Mining for Treasures:

Systematic Profiling of 8-, 9-, 10-, and 11-mer HLA-B*0702-restricted CD8+ T cell epitopes from the Influenza A/Puerto Rico/8/34 (H1N1) Virus Hemagglutinin with Potential Application in Vaccine Development

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Abstract

The Identification of T cell epitopes is of crucial importance for the development of cancer and viral T cell eliciting vaccines. Here, we define CD8+ T cell epitopes using a novel high-throughput system for the synthesis and screening of peptide libraries that include all dominant epitope lengths (8-11mers) for MHC class I molecules. These representative peptide libraries are tested for their ability to bind various MHC class I molecules in a competitive manner. Screening of a Hemagglutinin (HA) -derived peptide library revealed 63 HLA-B*0702-restricted high/medium affinity epitopes at various sizes. A number of these putative influenza epitopes coincide with previously identified peptides which are reportedly recognized by CTL. In conclusion, this study demonstrates that the rapid and methodical screening of T cell immune epitope libraries can help in detecting and monitoring infections as well as enabling immune intervention strategies with respect to treatment or prevention of the disease.

Introduction

Influenza A viruses (Inf A) are widely distributed in nature and can infect a variety of birds and mammals. These viruses are highly contagious, causing an airborne respiratory tract infection. Their genomes consists of eight separate segments of single-stranded, negative-sense RNA that code for 10 different proteins, one nucleoprotein (NP), three polymerase proteins (PA, PB1, and PB2), two matrix proteins (M1 and M2), two nonstructural proteins (NS1 and NS2), and two external glycoproteins, hemagglutinin (HA) and neuraminidase (NA). The viruses are classified on the basis of differences in the antigenic structure of HA and NA proteins. There are 16 HA and 9 NA subtypes known to exist, allowing many different combinations of HA and NA proteins representing unique virus subtypes that are further classified into specific strains. However, only a few subtypes of influenza A viruses have caused sustained outbreaks of disease in the human population. Inf A viruses of the H1, H2, and H3 HA and of the N1 and N2 NA subtypes have circulated in the human population in the 20th century. H1N1 viruses appeared in the pandemic of 1918, the “Spanish flu”. This subtype circulated until the Asian influenza pandemic of 1957, in which H2N2 viruses appeared. The influenza pandemic of 1968 started in Hong Kong and was caused by H3N2 viruses, replacing H2N2 viruses. H1N1 viruses reappeared in the human population in 1977 and continue to circulate with H3N2 viruses until the present day.

The ability of the immune system to recognize virus-infected cells has opened the door to the development of vaccines to treat or prevent various types of infectious diseases. It is known that elimination of most viral infections by the immune system depends on the recognition of viral antigen-derived peptides by cytotoxic T lymphocytes (CTLs) which are presented by HLA-A, -B and -C on the cell surface of infected cells. Consequently, identification and exact definition of molecular parameters involved in peptide-HLA class I interactions of putative CTL epitopes is of prime importance for the detection and monitoring of infectious diseases as well as the development of immunomodulating compounds.

Although major advances in our understanding of the immunology and ecology of the Inf A viruses have been made over the course of several decades, gaps remain in our understanding of the immunity to these viruses. Particularly the paucity of T cell epitopes directed against various influenza strains/subtypes encouraged us to further investigate the influenza protein Hemagglutinin (HA or H) which plays an important role in the propagation of the virus and in the infection of the host. Using a comprehensive high-throughput screening strategy, we revealed a large number of HLA-B*0702-restricted epitopes at various sizes of which only five were previously reported by others (Table 1).

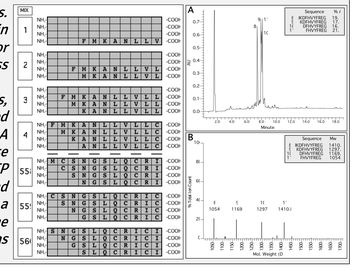
Table 1: Published HLA-B*07 class I epitopes for Inf A HA

Published Inf A Hemagglutinin Epitope									sHLA -B*0702 Binding Capabilities Measured	
MHC Peptide Sequence	Strain	Position	Sequence ID	Peptide length (aa)	Primary Allele	IC50 (Radioactivity Assay (nM)	Inhibition (%)	SE (%)	Affinity Category	Poly Screen
LPVRSNSYIV	A/PuertoRco/8/34(H1N1)	(08-097	P03451	10	HLA-B*07	2077.	55.	2.4	Low	8
YPKLRNSYV	A/PuertoRco/8/34(H1N1)	(17-181	P03451	9	HLA-B*07	176.	85.	1.1	High/Medium	8
TPLGAINSSL	A/PuertoRco/8/34(H1N1)	(29-307	P03451	10	HLA-B*07	34.	91.	1.4	High/Medium	8
CPKYVRSAKL	A/PuertoRco/8/34(H1N1)	(32-325	P03451	10	HLA-B*07	344.	72.	2.4	High/Medium	8
IPSIQSRGL	A/PuertoRco/8/34(H1N1)	(33-346	P03451	10	HLA-B*07	31.	86.	1.1	High/Medium	8

Method

Most HLA-based peptide binding assays currently used in basic immunological research are not suitable for industrial-scale screening. This situation has led us to the development of a specialized high-throughput assay that includes all dominant epitope lengths (8-11mers) for HLA class I molecules and is optimized for speed, efficiency, signal detection, and low reagent consumption. This comprehensive screening strategy begins with the synthesis of an overlapping truncated peptide library (PepSet™-Tc) containing equimolar mixtures of four peptides with a *common C terminus, but having a length of 8, 9, 10, or 11 residues. Cleaved PepSets comprise racks containing up to 94 peptides. Within each rack, two peptide controls were included which were checked for purity (by reverse phase HPLC), identity (by electrospray mass spectrometry) and quantity (by amino acid Analysis).*

To elaborate the binding of a peptide to MHC class I molecules, the technique of fluorescence polarization (FP) was utilized. FP is based on the principal that if a fluorescent-labeled peptide binds to a sHLA molecule of higher molecular weight, polarization values will increase due to the slower molecular rotation of the bound probe. Therefore, FP allows the direct measurement of the ratio between free and bound ligand in solution without any separation steps. Experimentally, a reference FITC-labeled peptide is incubated with activated sHLA in the presence or absence of a competitor and peptide/MHC interaction was monitored over time.

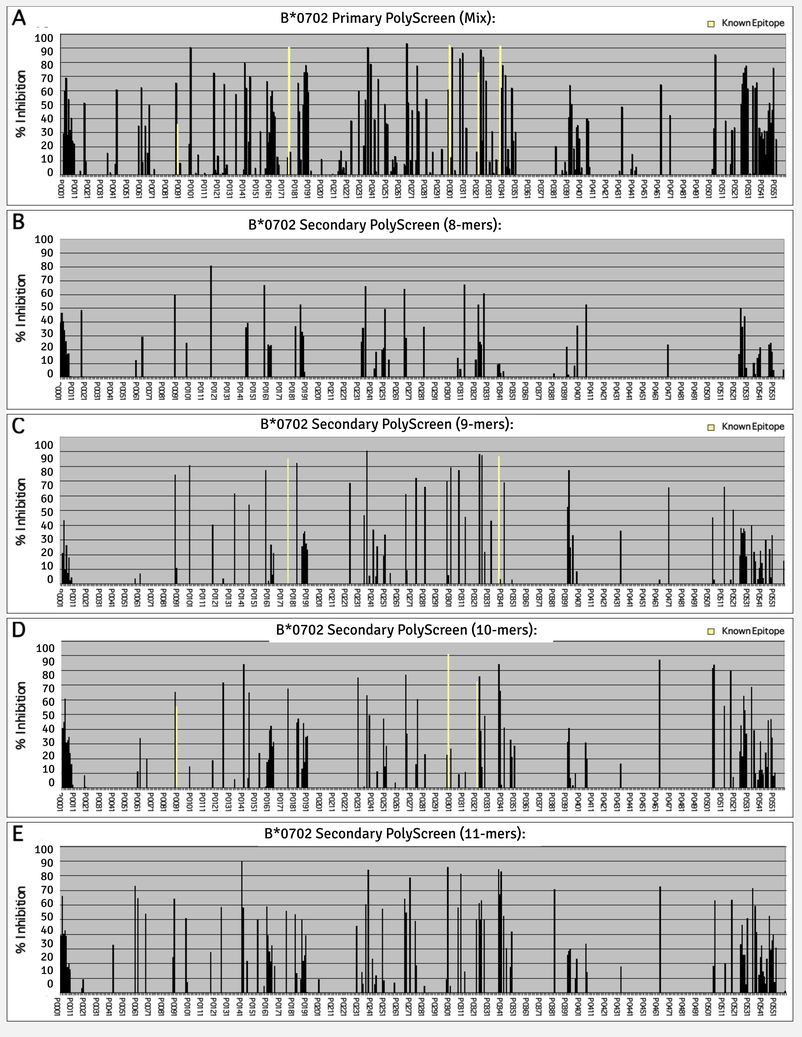


Results

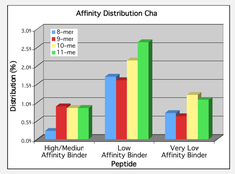
Despite the immune responses against influenza A virus having been intensively characterized over the course of several decades, there is still only a limited number of T cell epitopes reported for the various strains/subtypes. The means to rapidly screen and characterize the binding of epitopes derived from complex virus-associated antigens is therefore an important enabling technology. In order to create more complete maps of targets of immune responses in infectious pathogens, we have advanced our screening technology to allow the systematic identification of novel target epitopes. Using such a direct biochemical approach suggests that a much broader range of epitope candidates with potential of eliciting immune responses can be discovered, greatly enhancing the effectiveness of future vaccine designs.

PolyScreen I – Primary hit profiling using individual mixtures of 8-, 9-, 10-, and 11-mer peptides

The screening procedure itself is based on the capability of synthetic peptides to compete against a FITC-labeled reference peptide by inhibiting its binding to the sHLA protein. Final equilibrium polarization levels indicated the extent of binding to each allele, which were expressed as % inhibition. Out of the 560 peptide mixes (containing a total of 2234 peptides) that cover a length of 567 amino acids of the HA protein, 444 mixes (79.3%) were eliminated from further analysis as they were not able to sufficiently bind to HLA-B*0702 (<20% inhibition). Representative screening data from the primary B*0702 PolyScreen are shown in figure A below.



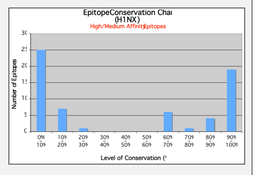
SUMMARY STATEMENT All Sizes			SUMMARY STATEMENT 8-mers			SUMMARY STATEMENT 9-mers			SUMMARY STATEMENT 10-mers			SUMMARY STATEMENT 11-mers		
Allele	B*0702		Allele	B*0702		Allele	B*0702		Allele	B*0702		Allele	B*0702	
# of High/Medium Affinity Binde	63	2.8%	# of High/Medium Affinity Binde	5	0.2%	# of High/Medium Affinity Binde	20	0.9%	# of High/Medium Affinity Binde	19	0.9%	# of High/Medium Affinity Binde	19	0.9%
# of Low Affinity Binde	181	8.1%	# of Low Affinity Binde	38	1.7%	# of Low Affinity Binde	36	1.6%	# of Low Affinity Binde	48	2.1%	# of Low Affinity Binde	59	2.6%
# of Very Low Affinity Binde	81	3.6%	# of Very Low Affinity Binde	16	0.7%	# of Very Low Affinity Binde	14	0.6%	# of Very Low Affinity Binde	27	1.2%	# of Very Low Affinity Binde	24	1.1%
# of No Binders	1909	85.5%	# of No Binders	501	22.4%	# of No Binders	489	21.9%	# of No Binders	464	20.8%	# of No Binders	455	20.4%
Total # of Peptides	2234	100.0%	Total # of 8-mer Peptid	560	25.1%	Total # of 9-mer Peptid	559	25.0%	Total # of 10-mer Peptides	558	25.0%	Total # of 11-mer Peptides	557	24.9%



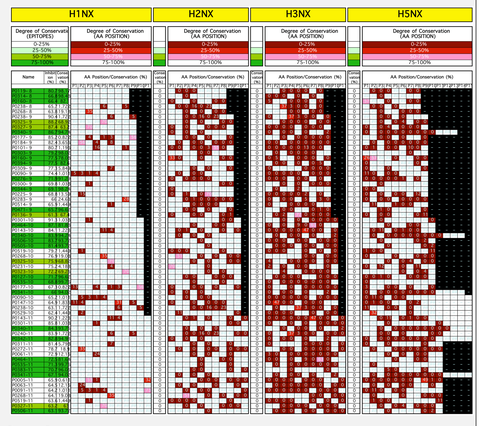
PolyScreen II – Secondary screen dissolving primary hit into individual response patterns

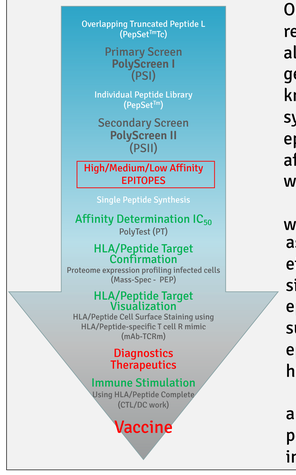
Since each of the four peptides within a mixture could be responsible for the activity observed within PolyScreen I, a secondary screen was performed utilizing an individually synthesized peptide library. Positive reactions were scored based on their inhibition capabilities into high/medium, low and very low affinity. 63 (2.8%) peptides reached inhibition above 60% and were categorized as high/medium affinity binders. The remaining peptide mixes were categorized as low (181/8.1%) and very low (81/3.6%) affinity binders. Looking at the affinity distribution between sizes, with the exception of a low number of 8-mers within the high/medium-affinity category, we did not observe a real preference for a particular size.

Conservancy Analysis of B*0702-restricted influenza HA epitopes



Identification of conserved CTL epitopes shared by multiple viral strains/subtypes, is thought to be a robust vaccine strategy against emerging influenza epidemics/pandemics. To study the degree of conservation of the B*0702 CTL epitopes identified herein, we assembled a collection of human influenza strains. The maximum identity level at which the epitope can be found in the given protein sequence was computed showing whether the residues involved in a conformational epitope are conserved in different sequences. A total of 5323 influenza strains including 1120 H1NX, 75 H2NX, 3927 H3NX, and 201 H5NX strains were selected for the analysis. Results show that about 1/3 of the A/Puerto Rico/8/ 34(H1N1) epitopes identified showed a very high degree of conservancy among all H1NX strains. However, compared to other subtypes, a very poor level of conservancy was observed, which seems to be a reflection of the high variability among the HA proteins themselves. This observation includes the H5NX subtype, which caused a heightened level of awareness because of recent outbreaks of highly pathogenic avian influenza in Asia and associated human infections.





Conclusion

One of the potential strategies for developing influenza vaccines relies on the identification of protective T cell epitopes. There has also been a resurgent interest in the study of influenza A virus in general and avian influenza H5N1 in particular. Based on current knowledge gaps, a proposed research agenda toward a more systematic and comprehensive collection of influenza immune epitopes is presented. We conclude that it is possible to identify high affinity epitopes utilizing truncated PepSet libraries in combination with FP-based peptide binding assays in a high-throughput fashion.

We believe that the definition of influenza-derived epitopes would be useful in detecting and monitoring infections as well as crucial to projecting potential cross-reactive immunity and efficacy against new strains. Conservancy analysis showed that significant levels of inter-strain cross-reactivity is available for T cell epitopes. However, the lack of conserved homology between subtypes points to the urgent need to focus on the identification of epitopes from other influenza proteins and subtypes responsible for human infections, in particular, avian influenza strains.

Overall, this new strategy promises to uncover targets for new and improved vaccines, therapies and diagnostic tools against potential bio-terror agents as well as emerging/re-emerging infectious diseases.