ALLAVGATK</u>	83	1
	LMETHLSSK	62	2		SIFEQWLQR	63	4		<u>ALNFPGSOK</u>	80	2
	VMSLHNLVH	59	8		KSYLEQASR	60	0		<u>QLRALDGGNK</u>	79	0
	VLLAFLOYRR	55	4		KFFAYLTAK	51	4		<u>QLVLHQILK</u>	72	3
	LLRRNQMR	48	1		MVPIPLYR	43	7		<u>GVSRLRTK</u>	72	2
	FLLSCLGCK	47	5		TQYESGMDK	36	3		QLRTKAWNR	62	1
	GLRRNQMR	46	2		FMGFNCGNCK	36	3		QVWGGQPVY	59	4
	MTVDSLUNK	42	1		CVSSKNLMEK	27	4		VSCQGLPK	58	10
	FQNSTFSR	41	8		LVSLCRHK	26	7		VLASLIYRR	54	2
	AIFDEWMKR	39	1		FFAYLTAK	22	1		TVSCQGGLPK	49	1
	VVISNRLLY	36	11		VSLCRHKRK	19	1		VVLASLIYR	45	3
	ILEHVRKEK	30	1		LLRWQEQIK	14	2		GALLAVGATK	38	1
	FLGALDLAK	27	0		ALLGSEIWR	2	0		HTMEVTVYH	35	4
	HVRKEKATK	27	3		SMHNALHIYM	0	2		ILKGGSGTY	30	2
	PLMETHLSSK	27	3		YMPVPIPLY	0	2		SLIYRRRLMK	30	7
	QVMSLHNLVH	21	6		NIFDLSAPEK	-1	1		LVLKRCLLH	29	7
	FLGALDLAKK	19	1		IPIGTYGQMK	-3	1		SSHWLRLPR	20	1
	NSMKLPTLK	14	1		QLSGRSCQ	-4	2		TVYHRRGSR	18	6
	SLVNKECCPR	7	1		DLSAPEKDK	-4	2		RQLRTKAWNR	15	3
	TLLGPORPY	3	1		HIYNGTMSQ	-4	3		QKRSFYVVK	14	4
	LLAFLOYRR	3	4		HPPRACVSSK	-4	1		LGVSRQLRTK	11	1
	CMTVDSLUNK	2	1		PLRRNPGNH	-4	1		LIYRRRLMKQ	9	3
	GFLSCLGCK	1	1		ALLAGLVSL	-5	2		QILKGGSGTY	7	1

Two hundred three predicted HLA-A3 ligands from six melanoma-associated proteins were selected based on three binding prediction algorithms. In an initial screen, peptides were screened at 5 μ M in a 45-hour FP competition assay format. Peptides in bold were chosen for further analysis to determine IC50 values. Underlined peptides represent previously described HLA-A3 ligands. Inhibition reflects the percentage reduction in polarization of the tracer peptide. Values represent mean $\pm$ SD of triplicate samples.

Table 3. Peptide library scanned for HLA-A3 binding

Protein	Sequence	Inhibition	SD	Protein	Sequence	Inhibition	SD	Protein	Sequence	Inhibition	SD
	SLSPQEREQF	0	3	Nodal	RLPSSADVEF	-5	2	Mart1	CWRGGQVSLK	6	4
	DIRDCLSLQK	0	1		PIGTYGQMK	-5	0		AESTGMTPEK	5	2
	FLNGTNALPH	0	1		WLLGAAMVG	-5	2		CQLVLHQILK	3	3
	TTNILEHVRK	-1	1		VLYCLLWSFQ	-5	1		YLAEADLSY	2	1
	AFLQYRRLRK	-1	1		LLAGLVSLLC	-6	1		AVVLASLIYR	1	1
	RLRKGYTPLM	-1	3		SYMVPFIPLY	-8	1		GLSIGTGRAM	0	3
	RKFHRTCK	-1	0		RLLVRRNIFD	-8	1		ILLVLMAVVL	-1	2
	QFLGALDLAK	-1	2		YDLFWWMHY	-8	0		ALQLHDPG	-1	1
	RLGAESANVC	-2	1		PLYRNGDFF	-9	1		AVGATKVPR	-2	1
	LVTLCNGTY	-3	1		NLLSPASFF	-9	2		AVVLASLIY	-2	3
	ILPGAQGF	-4	1		RLLVRRNIF	-9	2		TLIGANASF	-4	1
	FLQYRRLRK	-4	1		HAYIQSLIK	90	1		AVIGALLAVG	-4	1
	FVLLAFLQY	-4	1		SLYRDPLPR	88	1		AVIGALLAV	-4	1
	RLIGNESFA	-4	1		KTKPLSMILY	74	2		VLYRYGSFS	-4	1
	ALVGLFVLLA	-5	3		RVAGECWPR	67	3		YLEPGPVTA	-5	1
	KILPGAQGF	-5	1		VTRPLSKWLK	57	3		LISRALVVTH	-5	1
	ALDLAKKRVH	-6	1		ATVATALLR	56	1		LLVLMAVVL	-5	1
	TLISRSRF	-7	1		STCCAPVTK	54	3		TLISRALVV	-6	0
	SVYDFFVWLH	-7	0		ATNVLLMLY	44	9		QVLGGPVSG	-6	1
	RMYNMVPFFP	-7	1		LAYMLSLYR	38	8		QVPLDCVLY	-6	2
Tyrosinase-related protein 1	NILEHVRKEK	-7	2		WLKRPGALEK	36	8		PLLDGTATL	-7	1
	RLLYNATTN	-8	1		HAYIQSLLR	24	5		FLRNQPLTF	-7	2
	RLLYNATTNI	-8	1		IYPKQYNAY	14	2		KVLPDGOVIV	-7	2
	FWWLHYYSVR	-8	2		AIEIFHQPK	12	10		KVLPDGOVI	-8	2
	ASYLIRARR	83	2		VLEVTRPLSK	8	1		HVGTQCALTR	0	1
	SLPYWNEATR	77	4		LKRPGALEK	-1	1		AHFTYGPKK	-3	2
	GLFDTPPFY	60	3		ALLQAGAAT	-3	2		VLLIGCWY	-4	2
	LTWHRYHLLR	55	10		LLQAGAATV	-4	1		GVLLIGCWY	-4	1

Table 3. Peptide library scanned for HLA-A3 binding

Protein	Sequence	Inhibition	SD	Protein	Sequence	Inhibition	SD	Protein	Sequence	Inhibition	SD
	AVFEWLR	53	4		GWGSWIYPK	-4	2		RDSKVSLEK	-4	0
	WTHYYSVK	47	4		MLYSNLSQE	-5	1		DAHYGYPK	-5	1
	FVWTHYSVK	42	6		ALLQAGATV	-6	2		LLIGCWYCR	-8	3
	LPWNFATRK	18	7		SPLAYMLSLY	-6	3		LLIGCWYCR	-8	2
	NVARPMVQR	17	2		AVDGNWTF	-6	0				
	YSNSTNSFR	9	4		ALLTRGQP	-6	2				
	ALFGTASY	8	2		TVTISQVTF	-7	1				
	KLLSLGCF	2	1		LLMLYSNLSQ	-7	1				
	EIIAIVVG	-1	4		NHAYQSLLK	-8	2				
	FVRALDMAK	-3	1		NLIGGWSWI	-8	1				
	ALLVVALIF	-3	0		RQLGGSTLLW	-8	2				
	TLSPNSVF	-3	2		PLAYMLSLY	-8	1				
	FLNGTGGQTH	-3	2		PLAYMLSLYR	-10	1				
	ALLVVALIF	-3	1								
	ILGPDGNT	-4	1								
	AVRSLHNLAH	-4	3								
	NLLDSKEEK	-4	1								
	AVVGALLVA	-5	2								
	ALDMAKRTTH	-5	1								
	FVRALDMAKR	-5	1								
	HRYHLRLEK	-6	1								
	CIFFPLLFQ	-7	0								
	ALFGTASYL	-7	2								
	SIYNFVWTH	-8	1								

Chapter 6

Parallel detection of antigen-specific T-cell responses by multidimensional encoding of MHC multimers

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Nat Methods. 2009 Jul;6(7):520–6

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Parallel detection of antigen-specific T-cell responses by multidimensional encoding of MHC multimers

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The use of fluorescently labeled major histocompatibility complex multimers has become an essential technique for analyzing disease- and therapy-induced T-cell immunity. Whereas classical major histocompatibility complex multimer analyses are well-suited for the detection of immune responses to a few epitopes, limitations on human-subject sample size preclude a comprehensive analysis of T-cell immunity. To address this issue, we developed a combinatorial encoding strategy that allows the parallel detection of a multitude of different T-cell populations in a single sample. Detection of T cells from peripheral blood by combinatorial encoding is as efficient as detection with conventionally labeled multimers but results in a substantially increased sensitivity and, most notably, allows comprehensive screens to be performed. We obtained proof of principle for the feasibility of large-scale screening of human material by analysis of human leukocyte antigen A3-restricted T-cell responses to known and potential melanoma-associated antigens in peripheral blood from individuals with melanoma.

T cells recognize virus-infected cells and tumor cells by detecting the presence of disease-specific peptide-major histocompatibility complexes (pMHC) with their clone-specific T-cell receptor. Both for the monitoring of disease-specific immune responses and for the development of new immunotherapeutics, it is essential to specifically detect only those T cells that recognize a given pMHC complex within the large pool of irrelevant T cells¹. The use of soluble multimeric pMHC complexes to detect such antigen-specific T cells by flow cytometry was first described in 1996 (ref. 2) and has since then become a cornerstone of T-cell monitoring³. However, a major limitation in the use of this methodology is that at most, a few antigen specificities can be monitored in a single biological sample because of limitations on the number of available fluorochromes⁴.

Prior work has shown that it is feasible to detect antigen-specific T cells by the binding of two different fluorochrome-coded major histocompatibility complex (MHC) multimers containing the same

or a related peptide. This technology of double MHC multimer staining has been used to reveal the fine specificity of T cells for variants of single peptide epitopes⁵. If a large set of such dual-color-encoded pMHCs could be combined in a single sample without interfering with the ability of each single pMHC to detect specific T cells, such a technology could conceivably be used to detect a much larger number of T-cell specificities than is currently possible with classical MHC multimer analysis.

Here we demonstrate that a large number of antigen-specific T-cell responses (at least 25) can indeed be analyzed in parallel through the use of pMHC multimers that are each coupled to a unique combination of fluorochromes, with the same fluorescent label being used many times, but in each case in a unique combination with one or more other labels. We exemplify the value of this 'combinatorial encoding' by dissecting melanoma-associated T-cell responses in peripheral blood from individuals with melanoma.

RESULTS

The concept of combinatorial encoding

In this approach, a specific T-cell population is no longer defined by a single fluorescent signal, as is the case in standard MHC multimer stainings, but instead is visualized by the joint binding of multiple fluorochromes. Conceptually, the approach can be considered a self-assembling molecular coding scheme, in which unique codes are only assembled at the moment of T-cell binding (Fig. 1a). The power of such a scheme becomes evident with an increasing number of available fluorochromes (Fig. 1b). In this study, we explored two-dimensional combinatorial encoding with eight fluorochromes, which deliver 28 unique codes (Supplementary Table 1), and demonstrated the ability to expand to three-dimensional combinatorial encoding using the five most intense fluorochromes.

Feasibility of combinatorial encoding

We first determined the feasibility of using six different quantum dots (Qdots) as fluorescent labels for pMHC multimers, in addition

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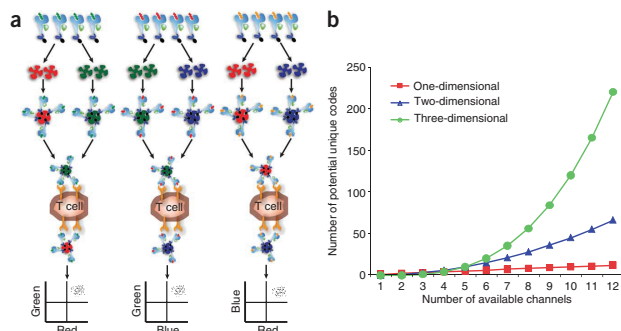


Figure 1 | Overview of the combinatorial encoding approach. (a) MHC molecules with different peptide specificities (green, red and orange peptides, top) are conjugated with different fluorochrome color codes (red, green and blue fluorochromes, second row) to generate differentially encoded pMHC multimers (third row). Combinatorial codes are then assembled on T-cell surfaces (fourth row) and these T-cell samples are analyzed by flow cytometry (bottom). (b) The theoretical number of unique color combinations that can be made using fluorochromes for the indicated numbers of available channels in either one-, two- or three-dimensional coding schemes.

to allophycocyanin (APC) and phycoerythrin (PE) fluorescent labels (**Supplementary Table 1**), which are more commonly used for the detection of antigen-specific T-cell responses. By analyzing peripheral blood samples for cytomegalovirus (CMV)-specific CD8⁺ T-cell responses, we established that pMHC complexes that were multimerized by coupling to streptavidin-conjugated Qdots, APC or PE could all be used to detect antigen-specific T cells (**Supplementary Fig. 1**). Subsequently we analyzed whether antigen-specific T-cell populations could reliably be identified by the binding of two pMHC multimers that contain the same antigenic peptide but that are coupled to a different fluorochrome (**Fig. 1a**). Testing of pMHC complexes conjugated to all 28 possible combinations of two different fluorochromes demonstrated that such dual encoding can in all cases reveal the appropriate T-cell population, with only limited variation in detection efficiency between the different color combinations (**Fig. 2a**). As expected, simultaneous staining of T cells with two differentially labeled pMHC multimers that contain the same antigenic peptide leads to a small reduction in fluorescence intensity for each channel (an expected factor of 2 at equimolarity). To limit this negative effect, we used the three Qdots that yielded the lowest intensity signal on our flow cytometric system (Qdots QD565, QD585 and QD800) in a 2:1 rather than 1:1 ratio relative to the other fluorochromes, and we did not use the Qdot combinations QD565 and QD585; QD565 and QD800; and QD585 and QD800 in subsequent experiments, thereby reducing the number of combinations to 25.

Sensitivity of combinatorial encoding

Antigen-specific T-cell populations can be present at very low frequencies, and pMHC multimers do show low-level background staining. To test whether the detection of antigen-specific T cells with combinatorial encoding affects background staining or the frequency of antigen-specific T cells detected, we stained peripheral blood mononuclear cells (PBMCs) containing a low-frequency T-cell response to the human leukocyte antigen (HLA)-A2-restricted CMV epitope NLVPMVATV (CMV-NLV) with HLA-A2 CMV-NLV multimers or with irrelevant HLA-A2 multimers and analyzed them by flow cytometry. Specifically, we incubated PBMCs either with the eight different single-color pMHC multimers in eight separate stainings or with the 25 dual-color-encoded MHC multimers in 25 separate stainings. We considered T cells positive for MHC multimer binding when detected above background either in

one channel (for single-color stainings) or when detected in both relevant channels (for dual-color encodings). Notably, the frequency of false positive cells observed upon staining with irrelevant HLA-A2 multimers was approximately tenfold lower when using a dual encoding scheme as compared to the traditional single-staining approach (**Fig. 2b**). This is because most background events are only positive in a single channel and are therefore excluded by combinatorial encoding.

Having established the feasibility of dual-color encoding, we examined whether multiple dual-color-encoded MHC multimer stainings can be performed in parallel on a single sample. To analyze T cells reactive with any of the dual-color-encoded pMHC multimers in a single sample, we developed a gating strategy. In brief, we identified single live CD8⁺ T cells (**Fig. 2c** and Online Methods) and analyzed T-cell populations reactive with any of the dual-color-encoded pMHC multimers by gating on each of the eight individual fluorochromes used to form the 25 different unique combinations (**Fig. 2c**), as exemplified by gating PE and QD705 double positive CD8⁺ T cells from both a relevant and an irrelevant mix of pMHC multimers. This strategy identified T cells with signal above background in a given combination of two channels and that are negative in the remaining six channels, thereby allowing the simultaneous analysis of 25 different combinations in one flow cytometry experiment.

Single-sample visualization of up to 25 T-cell responses

We generated 25 different pMHC multimers containing various viral and cancer epitopes for the human MHC alleles HLA-A1, -A2, -A3 and -B7 (**Supplementary Table 2**) by MHC peptide exchange⁶⁻⁸. Then, we coupled each of these pMHC multimers to two fluorochromes, generating a set of unique codes (**Supplementary Table 2**). To compare the data obtained by combinatorial encoding with conventional MHC-multimer analysis, we made PE-labeled pMHC multimers in parallel using the same T-cell epitopes. To determine the background of combinatorial encoding, we also prepared irrelevant pMHC multimers in all two-color combinations.

We analyzed PBMCs from three healthy donors covering all four HLA alleles (**Fig. 3a-c**) by (i) one single staining with the mix of dual-color-encoded viral and cancer epitope pMHC multimers, (ii) 25 separate stainings with all individual dual-color-encoded pMHC multimers, (iii) one single staining with the mix of dual-color-encoded irrelevant pMHC multimers or (iv) 25 separate stainings with all 25 PE-labeled pMHC multimers. We performed the experiment in a blinded fashion with respect to HLA haplotype

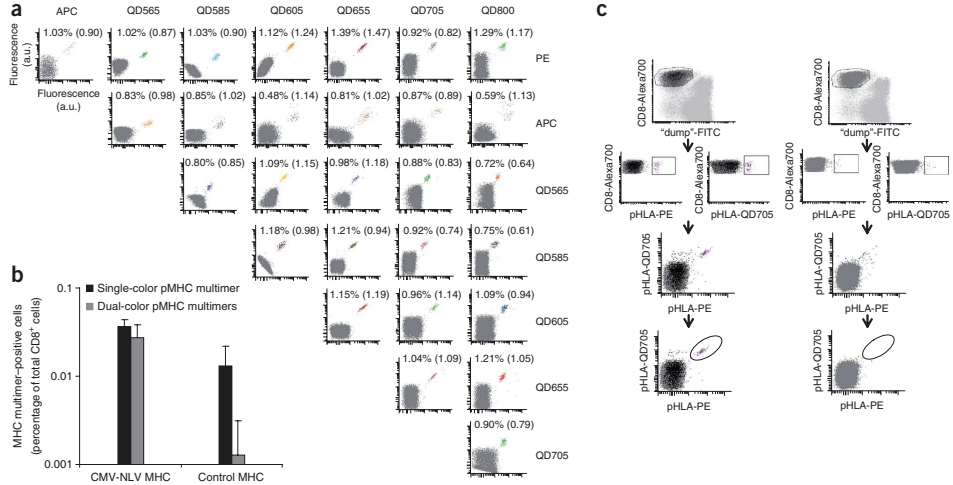


Figure 2 | Feasibility of T-cell staining with dual-color-encoded pMHC multimers. (a) Flow cytometry plots displaying fluorescence intensity for the fluorochromes listed at the top and right side of the plot matrix. PBMCs stained using the 28 possible two-color combinations of HLA-A2 CMV-NLV MHC multimers in separate stainings were analyzed, as indicated. Each plot is labeled with the percentage of HLA-A2 CMV-NLV-specific T cells out of total CD8⁺ cells and, in parentheses, the number of detected HLA-A2 CMV-NLV-specific T cells divided by the average number of HLA-A2 CMV-NLV-specific cells found for all color combinations (average of three experiments using three different donors). Dot plots were gated on approximately 10,000 CD8⁺ lymphocytes. Gray dots represent CD8⁺ T cells with no MHC multimer binding, colored dots represent MHC multimer-reactive CD8⁺ T cells (b) Percentage of MHC multimer-positive T cells found with the indicated techniques. Data are mean $\pm$ s.d., $n = 25$ for gray bars and $n = 8$ for black bars. (c) Schematic overview of the gating strategy used for identification of pMHC-specific T cells after staining with dual-color-encoded MHC multimers. PBMCs were stained with relevant pMHC complexes to visualize antigen-specific T cells (left) or with irrelevant pMHC complexes to indicate background staining (right). CD8⁺ and 'dump' (FITC) negative cells are selected (dark gray; top). Cells positive in two different MHC multimer channels (PE, QD705) are indicated in purple, and cells positive in any of the other multimer channels indicated in black (second row). Then one determines whether cells are double positive for the two selected colors (third row). Cells positive in three or more MHC multimer channels are removed (bottom). In typical final data plots, an antigen-specific T-cell population is indicated by fluorescence in two defined channels (bottom, left).

and to prior analysis of antigen-specific T-cell responses in these donors. Combinatorial encoding of pMHC multimers allowed the simultaneous visualization of a large number of antigen-specific T-cell populations in one single sample (Fig. 3 and Supplementary Fig. 2). Notably, we found the same virus-specific T-cell populations in each donor when analyzed by individual PE-multimer stainings. Furthermore, statistical comparison between staining strategies i, ii and iv revealed a high interclass correlation (interclass correlation coefficient (ρ) = 0.84 (95% confidence interval: 0.78–0.89)), describing an almost perfect agreement between the outcomes of the three strategies⁹. Individual comparison between the mix of 25 dual-color-encoded pMHC multimers in one sample (strategy i) with either the PE-tetramer stainings (strategy iv) or the same set of dual-color-encoded pMHC multimers in 25 separate stainings (strategy ii) reveal similarly high correlations (ρ = 0.79 (95% confidence interval: 0.68–0.86) and ρ = 0.83 (95% confidence interval: 0.74–0.89), respectively) (Fig. 4). Thus, the simultaneous detection of multiple antigen specificities with sets of pMHC multimers, in which each multimer is coupled to a distinct combination of fluorochromes, is feasible. Finally, comparison of the signals from the dual-color-encoded pMHC set containing virus and tumor-associated epitopes (strategy i) with the signals from the pMHC set containing 25 irrelevant MHC multimers (strategy iii) indicates that the sensitivity of the approach is high

and that T-cell populations as infrequent as 0.02% of CD8⁺ cells can be identified (Figs. 3a–c and 4a) (average background: 0.0012% of CD8⁺ T cells, s.d. = 0.0035, $n = 100$).

To validate the T-cell responses observed by MHC multimer staining using a non-flow cytometry-based method, we performed an INF γ enzyme-linked immunosorbent spot (ELISPOT) assay with these PBMCs using the same 25 peptides (Fig. 3a–c). All T-cell responses detected by MHC multimer analysis were confirmed by detection of INF γ secretion. Note that the magnitude of T-cell responses, as measured by INF γ secretion, was in some cases substantially lower than that measured by any of the MHC multimer approaches, consistent with the observation that a variable fraction of antigen-specific T cells acquires the capacity to secrete effector cytokines.

To provide proof of principle for higher-dimension encoding of antigen-specific T-cell responses, we generated eight different combinations of five high-intensity fluorochromes (PE, APC, QD605, QD655 and QD705), in which each antigen specificity was encoded by a unique combination of three different fluorochromes. Even though the frequency of antigen-specific T cells detected was lower as compared to two-dimensional encoding (0.29% versus 0.47% of CD8⁺ T cells), identification of CMV-NLV-specific T cells in healthy donor PBMCs was feasible using this three-dimensional encoding strategy (Supplementary Fig. 3).

Finally, combinatorial encoding can be combined with single-color antibody staining to determine the phenotype of pMHC-specific T cells. To illustrate this, we analyzed the phenotype of three virus-specific T-cell populations, HLA-A2 Inf-GIL,

CMV-NLV and EBV-GLC. As described previously^{10,11}, HLA-A2 CMV-NLV-specific T cells displayed a more differentiated phenotype (CD45RA⁺, CCR7⁻, CD62L⁻ and CD57⁺ or CD57⁻), as compared to HLA-A2 EBV-GLC and Inf-GIL T-cell populations

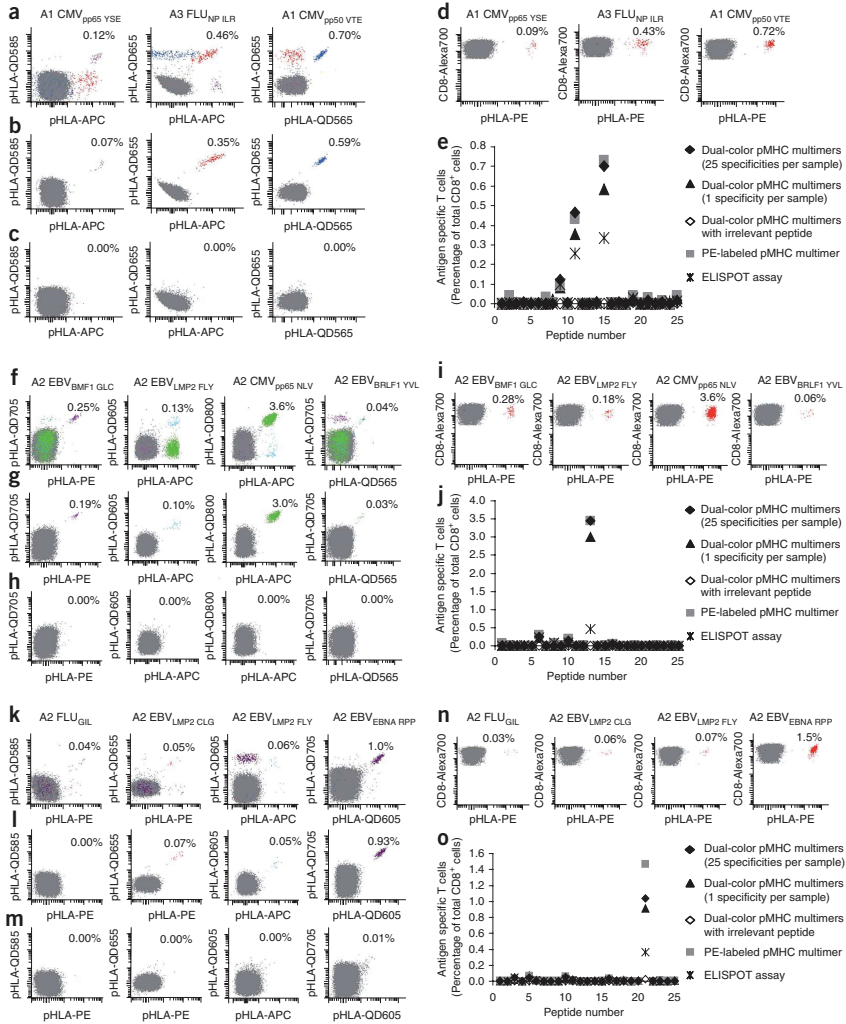


Figure 3 | Multiplex detection of virus-specific T-cell responses through combinatorial encoding. (a–e) Donor 1. (f–j) Donor 2. (k–o) Donor 3. Dot plots of antigen-specific T-cell populations detected at a frequency >0.03% are shown by staining one sample with a mix of 25 different dual-color pMHC multimers (a,f,k); by staining 25 individual samples with 1 of the 25 dual-color pMHC multimers (b,g,l); by staining one sample with a mix of 25 irrelevant dual-color pMHC multimers (c,h,m); and by staining 25 individual samples with classical PE-labeled pMHC multimers each containing one of the 25 peptides (d,i,n). All dot plots are shown with bi-exponential axes and display fluorescence intensity (a.u.) for the fluorochrome indicated on each axis. Graphical representation of the frequency of antigen-specific CD8⁺ T cells directed against the 25 epitopes (Supplementary Table 2), when measured using the four different flow cytometry-based strategies shown in a–d, f–l and k–n, and IFN γ ELISPOT assay (e,j,o).

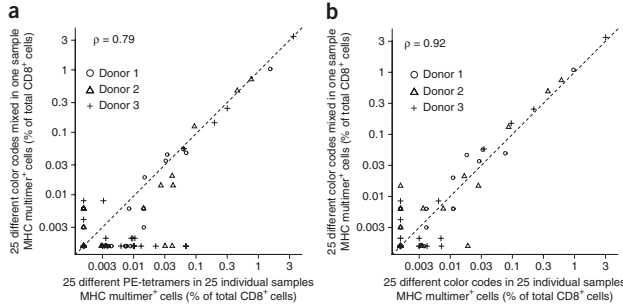


Figure 4 | Correlation between different T-cell detection strategies. (a) Correlation between antigen-specific T-cell frequencies as detected by classical PE-labeled MHC multimer staining and by dual-color-encoded MHC multimer staining with 25 specificities per sample. (b) Correlation between antigen-specific T-cell frequencies as detected by dual-color-encoded MHC multimers with one pMHC specificity per sample and by dual-color-encoded MHC multimer staining with 25 specificities per sample. Donors are the same as in Figure 3. ρ , interclass correlation coefficient. For transforming to logarithmic scale all zero values were converted to 0.0015. Dashed lines represent the optimal linear correlation.

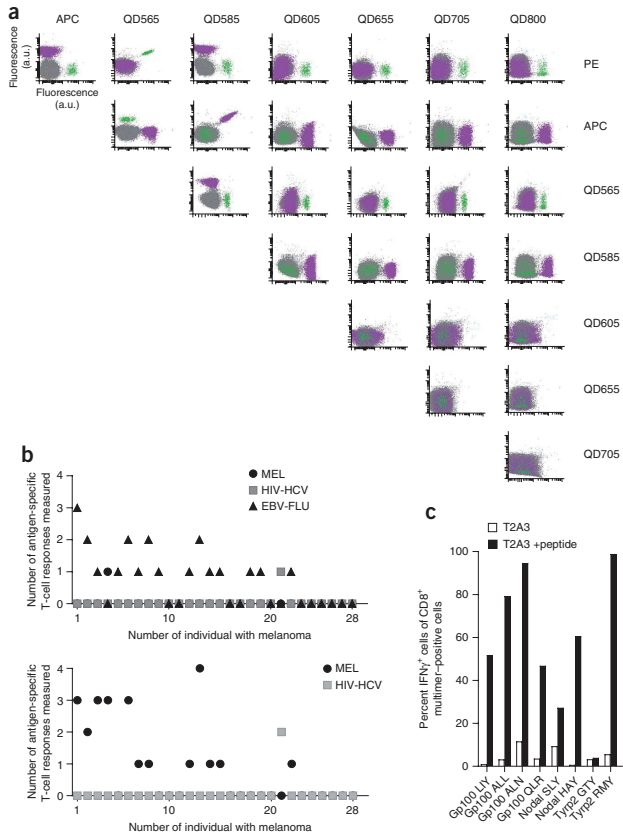
detected in the same sample (CD45RA⁺ or CD45RA⁻, CCR7⁺, CD62L⁻ and CD57⁻) (Supplementary Fig. 4).

Identification of melanoma-associated T-cell epitopes

Having established the feasibility of combinatorial encoding for the parallel measurement of a large number of antigen-specific T-cell populations in a single sample, we next determined the potential value of combinatorial encoding in epitope identification. In a recent screen⁸, we had identified 22 peptides from four different melanoma-associated proteins that display a high affinity for HLA-A3 (Supplementary Table 3). This set included all four previously described HLA-A3-associated epitopes as well as 18 potential new epitopes. To address the feasibility of screening small patient samples for responses to sets of potential epitopes, we generated MHC reagents by UV-light-induced peptide-exchange reactions⁶⁻⁸ for all 22 epitopes,

as well as for three HLA-A3-associated Epstein-Barr virus (EBV)-derived and influenza A-derived epitopes, and we encoded these reagents to form fluorescently labeled pMHC multimers as described in Supplementary Table 3. Subsequently, we screened

Figure 5 | T-cell responses to melanoma-associated peptides. (a) Dot plots show a representative example of pMHC multimer-enriched PBMCs from an individual with melanoma analyzed by combinatorial encoding. Antigen-specific T-cell populations were detected for gp100-LIY (PE and QD565) (green) and Nodal-SLY (APC and QD585) (pink). All dot plots are shown with bi-exponential axes and display fluorescence intensity (a.u.) for the indicated fluorochromes. (b) Summary of antigen-specific T-cell responses of 28 individuals to 22 melanoma-associated peptides (MEL), 20 HIV- and HCV-derived T-cell epitopes (HIV-HCV) and three HLA-A3-restricted virus-derived T-cell epitopes (EBV-FLU). The response of each individual is shown for detection directly *ex vivo* (top) and after enrichment and *in vitro* expansion (bottom). (c) T-cell cultures (from different individuals) that responded to the indicated peptide in an intracellular IFN γ staining assay. Assays were performed on sorted pMHC multimer-reactive T-cell populations incubated with T2-A3 cells pulsed with or without the indicated peptide. Data from representative cultures are shown for each epitope.



PBMCs from 28 HLA-A3–positive individuals with melanoma for T-cell responses to these potential antigens, either directly *ex vivo* or after antigen-specific T-cell enrichment. In the latter case, we only performed enrichments with pMHC multimers containing melanoma-associated antigens, to avoid potential overgrowth of virus-specific T cells during culture. As expected, we detected EBV- and influenza A-specific responses in the majority (15 out of 28) of the individuals with melanoma directly *ex vivo*. Additionally, we detected a single melanoma-associated antigen-specific response directly *ex vivo*. Notably, using parallel MHC multimer staining, we detected many T-cell responses specific for different potential melanoma-associated antigens after enrichment (Fig. 5a). In total, we detected 24 responses targeting eight distinct epitopes (Fig. 5b). Three of these epitopes consisted of previously described gp100-derived peptides that have been shown to be presented by tumor cells^{12–14}. In addition, we observed responses to five previously unknown T-cell epitopes from gp100 (QLRALDGGNK), Nodal (SLYRDPLRP and HAYIQSLK) and Tyrp2 (GTYEGLLR and RMYNMVPPF). In all cases, we confirmed peptide specificity of the T cells with subsequent analysis by conventional MHC multimer staining followed by MHC tetramer-based sorting. We assayed the functional activity of these T-cell populations by measuring IFN γ production upon incubation with peptide-loaded T2-A3 target cells; we confirmed the functional activity of 18 out of 22 cultures analyzed and for four out of the five newly identified epitopes (Fig. 5c). We performed the same type of combinatorial encoding analysis using HIV- and hepatitis C virus (HCV)-derived peptides (Supplementary Table 4), antigens to which the group of individuals we tested has presumably not been exposed. In these analyses, a sample from only one individual with melanoma showed reactivity against HCV-derived epitopes (HCV-GVA and GCV-KVF) (Fig. 5b). Although we could not attribute this single response to a known HCV exposure, the lack of T-cell responses against any of the other viral antigens in these individuals with melanoma suggests that the frequent detection of T-cell responses to the HLA-A3–restricted melanoma-associated antigens in this cohort is a consequence of disease state. Although it remains to be established whether T-cell responses against these antigens can contribute to tumor control, these data make a strong case for the value of combinatorial encoding in epitope identification.

DISCUSSION

With regard to the technical aspects of our approach, three points are noteworthy. First, the use of differentially labeled pMHC complexes carrying the same peptide led to a reduction in signal in each individual channel owing to competition for available binding sites, but such competition is apparently not a major factor. In a setup that used eight channels for encoding, we detected a signal that was clearly discernable above background for 25 out of 28 possible two-dimensional combinations. Based on these data, it seems plausible that with additional improvements in flow-cytometric detection, multiplex analysis will be possible for most or all potential combinations of fluorochromes even when moving to higher-dimension encoding. Second, the sensitivity of detection of antigen-specific T-cell responses by combinatorial encoding is as high or higher than that of classical MHC multimer staining. Background staining in MHC-based detection of T-cell responses can generally be attributed to interaction of cells with a single fluorescent marker or because of random interaction with all

fluorescent markers used. Thus, by restricting detection to those T cells that interact with a defined combination of two and only two of the fluorochromes used for encoding, such background events are effectively removed. Finally, a potentially serious issue with the use of combinatorial encoding is the fact that T cells are considered highly cross-reactive. As an example, if CMV-specific T cells would show a spurious cross-reactivity with any of the other pMHC complexes used, such T cells would be detected in more than two channels and thereby be excluded from analysis. Notably, for all antigen-specific T-cell responses that we analyzed in parallel by combinatorial encoding and classical MHC multimer staining, there was a full match between the antigen-specific T-cell populations detected by the two assays and the correlation between the assays was high. Thus, when using sets of dozens of unrelated pMHC multimers, a possible cross-reactivity of T cells appears not to be a measurable factor. We note that such cross-reactivity can be expected to become a relevant issue in the case of high-throughput analysis of T-cell responses with sets of pMHC multimers with substantial structural homology, for instance, involving peptide variants.

Prior work in animal models has demonstrated the value of MHC-based detection of T-cell responses using large sets of pMHC multimers by conventional single-channel analysis. Specifically, for all four tested pathogens (H5N1 influenza A, *Chlamydia trachomatis*, MHV-68 and *Toxoplasma gondii*^{6,15–17}), new epitopes had been identified in screens using 150–2,000 peptides. Notably, if the same screens were to be performed on human PBMCs, this would require approximately 300 ml to 4 l of peripheral blood. However, with the ability to analyze at least 25 antigen specificities per sample, this is reduced to substantially more realistic amounts. As a first demonstration of the potential value of large-scale screening of human T-cell responses, we analyzed cytotoxic T-cell responses to potential melanoma-associated antigens. Based on these data, it seems likely that MHC-based detection of T-cell responses will be of substantial value for large-scale screening efforts aimed at the identification of pathogen-, cancer- or auto-immune-associated epitopes. In addition, multiplexed measurement of antigen-specific immune responses could form a useful addition to current analyses of bulk T-cell frequencies in routine human diagnostics.

METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/naturemethods/>.

Note: Supplementary information is available on the Nature Methods website.

ACKNOWLEDGMENTS

We thank B. Rodenko and H. Ovaa (Netherlands Cancer Institute) for the kind gift of J. W. van de Kastele for help with cell culture, T. Wierfeldt for statistical assistance, and A. Pfauth, F. van Diepen, M. van der Hooft and G. de Roo for technical support with flow cytometry. This work was supported by the Danish Cancer Society grant DP06031 and the Carlsberg Foundation grant 2005-1-641 (to S.R.H.), Landsteiner Foundation of Blood Transfusion research grant 0522 and a Melanoma Research Alliance established investigator award (to T.N.S.) and Dutch Cancer Society grant UL 2007-3825 (to M.H.H. and T.N.M.S.).

AUTHOR CONTRIBUTIONS

S.R.H., A.H.B. and C.J.S. designed research, performed research, analyzed data and wrote the paper; R.S.A. performed research and analyzed data; J.v.V. performed research; P.H. and E.C. provided practical assistance; P.T.S., C.B. and J.B.H. contributed material from individuals with melanoma; M.H.H., designed research and analyzed data; T.N.S., designed research, analyzed data and wrote the paper.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at <http://www.nature.com/naturemethods/>.

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ONLINE METHODS

Generation of pMHC complexes. All peptides were synthesized in-house using standard Fmoc chemistry or purchased from Peptide Sciences (Peptide Sciences Presto BV). The UV light-sensitive building block J was synthesized as described^{6–8}. Recombinant HLA-A1, -A2, -A3 and -B7 heavy chains and human β_2m light chain were produced in *Escherichia coli*. MHC class I refolding reactions were performed as described¹⁸ and MHC class I complexes were purified by gel-filtration high-performance liquid chromatography in PBS (pH 7.4). Specific peptide-MHC complexes were generated by MHC peptide exchange^{6–8}. In brief, UV light-sensitive pMHC complexes (100 $\mu\text{g ml}^{-1}$) were subjected to 366 nm UV light (Camag) for 1 h in presence of the various peptides (200 μM). After exchange, samples were centrifuged at 16,000g for 5 min, and supernatants were used for pMHC multimer formation.

Generation of pMHC multimers. pMHC multimers were generated using eight different fluorescent streptavidin (SA) conjugates (Invitrogen): SA-QD565, SA-QD585, SA-QD605, SA-QD655, SA-QD705, SA-QD800, SA-PE and SA-APC. For each 100 μl of MHC monomer (concentration, 100 $\mu\text{g ml}^{-1}$) 7.08 μl of SA-Qdot conjugate (1 μM), 10.8 μl SA-PE (1 mg ml^{-1}) or 6 μl SA-APC (1 mg ml^{-1}) was added, followed by incubation on ice for 20 min. Assuming a 100% rescue after MHC peptide exchange, this would result in an occupancy of 30 MHC monomers per SA-Qdot and 4 monomers per SA-PE or SA-APC. To block residual binding sites, D-biotin (Sigma) was added to a final concentration of 26.4 μM , followed by incubation on ice for 20 min. PE- and APC-labeled complexes were diluted twofold in PBS with 0.02% (wt/vol) NaN_3 . For each pMHC complex, multimers were made with two different fluorescent labels according to the schemes in **Supplementary Tables 2–4**. For combinatorial T-cell staining, multimer complexes of the same specificity were mixed 1:1 for QD605-, QD655-, QD705-, PE- and APC-labeled complexes and 2:1 for QD565-, QD585- and QD800-labeled complexes in combination with any other color. Combinations of QD565, QD585 and QD800 with each other were excluded. Combined pMHC mixtures for analysis of T-cell responses by combinatorial encoding were generated by pooling and were stored with 0.02% NaN_3 (Sigma) at 4 °C as 50-fold concentrated ready-to-use stocks for T-cell staining (concentration was 100 $\mu\text{g ml}^{-1}$ based on initial monomer concentration). Before use, pMHC multimers were centrifuged at 17,000g for 2 min, and the supernatant was used. Although the stability of pMHC complexes can be expected to differ between individual pMHC complexes, we have successfully used pMHC mixtures stored in this manner for up to 2 months.

PBMC samples and T-cell staining. PBMCs were obtained from healthy individuals and from individuals with stage IV melanoma in accordance with local guidelines, with informed consent and with approval from the local ethics committee (METC). All T-cell staining was performed on cryopreserved material. For T-cell staining of approximately 1×10^6 PBMCs or 2×10^5 cultured T cells, 2 μl of single pMHC multimer, or 50 μl of dual-color-encoded pMHC collections (final concentration: 2 $\mu\text{g ml}^{-1}$ per distinct pMHC based on initial monomer concentration) was used. Final staining volume was 80 μl and cells were incubated for 10 min at 37 °C. Next, 20 μl of a fivefold concentrated antibody mix consisting of CD8-Alexa700 (Caltag MHCD0829) (final

dilution 1/200), CD4-FITC (BD 345768) (final dilution 1/8), CD14-FITC (BD 345784) (final dilution 1/32), CD19-FITC (BD 345776) (final dilution 1/16), CD40-FITC (Serotech MCA1590F) (final dilution 1/40), CD16-FITC (BD 347523) (final dilution 1/64) was added, and cells were incubated for 20–30 min at 4 °C. Before flow cytometry analysis, cells were washed twice and propidium iodide was added to allow dead-cell exclusion.

Flow cytometry. Data acquisition was performed on an LSR-II flow cytometer (Becton Dickinson) with FACS Diva software using the following 11-color instrument settings. For the 488-nm laser: PI, 685LP, 695/40; PE, 550LP, 575/26; FITC, 505LP, 530/30; SSC, 488/10. For the 633-nm laser: Alexa700, 685LP, 730/45; APC, 660/20. For the 405-nm laser: QD800, 770LP, 800/30; QD705, 680LP, 710/50; QD655, 635LP, 660/40; QD605, 595LP, 650/12. For the 355-nm laser: QD585, 575LP, 585/15; QD565, 545LP: 560/20. Approximately 200,000 lymphocytes were recorded for each analysis. To identify antigen-specific T cells, the following gating strategy was used. (i) Selection of live (PI-negative) single-cell lymphocytes (FSC-W/H low, SSC-W/H low, FSC/SSC-A). (ii) Selection of CD8⁺ and 'dump' (CD4, 14, 16, 19 and 40) negative cells. FITC was used as a 'dump channel' to gate away all irrelevant cells, as a means to reduce background¹⁹. (iii) Selection of CD8⁺ T cells that were positive in two MHC multimer channels and negative in the six other MHC multimer channels. Each cell population positive in two and only two MHC multimer channels received a unique color to facilitate analysis.

IFN γ enzyme-linked immunosorbent spot assay. We coated 96-well nitrocellulose plates (Multiscreen MAIP N45; Millipore) with 7.5 $\mu\text{g ml}^{-1}$ mouse monoclonal antibody to IFN γ (1-D1k; Mabtech) in 75 μl PBS overnight at room temperature. After six washes and blocking with 200 μl X-Vivo medium (Biowhittaker), lymphocytes were added in three concentrations: 1×10^6 , 5×10^5 and 2.5×10^5 cells per well, together with indicated peptide (5 $\mu\text{g ml}^{-1}$). After overnight incubation, plates were washed and biotinylated secondary monoclonal antibody to IFN γ was added (7-B6-biotin; Mabtech). After a 2-h incubation, plates were washed and avidin-enzyme conjugate (Life Technologies) was added to each well. After 1 h of incubation at room temperature, plates were washed and enzyme substrate (Dako) was added for 5–10 min. Reactions were terminated by addition of water, and the number of spots was assessed using an ELISPOT counter (Immunospot; CTL Inc.).

Enrichment of antigen-specific T cells. Antigen-specific T cells were stained with PE multimers (1.25 μl of a 100 $\mu\text{g ml}^{-1}$ stock of each individual PE-multimer for 10^7 PBMCs) for 1 h at 4 °C. Subsequently, cells were washed, and incubated with 20 μl magnetic beads coated with antibody to PE (Miltenyi). Cells were then isolated by MACS (Miltenyi), using an LS column, following the manufacturer's protocol. Eluted cells were washed and resuspended in 200 μl of T cell medium (IMDM (Gibco) with 10% human serum (Invitrogen), 100 IU ml^{-1} IL-2 (Proleukin) and 20 ng ml^{-1} IL-15 (Peprotech)) with 5,000 anti-CD3/CD28-coated Dynabeads (Invitrogen). Enriched cells were cultured in 96-well plates and resuspended the next day. Cultures were split and medium was refreshed at least twice a week. Note that residual PE-multimer staining from the enrichment step disappears

within two days of culture (data not shown). After 2–3 weeks, antigen-specific T-cell responses were measured by combinatorial encoding-based MHC multimer flow cytometry.

T-cell sorting and culture. T cells were stained with the relevant pMHC multimer and then sorted on a MoFlo (Dako) or FACSaria (Becton Dickinson) into 10^5 irradiated feeder cells (JY plus allogeneic PBMCs). Cells were spun and resuspended in IMDM with 10% human serum, 100 IU ml^{-1} IL-2 and 0.5 $\mu\text{g ml}^{-1}$ PHA (Biochrom AG). Cultures were restimulated every second week. Established cultures were tested for antigen-specificity by MHC multimer staining.

Cytokine release assay. T2-A3 cells were loaded with the indicated peptides for 1 h and washed once. Then, 1×10^5 T cells from indicated cultures were incubated with 1×10^5 T2-A3 cells for 4 h at 37 °C in IMDM with 10% human serum and protein transport inhibitor (BD GolgiPlug). Cells were stained with PE-conjugated antibodies to CD8 (clone SK1, BD) for 15 min at 25 °C, fixed

and permeabilized (BD Cytofix/Cytoperm kit), and stained with APC-conjugated antibodies to IFN γ (25723.11, BD) for 30 min at 4 °C. Samples were analyzed by flow cytometry (Cyan, Dako), data analysis was performed using FlowJo.

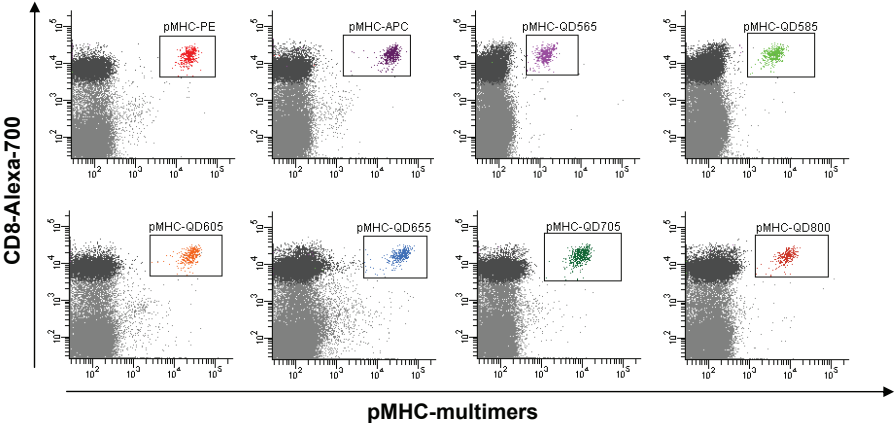
Statistics. The three different staining approaches: (i) one single staining with the mix of 25 dual-color-encoded viral and cancer epitope pMHC multimers, (ii) 25 separate stainings with all 25 PE-labeled pMHC multimers or (iii) 25 separate stainings with all individual dual-color-encoded pMHC multimers were compared to determine the interclass correlation, using a two-way mixed model (ICC3.1), calculated based on consistency. Interpretation of the results is based on reference 9.

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19. van Oijen, M. *et al.* On the role of melanoma-specific CD8+ T-cell immunity in disease progression of advanced-stage melanoma patients. *Clin. Cancer Res.* **10**, 4754–4760 (2004).

SUPPLEMENTARY INFORMATION

Supplementary Figure 1

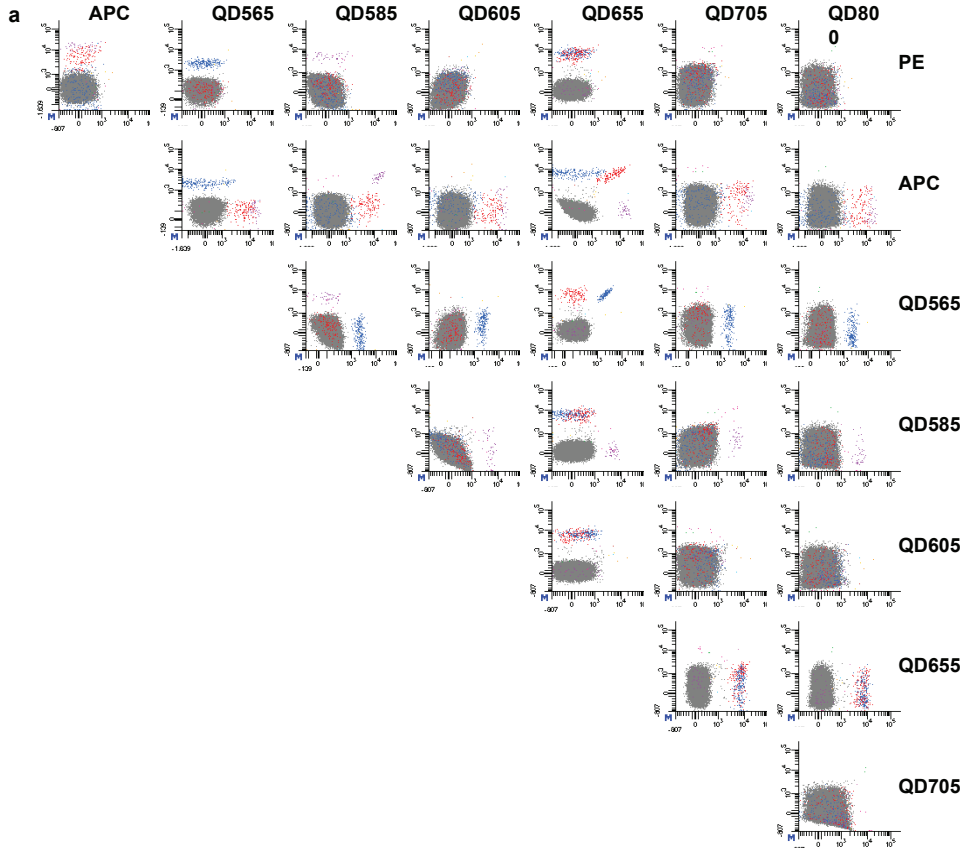
Detection of antigen-specific T cells using MHC multimers coupled to different fluorochromes



Staining of a clonal HLA-A2 CMV_{NLV}-specific T cell population mixed with an excess of HLA-A2-negative PBMCs using MHC multimers conjugated to eight different fluorochromes: PE, APC, QD565, QD585, QD605, QD655, QD705 and QD800. Dot plots are gated on live, single-cell lymphocytes.

Supplementary Figure 2

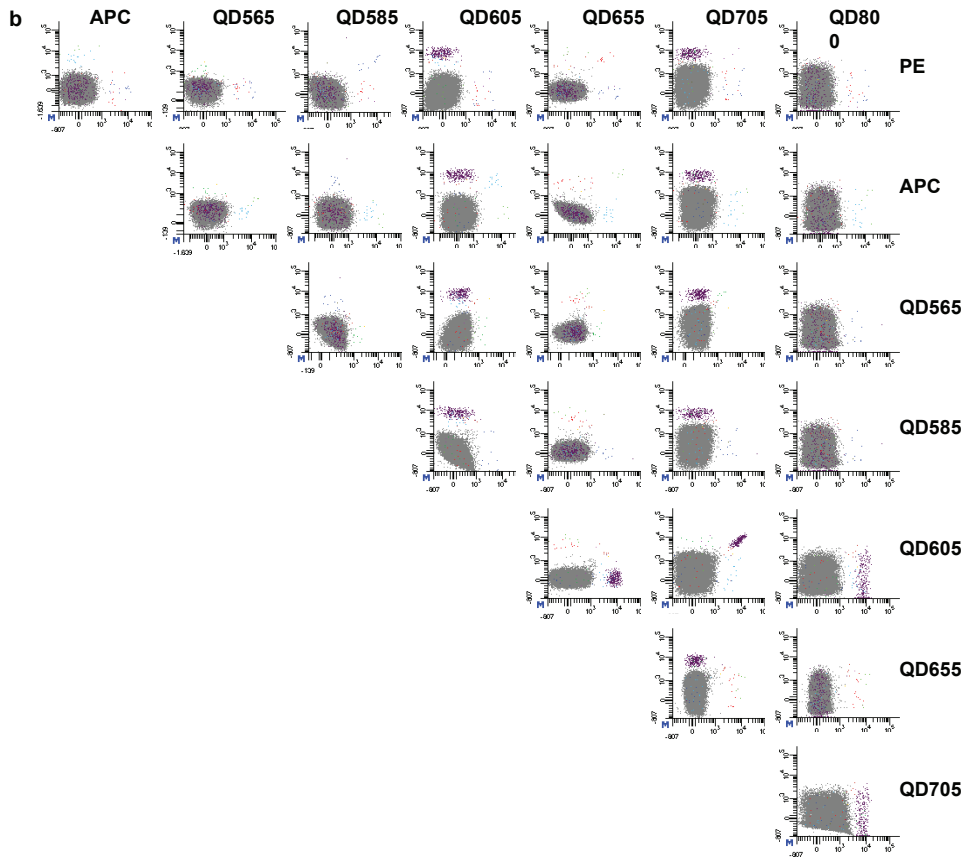
Multiplex detection of virus-specific T cell responses through combinatorial encoding



(a–c) Dot plots showing all 28 different 2–D representations of gated CD8⁺ T cells of three healthy donors analyzed by dual–color encoded MHC multimer staining using the peptide set described in **Supplementary Table 2** online. (a) Donor 1. (b) Donor 2. (c) Donor 3. (d) Representative example of staining of PBMCs from a healthy donor analyzed by dual–color encoded MHC multimer staining using irrelevant peptide–MHC multimers. (e) Dot plots for healthy donor 2 gated on all lymphocytes.

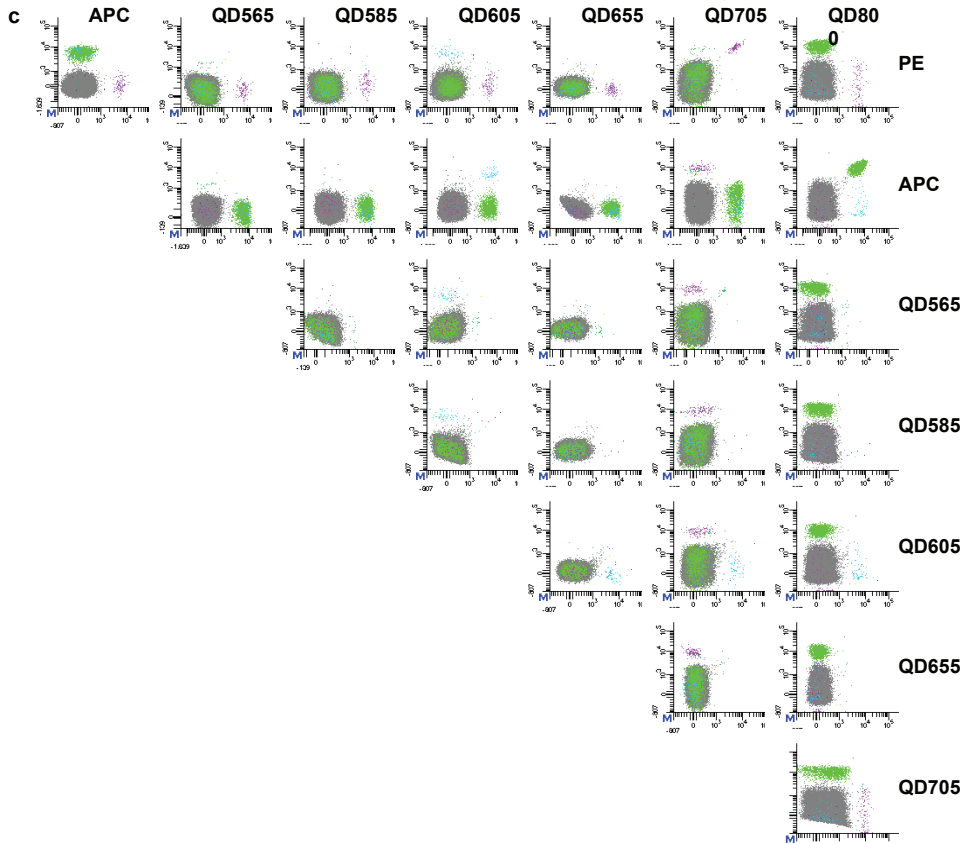
Supplementary Figure 2

Multiplex detection of virus-specific T cell responses through combinatorial encoding



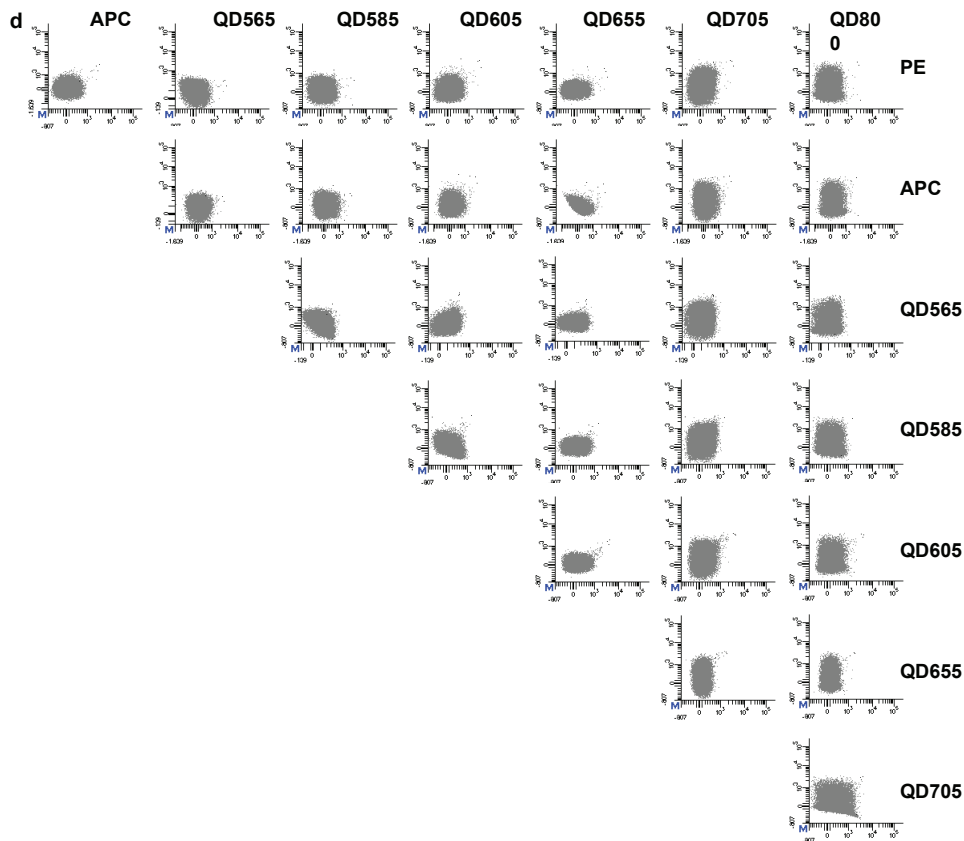
Supplementary Figure 2

Multiplex detection of virus-specific T cell responses through combinatorial encoding



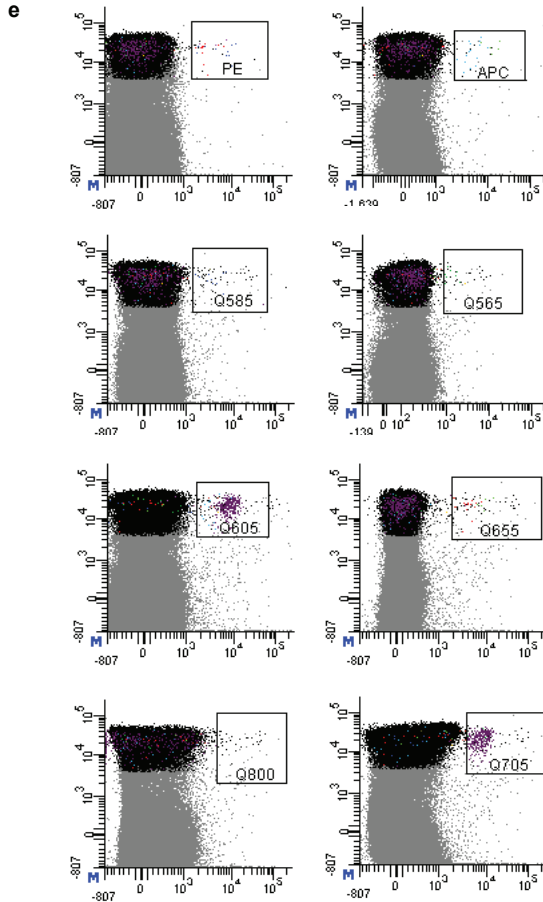
Supplementary Figure 2

Multiplex detection of virus-specific T cell responses through combinatorial encoding

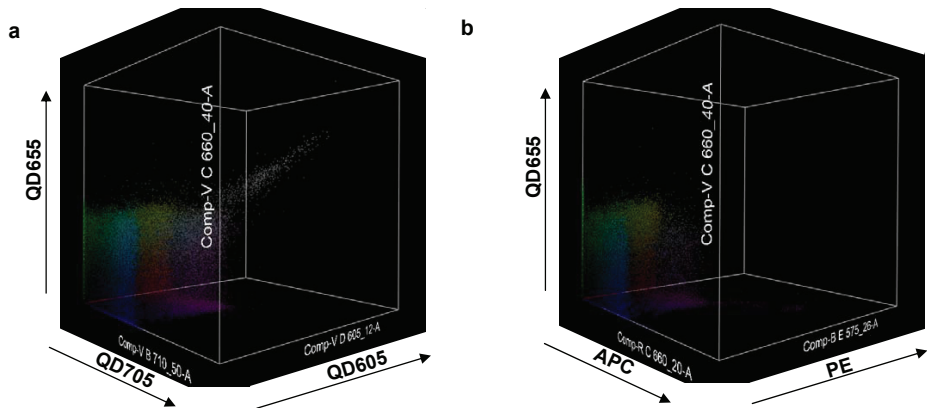


Supplementary Figure 2

Multiplex detection of virus-specific T cell responses through combinatorial encoding



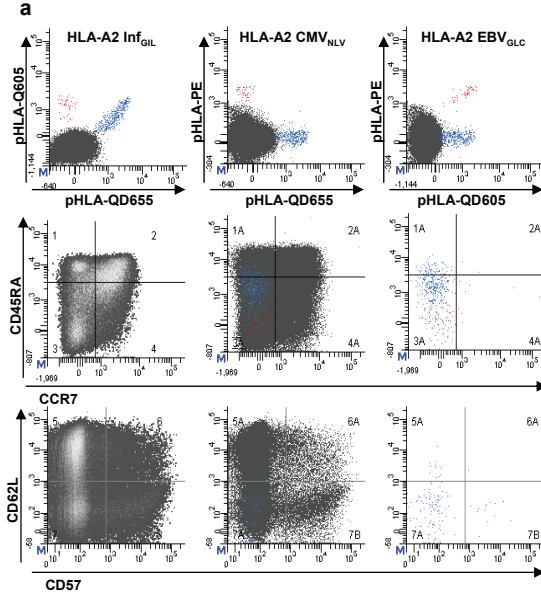
Supplementary Figure 3
Feasibility of 3D pMHC multimer staining



CMV_{NLV}-specific T cells are stained with a combination of QD605, QD655 and QD705 labeled CMV_{NLV}-MHC multimers. **(a)** The parameters QD605, QD655 and QD705, with the triple positive cells in white. **(b)** The parameters PE, APC and QD655 of the same sample. No triple positive cells were detected using this combination. 3D dot plots were made using FlowJo v7.5 (beta version).

Supplementary Figure 4

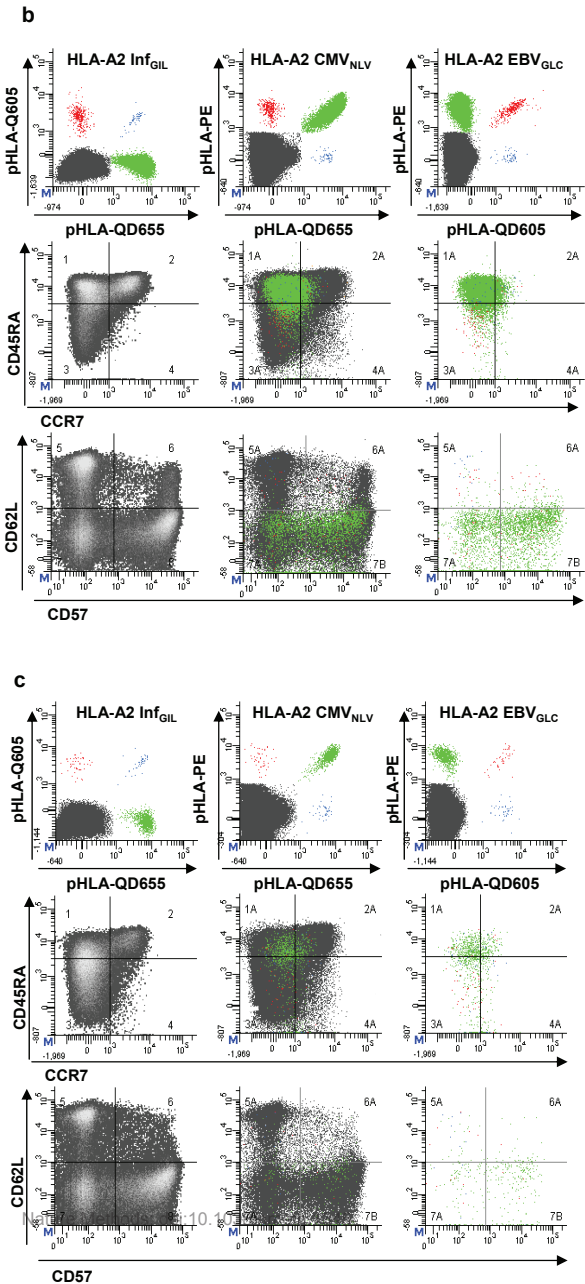
Phenotypic characterization of virus-specific T cells detected by combinatorial encoding



(**a–c**) dot plots from three healthy donors gated on approximately 200,000 CD8⁺ T cells after exclusion of 'triple' or 'single' pMHC multimer positive T cells. (**top row**) PBMCs were stained with pMHC–multimer complexes for HLA-A2 Inf_{GIL} (QD605 + QD655, blue), HLA-A2 CMV_{NLV} (PE + QD655, green), and HLA-A2 EBV_{GLC} (PE + QD605, red). (**middle row**) Phenotypic characteristics of virus-specific T cells based on CD45RA and CCR7 expression: a density plot gated on CD8⁺ T cells used to define quadrant gates (left), a dot plot showing all CD8⁺ T cells with pMHC positive T cells depicted in the indicated colors (middle), and a dot plot showing only pMHC positive T cells (right). (**bottom row**) Phenotypic characteristics of virus-specific T cells based on CD62L and CD57 expression: a density plot gated on CD8⁺ T cells used to define quadrant gates (left), a dot plot showing all CD8⁺ T cells with pMHC positive T cells depicted in the indicated colors (middle), and a dot plot showing only pMHC positive T cells (right).

Supplementary Figure 4

Phenotypic characterization of virus-specific T cells detected by combinatorial encoding



Supplementary Table 1

The 28 unique color combinations that can be made using a two-dimensional matrix of eight fluorochromes

	PE	APC	QD565	QD585	QD605	QD655	QD705	QD800
PE		x	x	x	x	x	x	x
APC			x	x	x	x	x	x
QD565				x	x	x	x	x
QD585					x	x	x	x
QD605						x	x	x
QD655							x	x
QD705								x
QD800								

Supplementary Table 2

Virus-derived and cancer-associated T cell epitopes

No.	Protein	HLA Restriction	Peptide	Coding
1	<i>HPV E6</i>	A2	TIHDIILECV	PE & APC
2	<i>CMV pp150</i>	A3	TTVYPPSSTAK	PE & Q565
3	<i>Influenza A M1</i>	A2	GILGFVFTL	PE & Q585
4	<i>gp100</i>	A2	IMDQVPFSV	PE & Q605
5	<i>EBV LMP2</i>	A2	CLGGLLTMV	PE & Q655
6	<i>EBV BMF1</i>	A2	GLCTLVAML	PE & Q705
7	<i>Tyrosinase</i>	A2	YMNGTMSQV	PE & Q800
8	<i>Survivin</i>	A2	LMLGEFLKL	APC & Q565
9	<i>CMV pp65</i>	A1	YSEHPTFTSQY	APC & Q585
10	<i>EBV LMP2</i>	A2	FLYALALLL	APC & Q605
11	<i>Influenza A NP</i>	A3	ILRGSVAHK	APC & Q655
12	<i>HA-2</i>	A2	YIGEVLVSV	APC & Q705
13	<i>CMV pp65</i>	A2	NLVPMVATV	APC & Q800
14	<i>CMV pp65</i>	B7	TPRVTGGGAM	Q565 & Q605
15	<i>CMV pp50</i>	A1	VTEHDTLLY	Q565 & Q655
16	<i>EBV BRLF1</i>	A2	YVLDHLIVV	Q565 & Q705
17	<i>HPV E7</i>	A2	YMLDLQPETT	Q585 & Q605
18	<i>EBV EBNA 3a</i>	A3	RLRAEAQVK	Q585 & Q655
19	<i>Influenza A BP1</i>	A1	VSDGGPNLY	Q585 & Q705
20	<i>CMV pp65</i>	B7	RIPHERNGFTV	Q605 & Q655
21	<i>EBV EBNA</i>	B7	RPPIFIRLL	Q605 & Q705
22	<i>HY</i>	A2	FIDSYICQV	Q605 & Q800
23	<i>CMV pp150</i>	A3	TVYPPSSTAK	Q655 & Q705
24	<i>CMV IE1</i>	A2	VLEETSVML	Q655 & Q800
25	<i>EBV BRLF1</i>	A3	RVRAYTYSK	Q705 & Q800

For each epitope MHC multimers were encoded by the indicated fluorochrome combination

Supplementary Table 3

Melanoma-associated HLA-A3 ligands

Protein	Peptide	Position	Coding
<i>Gp100</i>	IALNFPGSQK	86-95	PE & APC
	LIYRRRLMK	614-622	PE & Q565
	GTATLRLVK	460-468	PE & Q585
	ALLAVGATK	17-25	PE & Q605
	ALNFPGSQK	87-95	PE & Q655
	GVSRQLRTK	34-42	PE & Q705
	QLVLHQILK	551-559	PE & Q800
	QLRALDGGNK	221-230	APC & Q565
<i>Nodal</i>	SLYRDPLPR	46-54	APC & Q585
	HAYIQSLLK	293-301	APC & Q605
	KTKPLSMLY	317-325	APC & Q655
	RVAGECWPR	175-183	APC & Q705
<i>Tyr</i>	YMVPFIPLYR	425-434	APC & Q800
	SLLCRHKRK	497-505	Q565 & Q605
	VSSKNLMEK	25-33	Q565 & Q655
	GLVSLLCRHK	494-503	Q565 & Q705
<i>Tyrp1</i>	SLPYWNFATR	245-254	Q585 & Q605
	ASYLIRARR	497-505	Q585 & Q655
<i>Tyrp2</i>	TLLGPGRPYR	196-205	Q585 & Q705
	GTYEGLLR	301-309	Q605 & Q655
	RMYNMVPFF	461-469	Q605 & Q705
	VLLAFLQYR	521-529	Q605 & Q800
<i>Influenza A NP</i>	ILRGSAHK	265-273	Q655 & Q705
<i>EBV EBNA 3a</i>	RLRAEAQVK	603-611	Q655 & Q800
<i>EBV BRLF1</i>	RVRAYTYSK	148-156	Q705 & Q800

Three EBV- and influenza A-derived epitopes were included for direct *ex-vivo* stainings as a positive control. Sequences in bold are previously described T cell epitopes. Individual pMHC multimers were encoded by the indicated fluorochrome combination.

Supplementary Table 4

HIV- and HCV-derived HLA-A3 restricted T cell epitopes

Virus	Peptide	Position	Coding
<i>HIV</i>	QVPLRPMTYK	86-95	PE & APC
<i>HIV</i>	TVYYGVVPWK	614-622	PE & Q565
<i>HIV</i>	RLRDLLIVTR	460-468	PE & Q585
<i>HIV</i>	RLRPGGKKK	17-25	PE & Q605
<i>HIV</i>	KIRLRPGGK	87-95	PE & Q655
<i>Hepatitis C virus</i>	RVCEKMALY	34-42	PE & Q705
<i>Hepatitis C virus</i>	RLGVRATRK	551-559	PE & Q800
<i>Hepatitis C virus</i>	KTSESRQPR	221-230	APC & Q565
<i>Hepatitis C virus</i>	QLFTFSPRR	46-54	APC & Q585
<i>Hepatitis C virus</i>	RMYVGGVEHR	293-301	APC & Q605
<i>Hepatitis C virus</i>	LGFGAYMSK	317-325	APC & Q655
<i>Hepatitis C virus</i>	GAYMSKAHGV	175-183	APC & Q705
<i>Hepatitis C virus</i>	LIFCHSKKK	425-434	APC & Q800
<i>Hepatitis C virus</i>	GVAGALVAFK	497-505	Q565 & Q605
<i>Hepatitis C virus</i>	VAGALVAFK	25-33	Q565 & Q655
<i>Hepatitis C virus</i>	SLTPPHSAK	494-503	Q565 & Q705
<i>Hepatitis C virus</i>	CINGVCWTC	245-254	Q585 & Q605
<i>Hepatitis C virus</i>	HDGAGKRVY	497-505	Q585 & Q655
<i>Hepatitis C virus</i>	KVFPKALINK	196-205	Q605 & Q655
<i>Hepatitis C virus</i>	LLFLLADA	301-309	Q605 & Q800
<i>Influenza A NP</i>	ILRGSAHK	265-273	Q655 & Q705
<i>EBV EBNA 3a</i>	RLRAEAQVK	603-611	Q655 & Q800
<i>EBV BRLF1</i>	RVRAYTYSK	148-156	Q705 & Q800

Three EBV- and influenza A-derived epitopes were included for direct *ex-vivo* stainings as a positive control. Individual pMHC multimers were encoded by the indicated fluorochrome combination.

Chapter 7

Summary and discussion

SUMMARY AND DISCUSSION

The generation of antigenic peptides and the interaction between pMHC complexes and T cell receptors are two important hallmarks of T cell-mediated immunity. This thesis describes our advances in the understanding of how MHC ligands are generated by APCs (chapters 2 and 3) as well as the development and use of pMHC based high-throughput techniques to analyze human T cell populations (chapters 5 and 6). Here I will first discuss the progress that has been made in the last few years in the field of antigen presentation and how these results fit with the new insights that have been obtained by other groups. I will conclude with a description of the two techniques that we have developed and discuss the potential value of these methods for the analysis of T cell populations and identification of new T cell epitopes.

Generation of antigenic peptides from extracellular material in T cell-mediated immunity

In chapters 2 and 3 we investigate the mechanisms of various APC for generating antigenic peptides and describe new rules governing these mechanisms. Through the direct visualization of phagosomal protease activity in both macrophages and dendritic cells two new observations are made in chapter 2. First, the recruitment of cysteine proteases to a maturing phagosome after internalization of particulate material occurs sequentially in a time-dependent manner and not through bulk delivery. Additionally, there is a marked difference in protease recruitment between different APC types as well as between different cellular maturation states. These results indicate that there is distinct enzymatic regulation during phagosomal maturation with tight control on how extracellular material is processed. The observation that macrophages display

more rapid recruitment of proteases than DCs fits with the notion that the two cell types play rather distinct roles in antigen processing and immunity; Macrophages act as scavengers against invading pathogens and therefore destroy internalized material rapidly, while DCs process material more slowly and in such a manner that T cells will be activated and recruited. Since our observations, several further studies have strengthened this notion by showing tightly regulated lysosomal activation during dendritic cell maturation, and more rapid antigenic destruction in macrophages than in DCs (3).

In chapter 3 the question was asked whether the parameters governing cross-presentation and endogenous antigen presentation are equivalent. While antigens contained in different locations of a protein (signal peptide vs. carboxi-terminus) are presented comparably through endogenous presentation, this similarity is lost when the same protein is processed through the cross-presentation pathway. In that setting, only when antigens are contained in a portion of the mature protein with a relatively long half-life does cross-priming of T cells occur. These observations, together with those from the groups of Rock and Yewdell (4,5), were the first to show that long-lived cellular proteins rather than short-lived peptides are the major source of antigens through cross-presentation. In a later study our group also confirmed the relationship between protein half-life and the magnitude of a T cell response when comparing the effectiveness of DNA vaccines (6).

In this chapter we have presented evidence that the extracellular material internalized during cross-presented is proteinaceous, while chapter 2 describes

regulated delivery of proteases in the endocytic pathway (investigated in the context of MHC class II antigen presentation). Together these results suggest that there also is a similar method of regulation for the generation of antigenic peptides for cross-priming. When also taking into account that free cytosolic peptides have a half-life of only seconds (7) a model emerges where degradation of extracellular material needs to be tightly controlled in dendritic cells to ensure initiation of T cell responses through cross-priming. Furthermore, considering the above-mentioned notion that macrophages play a different role in immunity than DCs, this regulation on antigen generation should, at least in part, be DC specific and start right when material is internalized. We show in this thesis that part of this control is executed through organized delivery of proteases to the phagosome with clear differences between DCs and macrophages. Also, if mature proteins rather than peptides are the cross-presented material the processing should primarily take place inside the presenting APC. In the introduction I have discussed the different possible mechanisms of antigen transfer between cells during cross-presentation. Here, I will discuss the recent developments specifically in the field of phagosomal maturation and intracellular antigen processing after uptake of extracellular material by APCs, and in particular DCs.

A major factor in the processing of extracellular material is the gradual acidification of phagosomes. pH-sensitive propieces that occupy the enzymatic domain of many endocytic proteases ensure that these proteases only become active at the right acidic compartments, while there are also specific acidic ranges in which certain enzymes are functional (8). A new level of control in regards to phagosomal acidification was recently described in DCs by the groups of Mellman and Amigorena.

Trombetta et al. were the first to show regulated acidification in DCs by showing *in vitro* that immature DCs lack a fully assembled V-ATPase (1). The laboratory of Amigorena then went on to describe in several publications that immature DCs also actively lower the pH of endocytic organelles through fusion with 'inhibitory lysosome-related organelles', thereby preventing rapid degradation of phagosomal content. This process is mediated by Rab27a-dependent recruitment of NOX2, which actively pumps out protons of the organelle (9–11). Although a reduction of lysosomal acidification after LPS treatment has been observed in macrophages (12), alkalization through NOX2 does not occur in these cells (13). Thus, dendritic cells appear to be equipped with a unique active mechanism to control vesicular pH in order to regulate hydrolytic activity and thereby antigenic processing. This ensures that material is available long enough for binding to MHC molecules. Several studies have described the transfer of extracellular material to the cytosol from early endocytic vesicles, indeed well before low lysosomal pH is reached (14–16). The prolonged but transient period of low pH in early phagosomes of immature DCs followed by acidification of the vesicles to a level where proteases involved in the generation of peptides for MHC class II binding are active (8), therefore argues for a model where the processing of peptides involved in cross-priming CD8⁺ T cells occurs early in phagosomal maturation, followed by the generation of MHC class II associated peptides later on in the pathway (17,18).

As already mentioned in the introduction of this thesis, another recent development in the investigation on phagosomal antigen processing is the proposed contribution of the ER to the maturing phagosome. Although several publications have described the presence of ER-derived proteins in phagosomes, including the MHC

class I peptide loading machinery (15,19–22) this issue remains controversial (23). Resolving the controversy surrounding the contribution of the ER to phagosomes as part of the antigenic processing machinery would be a significant advance in the field of cross-presentation. In light of this, two elegant studies of the group of Cresswell provide compelling evidence that ER components, such as the retrotranslocation machinery, indeed play a role in the transfer of at least soluble extracellular material to the cytosol (24,25) and Burgdorf et al. have recently provided evidence that soluble ovalbumin enters the cytosol rapidly after internalization and then re-enters the phagosome in a TAP-dependent manner (16).

This brings us to the question whether different modes of uptake, such as for example is the case for soluble vs. particulate material, play a role in how material is processed by the DC. Presentation of soluble antigens has generally been assumed to be regulated by similar mechanisms as those involved in the processing of particulate antigens. However, it is becoming more clear that extracellular material does not enter a common default vesicle after uptake by an APC (26). Rather, the mode of uptake, cell-type and maturation state of the cell seem to be a determining factor as to how the material is processed. For example, material entering DCs through the DEC205 receptor primes T cells differently than the same antigens internalized via DCIR2 (27), where the first situation leads to both CD4⁺ and CD8⁺ T cell activation, while DCIR2-targeting only primes CD4⁺ T cells. Additionally, Burgdorf et al. recently showed that soluble material internalized via the mannose receptor is routed differently than material endocytosed via the scavenger receptor (28). This has led to renewed interest in the differential receptor expression between cell-types as well as altered receptor expression between

maturation states (17). The insights from these and future studies should have a large impact in the field of antigen-uptake through receptor-targeting that has seen been growing steadily in the last decade (29). Specifically, control of the onset and magnitude of T cell responses might be influenced through specific receptor-targeting of desired antigens. On the other hand, this extra level of cellular control might also make the unraveling of how extracellular antigens are processed inside a DC much more complicated.

Finally, receptors of the innate immune system might prove to also play a role in cross-presentation. It has been well established that TLR-triggering leads to DC maturation as well as the fact that TLR ligands increase protein immunogenicity. Recently Blander and Medzhitov have published several papers describing an important role for TLR-engagement in control of phagosome maturation (30–32). In those studies, internalization of bacteria leads to CD4⁺ T cell activation because TLRs are engaged, while apoptotic material does not lead to a T cell response. This latter could be reversed by addition of a TLR ligand to the apoptotic material. However, these findings remain controversial, since the lab of Russell failed to find a direct role for TLRs in phagosomal maturation and processing (33–35). It should be noted that all these studies have primarily examined CD4⁺ T cell activation. Given the various methods of control of phagosomal maturation and processing by DCs in generating MHC class I epitopes described above, it seems likely that there is also a role for TLRs in controlling how, when, and where MHC class I epitopes are generated after internalization of antigens, but further studies need to be performed to validate this.

Our research has given new insights on which extracellular material is processed for CD8⁺ T cell activation during cross-

priming and which proteases are active during phagosome maturation. Especially these latter findings fit with the growing realizations that DCs are the primary cell type for generating both CD4⁺ and CD8⁺ T cell responses and that antigenic processing after uptake is tightly regulated. Macrophages seem to be optimized for the destruction of pathogenic particles. The phagocytic pathway of DCs, on the other hand, differs from that of macrophages in the ability to tightly control mechanisms such as phagosomal acidification, transport of proteins from the phagosome to the cytosol, recruitment of ER-resident proteins to the phagosome and, possibly, TLR engagement during phagosomal maturation (13).

pMHC multimers in high-throughput T cell analysis

Chapters 4 through 6 describe a different aspect of antigen presentation than the previous chapters: the use of MHC multimers in the analysis of T cell responses. The field of MHC multimers exists since the landmark publication of Altman et al. in 1996 describing for the first time the use of multimerized MHC molecules in flow cytometry for T cell analysis (36). Since then this 'tetramer technology' has become a core experimental tool in the field of T cell immunity. In chapter 4 we discuss the different available methods to generate MHC class I and MHC class II multimers and provide a roadmap for the future development of MHC multimer technology. One of the aspects that are covered in chapter 4 is the limitation of high-throughput uses of MHC multimers in the analysis or selection of large sets of T cell populations. Due to the standard refolding and purification methods of generating peptide-specific MHC multimers, this technology is unsuited for the generation of large collections of different pMHC complexes, thereby precluding high-throughput applications.

To overcome this limitation, our lab developed a peptide exchange technique using UV-sensitive conditional ligands for MHC multimers (37). These ligands allow for efficient UV-mediated disintegration in the MHC-bound state under conditions that do not affect the integrity of the MHC molecule, while at the same time conferring efficient pMHC refolding and long-term stability when no trigger is applied. Indeed, a large array of multimers can be generated in only several hours from one batch of conditional pMHC complexes (38).

The initial description of this technique was limited to HLA-A2 and it remained unclear whether this technique could be extended to other human HLA gene products. In chapter 5 we describe the development and characterization of 4 new conditional ligands for the gene products HLA-A1, -A3, -A11, and -B7. We thereby establish that peptide exchange technology is indeed a generally applicable approach for several HLA gene products and provide more insight in the biochemical kinetics of the exchange reaction. We show that dissociation of cleaved conditional ligands after UV-exposure is rapid and show that residual material is a reaction product rather than uncleaved material and ultimately does not interfere with detection of T cell responses. With this study, the HLA complexes for which conditional ligands are now available cover at least one allele of over 90% of Western European individuals.

With the establishment of the exchange technology it has become possible to rapidly generate batches of MHC complexes presenting a wide range of epitopes. This should allow for the development of different high-throughput screening methods of antigen-specific T cells. In chapter 6 we describe the development, testing and use of one such high-throughput screening approach, where we multidimensionally encode a large pool of different pMHC

complexes in order to simultaneously analyze multiple T cell populations with flow cytometry. Specifically, we coupled a unique combination of two fluorochromes to specific pMHC complexes while using the same fluorescent labels multiple times, but each time in a different combination of two labels. Using such an encoding approach to label MHC complexes with unique multi-color tags allows to vastly increase the maximum available labeling combinations over the traditional use of single-color tags. For this, we use six of the recently developed quantum dots in combination with two traditional organic fluorochromes. Using MHC multimers that are each uniquely encoded by 2 fluorochromes we are able to detect and analyze up to 25 T cell populations simultaneously out of the 28 possible combinations. Combinatorial coding systems have been used in a number of settings to increase the number of analyses that can be performed on a single sample. For example combinatorial coding has been used to measure serum products such as cytokines using bead arrays in which encoding is performed by variation in bead size, fluorochrome and fluorochrome intensity (e.g. the cytometric bead arrays by BD (39)). In those cases, however, solutes are analyzed by binding to pre-encoded microbeads. Contrary to this, our technique differs through the involvement of *de novo* creation of a code during the assay.

In chapter 6 we validate combinatorial coding primarily using 2 dimensions. We also provide proof that an increase to 3 dimensions is feasible, and we have by now tested up to 4 dimensions successfully (data not shown). With an increase in available fluorochromes and better detection capacities of flow cytometers, higher coding dimensions would allow for a vast increase of T cell populations that could be detected. Even with the current available technical limitations, the use of 17 fluorochromes (the maximal number of different fluorochromes

that has to our knowledge been used in a single flow cytometric analysis (40)), our 2-dimensional coding system could already encompass up to 136 specific codes. The capacity to us higher dimensions would then allow for the encoding of many thousands of specificities.

The ability to use large sets of MHC multimers has been shown to be very instrumental in identifying novel epitopes by our lab and others (37,41–43), where epitopes were identified in screens in mouse models using sets of 150 to 2000 peptides. The development of the multidimensional encoding approach now also opens up this ability for analysis of human PBMCs, where sample size previously was the limiting factor. To demonstrate the value and possibility of large scale screening of human T cell responses directly, we analyzed cytotoxic T cell responses against a series of potential melanoma-associated antigens using both the techniques described in chapters 5 and 6. In chapter 5 we screen a library of 203 potential melanoma-associated peptides in the context of HLA-A3. The newly developed exchange technology allowed for rapid high-throughput binding assays, identifying 22 peptides that display high binding affinity for HLA-A3. In chapter 6 we use the parallel detection technique to simultaneously analyze cytotoxic T cell responses against these 22 peptides in blood of melanoma patients. With this approach we could identify reactivity against 8 epitopes, of which 5 were not previously identified. This could have therapeutic implications, since MHC-based selection of antigen-specific T cells has been proposed as a strategy to boost melanoma-specific T-cell responses in individuals with melanoma (44). Our identification of potential novel associated epitopes should allow for a similar strategy in HLA-A3 positive patients.

This melanoma-specific screen shows the potential value of combining

both the exchange technology and the multidimensional encoding strategy to identify pathogen-, cancer-, and autoimmune-associated epitopes.

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Nederlandse samenvatting

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NEDERLANDSE SAMENVATTING

Dit proefschrift gaat over het afweersysteem. Tenminste, over een bepaald gedeelte van het afweersysteem, namelijk het 'T-cel gemedieerde deel van het adaptieve immuunsysteem'. En dan ook nog eens over de vraag hoe binnen dat specifieke onderdeel bepaalde stukken eiwit door de cel gemaakt worden en hoe we deze zogenoemde epitopen zouden kunnen gebruiken in nieuwe technische toepassingen.

De voorgaande zinnen geven volgens mij gelijk aan dat het afweersysteem behoorlijk uitgebreid is en ingewikkeld in elkaar zit. Dat maakt het ook zo mooi om te onderzoeken. Maar het is daarom ook niet altijd makkelijk uit te leggen aan een niet-immunoloog hoe het afweersysteem precies werkt, laat staan welk onderdeel van het afweersysteem ik nou precies onderzoek. Toch wil ik een poging wagen in deze samenvatting, die daarom vooral gericht is op mensen buiten mijn vakgebied. Vakgenoten raad ik aan om (ook) het engelse gedeelte van dit boekje te lezen. Ik begin met een basisuitleg van het afweersysteem, en daarna leg ik uit wat mijn onderzoek dat in dit proefschrift beschreven wordt inhoudt.

Twee tactieken

Het afweersysteem, ook wel immuunsysteem genoemd, heeft als taak het lichaam tegen ziektemakers ('pathogenen') te beschermen. Om deze taak uit te voeren bestaan er vele verschillende types cellen die hierin samenwerken. De basis van alle verschillende afweermechanismen die deze cellen gebruiken ligt bij het vermogen om onderscheid te maken tussen 'zelf' en 'niet-zelf'. Lichaamseigen cellen moeten met rust gelaten worden terwijl lichaamsvreemd materiaal (inclusief eigen cellen met daarin

een ziektemaker) zo snel mogelijk herkend en opgeruimd moet worden. In het lichaam wordt dus continue nagegaan of iets bij het lichaam hoort, of niet.

Er bestaan grofweg twee algemene tactieken om infectie door pathogenen zoals virussen, bacteriën en parasieten tegen te gaan: het 'aangeboren' afweersysteem bestaat uit cellen die weten wat lichaamsvreemd precies inhoudt en daar tegen optreden, terwijl het 'adaptieve' immuunsysteem juist opereert door na te gaan wat lichaamseigen is, en alles wat daar niet aan voldoet opruimt. Om dit wat concreter te maken: cellen van het aangeboren immuunsysteem herkennen onderdelen van ziektemakers die niet in ons eigen lichaam voorkomen. Als ze die bepaalde moleculaire structuren tegenkomen dan is het dus duidelijk dat er iets mis is en kan er opgetreden worden. Zo hebben veel bacteriën een celwand die uit andere materialen bestaat dan de celwand van zoogdieren. Het aangeboren afweersysteem kan deze bacteriële celwanden herkennen en zal dus specifiek op basis van de aanwezigheid van die structuur in het lichaam een afweerreactie starten. Het adaptieve systeem werkt eigenlijk precies andersom. Cellen van dit systeem hebben geen idee hoe pathogenen of onderdelen daarvan er uit zien, maar ze weten wel wat lichaamseigen is. Als ze iets tegenkomen wat daar niet onder valt, dan wordt dit aangevallen en opgeruimd.

Aangezien mijn proefschrift over het adaptieve afweersysteem gaat en hoe het mechanisme werkt dat tot activatie van T cellen leidt, zal ik wat meer over dit specifieke onderdeel uiteen wijden.

T cellen, antigeen presentatie en APCs

T cellen zijn cellen die gespecialiseerd zijn in het vinden en opruimen van cellen die geïnfecteerd zijn met bacteriën en virussen. Er zijn twee types: helper T cellen die aan andere cellen signalen doorgeven dat er opgetreden moet worden en cytotoxische T cellen die zelf andere cellen kunnen doden. Beide types hebben een belangrijke receptor aan de buitenkant van de cel: de T cel receptor, of TCR. Elke T cel heeft een eigen, unieke TCR en omdat er vele miljoenen T cellen in het lichaam voorkomen, zijn er dus ook vele miljoenen verschillende T cel receptoren. Dit komt omdat tijdens het ontstaan van een T cel (wat in de thymus gebeurt) de TCR door mutaties en herschikkingen voor een gedeelte willekeurig in elkaar wordt gezet. Het is misschien interessant om te realiseren dat niet alle mutaties dus 'slecht' zijn. Sterker nog, deze (en andere) herschikkingen en mutaties in het lichaam zijn een cruciaal onderdeel van ons afweersysteem. Om te begrijpen waarom dit zo belangrijk is, moet ik eerst uitleggen wat deze receptoren precies herkennen.

Een belangrijke taak van T cellen is om na te gaan of een cel aan de binnenkant geïnfecteerd is met een pathogeen, maar een T cel kan natuurlijk niet letterlijk in een cel naar binnen kijken om te zien wat er aan de hand is. Het lichaam heeft hier een oplossing voor gevonden door een mechanisme te gebruiken dat aan de buitenkant een soort blauwdruk geeft van wat er binnenin een cel aan de hand is. Elke cel van het lichaam heeft hiervoor een eiwit complex op de buitenkant zitten, het MHC (Major Histocompatibility Complex) complex. Deze complexen kunnen kleine stukjes eiwit (liganden of epitopen genaamd) aan zich binden. Deze eiwit stukjes worden gevormd tijdens het proces van eiwitafbraak dat in de cel plaatsvindt.

Vrijwel van elk eiwit dat in een cel wordt afgebroken komt een klein gedeelte op een MHC complex terecht. Dit complex van MHC met stukje eiwit wordt nadat het gevormd is naar de buitenkant van de cel getransporteerd. En omdat eiwit productie en afbraak non-stop in een cel plaatsvindt, zal er ook continue aan de buitenkant van de cel dus een overzicht zijn van wat er binnenin gebeurt. Het handige van dit systeem is dat virussen en bacteriën ook gebruik maken van het eiwit productie mechanisme van cellen, inclusief het onderdeel dat eiwitten afbreekt. Na een infectie zullen er dus ook stukjes eiwit van een virus of bacterie aan de buitenkant van een cel aanwezig zijn in het MHC complex. En T cellen zijn in staat om deze complexen te herkennen. De TCR van een T cel bindt namelijk aan MHC complexen en als er een geschikte binding plaatsvindt, dan wordt het afweersysteem aangezet om alle cellen die de combinatie van een MHC complex met juist dat stukje eiwit aan de buitenkant hebben zitten op te ruimen. Dit principe geeft ook de naam aan mijn onderzoeksveld: de eiwitten waarvan de stukjes op een MHC complex terecht komen, worden 'antigenen' genoemd. En de MHC complexen 'presenteren' deze stukjes feitelijk aan de T cellen via binding met hun TCR. Het veld wordt dan ook het vakgebied van 'antigeen presentatie' genoemd.

Eerder heb ik uitgelegd dat T cellen onderdeel van het adaptieve systeem zijn en dus weten wat eigen is, en tegen alles wat daar niet onder valt optreden. Nu het principe van antigeen presentatie bekend is, kan ik uitleggen hoe het afweersysteem er voor zorgt dat T cellen weten wat lichaamseigen is. De TCR komt, zoals eerder gezegd, gedeeltelijk met willekeur tot stand. Welke eiwit structuur binnen een MHC complex de TCR zal herkennen staat van te voren niet vast en elke TCR herkent een andere structuur. Als de TCR van een

T cel gevormd is, bevindt de T cel zich nog in de thymus. In de thymus bevinden zich gespecialiseerde cellen met MHC complexen die ontzettend veel verschillende stukken eigen eiwit van het lichaam zelf presenteren. Van elke TCR wordt gekeken of hier op gereageerd wordt. Als dat zo is, dan wordt die specifieke T cel opgeruimd. Die TCR herkent immers een stukje van een lichaamseigen eiwit. Als er geen herkenning plaatsvindt, dan herkent de TCR dus 'iets anders'. Wat dat precies is maakt niet uit: alles wat niet lichaamseigen is, is tenslotte lichaamsvreemd. In tegenstelling tot de cellen van het aangeboren afweersysteem hebben T cellen dus absoluut geen idee hoe een virus of bacterie er uitziet. Ze weten alleen wel hoe het eigen lichaam er uitziet en reageren met de rest.

Naast T cellen spelen andere cellen ook een belangrijke rol in mijn onderzoek. Dit zijn cellen die in staat zijn om materiaal van buiten op te nemen en ook stukjes eiwit van dit materiaal aan hun MHC complexen te binden, naast de stukjes van hun eigen eiwitten. Deze cellen worden antigeen presenterende cellen, of kortweg APCs, genoemd. APCs kunnen bijvoorbeeld actief bacteriën opnemen en doden en tegelijkertijd via het MHC complex aan T cellen doorgeven dat andere cellen van het lichaam waarschijnlijk geïnfecteerd zijn. Ook kunnen ze helpen om bepaalde ontwikkelings mechanismen van virussen te omzeilen, als een virus bijvoorbeeld actief de MHC complexen van een cel uitschakelt, door gedode lichaamscellen die nog virus eiwitten bevatten op te nemen en deze eiwitten aan het MHC complex te binden. Maar APCs zijn vooral erg belangrijk om aan de T cellen door te geven dat er ergens anders in het lichaam een infectie plaatsvindt en er door de T cellen opgetreden moet worden. De APCs die dit doen heten dendritische cellen (DCs).

Dit proefschrift

Ik heb dus onderzoek aan antigeen presentatie gedaan. Waar we specifiek naar gekeken hebben zijn twee vragen: 1) hoe worden epitopen (dus de stukjes eiwit die aan het MHC complex binden) door een cel gemaakt, en welke regels en processen spelen hier een rol in? 2) Kunnen we epitopen en MHC complexen gebruiken om nieuwe technieken op te zetten om T cellen te analyseren? Het proefschrift is dan ook in twee onderdelen opgedeeld. Hoofdstukken 1 t/m 3 behandelen vraag 1, en hoofdstukken 4 t/m 6 beschrijven de bevindingen op het gebied van vraag 2.

Eerste gedeelte

In hoofdstuk 2 bekijken we hoe APCs het materiaal dat ze van buiten de cel opnemen afbreken. We onderzoeken specifiek de activiteit van de enzymen die deze afbraak uitvoeren en kijken hoe en wanneer na het opnemen van materiaal deze enzymen actief zijn. Meer kennis op dit gebied kan er voor zorgen dat we beter in staat zijn te begrijpen hoe epitopen gemaakt worden en waarom na opname door de cel het ene materiaal wel, en het andere materiaal niet tot T cel activatie leidt. In dit onderzoek hebben we ook naar verschillende APC types gekeken en kunnen twee conclusies trekken: de activiteit van de enzymen die we bekeken hebben is meer gereguleerd dan eerder werd gedacht, en er is een verschil in enzym activiteit tussen verschillende cellen. Op basis van dit verschil stellen we voor dat een van de onderzochte celtypes, de macrofaag, gespecialiseerd is in het snel afbreken en uitschakelen van opgenomen pathogenen, terwijl het andere celttype, de DC, het opgenomen materiaal juist langzaam afbreekt zodat er genoeg tijd overblijft om epitopen te maken om aan de MHC complexen te binden voor T cel activatie.

Zoals hierboven beschreven is, kunnen epitopen gegenereerd worden uit eiwitten die in een cel zelf voorkomen, en uit eiwitten nadat deze door APCs zijn opgenomen. In hoofdstuk 3 stellen we de vraag of deze beide processen volgens dezelfde regels werken. Dit wordt gedaan door model epitopen op verschillende plekken binnen een eiwit te plaatsen en te bekijken of deze epitopen wel of niet op MHC complexen terecht komen en T cellen activeren. Voor de vorming van epitopen door cellen waarin het eiwit zelf tot expressie komt, maakt het niet uit waar in het eiwit we het epitooop geplaatst hebben. Echter, als de epitopen gevormd worden door een APC nadat deze zijn opgenomen, dan blijkt dat alleen epitopen die onderdeel zijn van een stabiel deel van het eiwit aan het MHC complex gebonden worden. Epitopen in instabiele delen van het eiwit die snel afgebroken worden zijn niet in staat T cellen te activeren. Dit onderzoek heeft tot twee belangrijke ontdekkingen geleid. Ten eerste werd het duidelijk dat de regels van het maken van epitopen verschilt tussen directe presentatie door een cel zelf en presentatie na opname door een APC. De belangrijkste conclusie is echter dat de stabiliteit van een eiwit een erg grote rol speelt in het activeren van T cellen via APCs, wat nog niet eerder was aangetoond. Op basis van deze resultaten adviseren we dan ook dat bij de ontwikkeling van vaccins er zeker aandacht besteed moet worden aan de stabiliteit en levensduur van eiwitten.

Tweede gedeelte

Het tweede deel van dit proefschrift beschrijft en bouwt voort op een veelgebruikte standaard techniek in de immunologie, de MHC multimeer technologie. Hoofdstuk 4 geeft een overzicht van deze techniek en onze mening over hoe deze in de toekomst verder ontwikkeld kan worden. De techniek maakt gebruik van het

feit dat één specifieke TCR één specifieke combinatie van MHC complex met epitooop herkent. We zijn technisch in staat om MHC complexen met epitooop zelf in het lab te genereren. Als we meerdere van deze MHC complexen (elk met hetzelfde epitooop, en dus dezelfde specificiteit) samenvoegen en daar een fluorescerend molecuul aan koppelen ontstaat een zogenoemde 'MHC multimeer'. Wanneer een voorraad van deze multimeren aan een mix van T cellen wordt toegevoegd, binden de multimeren zich uitsluitend aan de T cellen met de juiste TCR die reageert met het eiwit stuk dat aan de MHC complexen gebonden is. Omdat de multimeren fluorescent zijn kunnen we die T cellen zichtbaar maken. Deze techniek stelt ons dus in staat om binnen een heel grote pool van verschillende T cellen met verschillende TCRs juist die T cellen te identificeren waar we in geïnteresseerd zijn. Deze techniek bestaat inmiddels ruim 10 jaar en is erg belangrijk geworden zowel in basaal onderzoek als bij verschillende klinische toepassingen.

Een erg groot nadeel van deze techniek is de tijdsduur. Het kost veel tijd en moeite om de MHC complexen te maken, en voor elke TCR die onderzocht wordt moet een aparte voorraad MHC complexen gemaakt worden. Het was dus lange tijd niet mogelijk om veel verschillende T cellen responsen tegelijk te analyseren. In hoofdstuk 5 bouwen we voort op een recente techniek die ons lab heeft ontwikkeld om dit probleem te omzeilen. Eerder onderzoek van ons lab heeft laten zien dat we chemische epitopen kunnen maken die na bestraling met UV-licht uit elkaar vallen. Als we MHC complexen die deze epitopen bevatten bestralen in een vloeistof waar ook andere, niet UV-gevoelige, epitopen aanwezig zijn blijken we in staat om het UV-gevoelige epitooop na het uiteenvallen te vervangen door het epitooop dat in de vloeistof aanwezig

is. Door een grote hoeveelheid UV-MHC complexen te maken en deze in kleine hoeveelheden te bestralen in aanwezigheid van verschillende epitopen kunnen we nu in zeer korte tijd erg veel verschillende MHC complexen maken, iets wat voor deze techniek onmogelijk was. In hoofdstuk 5 beschrijven we de ontwikkeling van 4 nieuwe UV-gevoelige epitopen voor deze techniek voor verschillende type menselijke MHC complexen. Hiermee bewijzen we dat deze aanpak met UV-gevoelige epitopen algemeen toepasbaar is voor meerder MHC types. Daarnaast hebben we de biochemische reactie van het uiteenvallen van het UV-gevoelige epitooop in het MHC complex beter onderzocht en geven daarmee meer inzicht in o.a. de snelheid van de reactie.

Aangezien we nu in staat zijn om in korte tijd zeer veel verschillende MHC complexen te maken door middel van de UV-techniek uit hoofdstuk 5 hebben we onderzocht of we ook daadwerkelijk in korte tijd simultaan veel verschillende T cel populaties konden analyseren. Een probleem bij de analyse van menselijke T cellen, en zeker die van patiënten, is de gelimiteerde hoeveelheid materiaal dat doorgaans beschikbaar is. In hoofdstuk 6 onderzoeken we de mogelijkheid om door middel van combinaties van de fluorescente moleculen van de MHC multimeren meerdere T cel responsen tegelijkertijd te analyseren dan tot nu toe mogelijk was. Dit doen we door een voorraad van één specifieke MHC multimeer in tweeën te splitsen en elk deel een ander fluorescent molecuul te geven. T cellen met de juiste TCR zullen dus fluoresceren met twee kleuren, in tegenstelling tot de tot nu toe gangbare enkele kleur. Dit stelt ons dan in staat om met een beperkte hoeveelheid fluorescente moleculen erg veel verschillende combinaties te maken. Als we voorheen 4 kleuren gebruikten, konden

we voorheen ook slechts 4 verschillende T cellen laten zien (bijvoorbeeld rood, groen, geel en blauw). Door MHC complexen met twee kleuren aan te duiden ben je in staat om kleuren te laten overlappen. Een T cel populatie die rood én groen kleurt is dan een andere populatie die rood én blauw kleurt. In dit voorbeeld van 4 kleuren zijn er bijvoorbeeld 6 combinaties mogelijk (rood-groen, rood-blauw, rood-geel, groen-blauw, groen-geel, blauw-geel), een winst van 2. In hoofdstuk 6 breiden we dit principe uit tot 8 kleuren en laten zien dat met deze '2-dimensionale code' van 2 kleuren we in staat zijn om in één experiment 25 (van de maximaal haalbare 28) verschillende T cel populaties aan te tonen. Deze techniek maakt het dus mogelijk om met het doorgaans weinige materiaal veel meer analyses uit te voeren.

Om aan te tonen dat beide technieken uit het laatste deel van dit proefschrift niet alleen correct werken maar ook toepasbaar zijn, hebben we ze toegepast in een screen om T cellen te ontdekken die reageren tegen tumor cellen van melanoom, een bepaalde vorm van huidkanker. Op basis van beschikbare computer modellen hebben we van 5 eiwitten die een rol spelen bij melanoom een lijst van 203 potentiële epitopen samengesteld. Vervolgens hebben we de UV-techniek van hoofdstuk 5 gebruikt om te bekijken welke van deze 203 epitopen daadwerkelijk aan het MHC complex kunnen binden, en met welke bindingssterkte. Dit leidde tot 22 mogelijke epitopen. Een test met deze aantallen was zonder de UV-techniek praktisch niet mogelijk geweest. Vervolgens hebben we MHC complexen van deze 22 verschillende epitopen gemaakt en met de techniek van hoofdstuk 6 bij bloed van melanoom patiënten onderzocht of er ook T cellen aanwezig waren die met deze epitopen (in het MHC complex) reageren. Zonder deze techniek was dit

Samenvatting

niet mogelijk geweest om alle 22 epitopen te testen vanwege de geringe hoeveelheid beschikbaar materiaal. Met deze test zijn we in staat gebleken om T cel populaties te identificeren tegen 8 van de 22 epitopen, waarvan er 5 niet eerder beschreven waren. Als deze T cel populaties inderdaad in staat zijn om tegen melanoom tumor cellen op te treden, wat momenteel wordt onderzocht, dan zouden ze in de toekomst als therapie gebruikt kunnen worden.

CURRICULUM VITAE

Arnold Hendrik Bakker werd geboren op 18 augustus 1977 te Haarlem. Op het Eerste Christelijk Lyceum in Haarlem behaalde hij in 1995 het gymnasium diploma, waarna hij een jaar in Amerika studeerde aan Knox College in Galesburg, Illinois. In 1996 werd begonnen met de studie Biologie aan de Universiteit van Amsterdam, waar hij in 2002 *cum laude* het doctoraal examen behaalde in Moleculaire en Cellulaire Technieken. Als onderdeel van deze studie werd stage gelopen aan de Universiteit van Amsterdam bij de vakgroep Moleculaire Biologie onder leiding van Prof. dr. Les Grivell en aan Harvard Medical School in Boston bij de afdeling Pathology in de groep van Prof. dr. Hidde Ploegh. Tijdens zijn studie maakte hij deel uit van de Opleidings Commissie en het Onderwijs Instituut van de faculteit, en bekleedde hij een jaar het voorzitterschap van de studievereniging CONGO. Van juni 2002 tot en met maart 2008 was hij werkzaam als onderzoeker in opleiding (OIO) in de groep van Prof. dr. Ton Schumacher op de afdeling Immunologie van het Nederlands Kanker Instituut (NKI) in Amsterdam, waarvan de resultaten in dit proefschrift te lezen zijn. Na zijn promotieonderzoek op het NKI heeft hij van mei 2008 tot en met januari 2009 gewerkt als projectleider voor de stichting Very Disco, waar hij verantwoordelijk was voor de organisatie van het o.a. door de EU en NWO gesponsorde festival Discovery 08. Sinds maart 2009 is hij werkzaam als onderzoeker in de groep van Prof. dr. Nilabh Shastri aan de University of California, Berkeley in Amerika.

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