

Rapid epitope identification from complex class-II-restricted T-cell antigens

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The identification of CD4⁺ T-cell epitopes is a laborious and tedious process. Various computer-assisted algorithms have been designed to assist in this process by predicting potential MHC-binding epitopes. Recently, the novel approach of using soluble MHC class II tetramers as a tool to identify T-cell epitopes has been developed successfully. We show that the T-cell epitopes identified using MHC tetramers are also predicted by a computer-assisted algorithm to bind to the same MHC molecule. We propose that epitope mapping using the MHC-tetramer-guided approach, in conjunction with predictive computer algorithms, will allow the rapid identification of T-cell epitopes from large proteins.

The trimolecular interaction between MHC, peptide and T-cell receptor (TCR) provides the structural basis for the antigen (Ag) specificity of T-cell recognition. There are two key energetic determinants of this specificity: the binding interaction between MHC and peptide; and the binding of the TCR to the combined MHC-peptide complex. The former interaction has been studied well using peptide-binding assays to delineate the structural determinants of a peptide that allow energetically favorable binding to particular MHC molecules. Data from these studies have led to the development of computer-assisted algorithms, by which the sequences of large proteins can be scanned to predict individual peptides that might bind to distinct MHC molecules¹⁻⁵.

The interaction of the TCR with MHC-peptide complexes has also been studied extensively, and has led to the recent development of MHC-tetramer technologies, in which soluble MHC-peptide complexes are used to bind to Ag-specific T cells present in heterogeneous lymphocyte populations⁶⁻¹⁰. In the past, studies relied on a working knowledge of a particular

peptide that binds to a specific MHC. Recently, soluble-MHC technology has been used to identify novel Ag-specific T cells and their restricted peptides in the absence of any knowledge of MHC-binding epitopes from the protein. MHC-mixed-peptide complexes were generated from a range of short, overlapping peptide sequences encompassing the whole protein, such that each MHC-mixed-peptide complex contains only a subset of all peptides. These complexes were used to identify and clone Ag-specific reactive T cells¹¹. Success in using this approach relies on the assumption that only a small fraction of the peptide sequences from any given protein is capable of binding to a particular MHC. This method of identifying Ag-specific T cells and their previously unknown epitopes has been named tetramer-guided epitope mapping (TGEM). In the light of these developments, we have examined how well the MHC-binding epitopes predicted by computer algorithms correlate with the T-cell epitopes identified using TGEM.

'...the binding of a peptide to a particular MHC pocket (a function of the residues forming that pocket) does not alter the binding characteristics of the other MHC peptide-binding pockets.'

We have used the TGEM technique and the computer algorithm TEPITOPE for the identification of human MHC-class-II-restricted epitopes from the 490 amino acid viral protein 16 (VP16) of herpes simplex virus 2 (HSV-2), using six different *HLA-DR* alleles. We suggest that the combination of these two approaches is sufficiently sensitive and specific to simplify greatly the identification of specific HLA-restricted T-cell epitopes from complex protein sequences, mixtures of sequences or whole proteomes. The identification of specific HLA-restricted T-cell epitopes is of particular relevance in the area of peptide-driven T-cell-mediated

immuno-modulation and/or tolerance therapies; it is applicable directly to therapies for HLA-correlated autoimmune disorders (e.g. insulin-dependent diabetes mellitus and rheumatoid arthritis) and foreign-Ag-driven diseases for which potential pathological agents have been identified (e.g. HIV and HSV-2).

The TEPITOPE program

The TEPITOPE program (<http://www.tepitope.com>) uses structural data from MHC-peptide complexes and MHC peptide-binding data to identify and predict the potential of peptides or proteins to bind to an MHC molecule. Both MHC-peptide structural data and extensive, experimental peptide-binding data for MHC molecules indicate that the binding of peptide to a particular MHC molecule is determined by the identity of only a few of the nine consecutive amino acid residues of the peptide that lie in the open-ended MHC class II groove. The region of the MHC surrounding a particular amino acid residue is called a binding pocket and comprises many MHC residues, most of which correspond to the polymorphic residues identified in the enormously diverse *MHC* genes. The preferential peptide-binding property of a particular MHC pocket depends on the energetic interactions between the MHC-pocket residues and the peptide amino acid residue. For example, the amino acid binding preference of pocket nine in HLA-DR4 molecules is dependent on the DRB1 polymorphic residues 9, 37, 57, 60 and 61. Other HLA-DR molecules having the same residues at positions 9, 37, 57, 60 and 61 are predicted to have the same amino acid binding preferences for pocket nine.

The TEPITOPE program assumes that the binding of a peptide to a particular MHC pocket (a function of the residues forming that pocket) does not alter the binding characteristics of the other MHC

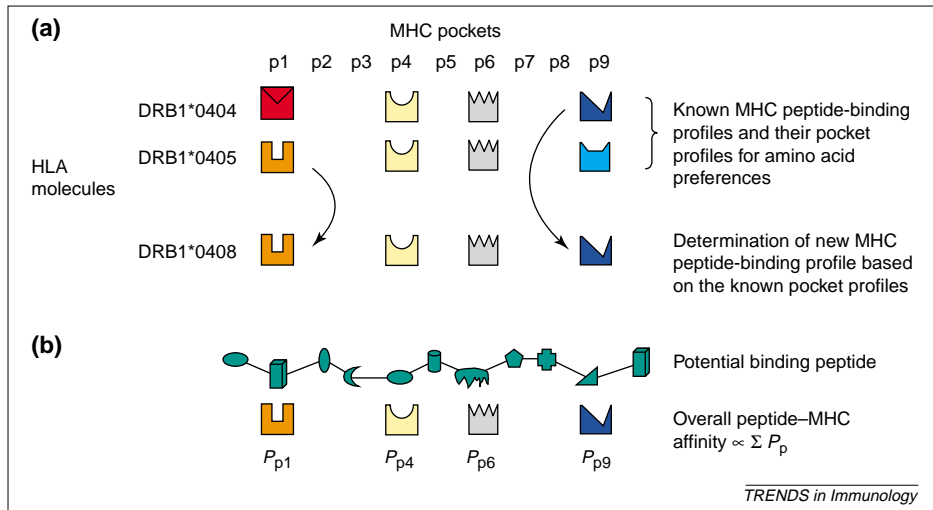


Fig. 1. Principles underlying a computer-generated algorithm used to predict the binding of peptides to MHC molecules. (a) Using experimentally obtained peptide-binding profiles for specific MHC molecules (DRB1*0404 and DRB1*0405) and the preferences of the MHC pockets (represented as colored shapes) for binding to particular amino acids, one can construct a peptide-binding profile for a new MHC molecule (DRB1*0408), for which the binding-pocket profile overlaps that of DRB1*0404 and DRB1*0405. (b) The binding probability (P) of a potential peptide to a particular MHC can then be calculated from the weighted sum of the pocket preferences for the amino acid residues (in green) of the potential binding peptide.

peptide-binding pockets. The overall ability of a particular peptide to bind to a particular MHC is the weighted sum of the amino acid preferences of the individual binding pockets. Generally, the topography of a specific pocket is independent of neighboring pockets, so that the constraints of individual pockets for binding to amino acid residues will be similar for different alleles; a principle which forms the basis for the derivation of 'virtual HLA class II matrices'³. By determining MHC sequence-dependent pocket profiles for a subset of known MHC alleles, overall binding characteristics of other polymorphic MHC molecules can be predicted (Fig. 1).

The application of this principle implies that it is not necessary to run peptide-binding assays for each MHC allele to predict its peptide-binding properties. In the TEPITOPE program, results from peptide-binding assays for a small number of HLA molecules are used to generate pocket profiles for a large number of HLA molecules. The combination of the different modular pocket profiles predicts the overall peptide-binding properties of a particular HLA molecule. These modular profiles can be assembled to generate virtual matrices for the prediction of binding of peptide to 51 different MHC molecules. Stringency in predicting peptide binding to a particular MHC molecule can be set at different threshold values, such that a

setting of 1% threshold implies that the peptides being selected are in the top 1% of the best binders amongst a pool of 8000 peptide frames in a database of random protein sequences. The TEPITOPE program has been used successfully to predict peptide epitopes¹²⁻¹⁴.

Tetramer-guided epitope mapping

Although MHC class II tetramers were generated initially with peptides covalently linked to the class II β chain^{9,15,16}, recent developments have demonstrated that Ag-specific tetramers can be generated from MHC class II molecules by the exogenous loading of peptide^{10,17}. The advantage of using noncovalently linked peptide-MHC complexes is that tetramers can be generated and loaded later with many different epitopes. The TGEM technique identifies antigenic epitopes for T cells rapidly by analyzing the ability of mixed-peptide-class-II tetramers to bind to T cells.

Two prerequisites of this epitope-mapping approach are that the HLA phenotype of the individual is known, and soluble HLA molecules of the restriction element of the T cells of interest are available. Overlapping peptides that correspond to the antigenic proteins of interest are synthesized and divided up into pools. Pools of peptides are loaded onto MHC class II molecules and subsequently, pooled-peptide tetramers

are used to stain peripheral-blood mononuclear cells (PBMCs) that have been stimulated previously with either whole Ag or a peptide library encompassing the whole protein. In a second step, peptides from positively staining pooled-peptide tetramers are loaded individually onto MHC class II molecules, and the staining of PBMCs with these tetramers is repeated¹¹. This second step identifies unique T-cell epitopes. The TGEM approach is illustrated in Figure 2.

Comparison of TEPITOPE algorithms with TGEM

Individuals that are infected with HSV-2 mount T-cell responses to viral Ags, including CD4⁺ T-cell responses to peptides from the VP16 tegument protein¹⁸. Using TGEM, peripheral-blood T cells from HSV-2-infected individuals carrying HLA class II DRB1*0101, DRB1*0401, DRB1*0402, DRB1*0404, DRB1*1104 or DRB1*1501 alleles were tested for their ability to bind to specific MHC-VP16-peptide tetramers. VP16 T-cell epitopes identified by TGEM were compared with those epitopes predicted by the TEPITOPE program to bind to the same MHC molecule. Table 1 lists the VP16 epitopes selected by the TEPITOPE program at a threshold level of 3%. The TEPITOPE program predicted promiscuous (epitopes that bind to multiple MHC molecules) and unique (allele-specific) sequences as candidate Ags for binding to each MHC molecule. Also listed in Table 1 are the specific epitopes determined by TGEM-mediated scanning of the entire VP16 sequence for all six MHC alleles. For this analysis, 60 peptides (p3-p62), each of 20 amino acids, were synthesized to cover the entire VP16 protein. These 60 peptides were divided into 12 equal pools. Each peptide pool was loaded onto MHC molecules and assembled into chromophore-labeled tetramers. T-cell-binding tetramer pools were identified by flow cytometry. Individual peptides from T-cell-staining mixed-peptide tetramer pools were then used to generate unique peptide-class-II tetramers, and the cytometry analysis was repeated. PBMCs from individuals carrying the same HLA-DR specificity as the peptide-loaded tetramers were incubated with fluorescent tetramers at 37°C and with labeled anti-CD4 antibodies. Results of the TGEM approach

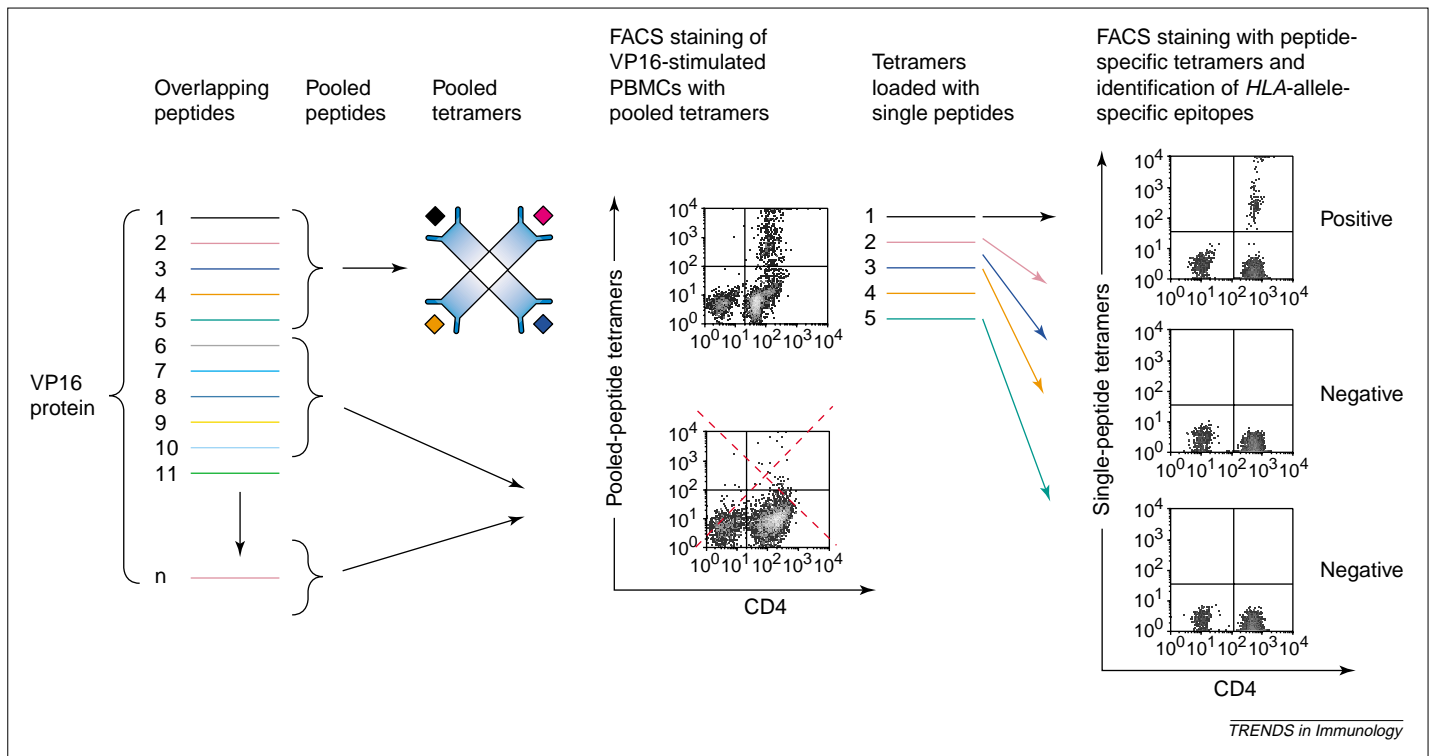


Fig. 2. Tetramer-guided epitope mapping (TGEM). A panel of overlapping peptides spanning the protein(s) of interest [e.g. viral protein 16 (VP16)] is divided into pools, with each pool containing five to ten peptides. Each peptide pool is loaded onto soluble MHC class II molecules to generate pooled-peptide tetramers, and used to stain peripheral blood mononuclear cells (PBMCs) that have been challenged previously with the appropriate antigen. Pooled tetramers that give positive staining are identified by fluorescence-activated cell sorting (FACS). Peptides from positively staining pooled-peptide tetramers are then loaded individually onto the class II molecules and the staining repeated. Tetramers that positively stain in the second round of FACS analysis enable the identification of the MHC-restricted epitopes.

in identifying the *DRB1*0101*-restricted VP16 epitopes recognized by PBMCs from a *DRB1*0101*⁺ subject are shown in Figure 3.

As indicated in Table 1, the TEPITOPE program identified major T-cell epitopes for all six HLA class II molecules examined. For *DRB1*0101*, *DRB1*0402* and *DRB1*1501*, the major epitopes identified by the TGEM approach were selected by the TEPITOPE program at the 1% threshold level. For *DRB1*0401* and *DRB1*1104*, the major epitopes were selected at the 2% threshold, whereas for *DRB1*0404* the major epitope was selected at the 3% threshold. Several of the *DRB1*0401*-restricted epitopes, and one *DRB1*0404*-restricted epitope identified by TGEM were not predicted by TEPITOPE at a threshold value of 3%. Thus, the sensitivity of correct prediction by computer algorithm varied for different MHC alleles, but in all cases, TEPITOPE predicted successfully at least one of the major epitopes identified by the TGEM approach. Because there is a large set of empirical data relating to the binding of

peptide to *DRB1*0401*, the HLA molecule that showed the most discordance between TGEM and TEPITOPE methods, we infer that the limitations of the algorithm are most probably due to intrinsic promiscuity within certain MHC pockets, whereby structural constraints for binding might be less stringent than predicted. All of the epitopes, with the exception of *DRB1*0401*-restricted VP16 233–252, were predicted by the TEPITOPE program at a relaxed threshold level of 10%.

The processing and presentation of antigenic peptides by MHC class II molecules is a complex process. The selection of epitopes depends not only on the affinity of the peptide for the MHC, but also, on the availability of the peptide, which is influenced by cellular compartmentalization of the Ag and specific proteolysis of the protein. Because virtual matrices predict potential epitopes based solely on the affinity of peptide binding to the MHC pockets, it is expected that the majority of these epitopes is not likely to be presented on the cell surface.

Indeed, even when using a TEPITOPE threshold level of 1%, most predicted peptides were negative for the T-cell recognition phenotype detected by TGEM. The TGEM approach, which involves the exogenous loading of peptides onto MHC molecules with high affinity, and selects directly those that are recognized by CD4⁺ T cells. Of the 13 VP16 T-cell epitopes identified by the TGEM approach, 12 were also predicted by the TEPITOPE program to be potential MHC-binding peptides, thus demonstrating a high degree of biological validation in using this particular predictive algorithm for binding to MHC.

It is possible that additional epitopes that have a low affinity for MHC are present, but avoid detection by both approaches. In a comparison of results from TGEM with the analysis of cytokine secretion by Ag-responsive T cells, the production of interferon γ by stimulated CD4⁺ PBMCs identified at least one epitope not detected by TGEM (Ref. 11). Thus, it might be possible to combine cytokine-screening approaches¹⁹ with TGEM analysis when the identification of additional or subdominant epitopes is desired. There are several other examples of low-affinity or subdominant epitopes that have partial agonist function on T cells, yet do not bind to MHC with sufficient avidity to show tetramer

Table 1. Comparison of T-cell epitopes identified using TGEM with those predicted by the TEPITOPE program^{a,b}

MHC allele	T-cell epitopes identified by the TGEM approach		T-cell epitopes predicted by the TEPITOPE program	
	Residue number	Amino acid sequence	Residue number	Amino acid sequence
DRB1*0101	209–228	IADRY <u>YRE</u> TARLARVLFHL	166–174	YRTVLANFC
			169–177	VLANFCSAL
			178–186	YRYLRASVR
			180–188	YLRASVRQL
			213–221	YYRE<u>TAR</u>LA
			228–236	LYLFLSREI
			229–237	YLFLSREIL
			261–269	WRQLACLFQ
			271–279	LMFINGSLT
			272–280	MFINGSLTV
			323–331	LOGNQARSS
334–342	FMLLIRAKL			
DRB1*0401	58–69	ALFNRLDDDLGF ^c		Not predicted
	73–92	PALCTMLDTWNEDLFSGFPT		Not predicted
	233–252	SREILWAAYAEQMMRPDLFD	166–174	YRTVLANFC
			178–186	YRYLRASVR
			213–221	YYRE <u>TAR</u> LA
			261–269	Not predicted
			272–280	WRQLACLFQ
	472–484	DFEFEQMFTDAMG	274–282	MFINGSLTV
			296–304	INGSLTVRG
			475–483	IREHLNLPL
				FEQMFTDAM
DRB1*0402	289–308	RLRELNHIREHLNLPLVRS <u>A</u>	180–188	YLRASVRQL
			228–236	LYLFLSREI
			231–239	FLSREILWA
			261–269	WRQLACLFQ
			272–280	MFINGSLTV
			296–304	IREHLNLPL
			300–308	LNLPLVRS <u>A</u>
			323–331	LOGNQARSS
			333–341	YFMLLIRAK
			336–344	LLIRAKLDS
			385–404	DDAPAEAGLVAPRMSF <u>LS</u> AG
DRB1*0404	225–244	FLHLYLFLSREILWAAYAEQ ^c	166–174	YRTVLANFC
	233–252	SREILWAAYAEQMMRPDLFD	178–186	YRYLRASVR
	443–455	FDLEMLGDVES <u>P</u> S	228–236	LYLFLSREI
			261–269	Not predicted
			272–280	WRQLACLFQ
			280–288	MFINGSLTV
			302–310	VRGVPVEAR
	475–483		445–453	LEMLGD<u>VES</u>
				FEQMFTDAM
DRB1*1104	25–44	GPKNTPAAPPLYATGRL <u>SQA</u>	35–43	LYATGRL<u>SQ</u>
	33–52	PPLYATGRL <u>SQA</u> QLMPSPPM		
	289–308	RLRELNHIREHLNLPLVRS <u>A</u>	185–193	VRQLHRQAH
			225–233	FLHLYLFLS
			231–239	FLSREILWA
			261–269	WRQLACLFQ
			272–280	MFINGSLTV
			333–341	YFMLLIRAK
			334–342	FMLLIRAKL
			336–344	LLIRAKLDS
DRB1*1501	289–308	RLRELNHIREHLNLPLVRS <u>A</u>	177–185	MAQFFRGEL
			201–209	LYRYLRASV
			224–232	LREMLRTTI
			226–234	LFLHLYLFL
			228–236	LHLYLFLSR
			231–239	LYLFLSREI
			272–280	FLSREILWA
			151–159	MFINGSLTV
			296–304	IREHLNLPL
			334–342	FMLLIRAKL
			336–344	LLIRAKLDS

^aAbbreviation: TGEM, tetramer-guided epitope mapping.

^bSequences in bold indicate epitopes selected by the TEPITOPE program that were identified as T-cell epitopes by the TGEM approach. Underlined residues indicate MHC-anchoring residues predicted by the TEPITOPE program. Colored sequences indicate peptides predicted to bind to MHC using threshold levels of 1% (pink), 2% (brown) or 3% (blue).

^cViral protein 16 (VP16)-specific epitopes restricted by DRB1*0401 and DRB1*0404 have been described earlier¹¹.

staining by flow cytometry, both for class I (Ref. 20) and class II (Refs 21,22) tetramers. It has been suggested that such avidity differences might help to fine-tune the immune response, for example by guiding the proliferation of naive or effector T cells²³, by amplifying T-cell activation to low concentrations of Ag (Ref. 24) or by mediating determinant spreading of a progressive T-cell-mediated response²⁵. The enhanced efficiency of binding of class II tetramers to T cells at 37°C (Ref. 22) is consistent with an activation-related clustering or internalization of TCRs as part of the requirement for binding and the detection of moderate- or low-avidity recognition.

Conclusion

The identification of T-cell epitopes is crucial for the development of effective vaccines against infectious agents and tumors, as well as in the design of Ag-specific modes of immunotherapy for autoimmune and immune-mediated diseases. Because T-cell epitopes only encompass a very small region of a complex protein, the mapping of the dominant T-cell epitopes of an Ag can be a difficult procedure. As a consequence, the T-cell epitopes of most medically important Ags remain unidentified.

Various approaches have attempted to simplify the mapping of T-cell epitopes. Because T cells recognize Ag in the context of a specific MHC allele, a phenomenon known as HLA restriction, the prediction of T-cell epitopes must involve the identification of peptides that can bind to MHC. The identification of MHC peptide-binding motifs during the past decade has facilitated greatly the design of predictive algorithms, using both quantitative matrices^{1-5,26-29} and neural-network approaches^{5,30}. Motifs for the binding of peptides to MHC class II molecules are less constrained than those for binding to MHC class I molecules and as a result, predicting class-II-restricted T-cell epitopes precisely is more problematic.

The TGEM approach using peptide libraries is based on the direct detection of binding of the TCR to the MHC-peptide complex. It has the advantage of reflecting the dominant T-cell specificity in the test sample, enabling it to be used with peripheral blood from patients or immunized subjects. It is best applied to

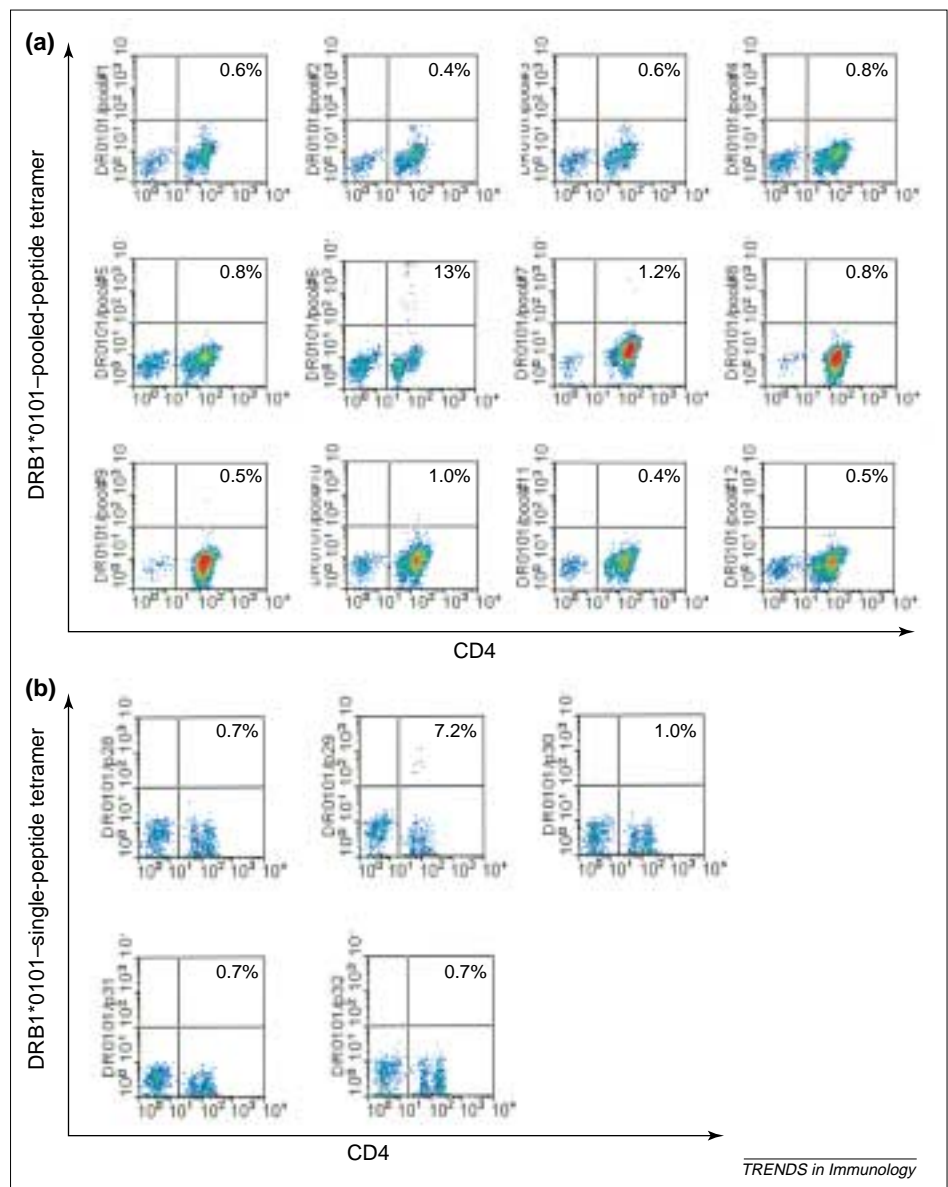


Fig. 3. Example of tetramer-guided epitope mapping (TGEM) analysis. (a) Peripheral blood mononuclear cells (PBMCs) from an HLA-DRB1*0101⁺ individual infected with herpes simplex virus 2 were stimulated with different peptide mixtures (pool#1–pool#12, each containing five peptides at a total concentration of 10 $\mu\text{g ml}^{-1}$). PBMCs were stained with the corresponding pooled-peptide tetramers and analyzed by fluorescence-activated cell sorting (FACS; Calibur, BD Biosciences) 12 days after stimulation. Pool #6 gave positive staining, and individual peptides from pool #6 were used to generate (b) a further set of tetramers loaded with individual peptides. Peptides p28, p29, p30, p31 and p32 correspond to viral protein 16 (VP16) residues 201–220, 209–228, 217–236, 225–244 and 233–252, respectively. These results identify p29 (VP16 209–228) as a major DRB1*0101-restricted epitope. Testing of a second individual (HLA-DRB1*0101⁺) produced identical results (not shown).

individual proteins, such as the HSV-2 VP16 Ag. However, the exhaustive screening of peptides with comprehensive libraries will be a large undertaking. To extend the scope of epitope mapping to very large proteins, mixtures of proteins or entire proteomes, we suggest that the combination of predictive algorithms for binding to MHC followed by TGEM analysis might prove fruitful. Analysis by computer algorithms, such as the TEPITOPE program, should reduce large

sets of sequence data to more tractable sets of predicted MHC-binding peptides, which can then be analyzed directly by TGEM methods. In this manner, it might be possible to analyze complex organisms or tissues based on cDNA sequence information for expressed proteins, in addition to the analysis of known, candidate molecular targets, as an efficient, sensitive and specific approach to the identification of unknown T-cell epitopes.

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Meeting Report

Setting a course for intervening in host–pathogen interactions

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The 25th Dageraad Symposium on The Molecular Mechanisms of Host–Pathogen Interactions in Infectious Disease: Towards Better Intervention Strategies? was held in Enkhuizen, The Netherlands, from 29 June to 3 July 2001.

A small group of scientists embarked from Enkhuizen on the clipper De Dageraad to plot a course on the latest insights into host–pathogen interactions in infectious

disease and tack towards better intervention strategies. They set out to discuss developments in human and pathogen genetics, research into the cell biology and immunology of host–pathogen interactions, and advances in the development of vaccines.

Human and pathogen genomics
Both human- and pathogen-genome-sequencing projects and the development

of high-throughput technologies have provided an enormous potential for studying infectious diseases, from which breakthroughs can be expected in unravelling the biological processes of disease susceptibility, pathogenicity, virulence and host specificity. The human genome project will have a major impact on the development of diagnostics, therapeutics and pharmacogenomics, through the tailoring of drug treatments