[Frontiers in Bioscience 16, 3014-3035, June 1, 2011]

Sources of diversity in T cell epitope discovery

Cynthia Xin Lei Chang[1,2], Lingyun Dai[1,3], Zhen Wei Tan[1,2], Joanna Ai Ling Choo[1], Antonio Bertoletti[4], Gijsbert M. Grotenbreg[1,2,3]

[1]National University of Singapore (NUS), Immunology Programme and Departments of Microbiology and Biological Sciences, Centre for Life Sciences, No 03-05, 28 Medical Drive, 117456 Singapore, [2]NUS Graduate School for Integrative Science and Engineering (NGS), Centre for Life Sciences, No 05-01, 28 Medical Drive, 117456, Singapore [3]Singapore–Massachusetts Institute of Technology Alliance (SMA), 4 Engineering Drive 3, 117576, Singapore, [4]Singapore Institute for Clinical Sciences, Agency for Science Technology and Research, Brenner Centre for Molecular Medicine, 30 Medical Drive, 117609, Singapore

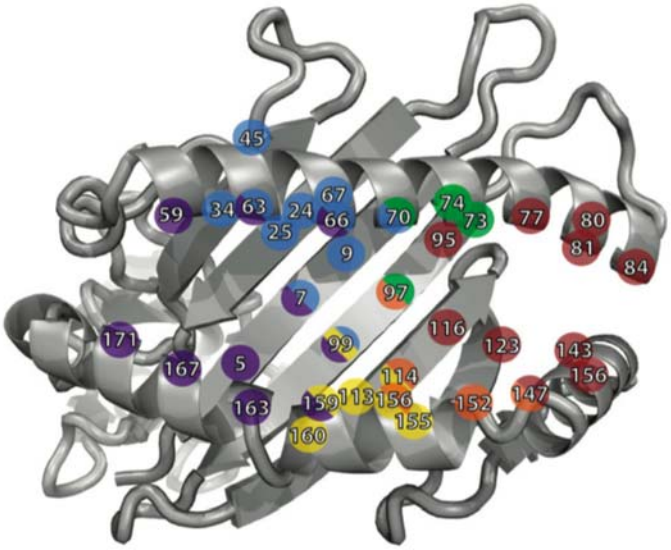
TABLE OF CONTENTS

1. ABSTRACT

CD8-positive T cells respond to small antigenic peptide fragments presented on class I major histocompatibility complexes (MHCs). Those specific T cell epitopes capable of precipitating a cellular immune response are either derived from (altered) self (*i.e.* they are autoimmune- or cancer-associated) or come from foreign sources (*i.e.* they are pathogen-associated). Identification of T cell epitopes provides elementary information that can be employed in technologies that monitor and predict the likely outcome of an immune response, as well as in therapeutic and vaccine development efforts. The coexistence between host and pathogen has largely driven the diversification of both their systems of immune surveillance and their antigenic determinants, respectively. In this review, we discuss the multitude of factors that introduce diversity to the T cell response from both sides of the host-pathogen interaction. Furthermore, we provide an overview of a variety of commonly employed methods and tools to characterize class I MHC restricted antigen presentation and recent endeavors towards the harmonization of reporting data concerning T cell responses.

2. INTRODUCTION

A complex interplay of various humoral and cellular immune effectors is required to effectively combat infections, malignancies, and autoimmune diseases. In order to eliminate foreign antigens, maintain immune homeostasis, and develop protective immunity, the host's adaptive immune system orchestrates a myriad of responses against specific pathogens. Whereas antibodies generally recognize intact antigens, T lymphocytes provide critical surveillance and protection by recognizing fragments of their cognate antigen presented to their T cell receptors (TCRs) by specialized molecules on the surface of the infected cell. An infectious insult activates the antigen-specific T cells to transiently undergo massive clonal expansion and effectuate the clearance of the infected cells (and the associated pathogen) through cell-mediated cytolysis and release of cytokines such as interferon-gamma (IFN-gamma) (1, 2). Numerous structurally diverse, and clonally distributed antigen-specific receptors are produced within a lymphocyte population in a bid to deal with the heterogeneity of presented antigens that the immune system has not previously encountered. The generation of a diverse T cell receptor repertoire is



Supertype	B pocket specificity		F pocket specificity	
	Description	a.a. residues*	Description	a.a. residues*
A01	Small and aliphatic	ATSVLIMQ	Aromatic and large hydrophobic	FWYLIM
A02	Small and aliphatic	ATSVLIMQ	Aliphatic and small hydrophobic	ATSVLIMQ
A03	Small and aliphatic	ATSVLIMQ	Basic	RHK
A24	Aromatic and aliphatic	FWYLIVMQ	Aromatic, aliphatic and hydrophobic	FWYLIVM
A01 A03	Small and aliphatic	ATSVLIMQ	Aromatic and basic	YRK
A01 A24	Small, aliphatic and aromatic	ASTVLIMQFWY	Aromatic and large hydrophobic	FWYLIM
B07	Proline	P	Aromatic, aliphatic and hydrophobic	FWYLIVM
B08	Undefined		Aromatic, aliphatic and hydrophobic	FWYLIVM
B27	Basic	RHK	Aromatic, aliphatic, basic and hydrophobic	RHKFWYLIVM
B44	Acidic	DE	Aromatic, aliphatic and hydrophobic	FWYLIVM
B58	Small	AST	Aromatic, aliphatic and hydrophobic	FWYLIVM
B62	Aliphatic	LIVMQ	Aromatic, aliphatic and hydrophobic	FWYLIVM

*a.a.residues = amino acid residues

Figure 1. Peptide binding groove of an HLA molecule. The numbers indicate the specific amino acid residues that line the six peptide binding pockets. (PDB:1BD2).

accomplished through the random recombination of variable (V), joining (J) and diversity (D) gene segments to a constant region (C) for the beta-chain of this heterodimeric complex, whilst the alpha-chain only undergoes V-J-C genetic recombination. Inaccuracy in the process of joining the gene segments, particularly the variable addition or subtraction of nucleotides, ensures additional diversity (3, 4). In the resulting type I membrane protein complex, the extracellular variable and constant domains for both the disulfide-linked alpha- and beta- or gamma- and delta-chains fold in immunoglobulin-like structures, that are followed by a transmembrane and a cytoplasmic domain. The hypervariability in the junctions of the alpha- and beta-variable domains is at the basis of TCR diversification, however, these complementarity-determining regions (CDR) have relatively modest affinity (□1–100 μM) for the presented antigen (5, 6). The counter-structure recognized by the TCR is a composite surface of a protein complex consisting of a pathogen-derived peptide and a major histocompatibility complex (MHC) molecule at the surface of the antigen-presenting cell. Typically, exogenously acquired antigens are presented on class II MHC products that in turn are capable of stimulating CD4-positive T lymphocytes, whereas the CD8-positive T cells respond to endogenously processed antigens presented on class I MHC. The class I MHC molecule is a heterotrimeric glycoprotein complex with characteristic immunoglobulin folds that consists of a polymorphic membrane-anchored heavy chain (~43kDa) which is non-covalently associated with an invariant light chain (beta2-microglobulin, beta2m, ~12kDa) and a peptide ligand (5). The heavy chain can be subdivided into three domains (i.e. alpha1, alpha2, and alpha3), where domains alpha1 and alpha2 form an antigen-presenting superdomain capable of binding small peptide fragments (~1kDa). Specifically, an eight stranded beta-sheet platform is traversed by two antiparallel alpha-helices, that form the sides of a ligand-binding groove. As the N- and C-terminal ends of the peptide are required to be firmly embedded in this closed MHC binding groove, their length is usually limited to 8–11 residues. Further constraints are imposed by six distinct binding pockets (A-F, see Figure 1) in this groove, where the amino acid residues lining these pockets define their exact geometry, chemical environment, and consequently the peptide

Table 1. Online available pathogen sequence databases

Database	Contents	URL
DNA Database of Japan	Primary sequence database	www.ddbj.nig.ac.jp
EMBL-ENA	Primary sequence database	www.ebi.ac.uk/ena
NCBI GenBank	Primary sequence database	www.ncbi.nlm.nih.gov/genbank
EuPathdb	Metadatabase for eukaryotic pathogens	http://eupathdb.org
Gemina	Metadatabase	http://gemina.igs.umaryland.edu
Genomes Online	Metadatabase	www.genomesonline.org
Influenza Research db	Metadatabase for Influenza	www.fludb.org
Los Alamos National Laboratory pathogen research databases	Metadatabase for HIV, HCV, Influenza, oral and STD pathogens	www.lanl.gov/science/pathogens
NIAID Bioinformatics Resource Centers	Metadatabase	www.pathogenportal.org
NMPDR	Metadatabase for food-borne pathogens and STD pathogens	www.nmpdr.org/FIG/wiki/view.cgi
PATRIC	Metadatabase for all bacterial species in the selected NIAID category A-C priority pathogens list	www.patricbrc.org
VectorBase	Metadatabase for invertebrate vectors of human pathogens	www.vectorbase.org
ViPR	Metadatabase for viral pathogens	www.viprbrc.org
varDB	Antigenic variation database	www.vardb.org/vardb

binding complimentarity. Peptides that are showcased to the immune system on class I MHC are derived from cellular proteins that have escaped complete destruction during normal intracellular protein turnover, where degradation of short-lived or defective proteins serves to maintain cellular protein homeostasis (7-9). The first step in the generation of antigenic peptides involves the posttranslational modification of a target protein substrate with an ubiquitin chain, which serves as a molecular signal for rapid degradation by the proteasome. The fraction of peptides that survives can be processed further by cytosolic proteases into even smaller peptides or their constituent single amino acids, or be translocated into the ER by the Transporter Associated with antigen Processing (TAP) complex. N-terminally extended precursors can there be trimmed to optimal length by endoplasmic reticulum (ER)-resident aminopeptidases (ERAP) before being loaded on a recipient class I MHC, thus releasing the final complex from its surrounding chaperones including tapasin, calreticulin, and the oxidoreductase ERp57, and initiating its migration through the Golgi to the plasma membrane (7, 10).

3. DIVERSITY IN T CELL EPITOPE DISCOVERY

The development of novel diagnostic, prognostic and eventually immunotherapeutic applications exploiting class I MHC antigen presentation, crucially depends on the exact definition of the antigen and the MHC restriction element, and subsequently on the monitoring of the ensuing antigen-specific T cell response. Some of the major obstacles that need to be overcome for a proper description of the cellular immune response involves dealing with the multiplicity of variables that are at its basis. In this review, we aim to give a broad overview of the challenges faced in this field, specifically due to the large diversity of pathogens encountered by the human immune system, the diversity of MHC molecules present in the individual host as well as in the population, and the plethora of technologies that immunologists currently have at their disposal. Finally, the recent drive to limit the diversity in reporting through the development of standards such as the Minimal Information About T cell Assays (MIATA) initiative will be highlighted.

3.1. Diversity of the pathogen

Antigen-independent diversification, through the stochastic process of V(D)J gene rearrangements, provides T cells with a near limitless repertoire of randomly generated antigen receptors in anticipation of pathogens that the adaptive immune system thus far has never encountered. Knowledge of the offending microbial genome represents the first step in describing the complexity of this arms race in diversity between both host and pathogen, as it essentially provides a catalogue of all the potential virulence factors and immunogens, and allows T cell epitope discovery to focus only on those translated products represented by the genome. In 1995, a landmark publication by The Institute for Genomic Research (TIGR) reported the sequencing of the first genome of a free-living organism, the bacterium *Haemophilus influenzae* (11). Whole genome sequencing, aided by novel sequencing technologies such as cyclic-array strategies that have been rapidly developed into commercial platforms and have proven successful in replacing the traditional Sanger biochemistry-based methods, has since dramatically accelerated (12, 13). The accumulation of genomic data on microorganisms of medical significance has generated a wealth of publicly available information, as illustrated by Table 1, where a number of web resources providing this sequence data as well as analysis software are listed. The enormous influx of information will continue to require solid bioinformatics infrastructures for the comprehensive analysis and extraction of useful *in silico* information of pathogen diversity (14-16). Archived data can provide a useful scaffold for the annotation of multiple related genomes, and permits the comparison of whole genome sequences on the level of phylogenetically related strains and species, as well as between higher order taxonomic ranks. The degree of sequence conservation and variability between genotypic variants of pathogens also provides direct insight into their proteomes and the inherent antigenic variation in immune responses they could provoke.

Apart from databases that catalogue the complete and intact source of the antigen, MHC-binding ligands have also been extensively documented and deposited in publicly available archives (17). This was

pioneered in 1995 with a relatively modest collection (as judged by current standards) of a few hundred peptide entries, mostly consisting of human ligands and T cell epitopes, that later were consolidated into the SYFPEITHI database (18, 19). Increasing demand from the immunological research community has driven the development of several databases (Table 2) with each of them addressing particular niche areas although considerable overlap exists between them. For example, there are repositories that focus exclusively on a particular pathogen (e.g. the Influenza Sequence and Epitope Database (20), or the Los Alamos HIV databases (21)), whereas others concentrate primarily on those data sets for which complete structural characterization of the pMHC complexes is available (e.g. the MHC-Peptide Interaction Database version T2 (MPID-T2) (22)). A more recently developed online repository, The Immune Epitope Database (IEDB), also aims to collate information on antibody and T cell epitopes for humans, as well as other species such as non-human primates and rodents. Experimental data, both positive and negative, is derived and curated from published data obtained from PubMed, as well as through direct submission of large data sets by individual investigators (23, 24). The data covers several immunological domains spanning from infectious diseases (excluding HIV), allergy, autoimmunity, transplantation, to cancer. Separate categories have been created for HIV, and orphan data that does not fit in any of those domains is classified as "Other". As with any archive, the data curation needs to apply transparent criteria for data inclusion and several levels of review to ascertain the antigenic entity, its source and the context of immune recognition, be it a description of a T cell response or qualitative and quantitative binding data for peptides to cognate MHC molecules. Furthermore, when querying and extracting information from epitope databases, it is paramount to be able to make a clear distinction between data that describes binding of a peptide sequence to a particular MHC molecule, and a T cell response against a defined peptide-MHC complex, which are fundamentally different parameters that cannot be used interchangeably. Finally, in order to stay current, these repositories need to deal with an exponential growth of immunologically relevant data making it "a work in progress" that requires continual updating of epitope data, and upgrading of the software interface.

Databases of immunologically relevant peptides and experimental details, combined with genomic data of a myriad of pathogens, have proven excellent resources for bioinformatics to develop tools to predict candidate epitopes, that can save valuable time and resources associated with experimental T cell epitope identification. The precise technological features of methods for epitope prediction have been extensively reviewed by the groups of Reche (25), Sette (17), Ren (26), Ranganathan (27), and Nielsen (28). We here provide a limited discussion of the more commonly used bioinformatic tools that are freely available as ready-to-use internet-accessible programs (Table 3). The main attraction of web-based epitope prediction programs is their easy and public access, even though they are not ideally suited for handling large data sets and their output data is not normalized making comparison between different methods difficult. Most methods in essence predict peptide binding to the MHC whereas predictive tools describing T cell reactivity for particular peptide-MHC combinations have, to our knowledge, not yet been reliably developed. The factors that influence the performance of prediction methods in terms of accuracy and sensitivity are primarily the type of data used to train the algorithm, and the nature of the predictive algorithm or model. The latter can be broadly categorized in qualitative and quantitative peptide sequence-based models, and structural modeling methods (25). The earliest attempts on predictions were epitope-data driven and comprised the qualitative description of motifs of amino acids commonly found at specific positions within the polypeptide sequence (18, 19, 29). This was improved upon by the definition of motif matrices, where complete tables were constructed with residue-specific coefficients for each position within the peptide chain, allowing the scoring and ranking of epitopes by summing, multiplying or averaging the individual coefficients derived from a peptide sequence (19). Ever more sophisticated platforms, including hidden markov models (HMMs), artificial neural networks (ANNs) and support vector machines (SVMs), have since been developed (25, 27, 28). Many of these computational strategies have also been applied to quantitative prediction models with the distinction that biophysical affinity data for peptide binding to the MHC serves as the training information (25). The incorporation of parameters such as the likelihood of proteasomal processing or TAP transport have further added theoretical sophistication, although the benefit of their inclusion has not been conclusively demonstrated (17), and predictive methods have yet to include variables such as rates of protein expression, protein localization, proteolytic processing, or the T cell repertoire to which the peptide is presented. Finally, three-dimensional atomic structures for various MHC molecules obtained by X-ray crystallography, each detailing the exact geometry and chemical environment of the peptide binding cleft of the specific MHC, have also been exploited for epitope prediction using strategies such as protein threading, homology modeling and peptide docking (25, 27).

Side-by-side comparison of prediction methods has revealed that artificial neural networks and consensus prediction emerge as the tools of choice, however, the principle factor influencing performance remains the size of the training data (30-32). Increasingly successful mathematical models and algorithms that will continue to guide and focus epitope identification efforts will require the integration of both novel *in silico* methods and experimentally validated data on peptide binding or T cell reactivity. It is noteworthy that there is considerable disparity in the quantity and quality of epitope-related data for different allelic variants of MHC molecules, with a strong bias towards the archetypical HLA-A*02:01 (Figure 2). The comprehensive dissection of cytotoxic T lymphocyte (CTL) responses across genetically heterogeneous human populations necessitates epitope identification efforts to focus on the less-studied yet commonly distributed MHC molecules.

Table 2. Online available MHC epitope databases

Database	Contents	URL
AntiJen	Peptide binding information	www.darrenflower.info/antijen
EPIMHC	Peptide binding information	http://imed.med.ucm.es/epimhc
IEDB	Peptide binding data and T cell responses	www.immuneepitope.org
ISED	Influenza epitope information	http://influenza.korea.ac.kr/ISED2
Los Alamos HIV databases	Published HIV epitopes	www.hiv.lanl.gov
MHCBN	Published epitopes	www.imtech.res.in/raghava/mhcbn
MPID-T2	Sequence-structure-function of MHC/TCR	http://biolinfo.org/mpid-t2
SYFPEITHI	Peptide motifs and ligand binding information	www.syfpeithi.de

Table 3. Online available MHC epitope prediction software and servers

Name	Prediction methods	Coverage	URL
BIMAS	QM	Class I MHC binding	www-bimas.cit.nih.gov/molbio/hla_bind
CBS Prediction Servers	Suite of prediction methods	Proteosome cleavage, class I and II MHC (pan-) specific binding	www.cbs.dtu.dk/services/
CEPP	Consensus prediction using published QM	Class I MHC binding	http://jura.wi.mit.edu/bioc/grotenbreg
Epidock	Homology modeling	Class I MHC binding	http://bioinfo-pharma.u-strasbg.fr/template/jd/pages/research/cheminformatics-tools.php#epidock
EpiJen	QM	Proteosome cleavage, TAP transport and class I MHC binding	www.darrenflower.info/EpiJen
EpiToolKit	Suite of prediction methods	Class I and II MHC binding, and Minor Histocompatibility Antigens	www.epitoolkit.org/
HLABIND	Structure-based modeling	Class I MHC binding	http://atom.research.microsoft.com/hlabinding
IEDB	Suite of prediction methods	Proteosome cleavage, TAP transport, and class I and II MHC binding	http://tools.immuneepitope.org/main/html/tcell_tools.html
Immunomedicine Group	Suite of prediction methods	Proteosome cleavage, class I and II MHC (pan-) specific binding	http://imed.med.ucm.es/Tools/index.html
KISS	Support vector machines (SVM)	Class I MHC binding prediction for alleles with few known binders	http://cbio.ensmp.fr/kiss
MAPPP	Combinations of cleavage models with QM and MMs	Proteosome cleavage, and class I MHC binding	www.mpiib-berlin.mpg.de/MAPPP
MHC-THREAD	Structure-based modeling	Class II MHC binding	www.csd.abdn.ac.uk/~gjlk/MHC-Thread
MHCPred	Quantitative Structure Activity Relationship (QSAR) models	TAP transport, and Class I and II MHC binding	www.darrenflower.info/mhcpred
MOTIF_SCAN	Sequence Motifs	Search Class I and II MHC for protein motifs.	www.hiv.lanl.gov/content/immunology/motif_scan/motif_scan
PAProC	Network-based model	Proteosome cleavage	www.paproc.de
PepDist	Distance Function	Class I MHC binding	http://www.pepdist.cs.huji.ac.il/
Peptidecheck	Matrices	Class I and II MHC binding	http://www.peptidecheck.org/
POPI	SVM	Class I and II MHC binding	http://iclab.life.nctu.edu.tw/POPI
PREDEP	Structure-based modeling	Class I MHC binding	http://margalit.huji.ac.il/Teppred/mhc-bind/index.html
PREDTAP	ANN, Hidden Markov model (HMM)	Tap binding peptides	http://antigen.i2r.a-star.edu.sg/predTAP
Raghava Group	Suite of prediction methods	Proteosome cleavage, class I and II MHC (pan-) specific binding	www.imtech.res.in/raghava/
SMM	SMM	Class I MHC binding	http://zlab.bu.edu/SMM
SVMHC	SVM	Class I and II MHC binding	http://www-bs.informatik.uni-tuebingen.de/Services/SVMHC
SVRMHC	PSSM	Class I MHC binding	www.sbc.su.se/~pierre/svmhc/new.cgi
SVRMHC	SVM	Class I and II MHC binding	http://svrmhc.biolead.org/
SYFPEITHI	PSSM	Class I and II MHC binding	www.syfpeithi.de/Scripts/MHCServer.dll/EpitopePrediction.htm

3.2. Diversity of the epitope

It is becoming increasingly clear that the diversity of peptides that are subjected to immune surveillance greatly exceeds what can be predicted from the coding capacity of the genome alone. From an immunity perspective, the exact source of an unconventional epitope must be subordinate to capturing any information that can enhance the capacity to differentiate friend from foe by widening the pool of potential antigens. The mechanisms reviewed below extend the sequence diversity beyond those peptides derived by normal cellular protein turnover, which has profound impact on epitope discovery efforts and highlights the potentially byzantine antigen diversity encountered by the immune system.

The term cryptic peptides, which refers to their atypical origin, is usually reserved for those peptides that arise from translation of sequences other than the primary DNA encoded open reading frame (8, 9, 33-36). Aberrant posttranscriptional regulation can, for example, result in the retention of intronic sequences in the final transcript (37), incomplete splicing events with novel epitopes located across an exon-intron junction (38), or exon extension with the concomitant introduction of novel translation initiation sites (39). The translation process itself provides yet another mechanism for generating unconventional gene products by using alternative sites of initiation. The ribosome can, for example, bypass the conventional initiation codon for a preferred site

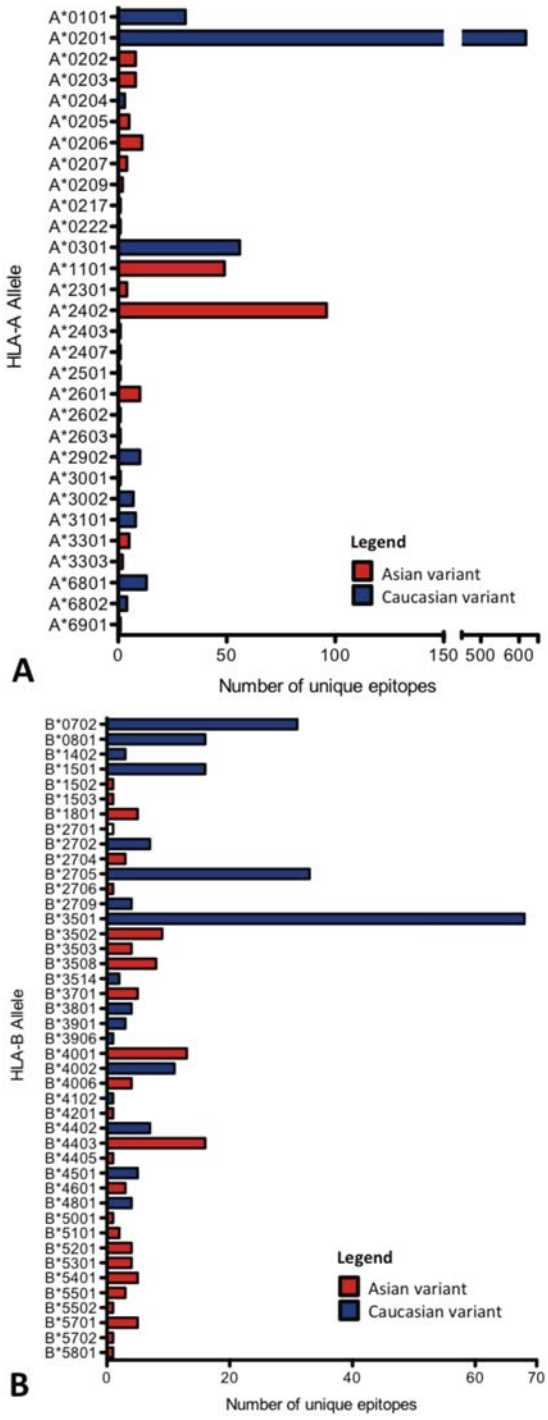


Figure 2. Number of unique CD8 T cell epitopes reported by IEDB (www.immuneepitope.org) for HLA-A and HLA-B molecules. Only epitopes for which the exact HLA-A (A) or HLA-B (B) restriction element was reported have been included. Further classification of HLA variants was performed for HLAs that are found more frequently in Asian populations ("Asian variant") as compared to Caucasian populations ("Caucasian variant").

downstream in an alternative frame, in a process known as initiation codon scan-through (40, 41). Moreover, the ribosome can commence translation from non-canonical codons (42-44), thus decoding a leucine residue instead of methionine as the first amino acid of the translated product, or include "untranslated" regions on either the 5'- or 3'-end of the transcript (43, 45), and many variations on this theme have been identified.

Epitopes derived from alternative reading frames have been observed repeatedly in malignancies (46) and in autoimmunity (47), as well as in T cell responses against pathogens with a high rate of nucleotide insertions and deletions such as influenza (40, 41) and human immunodeficiency virus (HIV) (48, 49). Remarkably, evolutionary pressure imposed by the immune response has been shown to drive viral adaptation to generate escape mutants of cryptic peptides, whilst leaving the coding sequence of the original reading frame unaffected, illustrating their relevance to immunity (48).

A variety of post-translational modifications (PTMs) contributes to protein stability and regulates their function, for example by determining the activation state of an enzyme (50). Fundamental cell signaling events, inflammation, cellular transformation, and apoptosis can all be accompanied by changes in PTM patterns, which in turn can result in immune recognition of the modified proteins or fragments thereof (51-53). Peptides derived from proteins involved in such processes can either retain these non-genetically encoded features, or incorporate them at some point in the antigen-processing pathway, subsequently eliciting an immune response that is capable of discriminating between these specific modifications. Antigenic peptides presented both by class I and class II MHC have been decorated with additional chemical moieties at their termini as well as on their side chains, with examples ranging from acetyl- (54), cysteinyl- (55), glycosyl- (56), methyl- (57), nitro- (58) to phosphate (59) groups. Alternatively, the chemical nature of the epitope side chains can be enzymatically converted in processes such as deamidation (60, 61) through the action of N-glycanases or transglutaminases, citrullination (35, 62), asparagine-bond isomerisation (63), or cysteine oxidation to form intramolecular disulphide bonds (64). Not only does chemical alteration of potential epitopes direct the T cell repertoire structurally by influencing peptide transport, binding to chaperones and the MHC, as well as pMHC-TCR affinity, they can also directly influence their processing (65, 66). For instance, deamidation of asparagine residues, occurring either spontaneously or during the removal of N-linked glycosyl groups, renders the sequence less vulnerable to proteolytic degradation by asparagine endopeptidases (67).

Protein (or peptide) splicing is arguably the most extreme form of PTM, and entails a primary amino acid sequence going through a series of proteolytic and peptide ligation events (68). This posttranslational "cut-and-paste" reaction yields noncontiguous peptide sequences effectively disrupting the genome coding sequence. In unicellular organisms, numerous examples of self-catalyzed protein splicing have been described where a polypeptide segment is excised from the original sequence

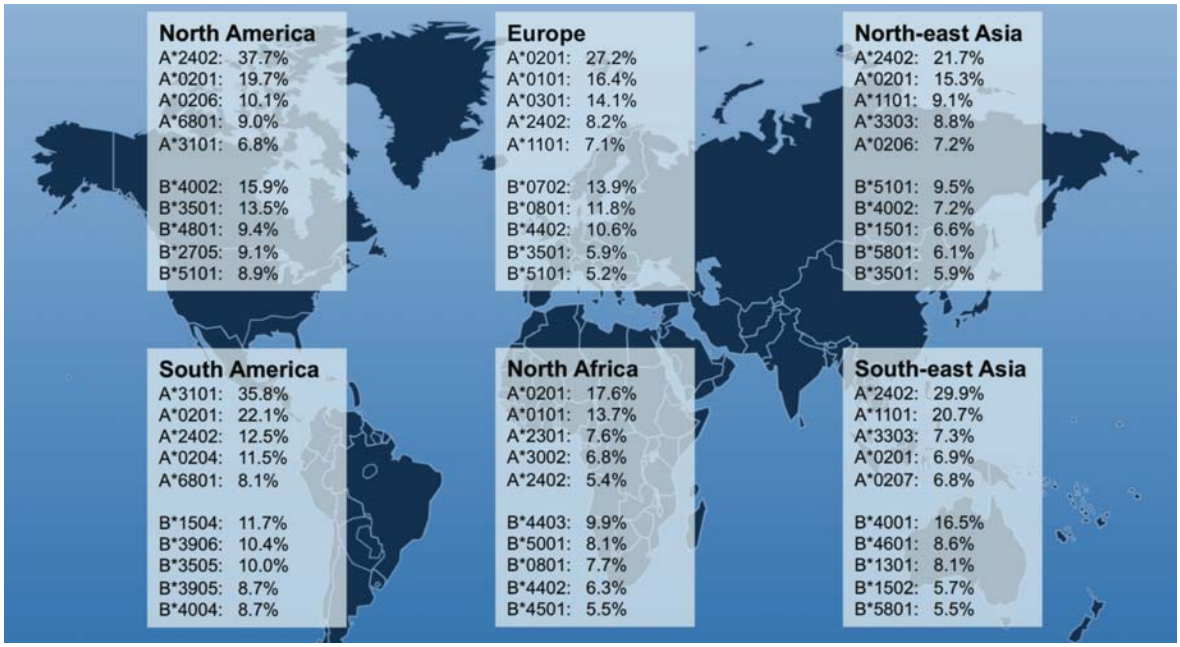


Figure 3. Most common HLA alleles found in human populations from separate regions. Only the top 5 most frequent HLA-A and HLA-B alleles are shown. Data was obtained from the dbMHC Anthropology Resources (www.ncbi.nlm.nih.gov/gv/mhc/main.cgi) in May 2010.

(an intein) and subsequently intramolecularly conjugated to a flanking sequence (the extein) without the help of proteases or other auxiliary factors (69). The predictability of this process make intein-based technologies increasingly popular in the field of protein-engineering (70). When the transpeptidation process is initiated by cysteine-, serine-, or threonine proteases, the first step of proteolysis involves the formation of an acyl-enzyme intermediate. Resolution of enzyme-substrate complex can occur through nucleophilic attack by the N-terminus of a polypeptide in close proximity, which is in direct competition with hydrolysis (68). A report by Hanada *et al* revealed that T cell epitopes could indeed be generated through protease-catalyzed protein splicing, thus highlighting that non-genetically encoded peptides could add significant complexity to epitope identification (71). Further studies cemented this notion by pinpointing the proteasome, a central player in the generation of antigenic peptide fragments, as the catalytic center where sequence scrambling of the peptide could occur in both linear and reverse order (72, 73). In this context, it would be intriguing to speculate about the existence of chimeric epitopes derived from multiple parent proteins being generated in similar fashion, although the likelihood of creating those in large abundance in a cellular context might be considered minimal.

In conclusion, the increased variety of epitopes that cannot be inferred directly from the genome sequence because of alternative modes of translation, through natural PTM of the primary peptide sequence, or through peptide splicing events, will both qualitatively and quantitatively affect the spectrum of T cell responses and therefore impact upon both immunity and disease.

3.3. Diversity of the host

In humans, MHC molecules are designated as Human Leukocyte Antigens (HLAs). The HLA gene locus, located in the 6p21 region of human chromosome 6, contains ~32 thousand known protein coding sequences, including the genes encoding the class I and class II HLA complexes (74). These HLA molecules are encoded by polygenic and exceptionally polymorphic gene families. The polygenicity is derived from clusters comprising classical genes (class I HLA-A, -B and -C; class II HLA-DP, -DQ, -DR), non-classical genes (class I HLA-E, -F, -G; class II HLA-DM and -DO) and various pseudogenes, and allows an individual to simultaneously present multiple antigens (75). Maternally and paternally inherited alleles add the final layer of HLA diversity, with a high probability for heterozygosity within offspring given the polymorphic nature of the HLA loci, thereby possibly providing a survival advantage against pathogens as discussed below (76-79). The allelic polymorphisms for each of those genes is primarily restricted to those residues that line the peptide-binding groove of these molecules, thereby largely defining the peptide-binding and presentation repertoire of the individual gene product. As per October 2010, the IMGT database lists the sequences of over 4300 class I and 1290 class II HLA molecules, with the highest polymorphism found in the classical HLA genes with 1381 HLA-A, 1927 HLA-B, 960 HLA-C and 924 HLA-DRB alleles, and these numbers are steadily increasing still (80). When clustered either by ethnicity or geographical location, prominent distributions of HLA allelic variants can be discerned, as illustrated by Figure 3 (81). HLA-A*02:01 is commonly found in Europeans (27%) and north Americans (20%), but is contrastingly low in southeast Asians (7%) and southwest Asians (12%). On

Table 4. Online available MHC databases

Database	Comment	URL
Allelefrequencies	Allele frequencies in human populations	http://www.allelefrequencies.net/
dbMHC	Platform for DNA and clinical data related to the human MHC	http://www.ncbi.nlm.nih.gov/gv/mhc/main.cgi
dbMHC anthropology	Allele frequencies in human populations	http://www.ncbi.nlm.nih.gov/gv/mhc/ihwg.cgi
IMGT	Genome, sequence and structure immunogenetics data.	http://www.imgt.org/
IMGT/HLA Database	Database for sequences of the HLA including the official sequences for the WHO Nomenclature Committee For Factors of the HLA System	http://www.ebi.ac.uk/imgt/hla/
HLA sequence data	Static database for HLA sequences	http://hla.alleles.org/data/index.html
ImmPort	DNA and protein sequences, sequence features, and population frequencies of MHC alleles	www.immport.org/
PyPop	Software for large-scale population genetic analyses	www.pypop.org/

the other hand, HLA-A*11:01 (21%), HLA-A*24:02 (30%) and HLA-B*40:01 (17%) alleles are widespread in southeast Asian populations whereas most of these HLA variants are rare in Europeans (7% of HLA-A*11:01; 8% of HLA-A*24:02, 5% of HLA-B*40:01) and north Americans (1% of HLA-A*11:01, 2% of HLA-B*40:01) (82). These are just a few examples for HLA frequency differences across populations, and the consolidated data is publicly available in online repositories listed in Table 4. On an individual level, disease susceptibility can be directly influenced by these genetic variations, as minor differences in the constellation of expressed HLA alleles can have crucial consequences during challenges to the immune system by infectious disease, as has been shown in dengue virus (DENV) (83), HIV (84, 85), hepatitis B and C virus (HBV and HCV) (86, 87), and human papilloma virus (HPV) (87). Because HLA diversity is a critical mediator in the generation of specific T cell responses, epitope identification should provide a basis of understanding of the requirements for developing protective immunity against these pathogens in various human populations.

Through the vast repertoire of allelic variants for HLA molecules present in the general population, each of them capable of binding distinct sets of peptides, a mechanism is created to deal with the large diversity of antigens for which no prior experience within the collective immune system exists. At the same time, significant cross-reactivity in peptide binding to different HLA variants has been observed. For example, it was found that the HLA molecules B*35:01, B*35:02, B*35:03, and B*54:01 share a consensus motif (88). Therefore, it has been proposed to cluster HLAs that bind largely overlapping collections of peptides into supertypes, in the above case the B7 supertype. A major driving factor behind this reductionists approach was the desire to design and develop subunit vaccines, which are non-infectious formulations consisting only of the immunogenic determinants without any irrelevant component. Vaccination with the antigen alone holds some inherent advantages, as it circumvents the safety concerns associated with whole pathogen vaccination schemes, the concentrated delivery of antigens could boost the vaccines efficiency, and it should provide control over the direction and breadth of the immune response. The conceptual reduction in complexity introduced through HLA supertypes can be a valuable tool in the search for cross-reactive antigens capable of providing appropriate protection over a heterogeneous group of HLAs. The initial classification, which so far is limited to allelic variants of HLA-A and -B, identified four HLA supertypes based on their ability to bind similar peptide sequences: their corresponding supermotifs (89). Detailed inspection of the pocket architecture, enabled largely through the availability of crystallographic data on a variety of MHC molecules (90-94), has revealed that peptide binding specificity is governed primarily by the physiochemical properties of the B and F binding pockets in coupled fashion (Figure 1). This scheme was thereafter expanded to nine HLA supertypes to accommodate the majority of HLA molecules identified at that time. The range of supertypes – A1, A2, A3, A24, B7, B27, B44, B58, B62 – was broadly inclusive of the HLA variants found in most ethnic groups worldwide (95). The refinement and development of novel immunotechnologies, as will be discussed in the following section, has continued to produce an abundance of novel T cell epitopes and spurred a third update and revision of the classification, yet, in its most recent incarnation no new HLA supertypes were identified (96). Moreover, based on overlapping peptide repertoires, some HLAs appeared not to belong to a single supertype but rather required to be categorized in several supertype families. In light of the polymorphic nature of HLA, this promiscuity in peptide binding is not difficult to envisage. Although the notion of HLA supertypes has been applied in a variety of disease settings, and has been evaluated as a diagnostic and prognostic parameter (89, 95, 96), it has its basis in peptide-MHC binding. Future studies shall provide insight whether the concept of clustering polymorphic HLA based on degeneracy in T cell recognition will continue to provide added value.

3.4. Diversity of immunotechnologies

Many technologies have been added to the immunologists' toolbox to establish the precise identity of $CD8^+$ T cell antigenic determinants, and the majority of those has been reviewed in-depth (97-101). Here, we highlight several selected assays (Table 5) categorized by those that probe peptide-binding to the MHC (as measured by the presence of a (labeled) ligand or by stabilization of the resulting protein complex) and assays looking at cellular responses (as measured by surface expression of specific TCRs or T cell function such as cytokine secretion).

Biophysical techniques allow assessment of epitope binding and stabilization characteristics for individual MHC products. They involve either systems with cell-lines expressing particular MHC molecules, or cell-free systems that require the use of soluble MHC molecules. Common cell-lines capable of antigen

Table 5. Examples of T cell epitope mapping technologies

Technology	Advantages	Disadvantages
• Cell-based peptide binding	• Established technology • Widely adopted technology • Robust	• Cell lines unavailable for several HLAs • Difficult to isolate sufficient quality and quantities of MHC proteins • Labor intensive
• Radio-labeled peptide binding	• Quantitative measure of peptide binding • Cell-free assay is possible	• Radioactive reagents • Indirect quantification of peptide binding • Labor intensive
• Fluorescence polarization	• Direct measurement of free and bound peptide • Real-time measurement • Fast and simple	• Requires the design and synthesis of fluorescent peptide probe • Peptide under investigation is modified with fluorescent moiety.
• MHC-stability ELISA	• Direct measurement • High throughput screening is possible	• No quantitative measurement • Multiple staining and incubation steps
• LC-MS/MS	• No prior knowledge of the antigen identity needed allowing unconventional epitopes to be identified • High sensitivity	• Only a small percentage of all possible ligands can be analyzed per run • Technically challenging
• MHC-peptide multimers	• Measures frequency of antigen-specific T cells • No requirement for *in vitro* expansion • Non-destructive for sample • Combination with other cell stainings is possible • High throughput screening is possible	• Does not measure effector function of T cells • Requires different multimer reagents for specific MHC-peptide combinations
• Intracellular Cytokine Staining	• Quantification of cytokine product • Multi-cytokine analysis is possible • High throughput screening is possible	• Identification is on based on function only • *In vitro* stimulation is required • Cell fixation is required
• T cell Specific Activation Markers	• Fast staining methods • Combination with other cell stainings is possible • High throughput screening is possible	• Some markers are not exclusive for T cell activation
• Proliferation (BrdU or CFSE)	• Measures function • Measures number of cell cycles (CFSE only) • Combination with other cell stainings is possible • *In vivo* application possible	• Cell fixation required for analysis (BrdU only) • Long *in vitro* culturing is required • Does not measure effector function
• ELISPOT	• Measures T cell function and frequency • High throughput screening is possible • Does not require *in vitro* expansion	• Measures a single cytokine • *In vitro* stimulation is required • Bystander activation not distinguishable
• Cytokine Secretion and Cell Surface Capture	• Protein-release is quantified • Can be used for cell sorting	• Multiple cell staining steps required • Require *in vitro* stimulation • Not applicable for high throughput assays
• Cytokine Secretion and Well Surface Capture	• Protein-release is quantified • High throughput assay	• Bystander activation not distinguishable • Measures a single cytokine • Require *in vitro* stimulation • Technically challenging
• MHC-Peptide Microarrays	• Simultaneous detection of T cell binding with cytokine secretion is possible • Simultaneous detection of multiple specificities	• Dependent on T cell diffusion to their target • Technically challenging
• Qrt-PCR	• Simultaneously measurement of different cytokine expression	• No quantification on protein- or cellular level • Technically challenging

presentation are the murine RMA-S cell-line (102, 103), human TAP-deficient T2 cell line (104) and EBV-transformed B cell lines (105, 106). Peptide binding assays employing these lines depend on the presence of "empty" class I MHC molecules on the cell surface to enable the exogenous addition of peptides. This is achieved in RMA-S cells by maintaining them at 26°C (102, 103), in transfected T2 cell-line because peptides derived from cytosolic antigens are not presented (104, 107), and in other cell types by transfection to induce an overexpression of HLA (108) or by a mild acid treatment to remove the peptide from the complex (105, 106). In the T2 or RMA-S peptide stabilization assays, peptides are added directly to these "empty" MHCs. The surface expression level of the stabilized complex is then probed with MHC-specific antibodies and quantified by flow cytometry (102, 109, 110). Another avenue pursued with these cell-lines are peptide competition assays, where the inhibition of binding of fluorescein-labeled (105, 106) or radio-labeled peptides (108) by non-labeled peptides-of-interest to the "empty" MHC is measured. This can be accomplished using either flow cytometry or gamma counting of metabolically labeled MHC molecules isolated from lysed cells by gel-filtration or immunoprecipitation. Peptide competition involving radioactive ligands has been demonstrated on pre-purified detergent-solubilized MHC molecules as well (111-115).

Assays involving cell-free systems that directly measure peptide-MHC binding come with a number of distinct advantages. First, the confounding effects of simultaneous surface expression of multiple MHC variants on a single cell are absent. Furthermore, less commonly studied MHCs are directly accessible even when cell-lines for those are not yet available. Finally, the difficult isolation of MHC proteins in sufficient quantity and purity from cultured cells is circumvented, as is the need for continuous maintenance of cells with the inherent potential for culture contamination. As a means of detection, fluorescence polarization has become increasingly popular in cell-free assays. Typically, a non-anchor amino acid residue in the peptide-of-interest is

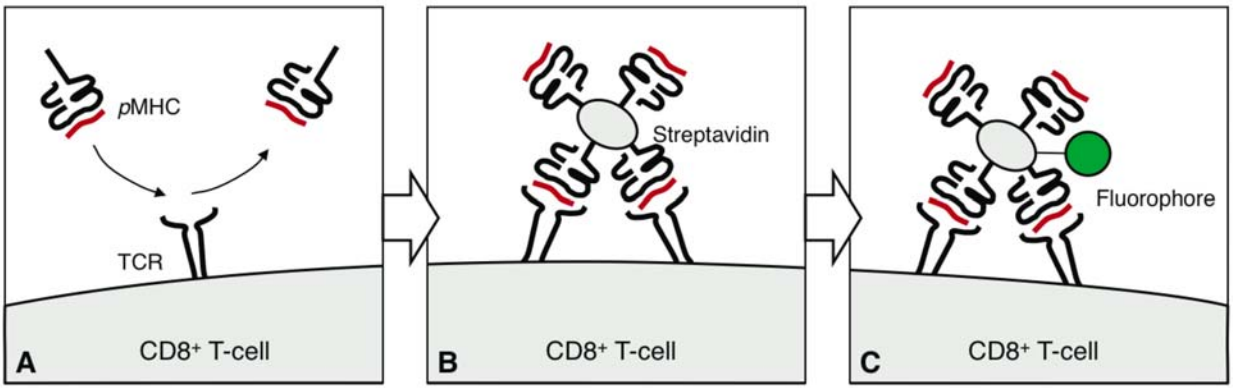


Figure 4. The principle of MHC tetramer staining reagents. (A) Class I pMHC complexes interact with the TCR on $CD8^+$ T cells with high specificity but with low affinity. (B) By attaching multiple pMHCs to a streptavidin core, the avidity of the complex for the T cell is significantly increased without loss of specificity. (C) Fluorescent labels attached to the core streptavidin make the MHC-tetramer (or "tetramer") a staining reagent for T cells of select specificity that can be employed in fluorescence microscopy, and flow cytometry analysis.

substituted for a FITC-conjugated lysine (100). The ratio of free and bound peptide-FITC probe to soluble MHC can then be measured directly by fluorescence anisotropy, from which the binding characteristics of the ligand can be determined without the need for any MHC separation steps (100). Peptide competition assays using this detection method are also feasible. Moreover, this method was successfully employed together with UV-mediated peptide exchange (*vide supra*), in which class II MHC was pre-loaded with a conditional ligand that fragmented upon UV-light exposure and then was exchanged for a fluorescent probe (116). Finally, the capability of non-labeled peptides to stabilize the MHC following UV-mediated peptide exchange can be tested with the MHC-stability ELISA assay. Peptide binding is indirectly ascertained with beta2m-specific antibodies by probing for its presence or absence, as this subunit will be lost too when a replacement peptide does not adequately stabilize the complex. Subsequent colorimetric development provides an assay read-out similar to traditional ELISA (117, 118).

Mass spectrometry (MS) based techniques provide a bottom-up approach to identify peptides that bind to cell surface MHC. Generally, the ligands are extracted by acid treatment and purified by high-performance liquid chromatography (HPLC). The eluted peptide fractions are then analyzed for their ability to induce T cell activation, and the bioactive fractions are probed using liquid chromatography in combination with tandem mass spectrometry (LC-MS/MS) for definitive peptide identification (97, 119-124). One major advantage of this technology is that it can operate without prior knowledge of the input antigen's identity. Cryptic epitopes or post-translational modifications, as discussed earlier, are thus readily identified. However, the technological sophistication that is required to run such experiments often precludes its wider and more frequent application.

As T cell expansion and function is the most direct measure of the host's immune response to infection, antigenic peptides identified through T cell-based assays are invaluable. T cells can be directly analyzed by flow cytometry using various labeling strategies that seek to distinguish them by their antigen-specific TCR, their function or combinations thereof. Such techniques are useful for both epitope discovery and for functional characterization of antigen-specific T cells. First described by Altman *et al*, the MHC tetramer is such an enabling technology for the visualization of antigen-specific $CD8^+$ T cells (125). As illustrated (Figure 4), recombinantly produced MHC molecules of defined specificity are multimerized, traditionally around a streptavidin core that is endowed with a fluorophore label of choice, to increase the avidity of the combined complex without loss of specificity (126-128). As the synthesis of tetramers is time- and labor-intensive, the construction of large tetramer libraries for epitope mapping has not been feasible until Toebes *et al* developed a peptide exchange strategy involving tetramers loaded with UV-sensitive conditional ligands (129). The initial ligand bound to the MHC contains a photocleavable residue which fragments the peptide backbone after longwave UV irradiation (129, 130). The resulting complex is unstable unless a rescue peptide is provided to occupy the emptied peptide-binding groove, consequently producing a novel peptide-MHC combination (Figure 5) (129). Adopting a similar peptide exchange strategy, chemosensitive ligands that cleave upon exposure to a chemical trigger can also be designed. Specifically, $NaIO_4$ has been employed to induce chemocleavage in a diol-containing conditional ligand (131). By using conditional ligands, the time spent with high-throughput production of MHC tetramers of distinct specificity can be vastly reduced and T cell epitope discovery has been achieved with MHC tetramer libraries for the murine MHC restriction elements H-$2K^b$, -D^b, and -L^d (132-135) and human HLA-A1, -A2, -A3, -A11 and -B7 (129, 136-138).

Epitope-identification based on T cell function can be accomplished by antigen-specific activation of T cells which results in a transient increase in cytokine

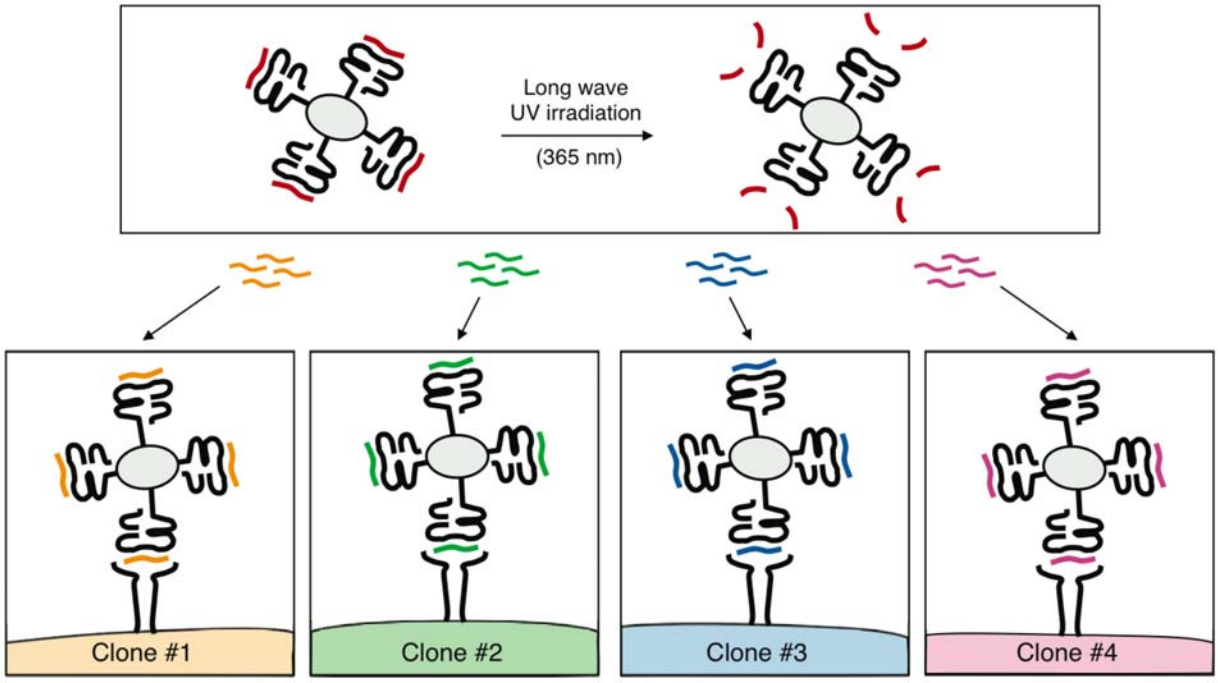


Figure 5. Conditional ligands for class I MHC tetramers. Tetramerized major histocompatibility complexes are occupied by a synthetic photocleavable ligand in the peptide binding groove. Upon UV irradiation, in the presence of putative peptide ligands, the conditional ligand is fragmented and the replacement peptide produces MHC tetramers of novel specificity capable of staining individual T cell clones.

expression such as IFN-gamma, IL-2 and TNF-alpha (139). These soluble factors can be visualized by intracellular cytokine staining (ICS) after administration of protein transport inhibitors, such as brefeldin A or monensin, to prevent their release into the extracellular milieu (140-143). The accumulated intracellular cytokines can then be detected through appropriate antibody staining of detergent-permeabilized cells (144, 145). The expression of surface proteins that mark particular cellular activation states also provides evidence of antigen-activated T cells. For example, CD107, a marker for activation-induced T cell degranulation, is found on the luminal side of cytotoxic granules but becomes part of the plasma membrane upon cytolytic degranulation. Antibodies specific for CD107a and -b allow the visualization, typically by flow cytometry, of this temporary increase on the cell surface which can occur in concert with IFN-gamma release (146). Further characteristic cell surface markers for activation include increased expression of CD137 (147), CD44 (148), CD69 (148-150), the late activation marker CD25 (150, 151) or low levels of CD62L (148). T cell proliferation is routinely demonstrated using ^{3}H-thymidine, bromodeoxyuridine (BrdU) (152, 153) and carboxyfluoresceine-diacetate–succinimidylester (CFSE). Similar to tritium-labeled thymidine, the non-radioactive thymidine analog BrdU is incorporated into the DNA during replication. As cell fixation is required to give BrdU-specific antibodies access to the nucleus, isolation of intact and vital cells for follow-up assays is not possible, and the number of cell cycles the cells have undergone can also not be measured. These limitations are not imposed when CFSE, a protein-reactive fluorescent dye, is used for tracking proliferating cells. Upon each cellular division, CFSE labeled cells lose half of their fluorescence intensity resulting in a series of spectral peaks (154). Alternatively, CFSE can be used to detect the site of antigen presentation, the origin of the antigen-presenting cells and the proliferation rate of T cells *in vivo* after CFSE-labeled T cells has been adoptively transferred to a recipient host (155, 156). A combination of MHC tetramer, cell activation and proliferation marker, and intracellular cytokine staining can be analyzed concurrently by multiparametric and polychromatic flow cytometry techniques, thus allowing a complete assessment of $CD8^{+}$ T cell antigen-specificity, activation state and function (153, 157-159).

T cell responses, specifically the release of cytokines like IFN-gamma, can also be visualized by assays other than flow cytometry such as the ELISPOT assay (160, 161). In brief, wells pre-coated with cytokine-specific antibodies are used to capture cytokines secreted in the local environment of sedimented T cells. Secondary antibodies are then used to probe for the presence of the captured cytokine, followed by color development steps, resulting in the occurrence of distinct spots in the well. Each of these spots corresponds to a single cytokine-producing cell when employing an appropriate dilution of cells (162). ELISPOT based on IFN-gamma secretion is a mainstay method for the identification of novel epitopes (163-165) and ensuing T cell functional studies (86, 165). The Cytokine Secretion and Cell Surface Capture (CSC) is a novel assay that measures the cytokine release of T cells

(166, 167). In CSC, the lymphocytes are labeled with a cytokine-specific "catch reagent", a bi-specific antibody that consists of an anti-CD45 antibody conjugated with an anti-cytokine antibody. This "catch reagent" captures the secreted cytokine onto the surface of the labeled cell. Cytokine-coated cells can then be detected with secondary anti-cytokine antibodies conjugated either to fluorochromes for flow cytometry analysis or to super-paramagnetic particles for magnetic cell sorting (MACS) enrichment (167-169). With this, CSC allows for the isolation of cytokine secreting antigen-specific T cells that can be cultured or used in subsequent experiments (167, 170, 171), and affords the possibility to isolate T cells for adoptive transfers (172-174), yet its application as high throughput assay is uncommon (99). Cytokine Secretion and Well Surface Capture (Cell-ELISA), on the other hand, allows for high throughput screening for antigenic peptides based on T cell cytokine release (175-177). Cytokines can furthermore be visualized by quantitative real-time polymerase chain reaction (qRT-PCR) to determine the cytokine mRNA expression, and has been employed as epitope mapping strategy (178, 179). Detection of T cell populations can also be accomplished with cellular array-based screening strategies (180-183). Various peptide-MHC dimers (181) or tetramers (182-184) and antibodies are immobilized in different "spots" to form a microarray. When a cell suspension is layered onto the array, these "spots" capture either antigen-specific T cells (182) or the cytokines produced by T cells activated by the peptide-MHC "on-the-spot" (180, 183). Occupied "spots" are visualized by direct inspection, differential interference contrast (DIC), or fluorescence microscopy (180-184). Common issues associated with methods that circumvent flow cytometry, as described above, are that the antigen-specific T cell frequency can be difficult to ascertain and the phenotype of the responding cells cannot be determined because of a lack of simultaneous multiparametric analysis (99).

3.5. Diversity in data reporting

Multi-institutional studies involving the enumeration and characterization of antigen-specific $CD8^+$ T cell responses have revealed that, although generally a good qualitative and quantitative agreement between results can be obtained, a significant degree of fluctuation in assay outcome between different laboratories remains inevitable (185-191). Numerous factors lie at the basis of this variability; input material and reagents, protocol choices and local laboratory practices, for example. The continual drive for technological innovation and modifications of some of the more common types of assays (i.e. ELISPOT, ICS and peptide-MHC multimer staining) also influence inter- and intra-laboratory findings. These results have highlighted an urgent need for the harmonization of data reporting in order to convince research subjects, patients, fellow investigators, or funding and regulatory agencies that the generated data conforms to the necessary standards and scientific rigor, and that the laboratory results are properly validated. Following in the footsteps of earlier "Minimal Information projects", in particular the Minimum Information about a Microarray Experiment (MIAME) (192), the ''Minimal Information About T cell Assays'' (MIATA) (193) project was conceived. Through several rounds of public consultation with experts in the field, associations and relevant authorities, as well as editors of scientific journals, MIATA aims to propose a broadly accepted framework of standards on the minimum information preferable for publishing T cell assay results that enable objective and thorough interpretation (194). The MIATA project is currently undergoing a careful vetting process and is yet to be finalized for publication. The basic scaffold that so far has been established as MIATA version 1 consists of five core modules. The first concerns the "Minimal Information of the Sample" and delineates the information on the source of cellular material, the method of cell collection and processing, the storage conditions such as temperature, media and crucial time-points, the quality of the sample in terms of recovery and viability of the material, as well as methods used in evaluating those parameters (195-200). Additional information regarding the donor is considered optional. The second module is "Minimal Information on the Assay", which proposes that information on cell counting methodology, cell recovery and viability ranges, medium and serum used, the procedures and assay, as well as controls are included. In the third module, "Data Acquisition", it is recommended that information on the equipment, the acquisition strategies, and analysis software used should be provided during reporting. The fourth module, "Results", defines important parameters for descriptions concerning how raw data were processed and interpreted, the accessibility of raw data and the response determination, statistical tests and empirical rules used during interpretation (201-204). Lastly, minimal information on the "Laboratory Environment", which again is optional, could include a description on the general laboratory operations, laboratory procedure standardization, and the status of assay qualification and validation. It is important to emphasize that MIATA guidelines specifically are not intended to impose specific standards on the operation of individual laboratories, but rather are intended to harmonize the reporting of data in publications, thereby enhancing the clarity of data presentation and quality.

4. PERSPECTIVE

Vaccines have provided a major contribution to the prevention of human disease. Regardless of the many success stories involving immunization strategies against small pox, polio or HBV, significant challenges from infectious diseases remain. Case in point is the ongoing search for a prophylactic HIV vaccine with the recent highly publicized failed efficacy trials for a vaccine based solely on evoking a T cell response (205-207). The question as to why sufficient levels of immune protection could not be reached is still debated, but the reasons are likely to be manifold as immunity is often best obtained when founded on several layers of protection, for example, through raising a combination of neutralizing antibodies and effective T cell responses. It would, however, be presumptuous to immediately disqualify the employed strategy as a whole, as it merely illustrates our limited understanding of what shapes an effective antiviral T cell repertoire.

The development of novel successful therapeutics and vaccines will need to build on the accurate description of the pertinent T cell responses, and those efforts will be confronted with the various layers of diversity as described in this review. Particular areas that in our opinion warrant systematic attention are the following. (A) There is no reason to assume that HLAs that are currently less studied (Figure 5), yet widely present in the general population (Figure 2), do not present a similar abundance of antigenic peptides as those select HLA molecules around which epitope identification has traditionally been centered. We believe that large gains can be made by focusing on HLA-variants that are commonly found in Asia, as with the two most populous countries (*i.e.* China and India) it accommodates well over halve the world's population and infectious diseases significantly burden the region. Coupled with the rapid development of Asian economies, which appear less affected by the recent global economic downturn, it can be reasonably expected that funding will continue to be available to support such efforts. (B) It has been demonstrated that host ethnicity influences the fine-specificity of the T cell response to HBV (86), HCV (208), HIV (209) and EBV (210), presumably as a result of host-pathogen co-adaptation which has driven both HLA and pathogen diversification (74, 87, 211, 212). Consequently, the HLA supertype classification, which primarily describes promiscuity in peptide binding, should be closely scrutinized for its ability to accurately describe the spectrum of protective T cell responses. (C) The shortage of identified T cell epitopes for a majority of HLA variants impacts on the confidence in corresponding predictive algorithms, as the outcome of epitope prediction for those alleles might become biased towards similar peptide motifs. Improvement of these bioinformatic methods needs to be driven by the generation of experimental data. Furthermore, we believe that particularly the consensus approach, which combines multiple predictive strategies each developed with independent training data, holds great promise. The potential of this method has been amply demonstrated for murine restriction elements together with large and complex pathogens (*i.e.* vaccinia virus (213), *Chlamydia trachomatis* (133), and murine gammaherpesvirus 68 (132)) and clearly needs to be extended to the prediction of HLA-restricted epitopes.

The above issues can all be addressed directly through epitope discovery, followed by definition of T cell hierarchy and functionality, employing large and clinically well-defined disease cohorts, and will be aided by the development of novel technologies and the harmonization of data reporting. Ultimately, this will allow the proper evaluation of the importance of T cell responses for immunity against a variety of pathological challenges.

5. ACKNOWLEDGMENTS

Due to scope of the topics discussed, it has not been possible to be fully inclusive of all the primary literature. The above should therefore not be considered an exhaustive review and any omission is entirely unintentional. The authors would like to thank Dr Stephan Gasser for important scientific input and critical reading of the manuscript. GMG is supported by the Singapore National Research Foundation (NRF) Research Fellowship (NRF2007NRF-RF001-226). CXLC and ZWT are NGS Graduate Scholarship recipients, and LYD is an SMA Graduate Scholarship recipient. The funders had no role in the preparation or the publishing of the manuscript.

6. REFERENCES

1. MA Williams, MJ Bevan: Effector and memory CTL differentiation. *Annu Rev Immunol* 25, 171-192 (2007)

2. JE Smith-Garvin, GA Koretzky, MS Jordan: T cell activation. *Annu Rev Immunol* 27, 591-619 (2009)

3. J Nikolich-Zugich, MK Slifka, I Messaoudi: The many important facets of T-cell repertoire diversity. *Nat Rev Immunol* 4, 123-132 (2004)

4. SJ Turner, PC Doherty, J McCluskey, J Rossjohn: Structural determinants of T-cell receptor bias in immunity. *Nat Rev Immunol* 6, 883-894 (2006)

5. MG Rudolph, RL Stanfield, IA Wilson: How TCRs bind MHCs, peptides, and coreceptors. *Annu Rev Immunol* 24, 419-466 (2006)

6.V Venturi, DA Price, DC Douek, MP Davenport: The molecular basis for public T-cell responses? *Nat Rev Immunol* 8, 231-238 (2008)

7. JM Vyas, AG Van der Veen, HL Ploegh: The known unknowns of antigen processing and presentation. *Nat Rev Immunol* 8, 607-618 (2008)

8. N Shastri, S Schwab, T Serwold: Producing nature's gene-chips: the generation of peptides for display by MHC class I molecules. *Annu Rev Immunol* 20, 463-493 (2002)

9. ES Trombetta, I Mellman: Cell biology of antigen processing *in vitro* and *in vivo*. *Annu Rev Immunol* 23, 975-1028 (2005)

10. PE Jensen: Recent advances in antigen processing and presentation. *Nat Immunol* 8, 1041-1048 (2007)

11. RD Fleischmann, MD Adams, O White, RA Clayton, EF Kirkness, AR Kerlavage, CJ Bult, JF Tomb, BA Dougherty, JM Merrick: Whole-genome random sequencing and assembly of Haemophilus influenzae Rd. *Science* 269, 496-512 (1995)

12. CW Fuller, LR Middendorf, SA Benner, GM Church, T Harris, X Huang, SB Jovanovich, JR Nelson, JA Schloss, DC Schwartz, DV Vezenov: The challenges of sequencing by synthesis. *Nat Biotechnol* 27, 1013-1023 (2009)

13. J Shendure, H Ji: Next-generation DNA sequencing. *Nat Biotechnol* 26, 1135-1145 (2008)

14. IN Okeke, J Wain: Post-genomic challenges for collaborative research in infectious diseases. *Nat Rev Microbiol* 6, 858-864 (2008)

15. BW Wren: Microbial genome analysis: insights into virulence, host adaptation and evolution. *Nat Rev Genet* 1, 30-39 (2000)

16. R Zhang, CT Zhang: The impact of comparative genomics on infectious disease research. *Microbes Infect* 8, 1613-1622 (2006)

17. N Salimi, W Fleri, B Peters, A Sette: Design and utilization of epitope-based databases and predictive tools. *Immunogenetics* 62, 185-196 (2010)

18. HG Rammensee, T Friede, S Stevanoviíc: MHC ligands and peptide motifs: first listing. *Immunogenetics* 41, 178-228 (1995)

19. H Rammensee, J Bachmann, NP Emmerich, OA Bachor, S Stevanović: SYFPEITHI: database for MHC ligands and peptide motifs. *Immunogenetics* 50, 213-219 (1999)

20. IS Yang, JY Lee, JS Lee, WP Mitchell, HB Oh, C Kang, KH Kim: Influenza sequence and epitope database. *Nucleic Acids Res* 37, D423-430 (2009)

21. C Kuiken, B Korber, RW Shafer: HIV sequence databases. *AIDS Rev* 5, 52-61 (2003)

22. JC Tong, L Kong, TW Tan, S Ranganathan: MPID-T: database for sequence-structure-function information on T-cell receptor/peptide/MHC interactions. *Appl Bioinformatics* 5, 111-114 (2006)

23. V Davies, K Vaughan, R Damle, B Peters, A Sette: Classification of the universe of immune epitope literature: representation and knowledge gaps. *PLoS ONE* 4, e6948 (2009)

24. R Vita, L Zarebski, JA Greenbaum, H Emami, I Hoof, N Salimi, R Damle, A Sette, B Peters: The immune epitope database 2.0. *Nucleic Acids Res* 38, D854-862 (2010)

25. E Lafuente, P Reche: Prediction of MHC-Peptide Binding: A Systematic and Comprehensive Overview. *Curr Pharm Des* 15, 3209-3220 (2009)

26. JC Tong, EC Ren: Immunoinformatics: Current trends and future directions. *Drug Discov Today* 14, 684-689 (2009)

27. JC Tong, TW, Tan, S Ranganathan: Methods and protocols for prediction of immunogenic epitopes. *Brief Bioinform* 8, 96-108 (2007)

28. C Lundegaard, O Lund, S Buus, M Nielsen: Major histocompatibility complex class I binding predictions as a tool in epitope discovery. *Immunology* 130, 309-318 (2010)

29. K Falk, O Rötzschke, S Stevanović, G Jung, HG Rammensee: Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. *Nature* 351, 290-296 (1991)

30. HH Lin, S Ray, S Tongchusak, EL Reinherz, V Brusic: Evaluation of MHC class I peptide binding prediction servers: applications for vaccine research. *BMC Immunol* 9, 8 (2008)

31. B Peters, HH Bui, S Frankild, M Nielson, C Lundegaard, E Kostem, D Basch, K Lamberth, M Harndahl, W Fleri, SS Wilson, J Sidney, O Lund, S Buus, A Sette: A community resource benchmarking predictions of peptide binding to MHC-I molecules. *PLoS Comput Biol* 2, e65 (2006)

32. H Zhang, C Lundegaard, M Nielsen: Pan-specific MHC class I predictors: a benchmark of HLA class I pan-specific prediction methods. *Bioinformatics* 25, 83-89 (2009)

33. JW Yewdell, HD Hickman: New lane in the information highway: alternative reading frame peptides elicit T cells with potent antiretrovirus activity. *J Exp Med* 204, 2501-2504 (2007)

34. O Ho, WR Green: Alternative translational products and cryptic T cell epitopes: expecting the unexpected. *J Immunol* 177, 8283-8289 (2006)

35. J Ireland, J Herzog, ER Unanue: Cutting edge: unique T cells that recognize citrullinated peptides are a feature of protein immunization. *J Immunol* 177, 1421-1425 (2006)

36. N Shastri, S Cardinaud, SR Schwab, T Serwold, J Kunisawa: All the peptides that fit: the beginning, the middle, and the end of the MHC class I antigen-processing pathway. *Immunol Rev* 207, 31-41 (2005)

37. PF Robbins, M El-Gamil, YF Li, EB Fitzgerald, Y Kawakami, SA Rosenberg: The intronic region of an incompletely spliced gp100 gene transcript encodes an epitope recognized by melanoma-reactive tumor-infiltrating lymphocytes. *J Immunol* 159, 303-308 (1997)

38. PG Coulie, F Lehmann, B Lethé, J Herman, C Lurquin, M Andrawiss, T Boon: A mutated intron sequence codes for an antigenic peptide recognized by cytolytic T lymphocytes on a human melanoma. *Proc Natl Acad Sci USA* 92, 7976-7980 (1995)

39. A Uenaka, Y Hirano, H Hata, S Win, T Aji, M Tanaka, T Ono, JCA Skipper, K Shimizu, E Nakayama: Cryptic CTL epitope on a murine sarcoma Meth A generated by exon extension as a novel mechanism. *J Immunol* 170, 4862-4868 (2003)

40. TN Bullock, LC Eisenlohr: Ribosomal scanning past the primary initiation codon as a mechanism for expression of CTL epitopes encoded in alternative reading frames. *J Exp Med* 184, 1319-1329 (1996)

41. TN Bullock, AE Patterson, LL Franlin, E Notidis, LC Eisenlohr: Initiation codon scanthrough versus termination codon readthrough demonstrates strong potential for major histocompatibility complex class I-restricted cryptic epitope expression. *J Exp Med* 186, 1051-1058 (1997)

42. S Malarkannan, T Horng, PP Shih, S Schwab, N Shastri: Presentation of out-of-frame peptide/MHC class I complexes by a novel translation initiation mechanism. *Immunity* 10, 681-690 (1999)

43. SR Schwab, KC Li, C Kang, N Shastri: Constitutive display of cryptic translation products by MHC class I molecules. *Science* 301, 1367-1371 (2003)

44. N Shastri, V Nguyen, F Gonzalez: Major histocompatibility class I molecules can present cryptic translation products to T-cells. *J Biol Chem* 270, 1088-1091 (1995)

45. A Uenaka, T Ono, T Akisawa, H Wada, T Yasuda, E Nakayama: Identification of a unique antigen peptide pRL1 on BALB/c RL male 1 leukemia recognized by cytotoxic T lymphocytes and its relation to the Akt oncogene. *J Exp Med* 180, 1599-1607 (1994)

46. I Saeterdal, J Bjørheim, K Lislerud, MK Gjertsen, IK Bukholm, OC Olsen, JM Nesland, JA Eriksen, M Møller, A Lindblom, G Gaudernack: Frameshift-mutation-derived peptides as tumor-specific antigens in inherited and spontaneous colorectal cancer. *Proc Natl Acad Sci USA* 98, 13255-13260 (2001)

47. X Saulquin, E Scotet, L Trautmann, MA Peyrat, F Halary, M Bonneville, E Houssaint: +1 Frameshifting as a novel mechanism to generate a cryptic cytotoxic T lymphocyte epitope derived from human interleukin 10. *J Exp Med* 195, 353-358 (2002)

48. CT Berger, JM Carlson, CJ Brumme, KL Hartman, ZL Brumme, LM Henry, PC Rosato, A Piechocka-Trocha, MA Brockman, PR Harrigan, D Heckerman, DE Kaufmann, C Brander: Viral adaptation to immune selection pressure by HLA class I-restricted CTL responses targeting epitopes in HIV frameshift sequences. *J Exp Med* 207, 61-75 (2010)

49. S Cardinaud, A Moris, M Février, PS Rohrlich, L Weiss, P Langlade-Demoyen, FA Lemonnier, O Schwartz, A Habel: Identification of cryptic MHC I-restricted epitopes encoded by HIV-1 alternative reading frames. *J Exp Med* 199, 1053-1063 (2004)

50. CT Walsh, S Garneau-Tsodikova, GJ Gatto: Protein posttranslational modifications: the chemistry of proteome diversifications. *Angew Chem Int Ed Engl* 44, 7342-7372 (2005)

51. SM Anderton: Post-translational modifications of self antigens: implications for autoimmunity. *Curr Opin Immunol* 16, 753-758 (2004)

52. VH Engelhard, M Altrich-Vanlith, M Ostankovitch, AL Zarling: Post-translational modifications of naturally processed MHC-binding epitopes. *Curr Opin Immunol* 18, 92-97 (2006)

53. J Petersen, AW Purcell, J Rossjohn: Post-translationally modified T cell epitopes: immune recognition and immunotherapy. *J Mol Med* 87, 1045-1051 (2009)

54. J Yagüe, I Alvarez, D Rognan, M Ramos, J Vázquez, JA de Castro: An N-acetylated natural ligand of human histocompatibility leukocyte antigen (HLA)-B39. Classical major histocompatibility complex class I proteins bind peptides with a blocked NH(2) terminus *in vivo*. *J Exp Med* 191, 2083-2092 (2000)

55. L Meadows, W Wang, JM den Haan, E Blokland, C Reinhardus, JW Drijfhout, J Shabanowitz, R Pierce, AI Agulnik, CE Bishop, DF Hunt, E Goulmy, VH Engelhard: The HLA-A*0201-restricted H-Y antigen contains a posttranslationally modified cysteine that significantly affects T cell recognition. *Immunity* 6, 273-281 (1997)

56. PE Van den Steen, P Proost, DD Brand, AH Kang, J Van Damme, G Opdenakker: Generation of glycosylated remnant epitopes from human collagen type II by gelatinase B. *Biochemistry* 43, 10809-10816 (2004)

57. J Yagüe, J Vázquez, JA López de Castro: A post-translational modification of nuclear proteins, N(G),N(G)-dimethyl-Arg, found in a natural HLA class I peptide ligand. *Protein Sci* 9, 2210-2217 (2000)

58. J Herzog, Y Maekawa, TP Cirrito, BS Illian, ER Unanue: Activated antigen-presenting cells select and present chemically modified peptides recognized by unique CD4 T cells. *Proc Natl Acad Sci USA* 102, 7928-7933 (2005)

59. MJ van Stipdonk, AA Willems, S Amor, C Persoon-Deen, PJ Travers, CJ Boog, JM van Noort: T cells discriminate between differentially phosphorylated forms of alphaB-crystallin, a major central nervous system myelin antigen. *Int Immunol* 10, 943-950 (1998)

60. SN McAdam, B Fleckenstein, IB Rasmussen, DG Schmid, I Sandlie, B Bogen, NJ Viner, LM Sollid: T cell recognition of the dominant I-A(k)-restricted hen egg lysozyme epitope: critical role for asparagine deamidation. *J Exp Med* 193, 1239-1246 (2001)

61. JC Skipper, RC Hendrickson, PH Gulden, V Brichard, A Van Pel, Y Chen, J Shabanowitz, T Wolfel, CL Slingluff, T Boon, DF Hunt, VH Engelhard: An HLA-A2-restricted tyrosinase antigen on melanoma cells results from posttranslational modification and suggests a novel pathway for processing of membrane proteins. *J Exp Med* 183, 527-534 (1996)

62. L Cao, R Goodin, D Wood, MA Moscarello, JN Whitaker: Rapid release and unusual stability of immunodominant peptide 45-89 from citrullinated myelin basic protein. *Biochemistry* 38, 6157-6163 (1999)

63. W Chen, NJ Ede, DC Jackson, J Mccluskey, AW Purcell: CTL recognition of an altered peptide associated with asparagine bond rearrangement. Implications for immunity and vaccine design. *J Immunol* 157, 1000-1005 (1996)

64. SI Mannering, LC Harrison, NA Williamson, JS Morris, DJ Thearle, KP Jensen, TWH Kay, J Rossjohn, BA Falk, GT Nepom, AW Purcell: The insulin A-chain epitope

recognized by human T cells is posttranslationally modified. *J Exp Med* 202, 1191-1197 (2005)

65. JA Hill, S Southwood, A Sette, AM Jevnikar, DA Bell, E Cairns: Cutting edge: the conversion of arginine to citrulline allows for a high-affinity peptide interaction with the rheumatoid arthritis-associated HLA-DRB1*0401 MHC class II molecule. *J Immunol* 171, 538-541 (2003)

66. H Arentz-Hansen, R Körner, O Molberg, H Quarsten, W Vader, YM Kooy, KE Lundin, F Koning, P Roepstorff, LM Sollid, SN McAdam: The intestinal T cell response to alpha-gliadin in adult celiac disease is focused on a single deamidated glutamine targeted by tissue transglutaminase. *J Exp Med* 191, 603-612 (2000)

67. CX Moss, SP Matthews, DJ Lamont, C Watts: Asparagine deamidation perturbs antigen presentation on class II major histocompatibility complex molecules. *J Biol Chem* 280, 18498-18503 (2005)

68. CR Berkers, A de Jong, H Ovaa, B Rodenko: Transpeptidation and reverse proteolysis and their consequences for immunity. *Int J Biochem Cell Biol* 41, 66-71 (2009)

69. H Paulus: Protein splicing and related forms of protein autoprocessing. *Annu Rev Biochem* 69, 447-496 (2000)

70. V Muralidharan, TW Muir: Protein ligation: an enabling technology for the biophysical analysis of proteins. *Nat Methods* 3, 429-438 (2006)

71. KI Hanada, JW Yewdell, JC Yang: Immune recognition of a human renal cancer antigen through post-translational protein splicing. *Nature* 427, 252-256 (2004)

72. N Vigneron, V Stroobant, J Chapiro, A Ooms, G Degiovanni, S Morel, P van der Bruggen, T Boon, BJ Van den Eynde: An antigenic peptide produced by peptide splicing in the proteasome. *Science* 304, 587-590 (2004)

73. EH Warren, NJ Vigneron, MA Gavin, PG Coulie, V Stroobant, A Dalet, SS Tykodi, SM Xuereb, JK Mito, SR Riddell, BJ Van den Eynde: An antigen produced by splicing of noncontiguous peptides in the reverse order. *Science* 313, 1444-1447 (2006)

74. T Shiina, K Hosomichi, H Inoko, JK Kulski: The HLA genomic loci map: expression, interaction, diversity and disease. *J Hum Genet* 54, 15-39 (2009)

75. R Horton, L Wilming, V Rand, RC Lovering, EA Bruford, VK Khodiyar, MJ Lush, S Povey, CC Talbot Jr, MW Wright, HM Wain, J Trowsdale, A Ziegler, S Beck: Gene map of the extended human MHC. *Nat Rev Genet* 5, 889-899 (2004)

76. RM Zinkernagel, PC Doherty: Restriction of *in vitro* T cell-mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system. *Nature* 248, 701-702 (1974)

77. P Hraber, C Kuiken, K Yusim: Evidence for human leukocyte antigen heterozygote advantage against hepatitis C virus infection. *Hepatology* 46, 1713-1721 (2007)

78. MR Thursz, HC Thomas, BM Greenwood, AV Hill: Heterozygote advantage for HLA class-II type in hepatitis B virus infection. *Nat Genet* 17, 11-12 (1997)

79. M Carrington, GW Nelson, MP Martin, T Kissner, D Vlahov, JJ Goedert, R Kaslow, S Buchbinder, K Hoots, SJ O'Brien: HLA and HIV-1: heterozygote advantage and B*35-Cw*04 disadvantage. *Science* 283, 1748-1752 (1999)

80. http://www.ebi.ac.uk/imgt/hla/stats.html

81. OD Solberg, SJ Mack, AK Lancaster, RM Single, Y Tsai, A Sanchez-Mazas, G Thomson: Balancing selection and heterogeneity across the classical human leukocyte antigen loci: a meta-analytic review of 497 population studies. *Hum Immunol* 69, 443-464 (2008)

82. www.ncbi.nlm.nih.gov/gv/mhc/main.cgi

83. HA Stephens: HLA and other gene associations with dengue disease severity. *Curr Top Microbiol Immunol* 338, 99-114 (2010)

84. M Carrington, SJ O'Brien: The influence of HLA genotype on AIDS. *Annu Rev Med* 54, 535-551 (2003)

85. Y Ghodke, K Joshi, A Chopra, B Patwardhan: HLA and disease. *Eur J Epidemiol* 20, 475-488 (2005)

86. AT Tan, E Loggi, C Boni, A Chia, AJ Gehring, KS Sastry, V Goh, P Fisicaro, P Andreone, C Brander, SG Lim, C Ferrari, F Bihl, A Bertoletti: Host ethnicity and virus genotype shape the hepatitis B virus-specific T-cell repertoire. *J Virol* 82, 10986-10997 (2008)

87. M Martin, M Carrington: Immunogenetics of viral infections. *Curr Opin Immunol* 17, 510-516 (2005)

88. J Sidney, M del Guercio, S Southwood, V Engelhard, E Appella, H Rammensee, K Falk, O Rötzschke, M Takiguchi, R Kubo: Several HLA alleles share overlapping peptide specificities. *J Immunol* 154, 247-259 (1995)

89. J Sidney, H Grey, R Kubo, A Sette: Practical, biochemical and evolutionary implications of the discovery of HLA class I supermotifs. *Immunol Today* 17, 261-266 (1996)

90. DH Fremont, M Matsumura, EA Stura, PA Peterson, IA Wilson: Crystal structures of two viral peptides in complex with murine MHC class I H-2Kb. *Science* 257, 919-927 (1992)

91. HC Guo, TS Jardetzky, TP Garrett, WS Lane, JL Strominger, DC Wiley: Different length peptides bind to HLA-Aw68 similarly at their ends but bulge out in the middle. *Nature* 360, 364-366 (1992)

92. DR Madden, JC Gorga, JL Strominger, DC Wiley: The three-dimensional structure of HLA-B27 at 2.1 A

resolution suggests a general mechanism for tight peptide binding to MHC. *Cell* 70, 1035-1048 (1992)

93. M Matsumura, DH Fremont, PA Peterson, IA Wilson: Emerging principles for the recognition of peptide antigens by MHC class I molecules. *Science* 257, 927-934 (1992)

94. MA Saper, PJ Bjorkman, DC Wiley: Refined structure of the human histocompatibility antigen HLA-A2 at 2.6 A resolution. *J Mol Biol* 219, 277-319 (1991)

95.A Sette, J Sidney: Nine major HLA class I supertypes account for the vast preponderance of HLA-A and -B polymorphism. *Immunogenetics* 50, 201-212 (1999)

96. J Sidney, B Peters, N Frahm, C Brander, A Sette: HLA class I supertypes: a revised and updated classification. *BMC Immunol* 9, 1 (2008)

97. F Klug, M Miller, HH Schmidt, S Stevanović: Characterization of MHC ligands for peptide based tumor vaccination. *Curr Pharm Des* 15, 3221-3236 (2009)

98. M Provenzano, MC Panelli, S Mocellin, L Bracci, G Sais, DF Stroncek, GC. Spagnoli, FM Marincola: MHC-peptide specificity and T-cell epitope mapping: where immunotherapy starts. *Trends Mol Med* 12, 465-472 (2006)

99. G Li Pira, F Ivaldi, P Moretti, F Manca: High throughput T epitope mapping and vaccine development. *J Biomed Biotechnol* 2010, 325720 (2010)

100 R Buchli, RS Vangundy, CF Giberson, WH Hildebrand: Critical factors in the development of fluorescence polarization-based peptide binding assays: an equilibrium study monitoring specific peptide binding to soluble HLA-A*0201. *J Immunol Methods* 314, 38-53 (2006)

101. M Harndahl, S Justesen, K Lamberth, G Røder, M Nielsen, S Buus: Peptide binding to HLA class I molecules: homogenous, high-throughput screening, and affinity assays. *J Biomol Screen* 14, 173-180 (2009)

102. W Chen, S Khilko, J Fecondo, DH Margulies, J McCluskey: Determinant selection of major histocompatibility complex class I-restricted antigenic peptides is explained by class I-peptide affinity and is strongly influenced by nondominant anchor residues. *J Exp Med* 180, 1471-1483 (1994)

103. TN Schumacher, MT Heemels, JJ Neefjes, WM Kast, CJ Melief, HL Ploegh: Direct binding of peptide to empty MHC class I molecules on intact cells and *in vitro*. *Cell* 62, 563-567 (1990)

104. NA Hosken, MJ Bevan: Defective presentation of endogenous antigen by a cell line expressing class I molecules. *Science* 248, 367-370 (1990)

105. JH Kessler, B Mommaas, T Mutis, I Huijbers, D Vissers, WE Benckhuijsen, GMT Schreuder, R Offringa, E Goulmy, CJM Melief, SH van der Burg, JW Drijfhout: Competition-based cellular peptide binding assays for 13 prevalent HLA class I alleles using fluorescein-labeled synthetic peptides. *Hum Immunol* 64, 245-255 (2003)

106. SH van der Burg, MJ Visseren, RM Brandt, WM Kast, CJ Melief: Immunogenicity of peptides bound to MHC class I molecules depends on the MHC-peptide complex stability. *J Immunol* 156, 3308-3314 (1996)

107. EL Huczko, WM Bodnar, D Benjamin, K Sakaguchi, NZ Zhu, J Shabanowitz, RA Henderson, E Appella, DF Hunt, VH Engelhard: Characteristics of endogenous peptides eluted from the class I MHC molecule HLA-B7 determined by mass spectrometry and computer modeling. *J Immunol* 151, 2572-2587 (1993)

108. M del Guercio, J Sidney, G Hermanson, C Perez, H Grey, R Kubo, A Sette: Binding of a peptide antigen to multiple HLA alleles allows definition of an A2-like supertype. *J Immunol* 154, 685-693 (1995)

109. F Boisgérault, V Tieng, MC Stolzenberg, N Dulphy, I Khalil, R Tamouza, D Charron, A Toubert: Differences in endogenous peptides presented by HLA-B*2705 and B*2703 allelic variants. Implications for susceptibility to spondylarthropathies. *J Clin Invest* 98, 2764-2770 (1996)

110. HJ Zeh, GH Leder, MT Lotze, RD Salter, M Tector, G Stuber, S Modrow, WJ Storkus: Flow-cytometric determination of peptide-class I complex formation. Identification of p53 peptides that bind to HLA-A2. *Hum Immunol* 39, 79-86 (1994)

111. A Bertoletti, A Costanzo, FV Chisari, M Levrero, M Artini, A Sette, A Penna, T Giuberti, F Fiaccadori, C Ferrari: Cytotoxic T lymphocyte response to a wild type hepatitis B virus epitope in patients chronically infected by variant viruses carrying substitutions within the epitope. *J Exp Med* 180, 933-943 (1994)

112. R Bertoni, J Sidney, P Fowler, R Chesnut, F Chisari, A Sette: Human histocompatibility leukocyte antigen-binding supermotifs predict broadly cross-reactive cytotoxic T lymphocyte responses in patients with acute hepatitis. *J Clin Invest* 100, 503-513 (1997)

113. A Sette, J Sidney, MF del Guercio, S Southwood, J Ruppert, C Dahlberg, HM Grey, RT Kubo: Peptide binding to the most frequent HLA-A class I alleles measured by quantitative molecular binding assays. *Mol Immunol* 31, 813-822 (1994)

114. AC Olsen, LO Pedersen, AS Hansen, MH Nissen, M Olsen, PR Hansen, A Holm, S Buus: A quantitative assay to measure the interaction between immunogenic peptides and purified class I major histocompatibility complex molecules. *Eur J Immunol* 24, 385-392 (1994)

115. J Ruppert, J Sidney, E Celis, RT Kubo, HM Grey, A Sette: Prominent role of secondary anchor residues in peptide binding to HLA-A2.1 molecules. *Cell* 74, 929-937 (1993)

116. GM Grotenbreg, MJ Nicholson, KD Fowler, K Wilbuer, L Octavio, M Yang, AK Chakraborty, HL Ploegh, KW Wucherpfennig: Empty Class II Major Histocompatibility Complex Created by Peptide Photolysis Establishes the Role of DM in Peptide Association. *J Biol Chem* 282, 21425-21436 (2007)

117.C Sylvester-Hvid, N Kristensen, T Blicher, H Ferre, SL Lauemoller, XA Wolf, K Lamberth, MH Nissen, LO Pedersen, S Buus: Establishment of a quantitative ELISA capable of determining peptide - MHC class I interaction. *Tissue Antigens* 59, 251-258 (2002)

118. SR Hadrup, M Toebes, B Rodenko, AH Bakker, DA Egan, H Ovaa, TN Schumacher: High-throughput T-cell epitope discovery through MHC peptide exchange. *Methods Mol Biol* 524, 383-405 (2009)

119. K Udaka, TJ Tsomides, HN Eisen: A naturally occurring peptide recognized by alloreactive CD8+ cytotoxic T lymphocytes in association with a class I MHC protein. *Cell* 69, 989-998 (1992)

120. AL Cox, J Skipper, Y Chen, RA Henderson, TL Darrow, J Shabanowitz, VH Engelhard, DF Hunt, CL Slingluff Jr: Identification of a peptide recognized by five melanoma-specific human cytotoxic T cell lines. *Science* 264, 716-719 (1994)

121. M Schirle, W Keilholz, B Weber, C Gouttefangeas, T Dumrese, HD Becker, S Stevanovic, HG Rammensee: Identification of tumor-associated MHC class I ligands by a novel T cell-independent approach. *Eur J Immunol* 30, 2216-2225 (2000)

122. K Antwi, PD Hanavan, CE Myers, YW Ruiz, EJ Thompson, DF Lake: Proteomic identification of an MHC-binding peptidome from pancreas and breast cancer cell lines. *Mol Immunol* 46, 2931-2937 (2009)

123. S Hofmann, M Gluckmann, S Kausche, A Schmidt, C Corvey, R Lichtenfels, C Huber, C Albrecht, M Karas, W Herr: Rapid and sensitive identification of major histocompatibility complex class I-associated tumor peptides by Nano-LC MALDI MS/MS. *Mol Cell Proteomics* 4, 1888-1897 (2005)

124. HD Hickman, AD Luis, R Buchli, SR Few, M Sathiamurthy, RS VanGundy, CF Giberson, WH Hildebrand: Toward a definition of self: proteomic evaluation of the class I peptide repertoire. *J Immunol* 172, 2944-2952 (2004)

125. JD Altman, PA Moss, PJ Goulder, DH Barouch, MG McHeyzer-Williams, JI Bell, AJ McMichael, MM Davis: Phenotypic analysis of antigen-specific T lymphocytes. *Science* 274, 94-96 (1996)

126. DSJ Allan, EJM Lepin, VM Braud, CA O'Callaghan, AJ McMichael: Tetrameric complexes of HLA-E, HLA-F, and HLA-G. *J Immunol Methods* 268, 43-50 (2002)

127. AH Bakker, TNM Schumacher: MHC multimer technology: current status and future prospects. *Curr Opin Immunol* 17, 428-433 (2005)

128. L Wooldridge, A Lissina, DK Cole, HA van den Berg, DA Price, AK Sewell: Tricks with tetramers: how to get the most from multimeric peptide-MHC. *Immunology* 126, 147-164 (2009)

129. M Toebes, M Coccoris, A Bins, B Rodenko, R Gomez, NJ Nieuwkoop, W van de Kasteele, GF Rimmelzwaan, JBAG Haanen, H Ovaa, TNM Schumacher: Design and use of conditional MHC class I ligands. *Nat Med* 12, 246-251 (2006)

130. B Rodenko, M Toebes, SR Hadrup, WJ van Esch, AM Molenaar, TN Schumacher, H Ovaa: Generation of peptide-MHC class I complexes through UV-mediated ligand exchange. *Nat Protoc* 1, 1120-1132 (2006)

131. B Rodenko, M Toebes, PH Celie, A Perrakis, TN Schumacher, H Ovaa: Class I major histocompatibility complexes loaded by a periodate trigger. *J Am Chem Soc* 131, 12305-12313 (2009)

132. S Gredmark-Russ, EJ Cheung, MK Isaacson, HL Ploegh, GM Grotenbreg: The CD8 T-cell response against murine gammaherpesvirus 68 is directed toward a broad repertoire of epitopes from both early and late antigens. *J Virol* 82, 12205-12212 (2008)

133. GM Grotenbreg, NR Roan, E Guillen, R Meijers, JH Wang, GW Bell, MN Starnbach, HL Ploegh: Discovery of CD8+ T cell epitopes in Chlamydia trachomatis infection through use of caged class I MHC tetramers. *Proc Natl Acad Sci USA* 105, 3831-3836 (2008)

134. DC Wilson, GM Grotenbreg, K Liu, Y Zhao, EM Frickel, MJ Gubbels, HL Ploegh, GS Yap: Differential Regulation of Effector- and Central-Memory Responses to Toxoplasma gondii Infection by IL-12 Revealed by Tracking of Tgd057-Specific CD8+ T Cells. *PLoS Pathog* 6, e1000815 (2010)

135. EM Frickel, N Sahoo, J Hopp, MJ Gubbels, MPJ Craver, LJ Knoll, HL Ploegh, GM Grotenbreg: Parasite Stage–Specific Recognition of Endogenous Toxoplasma gondii–Derived CD8 +T Cell Epitopes. *J Infect Dis* 198, 1625-1633 (2008)

136. AH Bakker, R Hoppes, C Linnemann, M Toebes, B Rodenko, CR Berkers, SR Hadrup, WJE van Esch, MHM Heemskerk, H Ovaa, TNM Schumacher: Conditional MHC class I ligands and peptide exchange technology for the human MHC gene products HLA-A1, -A3, -A11, and -B7. *Proc Natl Acad Sci USA* 105, 3825-3830 (2008)

137. SR Hadrup, AH Bakker, CJ Shu, RS Andersen, J van Veluw, P Hombrink, E Castermans, P Thor Straten, C Blank, JB Haanen, MH Heemskerk, TN Schumacher: Parallel detection of antigen-specific T-cell responses by

multidimensional encoding of MHC multimers. *Nat Methods* 6, 520-526 (2009)

138. EW Newell, LO Klein, W Yu, MM Davis: Simultaneous detection of many T-cell specificities using combinatorial tetramer staining. *Nat Methods*, 6, 497-499 (2009)

139. CE Lewis: Detecting cytokine production at the single-cell level. *Cytokine* 3, 184-188 (1991)

140. F Kern, IP Surel, C Brock, B Freistedt, H Radtke, A Scheffold, R Blasczyk, P Reinke, J Schneider-Mergener, A Radbruch, P Walden, HD Volk: T-cell epitope mapping by flow cytometry. *Nat Med* 4, 975-978 (1998)

141. AW Sylwester, BL Mitchell, JB Edgar, C Taormina, C Pelte, F Ruchti, PR Sleath, KH Grabstein, NA Hosken, F Kern, JA Nelson, LJ Picker: Broadly targeted human cytomegalovirus-specific CD4+ and CD8+ T cells dominate the memory compartments of exposed subjects. *J Exp Med* 202, 673-685 (2005)

142. T Jung, U Schauer, C Heusser, C Neumann, C Rieger: Detection of intracellular cytokines by flow cytometry. *J Immunol Methods* 159, 197-207 (1993)

143. LJ Picker, MK Singh, Z Zdraveski, JR Treer, SL Waldrop, PR Bergstresser VC Maino: Direct demonstration of cytokine synthesis heterogeneity among human memory/effector T cells by flow cytometry. *Blood* 86, 1408-1419 (1995)

144. C Prussin, DD Metcalfe: Detection of intracytoplasmic cytokine using flow cytometry and directly conjugated anti-cytokine antibodies. *J Immunol Methods*, 188, 117-128 (1995)

145. B Foster, C Prussin, F Liu, JK Whitmire, JL Whitton: Detection of intracellular cytokines by flow cytometry. *Curr Protoc Immunol* Chapter 6, Unit 6 24 (2007)

146. MR Betts, JM Brenchley, DA Price, SC De Rosa, DC Douek, M Roederer, RA Koup: Sensitive and viable identification of antigen-specific CD8+ T cells by a flow cytometric assay for degranulation. *J Immunol Methods* 281, 65-78 (2003)

147. M Wolfl, J Kuball, WY Ho, H Nguyen, TJ Manley, M Bleakley, PD Greenberg: Activation-induced expression of CD137 permits detection, isolation, and expansion of the full repertoire of CD8+ T cells responding to antigen without requiring knowledge of epitope specificities. *Blood* 110, 201-210 (2007)

148. AJ Zajac, JN Blattman, K Murali-Krishna, DJ Sourdive, M Suresh, JD Altman, R Ahmed: Viral immune evasion due to persistence of activated T cells without effector function. *J Exp Med* 188, 2205-2213 (1998)

149. JM Benson, KA Campbell, Z Guan, IE Gienapp, SS Stuckman, T Forsthuber, CC Whitacre: T-cell activation and receptor downmodulation precede deletion induced by mucosally administered antigen. *J Clin Invest*, 106, 1031-1038 (2000)

150. BA Mehta, VC Maino: Simultaneous detection of DNA synthesis and cytokine production in staphylococcal enterotoxin B activated CD4+ T lymphocytes by flow cytometry. *J Immunol Methods* 208, 49-59 (1997)

151. J Michalek, RH Collins, BJ Hill, JM Brenchley, DC Douek: Identification and monitoring of graft-versus-host specific T-cell clone in stem cell transplantation. *Lancet* 361, 1183-1185 (2003)

152. DW Houck, MR Loken: Simultaneous analysis of cell surface antigens, bromodeoxyuridine incorporation and DNA content. *Cytometry*, 6, 531-538 (1985)

153. J McGill, KL Legge: Cutting edge: contribution of lung-resident T cell proliferation to the overall magnitude of the antigen-specific CD8 T cell response in the lungs following murine influenza virus infection. *J Immunol* 183, 4177-4181 (2009)

154. AB Lyons, CR Parish: Determination of lymphocyte division by flow cytometry. *J Immunol Methods* 171, 131-137 (1994)

155. J Mintern, M Li, GM Davey, E Blanas, C Kurts, FR Carbone, WR Heath: The use of carboxyfluorescein diacetate succinimidyl ester to determine the site, duration and cell type responsible for antigen presentation *in vivo*. *Immunol Cell Biol* 77, 539-543 (1999)

156. O Kirak, EM Frickel, GM Grotenbreg, H Suh, R Jaenisch, HL Ploegh: Transnuclear mice with predefined T cell receptor specificities against Toxoplasma gondii obtained via SCNT. *Science* 328, 243-248 (2010)

157. PK Chattopadhyay, CM Hogerkorp, M Roederer: A chromatic explosion: the development and future of multiparameter flow cytometry. *Immunology*, 125, 441-449 (2008)

158. ML Precopio, MR Betts, J Parrino, DA Price, E Gostick, DR Ambrozak, TE Asher, DC Douek, A Harari, G Pantaleo, R Bailer, BS Graham, M Roederer, RA Koup: Immunization with vaccinia virus induces polyfunctional and phenotypically distinctive CD8(+) T cell responses. *J Exp Med* 204, 1405-1416 (2007)

159. PK Wallace, JD Tario Jr, JL Fisher, SS Wallace, MS Ernstoff, KA Muirhead: Tracking antigen-driven responses by flow cytometry: monitoring proliferation by dye dilution. *Cytometry A* 73, 1019-1034 (2008)

160. JM Versteegen, T Logtenberg, RE Ballieux: Enumeration of IFN-gamma-producing human lymphocytes by spot-ELISA. A method to detect lymphokine-producing lymphocytes at the single-cell level. *J Immunol Methods*, 111, 25-29 (1988)

161. C Czerkinsky, G Andersson, HP Ekre, LA Nilsson, L Klareskog, O Ouchterlony: Reverse ELISPOT assay for clonal analysis of cytokine production. I. Enumeration of gamma-interferon-secreting cells. *J Immunol Methods* 110, 29-36 (1988)

162. M Wulf, P Hoehn, P Trinder: Identification and validation of T-cell epitopes using the IFN-gamma ELISPOT assay. *Methods Mol Biol* 524, 439-446 (2009)

163. J Wen, Z Duan, L Jiang: Identification of a dengue virus-specific HLA-A*0201-restricted CD8+ T cell epitope. *J Med Virol* 82, 642-648 (2010)

164. LE Yauch, RM Zellweger, MF Kotturi, A Qutubuddin, J Sidney, B Peters, TR Prestwood, A Sette, S Shresta: A protective role for dengue virus-specific CD8+ T cells. *J Immunol* 182, 4865-4873 (2009)

165. CK Li, H Wu, H Yan, S Ma, L Wang, M Zhang, X Tang, NJ Temperton, RA Weiss, JM Brenchley, DC Douek, J Mongkolsapaya, BH Tran, CL Lin, GR Screaton, JL Hou, AJ McMichael, XN Xu: T cell responses to whole SARS coronavirus in humans. *J Immunol* 181, 5490-5500 (2008)

166. R Manz, M Assenmacher, E Pfluger, S Miltenyi, A Radbruch: Analysis and sorting of live cells according to secreted molecules, relocated to a cell-surface affinity matrix. *Proc Natl Acad Sci U S A* 92, 1921-1925 (1995)

167. H Brosterhus, S Brings, H Leyendeckers, RA Manz, S Miltenyi, A Radbruch, M Assenmacher, J Schmitz: Enrichment and detection of live antigen-specific CD4(+) and CD8(+) T cells based on cytokine secretion. *Eur J Immunol* 29, 4053-4059 (1999)

168. JD Campbell: Detection and enrichment of antigen-specific CD4+ and CD8+ T cells based on cytokine secretion. *Methods* 31, 150-159 (2003)

169. MJ Pittet, A Zippelius, DE Speiser, M Assenmacher, P Guillaume, D Valmori, D Lienard, F Lejeune, JC Cerottini P Romero: *Ex vivo* IFN-gamma secretion by circulating CD8 T lymphocytes: implications of a novel approach for T cell monitoring in infectious and malignant diseases. *J Immunol* 166, 7634-7640 (2001)

170. M Assenmacher, M Lohning, A Scheffold, RA Manz, J Schmitz, A Radbruch: Sequential production of IL-2, IFN-gamma and IL-10 by individual staphylococcal enterotoxin B-activated T helper lymphocytes. *Eur J Immunol* 28, 1534-1543 (1998)

171. M Oelke, T Kurokawa, I Hentrich, D Behringer, V Cerundolo, A Lindemann, A Mackensen: Functional characterization of CD8(+) antigen-specific cytotoxic T lymphocytes after enrichment based on cytokine secretion: comparison with the MHC-tetramer technology. *Scand J Immunol* 52, 544-549 (2000)

172. G Rauser, H Einsele, C Sinzger, D Wernet, G Kuntz, M Assenmacher, JD Campbell, MS Topp: Rapid generation of combined CMV-specific CD4+ and CD8+ T-cell lines for adoptive transfer into recipients of allogeneic stem cell transplants. *Blood* 103, 3565-3572 (2004)

173. G Li Pira, F Ivaldi, G Tripodi, M Martinengo, F Manca: Positive selection and expansion of cytomegalovirus-specific CD4 and CD8 T cells in sealed systems: potential applications for adoptive cellular immunoreconstitution. *J Immunother* 31, 762-770 (2008)

174. M Oelke, U Moehrle, JL Chen, D Behringer, V Cerundolo, A Lindemann, A Mackensen: Generation and purification of CD8+ melan-A-specific cytotoxic T lymphocytes for adoptive transfer in tumor immunotherapy. *Clin Cancer Res* 6, 1997-2005 (2000)

175. DM McKinney, R Skvoretz, M Qin, G Ishioka, A Sette: Characterization of an *in situ* IFN-gamma ELISA assay which is able to detect specific peptide responses from freshly isolated splenocytes induced by DNA minigene immunization. *J Immunol Methods* 237, 105-117 (2000)

176. G Li Pira, F Ivaldi, L Bottone, F Manca: High throughput functional microdissection of pathogen-specific T-cell immunity using antigen and lymphocyte arrays. *J Immunol Methods* 326, 22-32 (2007)

177. G Li Pira, F Ivaldi, C Dentone, E Righi, V Del Bono, C Viscoli, G Koopman, F Manca: Evaluation of antigen-specific T-cell responses with a miniaturized and automated method. *Clin Vaccine Immunol* 15, 1811-1818 (2008)

178. M Provenzano, S Mocellin, P Bonginelli, D Nagorsen, SW Kwon, D Stroncek: *Ex vivo* screening for immunodominant viral epitopes by quantitative real time polymerase chain reaction (qRT-PCR). *J Transl Med* 1, 12 (2003)

179. JB Lim, M Provenzano, OH Kwon, M Bettinotti, L Caruccio, D Nagorsen, D Stroncek: Identification of HLA-A33-restricted CMV pp65 epitopes as common targets for CD8(+) CMV-specific cytotoxic T lymphocytes. *Exp Hematol* 34, 296-307 (2006)

180. DS Chen, Y Soen, TB Stuge, PP Lee, JS Weber, PO Brown, MM Davis: Marked differences in human melanoma antigen-specific T cell responsiveness after vaccination using a functional microarray. *PLoS Med* 2, e265 (2005)

181. G Deviren, K Gupta, ME Paulaitis, JP Schneck: Detection of antigen-specific T cells on p/MHC microarrays. *J Mol Recognit* 20, 32-38 (2007)

182. Y Soen, DS Chen, DL Kraft, MM Davis, PO Brown: Detection and characterization of cellular immune responses using peptide-MHC microarrays. *PLoS Biol* 1, E65 (2003)

183. JD Stone, WE Demkowicz Jr, LJ Stern: HLA-restricted epitope identification and detection of functional T cell responses by using MHC-peptide and costimulatory microarrays. *Proc Natl Acad Sci U S A*, 102, 3744-3749 (2005)

184. GA Kwong, CG Radu, K Hwang, CJ Shu, C Ma, RC Koya, B Comin-Anduix, SR Hadrup, RC Bailey, ON Witte, TN Schumacher, A Ribas, JR Heath: Modular nucleic acid assembled p/MHC microarrays for multiplexed sorting of antigen-specific T cells. *J Am Chem Soc* 131, 9695-9703 (2009)

185. CM Britten, C Gouttefangeas, MJP Welters, G Pawelec, S Koch, C Ottensmeier, A Mander, S Walter, A Paschen, J Müller-Berghaus, I Haas, A Mackensen, T Køllgaard, P thor Straten, M Schmitt, K Giannopoulos, R Maier, H Veelken, C Bertinetti, A Konur, C Huber, S Stevanović, T Wölfel, SH van der Burg: The CIMT-monitoring panel: a two-step approach to harmonize the enumeration of antigen-specific CD8+ T lymphocytes by structural and functional assays. *Cancer Immunol Immunother* 57, 289-302 (2008)

186. CM Britten, S Janetzki, L Ben-Porat, TM Clay, M Kalos, H Maecker, K Odunsi, M Pride, L Old, A Hoos, P Romero, HLA-peptide Multimer Proficiency Panel of the CVC-CRI Immune Assay Working Group: Harmonization guidelines for HLA-peptide multimer assays derived from results of a large scale international proficiency panel of the Cancer Vaccine Consortium. *Cancer Immunol Immunother* 58, 1701-1713 (2009)

187. JW Gratama, M Boeckh, R Nakamura, JJ Cornelissen, RA Brooimans, JA Zaia, SJ Forman, K Gaal, KR Bray, GH Gasior, CS Boyce, LA Sullivan, PC Southwick: Immune monitoring with iTAgTM MHC tetramers for prediction of recurrent or persistent cytomegalovirus (CMV) infection or disease in allogeneic hematopoietic stem cell transplant (HCT) recipients: a prospective multicenter study. *Blood* 116, 1655-1662 (2010)

188. S Janetzki, KS Panageas, L Ben-Porat, J Boyer, CM Britten, TM Clay, M Kalos, HT Maecker, P Romero, J Yuan, WM Kast, A Hoos, Elispot Proficiency Panel of the CVC Immune Assay Working Group: Results and harmonization guidelines from two large-scale international Elispot proficiency panels conducted by the Cancer Vaccine Consortium (CVC/SVI). *Cancer Immunol Immunother* 57, 303-315 (2008)

189. S Janetzki, L Price, CM Britten, SH van der Burg, J Caterini, JR Currier, G Ferrari, C Gouttefangeas, P Hayes, E Kaempgen, V Lennerz, K Nihlmark, V Souza, A Hoos: Performance of serum-supplemented and serum-free media in IFNgamma Elispot Assays for human T cells. *Cancer Immunol Immunother* 59, 609-618 (2010)

190. A Mander, C Gouttefangeas, C Ottensmeier, MJP Welters, L Low, SH Burg, CM Britten: Serum is not required for *ex vivo* IFN-γ ELISPOT: a collaborative study of different protocols from the European CIMT Immunoguiding Program. *Cancer Immunol Immunother* 59, 619-627 (2010)

191. HT Maecker, JP Mccoy Jr, FOCIS Human Immunophenotyping Consortium, M Amos, J Elliott, A Gaigalas, L Wang, R Aranda, J Banchereau, C Boshoff, J Braun, Y Korin, E Reed, J Cho, D Hafler, M Davis, CG Fathman, W Robinson, T Denny, K Weinhold, B Desai, B Diamond, P Gregersen, P Dimeglio, F Nestle, M Peakman, F Villnova, J Ferbas, E Field, A Kantor, T Kawabata, W Komocsar, M Lotze, J Nepom, H Ochs, R O'Lone, D Phippard, S Plevy, S Rich, M Roederer, D Rotrosen, JH Yeh: A model for harmonizing flow cytometry in clinical trials. *Nat Immunol* 11, 975-978 (2010)

192. A Brazma, P Hingamp, J Quackenbush, G Sherlock, P Spellman, C Stoeckert, J Aach, W Ansorge, CA Ball, HC Causton, T Gaasterland, P Glenisson, FC Holstege, IF Kim, V Markowitz, JC Matese, H Parkinson, A Robinson, U Sarkans, S Schulze-Kremer, J Stewart, R Taylor, J Vilo, M, Vingron: Minimum information about a microarray experiment (MIAME)-toward standards for microarray data. *Nat Genet* 29, 365-371 (2001)

193. S Janetzki, CM Britten, M Kalos, HI Levitsky, HT Maecker, CJM Melief, LJ Old, P Romero, A Hoos, MM Davis: "MIATA"-minimal information about T cell assays. *Immunity* 31, 527-528 (2009)

194. www.miataproject.org

195. M Bull, D Lee, J Stucky, YL Chiu, A Rubin, H Horton, MJ McElrath: Defining blood processing parameters for optimal detection of cryopreserved antigen-specific responses for HIV vaccine trials. *J Immunol Methods* 322, 57-69 (2007)

196. LS Kierstead, S Dubey, B Meyer, TW Tobery, R Mogg, VR Fernandez, R Long, L Guan, C Gaunt, K Collins, KJ Sykes, DV Mehrotra, N Chirmule, JW Shiver, DR Casimiro: Enhanced rates and magnitude of immune responses detected against an HIV vaccine: effect of using an optimized process for isolating PBMC. *AIDS Res Hum Retroviruses* 23, 86-92 (2007)

197. CR Kreher, MT Dittrich, R Guerkov, BO Boehm, M Tary-Lehmann: CD4+ and CD8+ cells in cryopreserved human PBMC maintain full functionality in cytokine ELISPOT assays. *J Immunol Methods* 278, 79-93 (2003)

198. HT Maecker, J Moon, S Bhatia, SA Ghanekar, VC Maino, JK Payne, K Kuus-Reichel, JC Chang, A Summers, TM Clay, MA Morse, HK Lyerly, C DeLaRosa, DP Ankerst, ML Disis: Impact of cryopreservation on tetramer, cytokine flow cytometry, and ELISPOT. *BMC Immunol* 6, 17 (2005)

199 JG Smith, HR Joseph, T Green, JA Field, M Wooters, RM Kaufhold, J Antonello, MJ Caulfield: Establishing acceptance criteria for cell-mediated-immunity assays using frozen peripheral blood mononuclear cells stored under optimal and suboptimal conditions. *Clin Vaccine Immunol* 14, 527-537 (2007)

200. SG Smith, SA Joosten, V Verscheure, AA Pathan, H McShane, THM Ottenhoff, HM Dockrell, F Mascart: Identification of major factors influencing ELISpot-based monitoring of cellular responses to antigens from Mycobacterium tuberculosis. *PLoS ONE* 4, e7972 (2009)

201. JH Cox, G Ferrari, SA Kalams, W Lopaczynski, N Oden, MP D'souza, ECS Group: Results of an ELISPOT proficiency panel conducted in 11 laboratories participating in international human immunodeficiency virus type 1 vaccine trials. *AIDS Res Hum Retroviruses* 21, 68-81 (2005)

202. S Dubey, J Clair, TM Fu, L Guan, R Long, R Mogg, K Anderson, KB Collins, C Gaunt, VR Fernandez, L Zhu, L Kierstead, S Thaler, SB Gupta, W Straus, D Mehrotra, TW Tobery, DR Casimiro, JW Shiver: Detection of HIV vaccine-induced cell-mediated immunity in HIV-seronegative clinical trial participants using an optimized and validated enzyme-linked immunospot assay. *J Acquir Immune Defic Syndr* 45, 20-27 (2007)

203. MG Hudgens, SG Self, YL Chiu, ND Russell, H Horton, MJ McElrath: Statistical considerations for the design and analysis of the ELISpot assay in HIV-1 vaccine trials. *J Immunol Methods* 288, 19-34 (2004)

204. Z Moodie, Y Huang, L Gu, J Hural, SG Self: Statistical positivity criteria for the analysis of ELISpot assay data in HIV-1 vaccine trials. *J Immunol Methods* 315, 121-132 (2006)

205. AJ McMichael, P Borrow, GD Tomaras, N Goonetilleke, BF Haynes: The immune response during acute HIV-1 infection: clues for vaccine development. *Nat Rev Immunol* 10, 11-23 (2010)

206. BD Walker, DR Burton: Toward an AIDS vaccine. *Science* 320, 760-764 (2008)

207. DH Barouch: Challenges in the development of an HIV-1 vaccine. *Nature* 455, 613-619 (2008)

208. S Gaudieri, A Rauch, L Park, E Freitas, S Herrmann, G Jeffrey, W Cheng, K Pfafferott, K Naidoo, R Chapman, M Battegay, R Weber, A Telenti, H Furrer, I James, M Lucas, S Mallal: Evidence of viral adaptation to HLA class I-restricted immune pressure in chronic hepatitis C virus infection. *J Virol* 80, 11094-11104 (2006)

209. C Moore, M John, I James, F Christiansen, C Witt, S Mallal: Evidence of HIV-1 adaptation to HLA-restricted immune responses at a population level. *Science* 296, 1439-1443 (2002)

210. P de Campos-Lima, R Gavioli, Q Zhang, L Wallace, R Dolcetti, M Rowe, A Rickinson, M Masucci: HLA-A11 epitope loss isolates of Epstein-Barr virus from a highly A11+ population. *Science* 260, 98-100 (1993)

211. AT Tan, S Koh, V Goh, A Bertoletti: Understanding the immunopathogenesis of chronic hepatitis B virus: An Asian prospective. *J Gastroenterol Hepatol* 23, 833-843 (2008)

212. F Prugnolle, A Manica, M Charpentier, J Guégan, V Guernier, F Balloux: Pathogen-driven selection and worldwide HLA class I diversity. *Curr Biol* 15, 1022-1027 (2005)

213. M Moutaftsi, B Peters, V Pasquetto, DC Tscharke, J Sidney, HH Bui, H Grey, A Sette: A consensus epitope prediction approach identifies the breadth of murine T(CD8+)-cell responses to vaccinia virus. *Nat Biotechnol* 24, 817-819 (2006)

Abbreviations: MHC: major histocompatibility complex; TCR: T cell receptor; V: variable; D: diversity; J: joining; C: constant; CDR; complementarity-determining regions; CD: cluster of differentiation; TAP: Transporer Associated with antigen Processing; ER: endoplasmic reticulum; ERAP: endoplasmic reticulum-resident aminopeptidases; MIATA: Minimal Information About T cell Assays; TIGR: The Institute for Genomic Research; HIV: Human Immunodeficiency Virus; pMHC: peptide-Major Histocompatibility Complex; MPID-T2: MHC-Peptide interaction Database Version T2; IEDB: Immuno Epitope Database; HMM: Hidden Markov models; ANN: Artificial Neural Network; SVM: Support Vector Machines; CTL: cytotoxic T lymphocyte; DNA: Deoxyribonucleic acid; PTM: post-translational modification; HLA: Human Leukocyte Antigens; IMGT: the international ImMunoGeneTics information system; DENV: Dengue virus; HBV: Hepatitis B virus; HCV: Hepatitis C virus; HPV: human papilloma virus; EBV: Epstein-Barr virus; FITC: Fluorescein isothiocyanate; UV: Ultraviolet; ELISA: Enzyme-linked immunosorbent assay; MS: Mass spectrometry; HPLC: High-performance liquid chromatography; LCMS: Liquid chromatography-mass spectrometry; IFN-gamma: interferon-gamma; IL-2: Interleukin-2; TNF-alpha: Tumor necrosis factor-alpha; ICS: Intracellular cytokine staining; BrdU: bromodeoxyuridine; CFSE: carboxyfluoresceine-diacetate-succinimidylester; ELISPOT: Enzyme-linked immunosorbent spot; CSC: cytokine secretion and cell surface capture; MACS: magnetic cell sorting; qRT-PCR: quantitative real-time polymerase chain reaction; DIC: differential interference constrast; MIAME: Minimum Information about a Microarray Experiment

Key Words: Diversity, Epitope mapping, T cell response, Immunotechnologies, Review

Send correspondence to: Gijsbert Grotenbreg, Immunology Programme, Centre for Life Sciences No. 03-05, 28 Medical Drive, Singapore 117456, Tel: 6565166661, Fax: 6567782684, E-mail: grotenbreg@nus.edu.sg

http://www.bioscience.org/current/vol16.htm