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An efficient T-cell epitope discovery strategy using in silico prediction and the iTopia assay platform

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Abbreviations: CEA, carcinoembryonic antigen; HER2, human epidermal growth factor receptor 2; TERT, telomerase reverse transcriptase

Functional T-cell epitope discovery is a key process for the development of novel immunotherapies, particularly for cancer immunology. In silico epitope prediction is a common strategy to try to achieve this objective. However, this approach suffers from a significant rate of false-negative results and epitope ranking lists that often are not validated by practical experience. A high-throughput platform for the identification and prioritization of potential T-cell epitopes is the iTopia™ Epitope Discovery System™, which allows measuring binding and stability of selected peptides to MHC Class I molecules. So far, the value of iTopia combined with in silico epitope prediction has not been investigated systematically. In this study, we have developed a novel in silico selection strategy based on three criteria: (1) predicted binding to one out of five common MHC Class I alleles; (2) uniqueness to the antigen of interest; and (3) increased likelihood of natural processing. We predicted in silico and characterized by iTopia 225 candidate T-cell epitopes and fixed-anchor analogs from three human tumor-associated antigens: CEA, HER2 and TERT. HLA-A2-restricted fragments were further screened for their ability to induce cell-mediated responses in HLA-A2 transgenic mice. The iTopia binding assay was only marginally informative while the stability assay proved to be a valuable experimental screening method complementary to in silico prediction. Thirteen novel T-cell epitopes and analogs were characterized and additional potential epitopes identified, providing the basis for novel anticancer immunotherapies. In conclusion, we show that combination of in silico prediction and an iTopia-based assay may be an accurate and efficient method for MHC Class I epitope discovery among tumor-associated antigens.

Introduction

Cytotoxic T lymphocytes (CTLs) represent a key component of the immune system and play a crucial role in tumor surveillance. CTLs recognize short peptides derived from intracellular proteins and expressed on surface of target cells in complex with MHC Class I (MHC-I) molecules.[1] T-cell receptor (TCR)-mediated recognition of the epitope-MHC complex triggers the cytolytic activity of T cells, resulting in target cell lysis.[2] Immunization-induced or adoptively transferred CTLs have been associated with tumor regression in preclinical models and in the clinic.[3–5]

T-cell epitopes have been identified in multiple clinically relevant tumor-associated antigens (TAAs). However, most CTL epitopes are restricted by a single MHC-I allele, HLA-A2. Furthermore, some of the previously characterized epitopes may not be optimal candidates for cancer immunotherapy due to pre-existing tolerance or to a lack of specificity. Therefore, the need to identify additional candidate epitopes, particularly among those restricted to alleles other than HLA-A2, remains.

The introduction of the iTopia™ high-throughput assay[6,7] has the potential to improve the efficiency of the epitope discovery process. This platform allows for rapid prioritization of predicted epitopes based on MHC binding affinity and peptide:MHC complex stability. The latter factor has been recognized as a key factor in determining peptide immunogenicity in vitro and in vivo.[8,9] In fact, off-rate assays are key indicators of the relative complex stability as a potential predictor of the antigen-specific immune response.

In this study, we screened by means of the iTopia assay 225 known or predicted T-cell epitopes from three tumor-associated antigens, the carcinoembryonic antigen (CEA), the epidermal growth factor receptor 2 (HER2) and telomerase reverse transcriptase (TERT). CEA and HER2 are overexpressed in > 50% of colorectal cancers,[10,11] while TERT is upregulated in > 85% of all cancers.[12] The epitopes were restricted by five common

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MHC-I alleles, HLA-A1, A2, A3, A24, and B7, carried by over 85% individuals in many populations.[13] The immunogenicity of HLA-A2-restricted epitopes (n = 45) was evaluated in HLA-A2 transgenic (HHD) mice. Based on the large amount of data generated, we defined a accurate and cost-effective strategy for T-cell epitope discovery in protein antigens.

Results

In silico prediction of T-cell epitopes from CEA, HER2, and TERT. Potential T-cell epitopes were selected from the tumor-associated antigens CEA, HER2 and TERT based on (A) predicted binding affinity to the MHC-I molecules HLA-A1, -A2, -A3, -A24, or -B7; (B) increased likelihood of natural processing; and (C) uniqueness to the target antigen in the human genome, as described in Materials and Methods (**Fig. S1**). Known T-cell epitopes were included for comparison, if they satisfied the above criteria. Unlike binding affinity and natural processing, the uniqueness filter has not been commonly used, and it was included to reduce the likelihood of an off-target autoimmune response, as exemplified by the destruction of pancreatic islet β-cells following immunotherapy.[14] In addition, high-avidity T cells specific for widely expressed, shared epitopes may be deleted or functionally inactivated, potentially limiting vaccine efficacy.[15,16]

Thirty-one% of CEA- and 8% of HER2-derived fragments were identical to fragments of other human proteins. Notably, among them, 27 were known T-cell epitopes (17 from CEA, 10 from HER2), previously suggested as candidates for immunotherapy. For example, the well-known epitope CEA.24,[17] included as part of an epitope-based cancer vaccine in clinical development,[18] was 100% conserved in the proteins CEACAM1 and CEACAM6, which are expressed on normal cells.[19] The HLA-A2-restricted epitope CEA.233[9] and HLA-A3 restricted CEA.61[20] were found in CEACAM8 and CEACAM3, while the HLA-A24-restricted epitope HER2.907[21] was 100% conserved in the epidermal growth factor receptor (EGFR) and in HER3. The complete list of published CEA and HER2 T-cell epitopes found in other human proteins is provided in **Table S1**.

In contrast to the large number of shared epitopes in CEA and HER2, only one 9-mer fragment of TERT was found in another human protein, reflecting the absence of close homologs of this tumor antigen. One hundred and 23 fragments of the three proteins satisfying the binding affinity, natural processing and uniqueness criteria, were selected. For 98 of the peptides, one or two fixed-anchor analogs[9,22] were generated, for a total of 102 analogs. The often-higher immunogenicity of analogs and their ability to induce cross-reactive T cells make them suitable in situations in which the native epitope is not sufficiently immunogenic or may be subject to immunological tolerance.[23,24]

Determination of binding to MHC-I molecules. Two hundreds and 25 selected fragments from CEA, HER2, and TERT (45 per HLA allele) were synthesized and characterized for MHC binding and complex stability using the iTopia Epitope Discovery System.[25] To our knowledge, this is the largest such evaluation of the iTopia platform published to date. Eighty-four out of 123 native fragments and 102/102 analogs have been characterized for the first time in this study. To determine relative binding, peptides were incubated for 18 h at 21°C in MHC-coated wells as described in Materials and Methods. Each peptide was tested in duplicate on the same plate. The standard deviation was generally below 5% (data not shown). Peptide binding data, reported as a percentage of the positive control, are summarized in **Figure 1** and **Table S2**. A binding cut-off of 30% of the assay positive control was used to classify peptides as binders, as suggested by the manufacturer.

Twelve out of 25 (48%) native peptides selected based on predicted HLA-A1 binding were classified as binders in the initial binding assay. For HLA-A2, A3, A24, and B7, the percentage of binders among in silico selected fragments was 85%, 52%, 100%, and 76%, respectively. Cumulatively, 89/123 native peptides (72%) were binders to the corresponding MHC-I allele. In comparison, < 1% of random 9-mers were HLA-A1 binders using the same threshold,[6] 3–5% were HLA-A3 and -B7 binders, and 15–17% bound to HLA-A2 and -A24. The high frequency of binders among selected native candidate epitopes reflects the utility of the in silico step in enriching for MHC binders (**Fig. 2**; $p < 0.0001$ for each allele, Fisher's exact test). As expected, most analogs had significantly higher binding compared with the corresponding native peptide. Eighty-one out of 102 analogs (79%) had higher binding (**Fig. 4A**, left panel; $p < 0.0001$, paired signed-rank test), for a median increase in binding of 14%. Ninety out of 102 analogs (88%) were classified as binders.

We next determined the dissociation rates and half-lives ($T_{1/2}$) of peptide:HLA complexes formed by in silico-selected candidate epitopes. Decay curves for a representative sample of peptides are shown in **Figure 3**. The estimated $T_{1/2}$ are reported in **Figure 4** and **Table S2**. MHC complex stability varied considerably between alleles. HLA-A2-restricted peptides showed the highest stability, with 34/45 peptides (76%) forming stable complexes, here defined as $T_{1/2} > 4$ h. Twenty-six fragments had $T_{1/2} > 8$ h, including 6 peptides with $T_{1/2} > 40$ h. Fifteen out of 45 HLA-A3-restricted wild-type fragments and analogs formed stable complexes, including 12 peptides with $T_{1/2} > 8$ h and two with $T_{1/2} > 40$ h.

In contrast, HLA-A1, -A24, and -B7 restricted candidate epitopes frequently formed relatively unstable MHC complexes, with 45/45 HLA-A1- and A24-restricted and 41/45 B7-restricted peptides having $T_{1/2} < 4$ h. This finding is consistent with the relatively low stability of the assay positive controls ($T_{1/2} = 2.7$, 3.3, and 2.4 h for HLA-A1, -A24, and -B7, vs. 21.3 and 17.3 h for HLA-A2 and -A3, respectively).[6] In fact, among > 300 peptides tested by Bachinsky et al.[6] and Shingler et al.[7] none formed stable complexes ($T_{1/2} > 4$ h) with HLA-A1 and -A24, and only 4 peptides formed stable complexes with HLA-B7. Eighty-three out of 102 analogs (81%) formed more stable MHC complexes than the corresponding native peptide, with a median increase in $T_{1/2}$ of 65% (**Fig. 5**, right panel; $p < 0.0001$). The improvement in stability was evident across all alleles, with 73–95% peptides showing longer complex stability upon anchor residue replacement.

Immunogenicity of HLA-A2 restricted epitope candidates in HHD mice. We next sought to determine the immunogenicity of

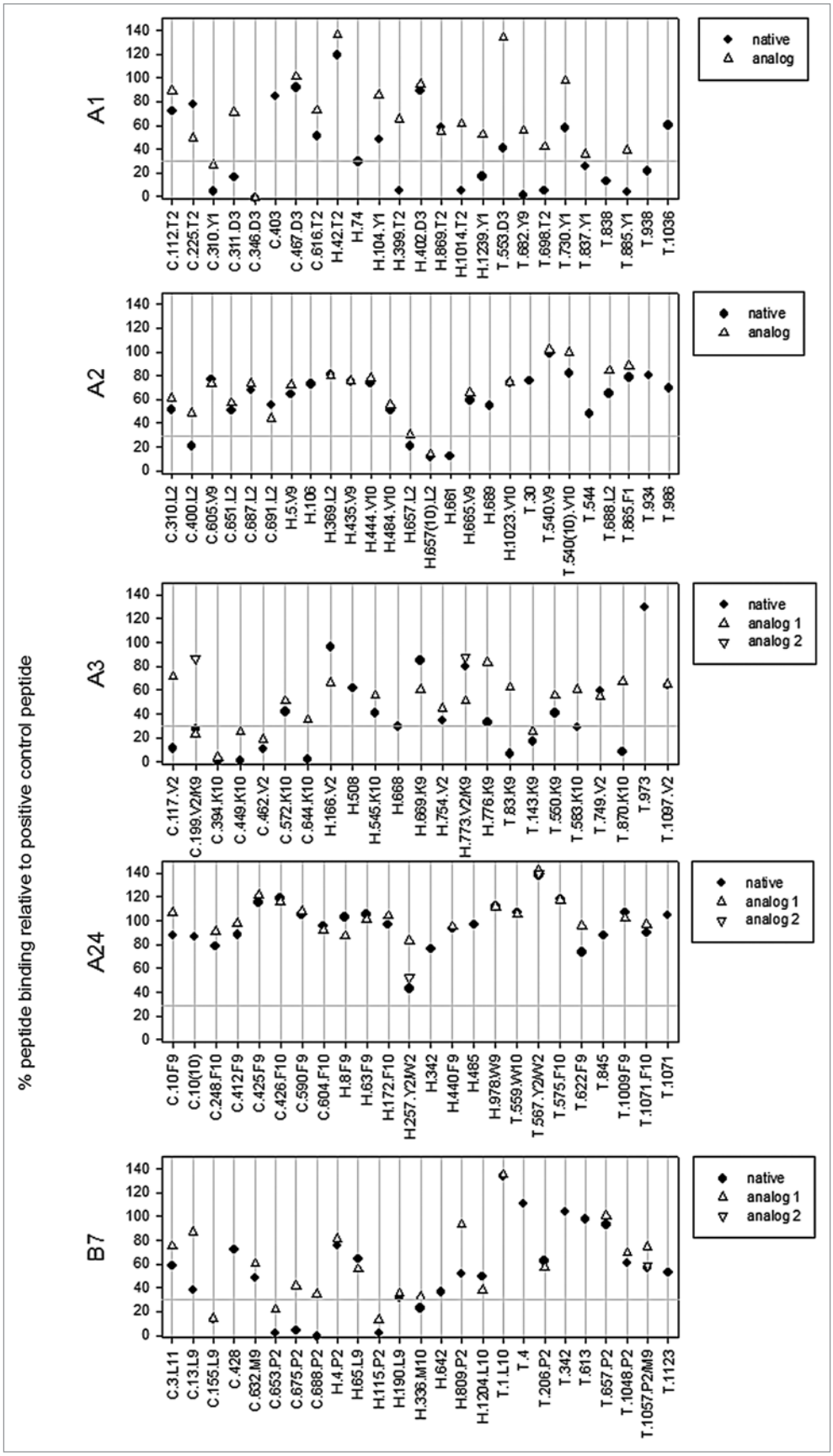

Figure 1. For figure legend, see page 1261.

Figure 1 (See opposite page). Binding of CEA, HER2 and TERT peptides and their analogs to five common MHC-I alleles. Binding of 123 native peptides and 102 analogs (45 peptides per allele) to HLA-A1, -A2, -A3, -A24, and -B7 is expressed as a fraction of the allele-specific positive control. The dotted line indicates a cut-off above which peptides are classified as binders. Native peptides (filled circles) and analogs (empty triangles) from CEA, HER2, and TERT are labeled as C., H., and T., respectively, following by the starting position in the protein sequence. Analogs are additionally labeled by the substitution and its position in the peptide sequence. Where multiple peptides begin at the same position, peptide lengths different from 9 are listed in parentheses. For instance, T.540(10) is the TERT 10-mer fragment starting at position 540. Each peptide was tested in duplicate on the same plate. This experiment has been performed twice with similar results.

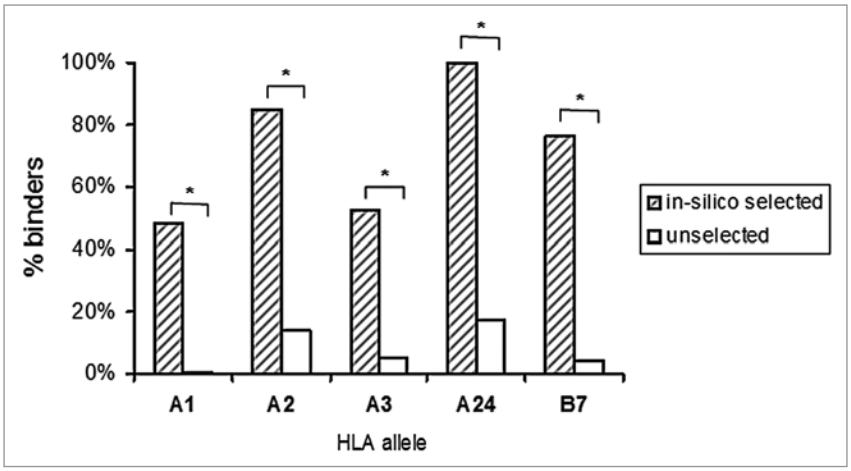

Figure 2. In silico selected native fragments of CEA, HER2, and TERT are enriched in MHC binders. Striped bars: fraction of peptides among the in silico selected native CEA, HER2, and TERT peptides which bound to > 30% of the allele positive control. Dotted bars: frequency of HLA binders among *arbitrary* 9-mer peptides, as estimated from a large number of peptides.[6] * $p < 0.0001$, Fisher's exact test

epitope candidates selected for binding to HLA-A2. HHD mice[26] express HLA-A*0201 and human β-2-microglobulin (β2M) and are deficient for D^b and murine β2M thus constituting an appropriate model for studying HLA-A2-restricted T-cell responses. Forty-five peptides, including 26 native fragments and 19 analogs, were tested in this model. Groups of 5–6 HHD mice were immunized with the individual peptides, and splenocyte responses were measured in each animal separately. The individual and group geometric mean responses are shown in **Figure 6** and **Table S2**. A cut-off of 0.1% $CD8^+$ cells secreting interferon γ ($CD8^+IFN\gamma^+$ cells) was chosen to indicate responders.

Sixteen out of 26 native peptides (62%) elicited CTL responses in HHD mice (**Fig. 6**). Among CEA peptides, the most immunogenic native peptide was CEA.605. This peptide generated significant cell-mediated immune responses in 6/6 mice, averaging over 1% $CD8^+IFN\gamma^+$ cells (range: 0.3–3.4%). Two additional fragments, CEA.687 and CEA.691, elicited significant responses in 4/6 animals each. Three other CEA peptides (CEA.310, 400, and 651) did not generate measurable CTL responses in most animals. This was despite significant binding at high peptide concentration but consistent with the relatively low HLA complex stability at 37°C (see Correlates of Immunogenicity section below).

The most immunogenic native HER2-derived peptide was HER2.435. All immunized mice developed significant CTL responses to this peptide, averaging 5% $CD8^+IFN\gamma^+$ cells (range: 3.1%–16.1%). Other highly immunogenic peptides were HER2.369 and HER2.665, recognized by 1.2% and 0.6% $CD8^+$ cells on average. Peptides HER2.5, HER2.689, HER2.106 and HER2.657 were moderately immunogenic, inducing 0.2–0.4% $CD8^+IFN\gamma^+$ cells on average. The TERT-derived peptides TERT.30 and TERT.934 were highly immunogenic, generating cell-mediated responses in all immunized mice, averaging 0.9–1.0% $CD8^+IFN\gamma^+$ cells. The peptides TERT.540, TERT.544, TERT.865 and TERT.986 were weakly to moderately immunogenic, generating an average of 0.1–0.3% $CD8^+IFN\gamma^+$ cells. Four out of 26 epitope candidates (HER2.369, HER2.484, HER2.689 and TERT.986) were identical to their mouse homologs. Intriguingly, we saw no evidence of immunological tolerance to these self antigens. In fact, 3/4 peptides (HER2.369, HER2.689 and TERT.986) generated significant immune responses in HHD mice, comparable to the overall rate of immunogenicity among native peptides in this study (62%). The lack of immunogenicity of HER2.484 may be explained by its relatively low HLA complex stability ($T_{1/2}$ = 3.1 h). In fact, no peptide for which the peptide:MHC-I complex half-life was less than 4 h induced significant CTL responses in HHD mice (see below).

Immunogenicity of HLA-A2-restricted epitope analogs in HHD mice. Additional groups of 5–6 HHD mice were immunized with 19 fixed-anchor analogs of the HLA-A2 restricted candidate epitopes. To evaluate the ability of analogs to induce cross-reactive responses, splenocytes from immunized mice were stimulated with the corresponding native peptides. The individual and group geometric mean responses are reported in **Figure 6A** and **Table S2**. Fourteen our of 19 analogs (74%) generated higher levels of native peptide-specific CTL than the corresponding wild-type peptides ($p = 0.04$, paired signed rank test). The median enhancement in the frequency of peptide-specific CTL using analogs was 2.0-fold (range: 0.3–117).

Four CEA-derived analogs induced increased CTL responses to the native peptide, including three that generated > 0.1% $CD8^+IFN\gamma^+$ cells on average. Two of them, CEA.605V9 and CEA.687L2, were analogs of known T-cell epitopes, with CEA.687L2 eliciting 3.4-fold higher CEA.687-specific T-cell responses. (Fixed anchor analogs are indicated by the amino acid substitution and its position in the epitope. E.g., analog CEA609.V9 has valine replacing the wild-type residue at position 9 of CEA.609.) The third analog, CEA.310L2, was the most immunogenic analog, and actually the most immunogenic peptide in this study. All CEA.310L2-immunized mice mounted significant

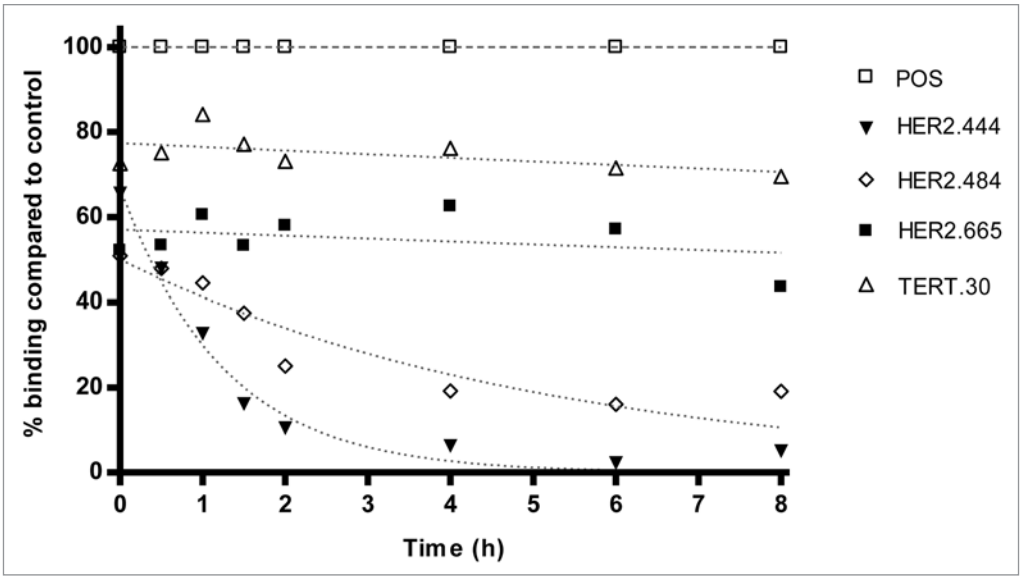

Figure 3. Off-rate curves for selected HLA-A2-restricted candidate epitopes. Binding after 0–8 h incubation after removal of free peptides is expressed as a percentage of the positive control at the same time point. This experiment has been performed twice with similar results.

CTL responses to CEA.310, averaging 5.8% $CD8^+IFN\gamma^+$ cells (range: 3.0–14.3%). In contrast, the wild-type CEA.310 peptide was not significantly immunogenic.

Seven HER2-derived analogs elicited specific CTL responses against the native peptide, with four analogs generating > 0.1% $CD8^+IFN\gamma^+$ cells on average: HER2.5V9, HER2.665V9, HER2.369L2, and HER2.657(10)L2.

Three TERT analogs generated consistent native epitope-specific CTL responses. Two of them, TERT.865F1 and TERT.540_{10}V10, exceeded the 0.1% average response cut-off for immunogenicity. Notably, TERT.865 contained optimal anchor residues at both primary anchor positions (L2, V9). Nonetheless, its F1 analog was able to increase the magnitude of T-cell responses further by 1.9-fold. While a tyrosine (Y) substitution at the N-terminus has been reported to increase HLA-A2 binding affinity and immunogenicity,[23,27] phenylalanine has recently emerged as a preferred N-terminal anchor residue based on the data from a combinatorial peptide library[28] and the analysis of a large set of binders.[29]

To assess whether the novel HLA-A2-restricted candidate epitopes identified with our procedure are indeed presented by target cells and to check if the elicited CTL effectors from vaccinated HHD mice are functional, we have performed CTL assays using HeLa-HHD cells overexpressing the target protein upon transduction with adenoviral vectors. In particular, HeLa-HHD cells were infected with Ad-CEA, Ad-hTERT, Ad-HER2 or Ad-GFP (negative control) or loaded with the immunogenic peptide (positive control) to evaluate the lytic activity of effectors derived from mice vaccinated with the peptides listed in **Table 1**. Notably, tested peptides and their corresponding analogs could induce CTL effectors that were able to kill HeLa-HHD cells (**Fig. 6B**). Both CEA.310 and CEA.687 were presented by Ad-CEA infected target cells. Importantly, effectors elicited upon vaccination with CEA.687L2 could recognize and kill $HLA\text{-}A^*0201^+/CEA^+$ (Colo705 and SW480) but not $HLA\text{-}A^*0201^+/CEA^-$ (Colo205) colon cancer cells. Similarly, TERT.865 and HER2.657-specific CTLs could efficiently recognize and lyse HeLa-HHD overexpressing TERT and HER2, respectively.

Correlates of peptide immunogenicity. We next investigated whether CMI responses in HHD mice could be predicted from the peptide in silico score, iTopia binding or MHC complex stability. Only native peptides were included in the correlation analysis. Since immune responses induced by the analogs were measured by stimulation with the native peptides, the bona fide immunogenicity of the analogs was presumably underestimated. Consistent with previous reports,[6] there was no correlation between in silico score and immunogenicity in vivo (**Fig. 7A**; Spearman rank correlation $p = 0.20$; $p > 0.1$). Another well-known epitope prediction algorithm, BIMAS,[31] similarly failed to show significant correlation between peptide scores and immunogenicity in HHD mice (**Fig. S2**). Binding in the iTopia initial screen was borderline significantly correlated with immunogenicity (**Fig. 7B**; Spearman $p = 0.41$; $p = 0.04$). The manufacturer's recommended binding cut-off of 30% did not effectively separate immunogenic and non-immunogenic peptides, as 15/22 binders and 1/4 non-binders were immunogenic [positive predictive value (+PV) = 68%, negative predictive value (-PV) = 75%, $p > 0.1$, Fisher's exact test].In contrast, MHC complex stability was significantly correlated with CMI responses in HHD mice (**Fig. 7C**; Spearman $p = 0.73$; $p < 0.0001$). Sixteen out of 20 peptides forming stable MHC complexes ($T_{1/2} > 4$ h) generated significant CMI responses, while 6/6 peptides forming less stable complexes were non-immunogenic (+PV = 80%, -PV = 100%; $p < 0.001$). Peptide selection based on initial binding and stability assays together had a higher positive predictive value than either screen alone. Fifteen out of 17 peptides that were binders in the initial assay and formed stable complexes were immunogenic, while 8/9 peptides that failed either screen were non-immunogenic (+PV = 88%, -PV = 89%; $p < 0.001$).

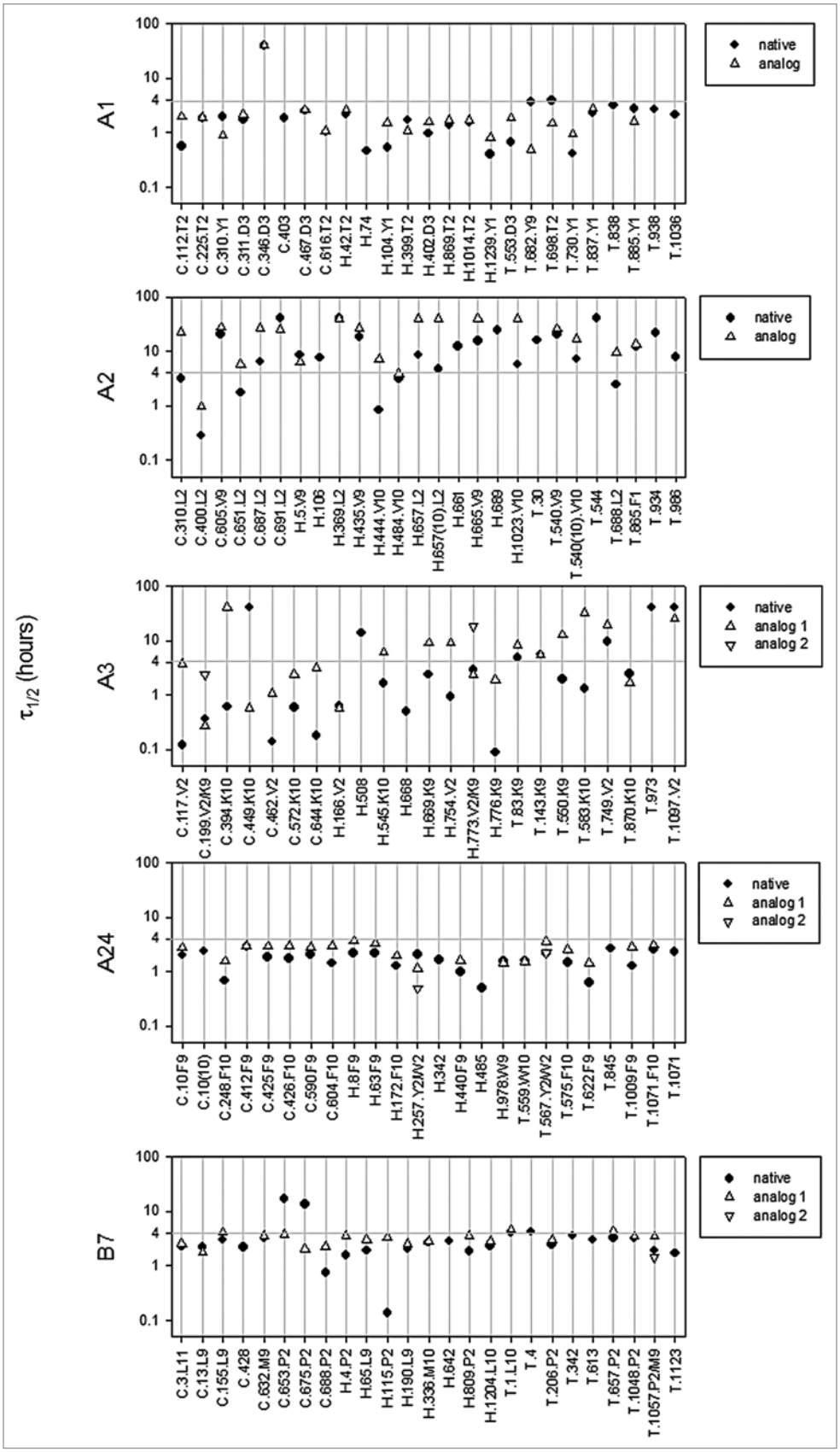

Figure 4. Stability of HLA complexes formed by the *in silico* selected native peptides and analogs. Complex stability is estimated from binding measurements done at 8 time points over an 8 h incubation period. The half-life, or $T_{1/2}$, is interpreted as the time required for the relative binding to diminish by 50%. Peptide labels are the same as in **Figure 1**.

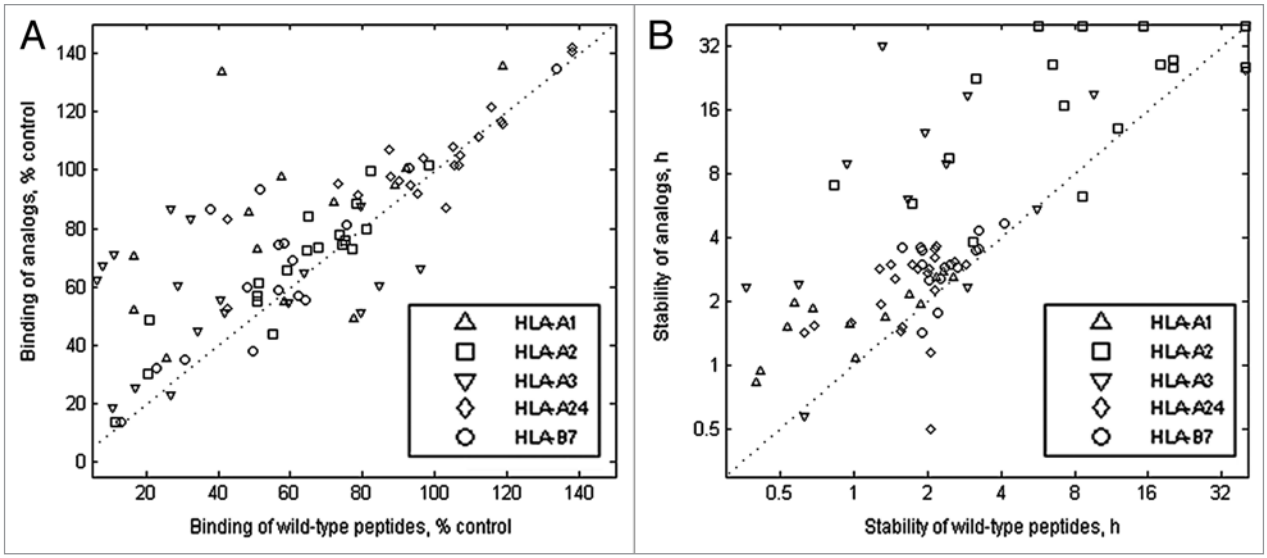

Figure 5. Fixed-anchor analogs have higher MHC binding and complex stability than the corresponding native peptides. A comparison of MHC binding (left) and complex stability (right) of native peptides (x-axis) vs. fixed-anchor analogs (y-axis). 81/102 (79%) and 83/102 (81%) analogs have higher MHC binding and longer MHC complex half-life, respectively ($p < 0.0001$ in both cases, paired signed-rank test).

While the iTopia stability assay was useful in predicting CMI responses in HHD mice, it required a significant amount of reagents. We therefore investigated whether comparable prediction accuracy could be achieved in a more resource-sparing manner. To that end, we evaluated MHC:peptide binding at the individual time points in the stability assay as potential predictors of peptide immunogenicity. **Figure 7D** shows the association between CMI responses and binding at 8 h incubation. The correlation was highly significant (Spearman $p = 0.74$, $p < 0.0001$) and comparable to that between immunogenicity and MHC complex stability. A binding cut-off of 30% of control at 8 h incubation provided an accurate threshold for separating immunogenic and non-immunogenic peptides. Fifteen out of 16 peptides binding above this cut-off elicited significant CMI responses in HHD mice, compared with 1/10 peptides binding to < 30% of control (+PV = 94%, -PV = 90%, $p < 0.001$, Fisher's exact test).

Binding after 12 or 24 h incubation was equally highly correlated with immunogenicity (Spearman $p = 0.72$). In contrast, very short incubation resulted in less accurate separation of immunogenic and non-immunogenic peptides. Spearman's rank correlation between CMI responses and binding after 1 h and 0 h incubation at 37°C without free peptide was 0.60, and 0.36, respectively. In summary, prediction of immunogenic peptides could be obtained by measuring binding at incubation as short as 2 h or as long as 24 h (Spearman $p = 0.72–0.74$, $p < 0.0001$).

Discussion

The iTopia assay is a valuable platform for the prioritization of potential immunogenic peptides in a high-throughput fashion and it has been used to identify several novel T-cell epitopes.[7] Prior to the introduction of iTopia, in silico selection was the only high-throughput selection method of which we are aware with a proven track record.[30] Therefore, it was natural to ask whether a combination of these two methodologies can further improve the efficiency of the epitope identification process.

The in silico selection strategy adopted in this study was based on three criteria: (1) predicted binding to one of five common MHC-I alleles; (2) uniqueness to the antigen of interest; and (3) increased likelihood of natural processing. In silico identification of MHC-I binders by algorithms such as BIMAS[31] or Syfpeithi[32] has been a key part of the reverse immunology approach.[30,33] Using another binding prediction algorithm,[14] we obtained 5- to 50-fold enrichment in MHC-I binders compared with a reference set (**Fig. 2**), confirming the utility of an in silico step.

It should be noted that most, if not all, epitope prediction algorithms suffer from a potentially significant rate of false negatives: peptides that form stable HLA complexes and may be immunogenic, yet are scored relatively low by the software. For example, in Shingler et al.,[7] 14 fragments of the tumor antigen 5T4 formed stable HLA-A2 complexes ($T_{1/2} > 4$ h in the iTopia stability assay) and would likely be immunogenic in vivo based on the relationship between complex stability and immunogenicity (**Fig. 7**). However, only 7/14 stable peptides (50%) were ranked in the top 10% of 5T4 fragments by BIMAS or ANN[34] algorithms, the latter considered the most accurate publicly available software for the HLA-A2 binder prediction.[35] Similarly, only 8/14 stable 5T4 fragments (57%) were ranked in the top 10% of 5T4 fragments using our algorithm. It is therefore important to consider complementary and experimental epitope identification methods, such as epitope mapping[36] or proteomic identification of peptides eluted from cell-surface HLA molecules.[37,38]

Candidate epitopes were further screened for the uniqueness to the target antigen in the human genome. This filter is not currently considered as a standard practice. As a result, several epitopes previously suggested as candidates for immunotherapy

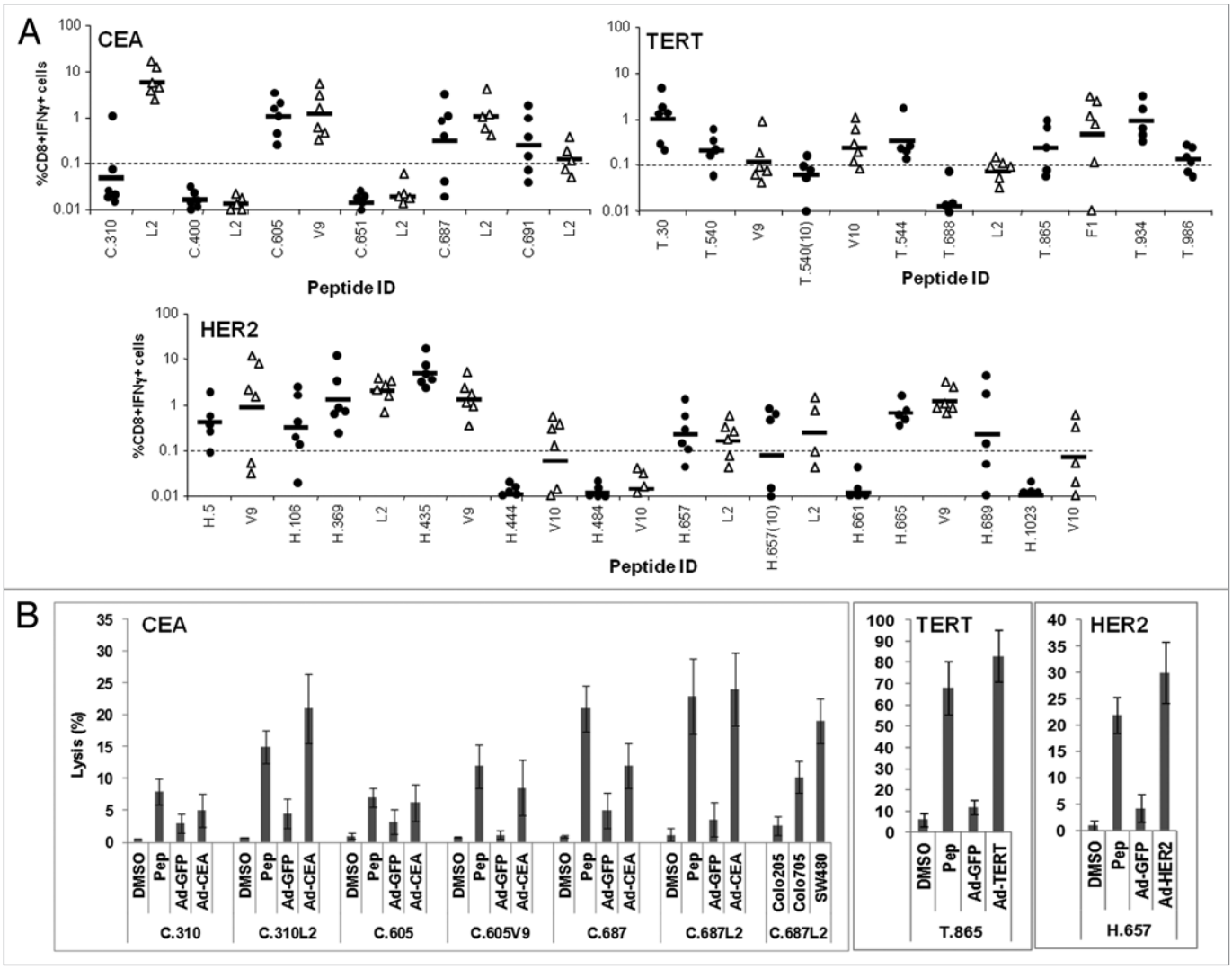

Figure 6. Immunogenicity of HLA-A2-restricted epitopes and analogs in HHD mice. Groups of 5–6 HHD mice were immunized with individual peptides. (A) Immunogenicity of CEA-, HER2-, and TERT-derived native peptides and analogs was determined by stimulating splenocytes with the native peptide. This vaccination has been performed twice with comparable outcome. Circles: responses of individual mice. Lines: group geometric means. Dashed horizontal lines: a cut-off of 0.1% $CD8^+IFN\gamma^+$ cells chosen to indicate significant immunogenicity. (B) CTL assays. HHD effectors from mice vaccinated with CEA, TERT and HER2 peptides were stimulated as described in the methods section and incubated with HeLa-HHD infected 48hrs before with the indicated Ad vectors at vp:cell ratio = 100. The E:T ratio was 100. Each bar represents the lysis obtained with effectors from each group vaccinated with the indicated peptide. SW480 and Colo-705 (CEA^+) were susceptible of CTL lysis while Colo205 (CEA^-) human colon adenocarcinoma cells were not efficiently killed.

were found in normal human proteins. One of these epitopes, CEA.24, had been included in an epitope-based cancer vaccine in clinical development,[18] which has recently been discontinued.

The final in silico selection filter was for the absence of a motif associated with poor natural processing. Algorithms to predict proteasomal cleavage sites, such as PAProC,[39] exist but have been poorly successfull,[40] probably due to the inherently non-specific nature of proteasomal processing. Nonetheless, naturally processed peptides possess motifs within their flanking residues which can be leveraged for the purpose of in silico selection. One of the most prominent such motifs is the lack of prolines at the N-terminus and immediately upstream of it,[41] reflecting cleavage preferences by endoplasmic reticulum aminopeptidases.[42,43] Ten % of candidate epitopes that passed the MHC binding and uniqueness filters were rejected due to their low likelihood of being naturally processed.

In total, we have selected in silico and characterized by means of iTopia 225 candidate epitopes, 45 of which were further tested for immunogenicity in transgenic mice. To our knowledge, this is the largest panel of peptides characterized by iTopia and in vivo in parallel in a single experiment. The size of our panel allowed us to make several conclusions about the utility of iTopia platform:

• The initial binding screen was at best marginally useful as a means of prioritization of the in silico selected candidates (**Fig. 7B**). For example, most A2-selected peptides were classified as binders and, within the subset of binders, there was little

Table 1. Novel epitopes

Epitope candidate								Corresponding native peptide	
Peptide Label	Sequence	Initial binding, % pos control	Binding after 8 h incubation w/o free peptide, % pos control	MHC complex stability (T1/2), h	CMIR, % $CD8^+IFN\gamma$ cells*	Fractions of responders†	Fold increase over native peptide immuno-genicity‡	CMIR, % $CD8^+IFN\gamma^+$ cells	Fraction of responders†
HER2.657	AVVGILLVV	20.6	12.0	8.6	0.23	5/6			
TERT.544	FLHWLMSVYV	48.0	74.4	40.0	0.33	5/5			
TERT.934	LLDTRTLEV	80.2	66.3	21.6	0.89	5/5			
TERT.986	FLDLQVNSL	69.5	54.5	7.8	0.13	4/6			
CEA.310L2	RLTVTTITV	61.2	64.4	25.4	5.82	6/6	122.8	0.05	1/6
CEA.605V9	YLSGANLNV	73.2	78.5	27.4	1.17	6/6	1.1	1.02	6/6
CEA687L2	ALVGIMIGV	73.7	76.2	26.3	1.06	5/5	3.4	0.31	4/6
HER2.5V9	ALCRWGLLV	72.3	33.9	6.2	0.82	4/6	2.1	0.39	4/5
HER2.369L2	KLFGSLAFL	79.7	114.9	40.0	2.06	5/5	1.7	1.24	6/6
HER2.657(10)L2	ALVGILLVVV	13.8	17.8	N/D§	0.24	2/4	3.2	0.08	3/6
HER2.665V9	VVLGVVFGV	65.8	72.7	40.0	1.22	6/6	1.9	0.63	6/6
TERT.540(10)V10	ILAKFLHWLV	99.8	88.8	16.8	0.23	5/6	4.0	0.06	1/5
TERT.865F1	FLVDDFLLV	88.4	76.2	13.2	0.44	5/6	1.9	0.24	4/6

*Splenocytes from native- or analog peptide-immunized HHD mice were stimulated with the native peptide as described in **Materials and Methods**. †Defined as geomean $\%CD8^+IFN\gamma^+$ cells > 0.1% after splenocype stimulation with the corresponding native peptide. ‡Ratio of CMI respondes to the w.t. peptide in mice immunized with the analog vs. w.t. peptide. §T1/2 was not determined for peptides binding to <15% of control at T = 0 h. Novel HLA-A2-restricted T-cell epitopes and immunogenic analogs in CEA, HER2, and TERT. Immunogenicity was determined by stimulating splenocytes from immunized mice with the native peptide as described in Materials and Methods.

correlation between binding and immunogenicity. In the future, it may be warranted to skip the iTopia initial binding assay when screening peptides selected using algorithms with low false-positive rates (e.g., the HLA-A2, A24 and B7-binding motifs used in this study). These findings were further validated using the BIMAS prediction algorithm (**Fig. S2**).

• The HLA complex stability ("off-rate") assay provided an excellent correlate of immunogenicity in HHD mice. Sixteen out of 20 peptides with $T_{1/2} > 4$ h were immunogenic, compared with 0/6 peptides with $T_{1/2} < 4$ h (**Fig. 7C**). However, the off-rate calculation required 16 wells per peptide, according to the manufacturer's protocol.[25] Notably, measuring binding after 8 h incubation without free peptide provided a similarly accurate method of peptide selection. In fact, 15/16 immunogenic and only 1/10 non-immunogenic peptides bound to a level > 30% of control at 8 h incubation (**Fig. 7D**). Longer incubation periods enabled equally accurate ranking (but did not improve accuracy) of peptide immunogenicity in HHD mice.

Our data suggest that binding at 8 h incubation after removing free peptides is an accurate and cost-effective predictor of peptide immunogenicity in HHD mice. Retrospectively, if we had chosen for in vivo immunogenicity screening only peptides binding at > 30% of control at 8 h incubation, we would have tested 35% fewer native peptides while excluding only one immunogenic peptide. Moreover, 16/17 in silico selected peptides that bound at > 30% control at 8 h incubation were immunogenic in HHD mice.

A second goal of our study was to identify additional immunogenic epitopes among such clinically relevant TAAs. Thus, we discovered four novel HLA-A2-restricted native peptides and nine immunogenic analogs of published HLA-A2 epitopes (**Table 1**). For some of them, we were able to demonstrate epitope processing and presentation on MHC-I as well as the recognition by CTLs (**Fig. 6B**). The data generated in this study can be used to select potential T-cell epitopes restricted to other common MHC-I alleles.

Fifteen out of 26 native peptides tested in HHD mice had previous evidence of immunogenicity in vitro or in vivo (see **Table S2** for a complete set of references). We confirmed the immunogenicity of 12 of them in HHD mice. The remaining three peptides, previously reported to expand specific T cells from peripheral blood mononuclear cells (namely, HER2.444 and HER2.484)[44] or tumor-infiltrating lymphocytes (i.e., HER2.1023)[45] of cancer patients, did not induce significant cell-mediated responses in our study. The lack of immunogenicity of HER2.444 and 484 was consistent with a low HLA complex stability. In contrast, HER2.1023 formed a relatively stable complex, with $T_{1/2} = 5.7$ h. Notably, all three peptides showed <

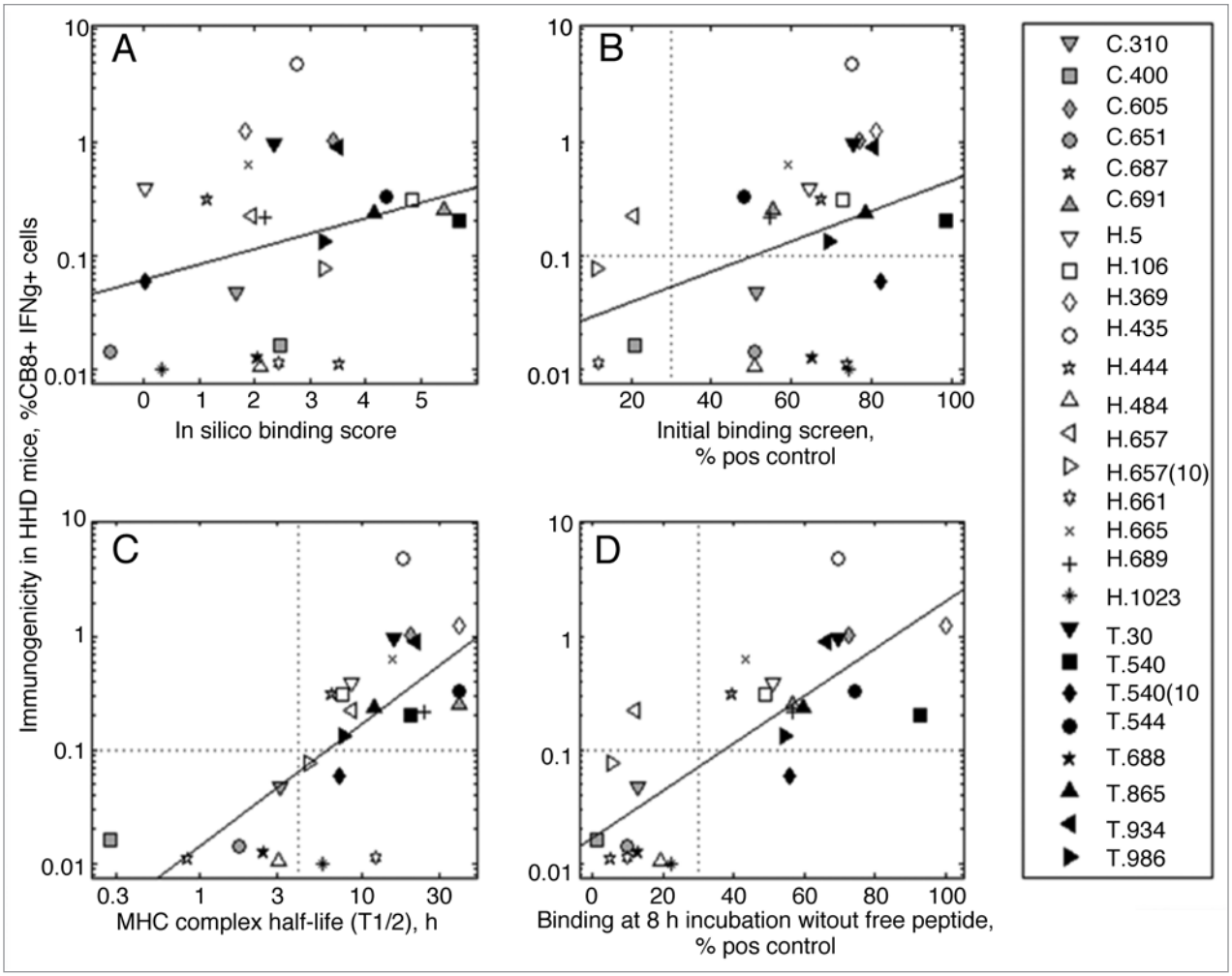

Figure 7. MHC complex half-life and relative binding at 8 h incubation after removal of free peptides, but not in silico score or initial binding, are predictive of immunogenicity in HHD mice. The geometric mean CMI response to 26 HLA-A2 candidate epitopes is plotted against in silico binding score (**A**); initial binding (**B**); MHC complex half-life (**C**); and binding at 8 h incubation without free peptide (**D**). Spearman's correlation coefficients are 0.20 ($p > 0.1$), 0.41 ($p = 0.04$), 0.73 ($p < 0.0001$), and 0.74 ($p < 0.001$), respectively. Vertical lines correspond to binding of 30% of allele positive control (**B and D**) and $T_{1/2} = 4$ h (**C**). Horizontal dotted lines correspond to 0.1% $CD8^+IFN\gamma^+$ cells.

30% relative binding compared with allele positive control at 8 h incubation after removal of free peptide.

A third goal of the paper was to quantify the degree of immunogenicity enhancement by fixed-anchor analogs. In contrast to several earlier large-scale experiments,[9] we tested the analogs and native peptides side by side to enable a direct comparison. As anticipated by earlier studies,[27,46] most single-substitution analogs improved binding, complex stability, and immunogenicity compared with the corresponding native peptides. However, the degree of immunogenicity enhancement was generally modest, with 11/19 analogs providing < 2-fold increase in the magnitude of responses to the native peptide. This increase in immunogenicity may be offset further by a potential decrease in CTL avidity.[47] Nonetheless, the use of analogs may be warranted in cases in which the analog is significantly more immunogenic than the native peptide.[45,48] For example, analogs of CEA.310 and TERT.540(10) (**Table 1**) and the analogs of CEA.411 and CEA.589 that we have previously described[49] are immunogenic while the native peptides are not.

Four of the peptides tested in HHD mice were 100% conserved in the mouse and may be considered self-antigens. In an earlier peptide immunization study,[48] self-antigens showed markedly lower immunogenicity in HHD mice despite forming stable MHC complexes. In contrast, 3/4 self-antigen peptides in our study generated significant CMI responses. In fact, HER2.369 (identical to mouse HER2.370) was one of the most immunogenic peptides. Notably, the murine variant of HER2 is widely expressed[50] and mHER2.370 is likely naturally processed, similar to its human homolog,[9,51] due to the identical flanking residues. This suggests the potential of our immunization protocol, which included the use of CpG oligonucleotides as adjuvants, to break immunological tolerance to self-antigens.

Some evidence[23] suggests that many, if not most, peptides with measurable MHC-I binding may be processed and presented by cells at a sufficient level to be recognized by vaccine-induced effector CTLs. As shown in **Figure 6B**, CEA.310, CEA.605, CEA.687, TERT.865 and HER.657 are indeed

processed and presented by the MHC-I machinery. Importantly, CTL effectors are able to recognize and kill antigen-expressing cells. Nonetheless, the in vivo antitumor potential of the novel epitopes remains to be established.

In conclusion, we have identified four novel HLA-A2 restricted epitopes and nine fixed-anchor analogs that generated greater cell-mediated immune responses than the corresponding native peptides when used as immunogen. One of the analogs contained a phenylalanine substitution at position 1, which has not been previously reported to produce HLA-A2 epitope analogs. In addition, we have characterized MHC binding and complex stability of a large number of potential CEA, HER2, and TERT epitopes restricted to other common MHC-I alleles, specifically HLA-A1, A3, A24 and B7. Finally, we have found that binding at 8 h incubation after removal of free peptides using the iTopia assay has similar predictive utility as the entire off-rate assay while requiring up to 90% less reagents. The combination of in silico epitope prediction and this in vitro screen has the potential to be an efficient means of T-cell epitope discovery in tumor and microbial antigens.

Materials and Methods

In silico prediction of T-cell epitopes and fixed-anchor analogs. Candidate epitopes were selected from tumor-associated antigens CEA, HER2, and TERT based on three filters: (A) predicted binding to MHC-I alleles HLA-A1, -A2, -A3, -A24 and -B7; (B) uniqueness in the human genome; and (C) increased likelihood of natural processing. MHC binding was predicted using a custom set of allele-specific binding matrices.[29] Unique peptides (i.e., fragments that occurred only in the target antigen and no other human protein) were identified by scanning the human proteome using PattInProt[41] in batch mode. Peptides containing proline at the N-terminus or immediately upstream of it in the protein sequence were excluded as unlikely to be naturally processed.[52] Fixed-anchor analogs were generated by replacing an amino acid in one of the MHC anchor positions with an optimal anchor residue in that position, as described.[28,53–56] The optimal anchors for each of the MHC-I alleles are as follows:

- HLA-A1: Y at the N-terminus (NT); T at P2; D at P3; Y at the carboxyl-terminus (CT)
- HLA-A2: Y or F at the NT; L at P2; V at the CT
- HLA-A3: V at P2; K at the CT
- HLA-A24: W at P2; F or W at the CT
- HLA-B7: P at P2; L or M at the CT.

In vitro binding assays. Two hundred 25 peptides, 45 per MHC-I allele, were synthesized at JPT. MHC binding and complex stability of each peptide was characterized using iTopia™ Epitope Discovery System (Beckman Coulter) as previously described.[6,25] Briefly, avidin-coated microtiter plates containing biotinylated MHC-I monomers (A*0101, A*0201, A*0301, A*2401 and B*0702) loaded with β2-microglobulin (β2M) and placeholder peptides were used to evaluate peptide binding and complex stability. The monomer-coated plates, assay buffers, FITC-conjugated anti-HLA-ABC monoclonal antibody (mAb) B9.12.1, β2M and allele-matched positive control peptides were obtained as part of the iTopia kits. Monomer-coated plates were first denatured, releasing the placeholder peptide and leaving only the MHC heavy chain bound to the plate. Test peptides were introduced under optimal folding conditions, along with the anti-HLA-ABC-FITC monoclonal tracer antibody. The denaturation/renaturation concept is the basis of the iTopia system. All peptides were first evaluated in the initial binding assay (11 μM peptide incubated for 18 h at 21°C with β2M, anti-MHC monoclonal antibody and plate-bound MHC heavy chain). Irrespective of the initial binding, all peptides were also screened in the stability assay to determine MHC complex half-life ($T_{1/2}$; time taken for 50% reduction in binding at 37°C after removing peptide from the assay buffer). Briefly, peptides were incubated overnight at 21°C in MHC-coated wells. Assay buffer was replaced with fresh buffer containing no peptide. Plates were then incubated at 37°C and read at multiple time points over a 24 h period. Dissociation rates were calculated by GraphPad Prism software using a single-phase exponential decay equation fitted to the first 8 h observation data, as recommended by the manufacturer and for consistency with earlier studies using this assay.[6,7] All assay plates were read on Cytofluor II fluorometer (PerSeptive Biosystems).

Peptide immunizations. HHD mice[26] were provided by Institute Pasteur, Paris France. Mice were injected subcutaneously at the base of the tail with an individual T-cell epitope (100 μg), admixed with 140 μg of HBVcore-derived helper peptide (TPPAYRPPNAPIL)[57] and 50 μg of the TLR9 agonist CpG ODN, emulsified in incomplete Freund's adjuvant. A second injection with the same components was given 15 d later. Two weeks after the second injection, splenocytes were recovered for immunological assays.

Interferon γ intracellular staining. Cell-based immune responses were measured in each animal separately as previously described.[58] Briefly, 1–2 × 10^6 splenocytes in 0.6 mL RPMI 10% FCS were incubated with the test peptide (5 μg/mL final concentration) and brefeldin A (1 μg/mL; BD PharMingen) at 37°C and 5% CO2 for 12–16 h. Cells were then washed with FACS buffer (PBS 1% FBS, 0.01% NaN3), incubated with purified anti-mouse CD16/CD32 Fc block (BD PharMingen) for 15 min at 4°C, washed and stained with PE-conjugated anti-mouse CD4 (BD PharMingen), PerCP-conjugated anti-mouse CD8 (BD PharMingen) and APC-conjugated anti-mouse CD3e (BD PharMingen), for 30 min at room temperature in the dark. After washing, cells were fixed and permeabilized with Cytofix-Cytoperm Solution (BD PharMingen) for 20 min at 4°C in the dark. After washing with PermWash Solution (BD PharMingen) cells were incubated with IFNγ-FITC antibodies (BD PharMingen). Cells were then washed, fixed with formaldehyde 1% in PBS and analyzed on a FACSCalibur flow cytometer using CellQuest software (Becton Dickinson).

In vitro cytotoxicity. Assays were done according to the standard protocols.[59] Briefly, lymphocytes were isolated from harvested spleen two weeks after the final vaccination, and these cells (2 × 10^6/mL) were stimulated with the native peptide along with 20 IU/mL recombinant human IL-2 (Sigma). On

day 5, these in vitro stimulated cells were used as CTL effector cells, and the CTL activity was determined by a standard 6 h ^{51}Cr-release cytotoxicity assay using the indicated cell lines as targets. Target cells were incubated with effectors at effector-to-target cell (E:T) ratio = 100. HeLa-HHD cells (kindly provided by Dr Lemmonier, Institute Pasteur) were either loaded for one hour with the peptide of interest or infected 48 h before with Ad-CEA,[60] Ad.hTERT,[61] Ad-HER2[62] or Ad-GFP at vp:cell ratio = 100. Colo-205, Colo-705 and SW-480 were obtained from ATCC. All cells were cultured in RPMI-1640 supplemented with 10% FCS. Specific lysis was calculated as (experimental ^{51}Cr release – spontaneous ^{51}Cr release)/(maximal ^{51}Cr release – spontaneous ^{51}Cr release) × 100.

Statistical analysis. MHC binder frequency among in silico predicted vs. non-selected peptides was compared by Fisher's exact test. MHC binding and immunogenicity of native peptides and fixed-anchor analogs were compared by the Wilcoxon signed-rank test. The significance of association between peptide in silico scores, MHC binding, and immunogenicity was determined using Spearman's correlation. p values lower than 0.05 were considered statistically significant. Calculations were performed using Matlab 7.5 (The MathWorks).

Disclosure of Potential Conflicts of Interest

A.F., A.C.F., I.P., A.B. and D.R.C. are employees of Merck and Co., Inc. N.L.M. is employed at Idera Phamaceuticals.

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Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/oncoimmunology/article/21355/

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