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Conditional ligands for Asian HLA variants facilitate the definition of CD8$^+$ T-cell responses in acute and chronic viral diseases

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Conditional ligands have enabled the high-throughput production of human leukocyte antigen (HLA) libraries that present defined peptides. Immunomonitoring platforms typically concentrate on restriction elements associated with European ancestry, and such tools are scarce for Asian HLA variants. We report 30 novel irradiation-sensitive ligands, specifically targeting South East Asian populations, which provide 93, 63, and 79% coverage for HLA-A, -B, and -C, respectively. Unique ligands for all 16 HLA types were constructed to provide the desired soluble HLA product in sufficient yield. Peptide exchange was accomplished for all variants as demonstrated by an ELISA-based MHC stability assay. HLA tetramers with redirected specificity could detect antigen-specific CD8$^+$ T-cell responses against human cytomegalovirus, hepatitis B (HBV), dengue virus (DENV), and Epstein-Barr virus (EBV) infections. The potential of this population-centric HLA library was demonstrated with the characterization of seven novel T-cell epitopes from severe acute respiratory syndrome coronavirus, HBV, and DENV. Posthoc analysis revealed that the majority of responses would be more readily identified by our unbiased discovery approach than through the application of state-of-the-art epitope prediction. This flow cytometry-based technology therefore holds considerable promise for monitoring clinically relevant antigen-specific T-cell responses in populations of distinct ethnicity.

Keywords: CD8$^+$ T-cell response · Conditional ligand · Epitope mapping · HLA polymorphism · Immunotechnology

Additional supporting information may be found in the online version of this article at the publisher's web-site

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Introduction

Antigen-specific T-cells recognize infected or neoplastic cells and respond by target lysis, the production of cytokines, and proliferation. This diverse set of effector functions generally contributes to clearance of infection but can also lead to immunopathology. Circulating $CD8^+$ T lymphocytes derive their specificity from highly variable but clonally distributed T-cell receptors (TCRs) that inspect class I major histocompatibility complex (MHC) glycoproteins. The composite surface of the surveyed molecule consists of an intracellularly derived antigenic peptide ligand that is presented by a protein heterodimer comprising the MHC heavy chain as well as an invariant beta-2 microglobulin (β2m) light chain. For humans, the class I gene products of the human leukocyte antigen (HLA) family of restriction elements are classical transplantation antigens encoded by three separate loci (i.e. HLA-A, -B, and -C) that are highly polymorphic. HLA variability and geographical distribution are significantly correlated, and allelic variants mark distinct ethnic groups. As a consequence, host ethnicity shapes the fine specificity of T-lymphocyte reactivity to several viral afflictions such as EBV [1], hepatitis B (HBV) [2], HCV [3], and HIV [4].

For the monitoring of T-cell-mediated immune responses, MHC multimer reagents [5] have been successfully exploited for the identification, enumeration, phenotypic characterization, and isolation of $CD8^+$ T cells [6, 7]. Several methodologies emerged to manage the variables affecting MHC antigen presentation, and enable multiparametric analysis of antigen-specific T-cell responses [8, 9]. Combinatorial coding of MHC multimers, for example, has allowed parallel detection of multiple T-cell specificities using limited numbers of fluorochromes in high-throughput flow cytometry [10, 11]. Another technological breakthrough came with the development of conditional ligands both for HLA complexes [12, 13] and murine MHCs [14–16], which facilitate the production of peptide-MHC reagents of diverse specificity from a common precursor through a peptide-exchange strategy. Originally, these reagents were designed for the archetypical HLA-A*02:01 [12], and were later expanded to A*01:01, A*03:01, A*11:01, B*07:02 [13], as well as B*57:03 [17], thereby providing significant coverage for the Western European population. Over half of the world's population, however, inhabits regions within Asia and Oceania or has its origins there (World Population Data sheet 2011, http://www.prb.org/Publications/Datasheets/2011/world-population-data-sheet/data-sheet.aspx). Paradoxically, the accurate definition of T-cell epitopes for Asian HLA variants has long trailed its European counterparts. Therefore, we report the peptide-exchange technology for seven HLA-A, five HLA-B, and four HLA-C restriction elements commonly associated with Asian ancestry to enable comprehensive assessment of the corresponding $CD8^+$ T-cell responses.

We demonstrate the utility of these diagnostic reagents with the precise definition of novel antigens derived from infectious agents such as dengue virus (DENV), severe acute respiratory syndrome coronavirus (SARS-CoV), and HBV in the relevant South East Asian (SEA) population. Our discovery pipeline comprises an initial enzyme-linked immunosorbent spot (ELISPOT) screen with pools of overlapping 15-mer peptides. The caged HLA complexes subsequently facilitate the rapid determination of peptide fine specificity and HLA restriction, thus alleviating an epitope discovery bottleneck while conserving PBMC samples in the process. Retrospective assessment of our screening demonstrates that the majority of these epitopes would not have been discovered if only select restriction elements or antigenic proteins had been considered, or bioinformatic selection of peptides applied; the restrictions that are commonly imposed [10, 18–20].

Results

Design and synthesis of the conditional ligands

Conditional ligands, as introduced by Toebes et al. [12], have a UV-light sensitive 3-amino-3-(2-nitro)phenyl-propionic acid group (Anp, J) incorporated into an HLA-binding peptide. Long-wave UV exposure triggers the photolysis of the Anp residue, resulting in the fragmentation of the ligand, while not affecting other amino acids of the complex. Several designs have been published [12, 13, 17] as variability in peptide-binding motif often prohibits interchangeable use between HLA products [21]. We aimed to develop conditional ligands for the principal SEA variants consisting of HLA-A*02:03, -A*02:06, -A*02:07, -A*02:11, -A*11:01, -A*24:02, -A*33:03, -B*15:02, -B*40:01, -B*46:01, -B*55:02, -B*58:01, -C*03:04, -C*04:01, -C*07:02, and -C*08:01. These cover the majority of HLA-A, -B, and -C molecules having allele frequencies of more than 5% in Asia (Fig. 1) with negligible occurrence in Caucasians. In fact, A*11:01, A*24:02, B*40:01, C*03:04, and C*08:01 dominate in SEA populations with allele frequencies at least 10% higher than in Europe.

We selected 16 parent peptides (Supporting Information Table 1) for the pertinent HLAs. Strategic amino acid substitutions were aimed at generating a ligand with unperturbed HLA affinity and several designs were tested (Fig. 2 and Supporting Information Fig. 1A). The Anp introduction in central positions of the peptide endeavored effective liberation of the peptide-binding cleft after UV-induced fragmentation. After their synthesis, the identity of the conditional ligands was ascertained with LCMS analysis (Supporting Information Fig. 1B). Each peptide was subsequently refolded with its corresponding heavy and light chain, biotinylated, and purified by size-exclusion chromatography to produce HLA monomers [22]. Although comparing yields is contentious, as the refolding concerns distinct molecular complexes, several trends were discernible. For example, the HLA-A products with an average yield of approximately 10 mg/L outperformed HLA-B that furnished only approximately 3.2 mg/L. Variation could be as high as 300-fold between HLA products, with A*24:02 providing the highest (~45 mg/L) and A*33:03 (~0.15 mg/L) the lowest yield, presumably reflecting differences both in ligand affinity as well

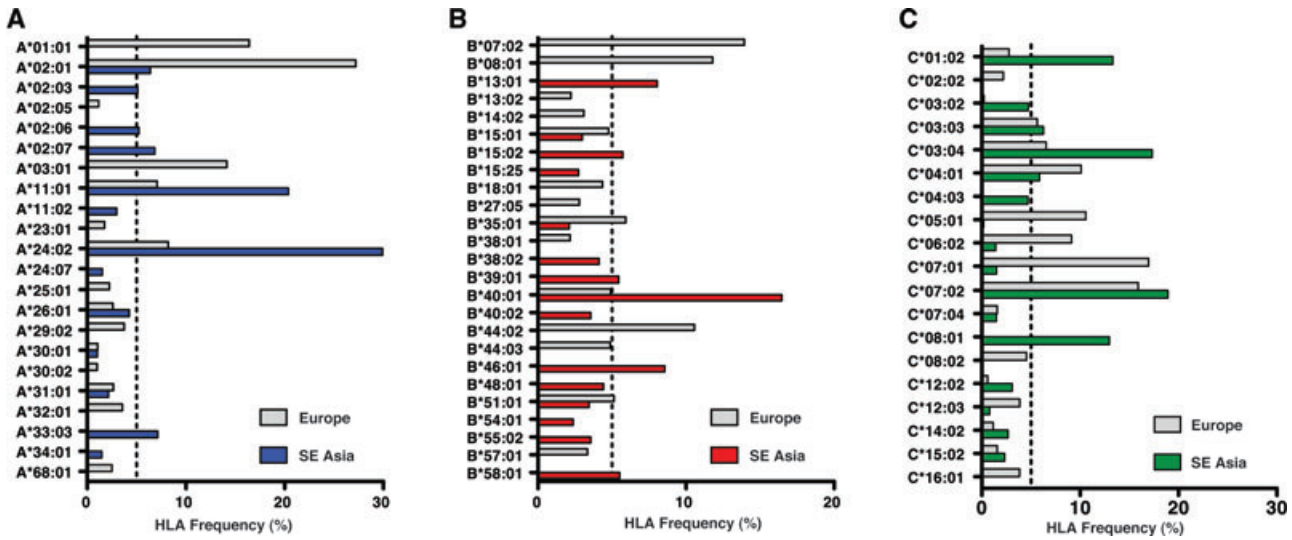

Figure 1. Frequency of HLA-A, HLA-B, and HLA-C alleles in the European and South East Asian population. (A) The allele frequencies for 22 HLA-A loci, based on data from NCBI (dbMHC anthropology resources alleles and haplotype frequencies website URL: http://www.ncbi.nlm.nih.gov/gv/mhc/) pooled from 95 published reports, were plotted for both European and South East Asian populations. Alleles with frequencies less than 2% were omitted. The dotted line indicates a 5% cut-off value. (B) The allele frequencies for 25 HLA-B loci are shown as in panel A. (C) The allele frequencies for 19 HLA-C loci are shown as in panel A.

HLA	p1	p2	p3	p4	p5	p6	p7	p8	p9	p10	Yield (mg/L)
A*02:01	G	I	L	G	F	V	F	●	L		4.35
A*02:03	S	V	R	D	●	L	A	R	L		12.00
A*02:06	L	T	A	●	F	L	I	F	L		4.90
A*02:07	L	L	D	S	D	●	E	R	L		6.35
A*02:11	K	M	D	I	●	V	P	L	L		5.50
A*11:01	A	I	F	Q	S	S	●	T	K		14.60
A*24:02	V	Y	G	●	V	R	A	C	L		45.15
A*33:03	F	Y	V	●	G	A	A	N	R		12.90
B*15:02	I	L	G	P	P	G	●	V	Y		5.70
B*40:01	T	E	A	D	V	Q	●	W	L		3.50
B*46:01	K	M	K	E	I	A	●	A	Y		1.55
B*55:02	K	P	W	D	●	I	P	M	V		5.85
B*58:01	I	S	A	R	G	Q	●	L	F		3.00
C*03:04	F	V	Y	G	●	S	K	T	S	L	2.20
C*04:01	Q	Y	D	●	A	V	Y	K	L		1.80
C*07:02	V	R	I	●	H	L	Y	I	L		11.20
C*08:01	K	A	A	●	D	L	S	H	F	L	14.7

Figure 2. Design of the UV-sensitive conditional ligands. Epitopes known to bind to their respective HLA molecule were chosen as parent peptide and one amino acid residue within the chosen ligand was substituted with 3-amino-3-(2-nitro)-phenylpropanoic acid (Anp) at a central position. Amino acid position 1 at the N-terminus to position 10 at the C-terminus of the peptide is indicated by p1–p10, respectively. The conditional ligands were synthesized by solid phase peptide synthesis and used for refolding reactions with HLA heavy and light chains. The yield represents the weight of HLA monomer successfully refolded with the respective conditional ligand per liter of refolding reaction.

as the overall stability of the complex. For analogs designed from the same parent peptide, 100-fold differences could be observed (~13 mg/L to ~0.15 mg/mL for A*33:03). The highest yielding conditional ligand for each HLA was selected for the following experiments (Fig. 2).

HLA stability and peptide-exchange

Conditional ligands permit manipulation of the HLA's peptide cargo that can be exploited to define HLA restriction and peptide fine-specificity of novel $CD8^+$ T-cell epitopes (see below) without consuming valuable peripheral blood mononuclear cell (PBMC) samples [12, 13, 23]. We evaluated the structural integrity of our HLA complexes by monitoring for the presence of the noncovalently associated β2m subunit. The in vitro HLA-stability ELISA allowed us to verify the extent of HLA disintegration during various stages of the photocleavage reaction as indicator for effective peptide exchange [22]. For the 16 pertinent HLAs, no appreciable degradation of conditional ligand-loaded complexes was observed in the absence of UV irradiation (Fig. 3). Exposure to UV light resulted in near-quantitative loss of signal for the majority of HLA products examined. However, A*11:01, A*24:02, C*07:02, and C*08:01 displayed exceptional stability with up to 39% of β2m still present after cleavage (Fig. 3) that is reminiscent of the production yield of those complexes (Fig. 2). After UV fragmentation [12], the unfolding, aggregation, and precipitation of the complex could be prevented by inclusion of peptides capable of reoccupying the peptide-binding groove, thus conferring stability, and delivering novel specificity in the process. For antigens derived from EBV, human cytomegalovirus, HBV, DENV, and

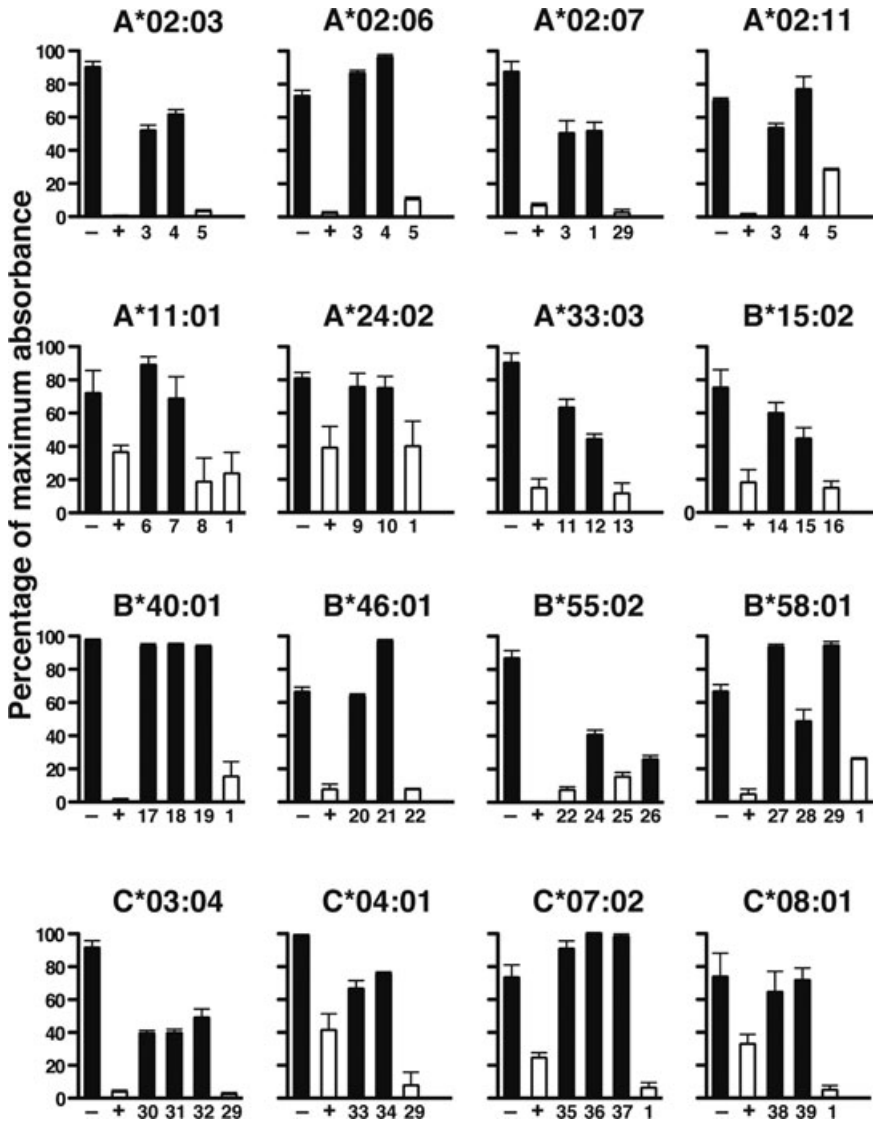

Figure 3. Stability of the recombinant HLA with conditional ligand exposed to UV-mediated peptide exchange. An ELISA assay was used to compare the stability of biotinylated HLA complexes without any treatment (–), after 15 min UV irradiation in the absence of rescue peptide (+), or in the presence of indicated peptides (1, 3–39, Supporting Information Table 2). The immobilized HLA complexes were probed with anti-human β2m antibody for the presence of the noncovalently associated β2m subunit. Horseradish peroxidase catalyzed 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) development was tracked by measurement of its absorbance (λ = 415 nm). Independent assays were conducted for each HLA, with various peptides being efficient at stabilizing the HLA (black bars) and others not capable of maintaining its integrity (white bars), as indicated. The data were normalized, are shown as the mean + SEM of four replicate measurements, and are representative of three experiments performed.

influenza A virus that conform to the reported restriction elements, restoration of each of the 16 complexes with at least two separate ligands proved feasible in individual exchange reactions (Fig. 3 and Supporting Information Table 2). However, peptides lacking the proper complementarity failed to rescue the HLA after UV irradiation, especially when mismatched in HLA supertype [21]. For example, the EBV-derived $LMP2_{426–434}$ (CLGGLLTMV) restricted by A*02:01 (i.e. HLA-A2 supertype) proved unable to conserve the integrity of B*40:01. HLA micropolymorphisms also affect peptide binding, where both $Env_{183–191}$ (FLLTKILTI) and $Pol_{455–463}$ (GLSRYVARL) associate well with A*02:01 [2], but only $Env_{183–191}$ proved capable of stabilizing the closely related variants A*02:03, A*02:06, and A*02:11 (Fig. 3). Collectively, this demonstrates successful temporary stabilization and peptide-exchange using conditional ligands tailored to Asian HLA variants.

Functional performance of HLA tetramers

Although emptied HLA products could be rescued from decay, the question remained whether the resulting complexes present their antigenic cargo indistinguishably from naturally loaded HLAs. This was addressed by staining antigen-specific T-cell populations with Asian HLA tetramers. We recruited 45 healthy volunteers and genotyped their HLA-A, -B, and -C alleles (Supporting Information Table 3). Fluorescently labeled HLA tetramers for EBV, human cytomegalovirus, HBV, DENV, and influenza A virus epitopes were generated by UV-exchange (Supporting Information Table 2). HLA-matched tetramers were used for direct ex vivo staining of fresh PBMCs from healthy volunteers and an HLA-B15 donor responding to the EBV epitope GQGGSPTAM. Flow analysis of the ex vivo isolated T cells revealed in the majority of samples no detectable responses (Supporting Information Fig. 2A and B),

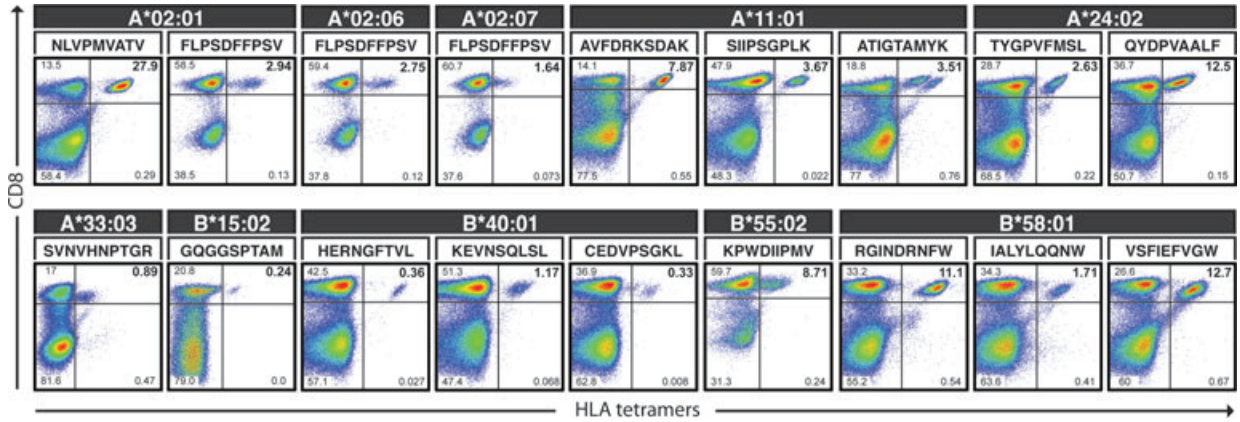

Figure 4. HLA tetramer staining of virus-specific CD8$^+$ T cells in PBMCs from healthy donors. PBMCs were expanded for 14 days with their respective peptide, with the exception of FLPDFFPSV-specific T cells that were engineered by TCR gene transfer of lymphocytes [24]. Cells were stained for viability as well as with HLA tetramers presenting the same peptides as used for stimulation, and costained with anti-human CD8a antibodies. Numbers shown in the flow cytometry analysis are percentages of total live cells for each gated quadrant. Cells in the top-right quadrant represent HLA tetramer and CD8 double-positive cells. The complete HLA genotyping of PBMC donors D01–D08, and D12 and D16 is listed in Supporting Information Table 3. The data shown is representative of at least two independent experiments for each peptide-HLA combination.

except for a distinct HLA-A*1101 AVFDRKSDAK tetramer positive population (0.046% of total CD8$^+$ T cells).

We therefore enriched the antigen-specific CD8$^+$ T cells by in vitro culturing them for 14 days following antigenic stimulation. The amplification allowed visualization of antigen-specific CD8$^+$ T cells by HLA tetramers after 10 days (Supporting Information Fig. 2C). The propagated lymphocytes were successfully visualized by A*02:01, A*11:01, A*24:02, A*33:03, B*40:01, B*55:02, or B*58:01 tetramers, and represented a significant proportion – on average 5.65% – of cells that were well separated from the principal population (Fig. 4). EBNA3A$_{831–839}$-specific T-cells were first observed 8 days poststimulation by HLA-B*15:01 tetramers, and could also be detected with B*15:02 tetramers. Moreover, we subjected HLA-A*02:01 restricted FLPSDFFPSV-specific cells, engineered by TCR gene transfer [24], to staining with HLA-A*02:03, -A*02:06, -A*02:07, and -A*02:11 tetramers loaded with the HBV Core 18–27 peptide to evaluate the capacity of the TCR to discriminate between micropolymorphisms in HLA-A2 subtypes common in the SEA population (Fig. 1). Cross-specific recognition was observed for HLA-A*02:06 and -A*02:07 presenting the HBV peptide, yet no positive staining was obtained with HLA-A*02:03 or -A*02:11 tetramers. The dearth of known epitopes for Asian HLA variants precluded confirmation of tetramer functionality for HLA-A*02:03, -A*02:11, and -B*46:01. HLA-C-restricted responses were similarly absent in our donor cohort.

Identification of novel epitopes restricted by Asian HLA variants

After validating our HLA tetramer library, we employed it for antigen identification, as epitopes for Asian variants are scarce compared to their Caucasian counterparts and often not fully characterized with regard to fine specificity and HLA restriction [8, 9]. We screened for CD8$^+$ T-cell responses in individuals with a history of acute HBV, exposure to DENV, or SARS-CoV. PBMCs were in vitro stimulated with peptide pools of 15-mers overlapping by ten amino acids, thus covering the entire viral proteome of DENV-2 (660 peptides), HBV genotype B (313 peptides), and the SARS-CoV NP (83 peptides), and 3a protein (53 peptides) [2, 25]. IFN-γ secretion was first assessed by ELISPOT, and positive pools were deconvoluted by stimulating the PBMCs with individual peptides followed by measuring IFN-γ production by intracellular cytokine staining. We identified novel CD8$^+$ T-cell responses against 15-mers derived from HBV polymerase (Pol33) and (Env72, Env34), DENV nonstructural protein 3 (NS3–108), as summarized in Fig. 5A, together with antigens embedded in the SARS-CoV nucleocapsid and 3a proteins (NP44, NP65, 3a2) [25]. Based on the HLA genotyping of the short-term T-cell lines (Supporting Information Table 3) and the HLA-binding motifs [21], we anticipated the restriction elements of the HBV Pol33 and Env72 lines to be B*58:01, and the DENV NS3–108 line to be B*55:02. The SARS-CoV NP65 and 3a2 peptides were known to be presented by B*40:01 and B*58:01, respectively. To move away from inferring HLA restriction from panels of peptide-pulsed B lymphoblastoid cell lines (B-LCLs), which is consumptive on samples, we derived 18 distinct overlapping 9-, 10-, and 11-mer peptides from each 15-mer epitope (Supporting Information Fig. 3A). The HLA-stability ELISA allowed us to rapidly screen the ability of each peptide to rescue various HLA complexes from denaturation [22]. The SARS NP44 15-mer [25], for example, had three embedded peptides restricted by either A*02:01 or B*40:01 (Fig. 5B–E). For the SARS-CoV, DENV, and HBV 15-mer antigens, we found at least one peptide in each 18-member set that stabilized an HLA product from our library. These 9- and 10-mers mostly bound to the anticipated HLAs (Supporting Information Fig. 3B–I), and were often positioned at the N- or C-terminus of the parent peptide (Fig. 5A),

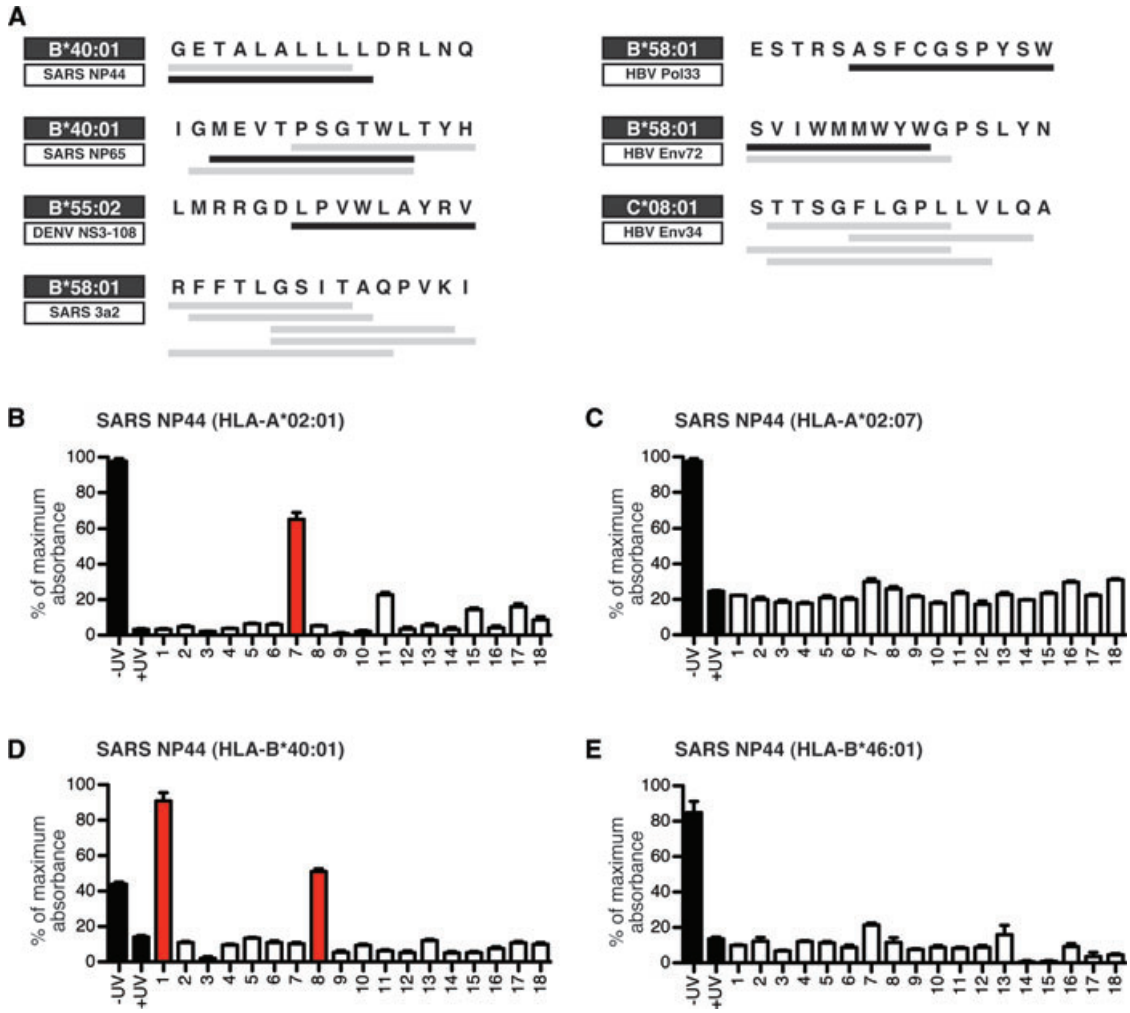

Figure 5. Peptide binding to Asian HLA variants. (A) Seven 15-mer peptides capable of stimulating T-cells reactive toward SARS-CoV, HBV, or DENV antigens were discovered by ELISPOT screening. All 18 possible 9-, 10-, and 11-mer truncated peptides imbedded in the 15-mer epitope that either tested positive in the HLA-stability assay (gray bars) or gave HLA tetramer staining (black bars) are indicated below the sequence. Numbering of the peptide fragments is provided in Supporting Information Fig. 3A. (B) Soluble HLA-A*02:01 molecules were ligand exchanged with truncated peptides from SARS NP44 15-mer peptide. The resulting complexes were captured on streptavidin-coated plates and probed for β2m as a marker for HLA complex stability. As controls, the photocleavable HLA molecules were treated with (+) or without (–) UV irradiation in the absence of rescue peptide (black bars). Peptides are labeled as capable (red bars) or incapable (white bars) of stabilizing the corresponding HLA. (C) Stabilization of soluble HLA-A*02:07 by truncations of the SARS NP44 15-mer peptide was assessed as in (B). (D) Stabilization of soluble HLA-B*40:01 by truncations of the SARS NP44 15-mer peptide was assessed as in (B). (E) Stabilization of soluble HLA-B*46:01 by truncations of the SARS NP44 15-mer peptide was assessed as in (B). (B–E) Data are shown as mean + SEM of four replicates.

underscoring the importance of the overlapping peptide screening approach.

We confirmed the fine specificity and HLA restriction for the curtailed list of peptides with tetramer staining. Employing a SARS NP44 T-cell line [25], we found that both 9- and 10-mer peptides (i.e. GETALALLL and GETALALLLL) stabilize B*40:01 (Fig. 5D), yet the cell line stained positive only with B*40:01 tetramers loaded with the 10-mer (Fig. 6). We frequently observed that the extension or contraction by a single amino was not detrimental to HLA binding of a peptide, yet only optimal epitopes were recognized by their corresponding TCRs. Short-term expanded SARS-CoV NP65, DENV NS3–108, and HBV Pol33 and Env72-specific lines stained with their corresponding B*40:01, B*55:02, or B*58:01 tetramers (Fig. 6B) but not PBMCs from unrelated controls (Fig. 6A). For the SARS 3a2 and HBV Env34 lines, the peptides preselected by ELISA failed to yield functional staining reagents. Comprehensive tetramer screens with all 18 truncated peptides; however, revealed SITAQPVKI and FLGPLLVLQA to be the optimal antigens presented by B*58:01 and C*08:01, respectively. For the seven characterized epitopes (Table 1), T cells bound their HLA counterpart with moderate affinity, as judged by the MFI shift for the individual tetramers. Only the B*58:01-presented HBV Pol33 9-mer and HBV Env34 10-mer being displayed by C*08:01 provided staining such that antigen-specific cells were clearly resolved from the main $CD8^+$ T-cell population. The functionality of all lines, established by IFN-γ production and

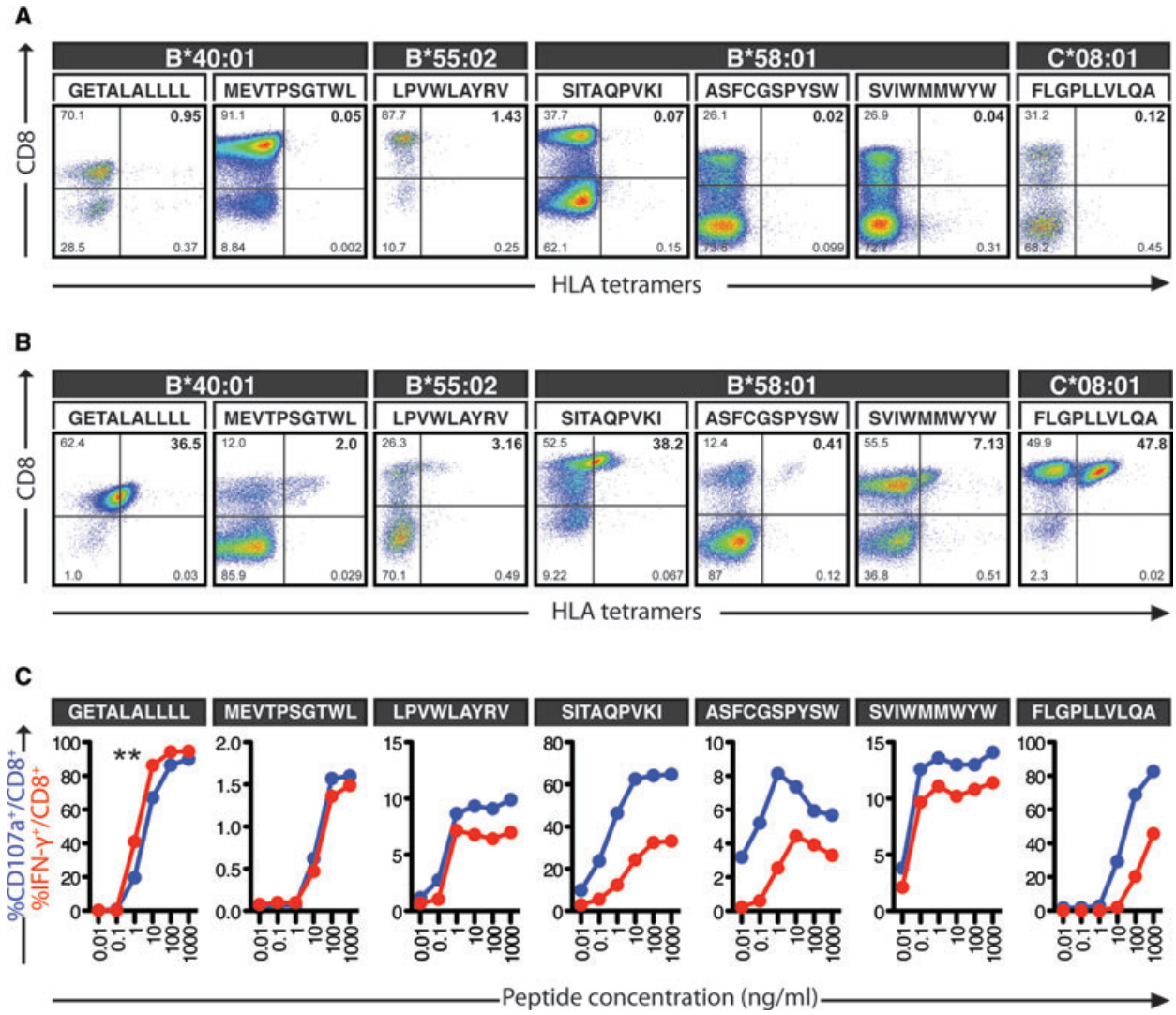

Figure 6. Identification of novel SARS-CoV, HBV, and DENV epitopes with photocleavable tetramers. (A) Tetramer staining of PBMCs from HLA-matched volunteers. The cells were stained with HLA-B*40:01, -B*55:02, -B*58:01, or -C*08:01 tetramers loaded with the indicated peptide. Numbers shown in the flow cytometry analysis are percentages of cells for each gated quadrant. (B) Tetramer staining of SARS-CoV, HBV, or DENV-specific lines. The cells were stained with HLA-B*40:01, -B*55:02, -B*58:01, or -C*08:01 tetramers loaded with the indicated peptide. Numbers shown in the flow cytometric analysis are percentages of cells for each gated quadrant. (A, B) The data shown is representative of two independent experiments for the peptide-HLA combinations. (C) Dose response curves of T-cell lines to the optimal epitope following 5 h peptide stimulation. The percentage of $CD107a^+$ (blue) or IFN-γ^+ (red) cells of the total $CD8^+$ T-cell population is plotted against peptide concentration. The data shown derives from a single experiment for each peptide-HLA combination. The complete HLA genotyping of PBMC donors D09–D15 is listed in Supporting Information Table 3. **The data for GETALALLLL-specific T cells have been reported [25].

CD107 surface expression upon peptide stimulation, confirmed the HLA-tetramer results (Fig. 6C).

Posthoc analysis of T-cell epitope discovery

We deliberately chose not to employ bioinformatic predictions to select our candidate antigens even though their adoption is commonplace [8, 18, 19, 26, 27], but we were curious to assess whether state-of-the-art algorithms could reproduce the outcome of our discovery efforts. We selected *NetMHCpan*, which (i) has the ability to compute the binding capability of peptides even for HLA products for which experimental data is scarce, (ii) can accommodate epitopes of variable length, and (iii) is publicly accessible [28–30]. We performed IC_{50} predictions for all possible 8, 9, 10, and 11-mer epitopes (encompassing 2716 SARS-CoV, 6256 HBV, and 12690 DENV peptides) of each HLA variant of the donor's genotype. For data inclusion, we imposed a 1000 nM affinity threshold, which is less stringent than the conventional IC_{50} cutoff of 500 nM, while providing an estimated specificity of 0.95 and sensitivity of 0.74 [29]. Peptides were ranked by IC_{50} value, and for each experimentally derived epitope the rank was determined

Table 1. Sequences of newly identified epitopes[a)]

Epitope sequence	HLA	Source			Possible Strain
		Organism	Protein	Location	(Accession no.)
GETALALLLL	B*40:01	SARS coronavirus	Nucleocapsid protein	216–225	Sin2774 (AY283798)
MEVTPSGTWL	B*40:01	SARS coronavirus	Nucleocapsid protein	323–332	Sin2774 (AY283798)
SITAQPVKI	B*58:01	SARS coronavirus	3a protein	12–20	Sin2774 (AY283798)
SVIWMMWYW	B*58:01	Hepatitis B virus	Envelope protein	356–364	Japan/Ry30/2002
ASFCGSPYSW	B*58:01	Hepatitis B virus	Polymerase protein	161–170	Genotype B (Q8JXB9)
LPVWLAYRV	B*55:02	Dengue virus	Polyprotein (Nonstructural 3 protein)	2017–2025 (542–550)	Genotype B (AF121243) Dengue 2 (NC_001474)
FLGPLLVLQA	C*08:01	Hepatitis B virus	Envelope protein	171–180	Genotype C (AB112063)

[a)] Novel HLA-B*40:01, -B*55:02, -B*58:01, or C*08:01 restricted $CD8^+$ T-cell epitopes and their HLA restriction element, organism, and protein source of the peptide, location within the protein, and possible virus strain or genotype.

within the complete HLA constellation of the PBMC donor as well as for the correct restriction element (Table 2). Even this liberal strategy would instantly preclude the discovery of two novel epitopes. Peptides SITAQPVKI and FLGPLLVLQA were predicted not to bind HLA-B*58:01 and HLA-C*08:01, respectively, with IC_{50} values 10–20 fold above the cutoff. Moreover, predictions can be misguiding, as FLGPLLVLQA was predicted to have affinity for HLA-A*02:01 (20.44 nM), while for its true restriction element HLA-C*08:01 the IC_{50} of 22942.68 nM would have excluded it from consideration. Given that predictive methods aim to reduce the magnitude of the peptide collection under investigation, a full proteome-based bioinformatics screen should encompass no more peptides than an overlapping peptide library. In this scenario, the total number of peptides to screen with *NetMHCpan* would at most be equal to the collection of our ELISPOT screens (i.e. 313 peptides for HBV, 136 peptides for SARS-CoV, and 660 peptides for DENV). The conditional tetramer reagents developed here comprise 17 different HLA molecules, including the reported HLA-A*02:01 [12], and therefore the number of highest ranking peptides to screen per HLA will be 18 for HBV, eight for SARS-CoV, and 38 for DENV. Following these criteria, three of the seven novel epitopes would not have been discovered based on HLA-specific rankings (i.e. SITAQPVKI, SVIWMMWYW, FLGPLLVLQA, Table 2). If the *NetMHCpan* algorithm would have been employed to curtail the peptide collection to half the size of an ELISPOT screen, the peptides to screen per HLA would be nine for HBV, four for SARS-CoV, and 19 for DENV, and the majority of novel antigens (i.e. MEVTPSGTWL, SITAQPVKI, SVIWMMWYW, FLGPLLVLQA) would have been missed. Although other methods might well outperform on individual allele-specific predictions, we submit that employing *NetMHCpan* is the most appropriate strategy for PBMC donor cohorts with high HLA polymorphism [28–30], particularly in populations with a preponderance of class I HLA variants for which limited experimentally verified data is available. This exposes current limitations with the deployment of predictive algorithms and validates our approach of using overlapping peptide libraries together with high-throughput tetramer screening to identify and characterize novel T-cell epitopes.

Table 2. Bioinformatic prediction analysis

Identified sequence	Identified HLA[a)]	NetMHCpan prediction				Remarks
		Predicted HLA	Predicted IC_{50} (nM)[b)]	Rank (pan-HLA)	Rank (HLA-specific)	
GETALALLLL	B*40:01	B*40:01	8.28	9 / 160	3 / 13	Correct prediction
MEVTPSGTWL	B*40:01	B*40:01	42.57	58 / 263	7 / 13	Requires 58 peptide screen
SITAQPVKI	B*58:01	B*58:01	14252.45	–	–	Excluded by prediction
SVIWMMWYW	B*58:01	B*58:01	43.18	66 / 680	28 / 166	Requires 66 peptide screen
ASFCGSPYSW	B*58:01	B*58:01	3.63	4 / 908	2 / 181	Correct prediction
LPVWLAYRV	B*55:02	B*55:02	67.82	254 / 1329	10 / 64	Requires 254 peptide screen
FLGPLLVLQA	C*08:01	C*08:01	22942.68	–	–	Excluded by prediction

[a)] The complete HLA genotyping of donors D09–D15 is listed in Supporting Information Table 3.
[b)] The IC_{50} (nM) values for peptide binding was predicted using the *NetMHCpan* version 2.3 algorithm for all possible 8-, 9-, 10-, 11-mers within the viral proteins for each HLA-A, -B, and -C alleles of the PBMC donor, and ranked accordingly. Peptides with $IC_{50} > 1000$ nM were excluded.

Discussion

Monitoring cellular immune responses requires the disentanglement of their heterogeneity and is at the basis of various diagnostic and therapeutic applications. Conditional ligands integrate the use of MHC-based multimer arrays with high-throughput flow cytometric analysis of antigen-specific T cells, as they allow the rapid synthesis of MHC molecules of distinct specificity through a peptide-exchange protocol. This strategy can be applied for screening of class I MHC-associated antigens. We sought to extend this technology specifically for Asian populations, and therefore designed and synthesized 30 UV-light sensitive conditional ligands, and constructed the corresponding HLA molecules. Thus, we expanded the portfolio of conditional ligand-loaded HLAs nearly threefold and for the first time included HLA-C allelic variants. Despite the polymorphic nature of HLA, this library includes the majority of alleles in Asia (Fig. 1), the world's most populous region. The stability of the complexes was validated, and all HLA variants proved cooperative with ligand replacement (Fig. 2). The corresponding tetramers could also detect common virus-specific $CD8^+$ T-lymphocyte responses in healthy volunteers (Fig. 4).

For the identification of novel epitopes for Asian HLA variants, we combined ELISPOT and conditional ligand-based assays (i.e. the HLA-stability ELISA and HLA tetramer arrays), thereby building on the key features each technology affords while circumventing their limitations. The ELISPOT assay, which quantifies the active secretion of cytokines, is recognized for its high sensitivity compared with assays such as bead-based assays or intracellular cytokine staining [31, 32]. It also permits screening with pooled overlapping peptides, usage of clinical samples is economical, and lymphocytes maintain their viability, making it amenable for high-throughput proteome-wide antigen screening [31, 32]. In ongoing epitope identification efforts for HBV, SARS-CoV, and DENV infection, we identified seven 15-mer peptides capable of stimulating T-cell responses in Singaporean donors (Fig. 5 and 6). The HLA restriction and fine specificity of the epitopes would commonly be inferred from cellular assays. These, however, suffer from simultaneous expression of various HLA variants that complicates data interpretation. We used caged Asian HLAs to measure their receptiveness to accommodate shorter peptides embedded in the parent 15-mer antigen. This rapidly pinpointed the likely antigenic sequences, while utilizing low amounts of HLA reagents and sparing precious PBMC samples. Ultimate confirmation of the peptide fine specificity and HLA restriction element responsible for engendering the T-cell response was provided by staining with arrays of HLA tetramers, which allowed rapid visualization and quantification of in vitro cultured, cryopreserved samples or freshly isolated antigen-specific $CD8^+$ T cells with high specificity and sensitivity. HLA tetramers thus not only permit the monitoring and isolation of antigen-specific T lymphocytes [5–7], but their usage extends to screening for novel $CD8^+$ T-cell antigens [10, 12–16]. In seven of seven times, the antigenic epitope was correctly identified, validating our approach to reduce the number of epitopes by biophysical means. The construction of the 16-membered peptide-exchangeable HLA library gives sufficient coverage for epitope screening in Asia where the probability of matching the HLA profile of a lymphocyte donor with at least one caged HLA is high for SEA (HLA-A: 93%, -B: 63%, -C: 79%) as well as the closely related populations from Oceania (HLA-A: 88%, -B: 46%, -C: 82%). This contrasts with the European (HLA-A: 30%, -B: 11%, -C: 54%) and North American regions (HLA-A: 74%, -B: 5%, -C: 74%) for which equivalent molecules have already been designed [12, 13]. Similar efforts in Sub-Saharan Africa (HLA-A: 11%, -B: 23%, -C: 42%) or Southwest Asia (HLA-A: 53%, -B: 10%, -C: 38%), where HLA diversity is more pronounced, would need a suite of reagents that is proportionally extended. Finally, we found through retrospective analysis that bioinformatic strategies for the prediction of HLA-restricted epitopes would likely have failed to identify a considerable portion of the novel CD8 T-cell epitopes described herein.

Advances in cytometry compel HLA tetramer technology to keep pace with equivalent improvements. Higher dimensional staining for distinct antigen-specific T-cell subsets has become feasible by labeling HLA multimers with unique combinatorial codes [10, 11], or passing the cells through microarray platforms for on-surface inspection [8]. An emerging technology that labels cell surface markers with heavy metal ions for CyTOF analysis that, among its advantages, eliminates the need for fluorescence compensation, enables simultaneous immunophenotypic tracking of large cellular subsets [6, 33, 34]. For various pathologies where T-cell immunity strongly correlates with protection, the exploration of their therapeutic value is both warranted and increasingly met with clinical success [35–37]. Redirected T cells that target virally infected or malignant cells may be generated either by in vitro expansion of autologous lymphocytes [35, 38], or by genetic modification to drive TCR expression of the desired specificity, before being infused into patients. All such developments rely on accurate definition of antigen-specific T-lymphocyte responses, and the HLA libraries for Asian variants reported here are essential for the characterization and enumeration of clinically relevant antigen-specific $CD8^+$ T cells [8].

Materials and methods

Synthetic peptides

Conditional ligands were manually constructed by standard Fmoc-based solid-phase peptide synthesis. Deprotection of amino acid side chains and release from the resin was achieved with 95% trifluoroacetic acid, 2.5% water, and 2.5% triisopropyl silane, followed by precipitation in cold diethyl ether. The peptide identities were confirmed by IT-TOF LC/MS analysis (Shimadzu). Peptides used for screening were supplied in crude lyophilized form (Mimotopes), or supplied in lyophilized form at >70% purity (GenScript). These were dissolved in 80% dimethyl sulfoxide (Sigma-Aldrich) in MilliQ water containing 10 mM tris(2-carboxyethyl)phosphine (Sigma-Aldrich) and stored at –20°C.

Generation of HLA complexes and peptide exchange

HLA products were produced as described [22]. Briefly, the genes were codon-optimized for *Escherichia coli* (Supporting Information Table 4) and plasmids encoding human β2m or any of the HLA-heavy chains were synthesized (GenScript). The heavy chains contain a C-terminal BirA-sequence, and were overexpressed in *E. coli* BL21 upon isopropyl β-D-thiogalactopyranoside induction. The proteins were isolated from inclusion bodies and denatured with 8 M urea. The solubilized heavy and light chains were mixed with a 10–30-fold molar excess of the conditional ligand for 36–72 h, dialyzed into 10 mM Tris-Cl buffer (pH = 8), concentrated, and enzymatically biotinylated with BirA. HLA monomers were purified by S200 size-exclusion chromatography, and conjugated with Streptavidin-PE or -allophycocyanin at a 4:1 molar ratio. The HLA tetramers were diluted to 40 μg/mL in PBS containing 200 μg/mL of replacement peptide, followed by 15-min long wave (365 nm) UV exposure by an Ultraviolet Cross-linker (UVP). After 1 h of incubation, the tetramers were centrifuged at 13 000 rpm and the supernatant was used for cell staining. For HLA-B*1502, the conditional ligand was exchanged with replacement peptide before HLA tetramer generation at a concentration of 25 μg/mL [39].

HLA-stability ELISA

Following established protocols [22], a 384-well microplate (Corning) was coated with 2 μg/mL streptavidin (Invitrogen) in PBS (2 h at 37°C) and washed with 0.05% PBS-Tween 20 (Sigma-Aldrich). Two percent of BSA in PBS was added and incubated for 30 min at room temperature (RT). The peptide exchange, initiated by 15 min UV irradiation, was performed in tubes containing 50 μM peptide, and 0.5 μM recombinant HLA in PBS at 4°C. The solution was then diluted to 1.6 nM HLA, added to each well, and incubated for 1 h at 37°C. Plates were washed with 0.05% Tween 20 in PBS, 1 μg/mL HRP-conjugated anti-β2m antibody (Clone D2E9, Abcam) was added, followed by 1 h incubation at 37°C. Plates were washed, and ABTS solution (Invitrogen) was added. After 15 min development at RT, the reaction was stopped with 0.1% sodium azide (Sigma-Aldrich) in 0.1 M citric acid. Absorbance (415 nm) was measured using a Spectramax M2 (Molecular Devices).

In vitro stimulation of PBMCs and novel T-cell lines

Approval for the study protocols, which conformed to the principles set out in the WMA Declaration of Helsinki and the NIH Belmont report, was provided by the Institutional Review Board of the National University of Singapore. Healthy volunteers were recruited with written informed consent and HLA-genotyped. Blood donations from suitable HLA-types were collected. PBMCs were isolated from fresh heparinized blood by Ficoll-Hypaque centrifugation. Cells, 10^6/mL, were cultured in 5% human AB serum (Invitrogen) or 5% autologous plasma with RPMI 1640 media containing 2.05 mM L-glutamine (Invitrogen) supplemented with 40 μM 2-mercaptoethanol (Sigma-Aldrich) and 100 IU/mL penicillin/streptomycin (Invitrogen) at 37°C, 5% CO_2. Antigenic peptides were added at 10 μg/mL, and 25 U/mL of IL-2 (R&D systems Inc.) was added 2 days poststimulation. Half media change and IL-2 supplementation was done every 2–3 days.

The SARS-CoV-specific T-cell lines and HBV Core 18–27 TCR-redirected T-cells were obtained in previous studies [24, 25]. The HBV Pol33 and HBV Env72-specific lines and DENV NS3–108- and DENV NS5–66-specific lines were developed as follows. Short-term T-cells lines were grown from frozen PBMCs that were previously stimulated with 15-mer peptides. For restimulation, EBV-transformed B-cells were pulsed with 5 μg/mL 15-mer in R10 media for 1 h. The cells were cultured with gamma-irradiated feeder PBMCs and peptide-pulsed EBV-transformed B cells in AIM-V medium (Invitrogen) with 2% pooled human AB serum, supplemented with 20 U/mL of IL-2, 10 ng/mL IL-7, and 10 ng/mL IL-15 (R&D systems) for 10 days at 37°C in 5% CO_2. Lines that upregulated CD107a and produced IFN-γ after 6 h peptide stimulation were used for epitope characterization. EBV-derived $EBNA3B_{831–839}$-specific T cells were obtained from an HLA-B15 donor recruited in a previous study [40]. For the enrichment of $EBNA3B_{831–839}$-specific T cells, PBMCs were pulsed with GQGGSPTAM for 2 h at RT in 200 μL X-vivo medium (Lonza) without serum. Afterward, cells were cultured in X-Vivo with 5% human serum at 2×10^6 cells/mL and incubated at 37°C, 5% CO_2. IL-2 was added to a total concentration of 40 U/mL IL-2 (Peprotech) the following day, and the cells were cultured for 8 days.

HLA tetramer staining

PBMCs were washed with PBS and cell viability was ascertained with LIVE/DEAD® fixable near-IR stain (Invitrogen) prior to tetramer staining. Cells were washed and stained with 1 μg/50 μL of PE- or allophycocyanin-conjugated HLA tetramers in PBS with 0.1% sodium azide on ice for 20 min. Cells were subsequently stained with anti-CD8-Pacific Blue™ (Clone RPA-T8, BD Biosciences) or anti-CD8-FITC (Clone HIT8A, BD Biosciences) for 15 min, washed and fixed with 1% paraformaldehyde in PBS. Cells from the HLA-B15 donor were stained at 1 μg/80 μL concentration of PE-conjugated HLA tetramers at 37°C for 15 min, followed by the addition of 20 μL of an antibody mix consisting of: CD8-Alexa Fluor 700 (clone HIT8a, Biolegend), CD4-FITC, CD14-FITC, CD16-FITC, CD19-FITC (Clone Sk3, MφP9, Leu11a, 4G7, respectively, BD), and CD40-FITC (Clone LOB7/6, Serotech) and a viability marker (LIVE/DEAD® fixable near-IR stain, Invitrogen) for 30 min at 4°C. Data were acquired with a BD LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo (Treestar Inc). Supporting Information Fig. 2A displays the gating strategy that was applied.

Intracellular cytokine staining

The cells were grown at 10^6/mL in RPMI 1640 (Invitrogen) supplemented with 5% pooled human AB serum (Invitrogen).

CD107a-FITC antibody (Clone H4A3; BD Biosciences) and 10 μg/mL of Brefeldin A (Sigma-Aldrich) were added, followed by stimulation with 10 μg/mL peptide or a mix of 100 ng/mL PMA and 10 ng/mL Ionomycin (Sigma-Aldrich) for 5 h at 37°C, 5% CO_2. The cells were washed, and HLA tetramer and surface-marker staining was performed as above. Intracellular cytokine staining was done using anti-human IFN-γ-PE antibodies (Clone 25723, R&D Systems) and a Human Intracellular Cytokine Staining Starter Kit (BD Biosciences) according to the manufacturer's specifications.

Bioinformatics

The NetMHCpan-2.3 algorithm was used to predict IC_{50} values of all 8-, 9-, 10-, and 11-mer peptides found in the proteomes of HBV, DENV, and SARS-CoV. This includes the core, X, envelope, and polymerase proteins of HBV Genotype B (NCBI accession AF121243), as well as HBV Genotype B (NCBI accession EU796066) and HBV Genotype C (NCBI accession AB112063) envelope protein. Furthermore, the capsid, membrane glycoprotein, envelope protein, NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5, proteins, and the 2 k peptide for DENV (NCBI accession NC_001474), and the NP and 3a proteins of SARS-CoV (NCBI accession AY283798) were included. Predictions were made for all HLA-A, -B, and -C alleles per donor. The output IC_{50} of the peptides with an affinity threshold of 1000 nM enabled ranking.

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Abbreviations: **DENV**: dengue virus · **HBV**: hepatitis B · **SARS-CoV**: severe acute respiratory syndrome coronavirus · **SEA**: South East Asian

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