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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 consist 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. H3N1 viruses reappeared in the human population in 1977 and continue to circulate with H2N2 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 the 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 MHC class I molecules and are recognized by CD8+ T-cell receptors. The extent of viral infection is consequently identified and specific 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 in 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 has hampered our ability to further understand 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).

Method

Most HLA-bound peptide binding assays currently used in basic immunological research are not suitable for industrial-scale screening. This situation has led to the development of a specialized high-throughput assay that includes all epitope screening (PolyScreen). For the PolyScreen assay, we developed an assay format that enables a high-throughput, rapid, and comprehensive screening of epitopes without the need for specialized equipment and is optimized for high-throughput screening. The PolyScreen assay is based on the principle that a fluorescent-labeled peptide binds to a sublibrary molecule of higher molecular weight. Polyscreen technology will allow the simultaneous identification of new target epitopes. Using such a direct biochemical approach suggests that a much broader range of epitope candidates with potential of generating CD8+ T-cell immune responses can be discovered, greatly enhancing the effectiveness of future vaccine designs.

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 influenza strains/subtypes. It is anticipated that the PolyScreen technology will allow the systematic identification of new target epitopes. Using such a direct biochemical approach suggests that a much broader range of epitope candidates with potential of generating CD8+ T-cell 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 HLA protein. Final equilibrium polarization levels are measured binding contributions between sizes, which are emphasized as % inhibition. Out of the 560 peptide mixtures (containing a total of 2324 peptides) that cover a length of 567 amino acids of the HA protein, 444 mixtures (79.3%) were eliminated from further analysis as they were not able to sufficiently bind to HLA-B*0702 (≥90% inhibition). Representative screening data from the primary B*0702 PolyScreen are shown in Figure A below.

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. Possible replacement of epitopes within a further hit/mixture capability into high/middle, low, and very low affinity. 63 (2.8%) peptides reached inhibition above 60% and were categorized as high affinity binders. The remaining peptide mixtures were categorized as low (18/8.1%) and very low (15/6.4%) affinity binders. Looking at the affinity distributions of the epitopes and with the exception of a low number of 8mers within the high/middle-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 conservancy of the B*0702 CTL epitopes identified herein, we assembled a collection of human influenza strains. The maximum identity level at which the epitope was found in the given protein sequence was computed showing whether the variants involved in a conformational epitope are conserved in different sequences. A total of 3323 influenza strains including 1210 H3N2, 75 H2N2, 3927 H3N2, and 201 H5N1 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 level of conservancy among all H1N1 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 H5N1 strain, 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 as a 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. However, the lack of conserved homology between the peptides points to the urgent need to focus on the identification of epitopes from other influenza strains 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, including potential bio-terror agents as well as emerging/re-emerging infectious diseases.