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Solid-Phase Epitope Recovery: A High Throughput Method for Antigen Identification and Epitope Optimization

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Self tolerance to MHC class I-restricted nonmutated self Ags is a significant hurdle to effective cancer immunotherapy. Compelling evidence is emerging that altered peptide ligands can be far more immunogenic than their corresponding native epitopes; however, there is no way to reliably predict which modifications will lead to enhanced native epitope-specific immune responses. We reasoned that this limitation could be overcome by devising an empirical screen in which the nearly complete combinatorial spectrum of peptides of optimal length can be rapidly assayed for reactivity with a MHC class I-restricted cytotoxic T cell clone. This method, solid-phase epitope recovery, quantitatively ranks all reactive peptides in the library and allows selection of altered peptide ligands having desirable immunogenic properties of interest. In contrast to rationally designed MHC anchor-modified peptides, peptides identified by the present method are highly substituted in predicted TCR contact residues and can reliably activate and expand effector cell populations in vitro which lyse target cells presenting the wild-type epitope. We demonstrate that solid-phase epitope recovery peptides corresponding to a poorly immunogenic epitope of the melanoma Ag, gp100, can reliably induce wild-type peptide-specific CTL using normal donor T cells in vitro. Furthermore, these peptides can complement one another to induce these responses in an overwhelming majority of normal individuals in vitro. These data provide a rationale for the design of superior vaccines comprising a mixture of structurally diverse yet functionally convergent peptides. The Journal of Immunology, 2002, 169: 2414–2421.

One goal of cancer immunotherapy is to elicit potent tumor rejection responses by vaccination with tumor-associated Ags. The majority of cancer vaccines currently in development are based on nonmutated self Ags that are preferentially or aberrantly expressed in tumor cells. Thus far, self Ags have demonstrated limited immunogenicity in clinical trials and often do not lead to potent or durable clinical responses (1–3). A significant limitation impacting the clinical utility of vaccines comprising this class of Ag is the requirement that they must achieve reversal of tolerance. Recent reports that heteroclitic altered peptide ligands are capable of reversing peripheral tolerance and/or anergy in animal models have provided a rationale for designing more immunogenic synthetic vaccines (4–6).

CD8+ cytotoxic T lymphocytes recognize short 8- to 10-aa peptides comprising the minimal essential epitope encoded by tumor Ags. Heteroclitic peptide derivatives of native epitopes achieve reversal of tolerance through the activation of CTL that have the ability to interact productively with the native epitope, indicating that they have escaped thymic deletion and peripheral tolerance, despite their specificity for self Ags (7, 8). The phenomenon of antigenic molecular mimicry whereby a foreign pathogen can break tolerance to a self epitope and give rise to autoimmune aggression represents a naturally occurring example of peripheral or central tolerance reversal by heteroclitic epitope vaccination (9–11). However, because the molecular definition of the determinants that confer immunogenicity is poorly understood, it is not possible to reliably identify such epitope mimics based on native epitope sequences through rational design (12–15).

Because epitope mimics that dependably give rise to T cells that effectively cross-react with the corresponding native epitope cannot be rationally designed in a reliable way, we have developed an empirical screening method for the optimization of MHC class I-restricted CTL epitopes. This technique, termed solid-phase epitope recovery (SPHERE),2 allows the identification of individual peptides of desired activities amid tens of millions of peptides. Several groups have reported screening complex combinatorial peptide libraries to identify reactive T cell epitope mimics (16–22). These approaches use either solid-phase or solution-phase peptide libraries. Groups using solid-phase combinatorial peptide libraries have, for practical reasons, reported screening only a small fraction of their libraries. Although these approaches identify reactive peptides, it is statistically likely that more effective peptide derivatives that were not identified, owing to the fact that the majority of possible library species were not tested. Those methods using complex combinatorial solution-phase libraries seek to identify a motif derived from reactivities present in multiple sublibraries. These motifs require further interrogation by synthesizing and reassaying tens or hundreds of peptides to identify the most desirable ones that conform to the motif. In contrast, we have limited our library complexity by synthesizing a library custom tailored for screening particular MHC-restricted T cell lines or clones. Because it is a prerequisite that strongly immunogenic peptides bind well to their restricting MHC molecule, we have engineered our library with fixed residues at positions previously shown to confer high-affinity binding to the HLA-A2 molecule, thereby reducing the library complexity by nearly 7000-fold compared with a fully

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2 Abbreviations used in this paper: SPHERE, solid-phase epitope recovery; DC, dendritic cell; F-moc, 9-fluorenylmethyloxycarbonyl.
degmene 9-mer library. Consequently, a relatively high percentage of our library peptides can potentially bind well to the HLA-A2 molecule yet still display >47 million different sequences to the TCR of HLA-A2-restricted T cells. The recursive deconvolution strategy we use easily allows screening of an entire library of nearly 50 million peptides yielding both the peptide sequences and their relative activities directly from the screening process. Therefore, our method stands in distinction in that it identifies highly reactive altered peptide ligands with precisely defined structures and with no need for further optimization.

In this study, we apply SPHERE to identify and characterize heteroclitic peptides reactive with a previously reported HLA-A2-restricted T cell line that recognizes an epitope encoded by the human melanoma-associated Ag, gp100 (23).

**Materials and Methods**

**Cell lines and reagents**

TIL1520, TIL620-10, and TIL1235 were generously provided by M. Nishimura (National Institutes of Health, Surgery Branch, Bethesda, MD). These T cell lines were maintained in AIM V medium (Life Technologies, Carlsbad, CA) supplemented with penicillin, streptomycin, 10% human AB serum (Sigma-Aldrich, St. Louis, MO), and 6000 IU/ml human IL-2 (Proleukin; Chiron, Emeryville, CA). AS49 and T2 cells were obtained through American Type Culture Collection (Manassas, VA) and maintained in DMEM/10% FBS and RPMI 1640/10% FBS (JRH Biosciences, Lenexa, TX), respectively.

**Peptide synthesis**

Peptides were synthesized by standard 9-fluorenylmethyloxycarbonyl (Fmoc) chemistry and purified to >95% by reverse-phase HPLC (New England Peptide, Fitchburg, MA). Amino acid analysis and mass spectrometry were performed to accurately quantitate the amount and purity of each peptide. All peptides were resuspended in 100% DMSO (Sigma-Aldrich) at a concentration of 10 mg/ml and used at the indicated dilutions.

**Peptide library synthesis**

A solid-phase combinatorial peptide library was synthesized by standard Fmoc chemistry according to the split-synthesis method previously reported (24) using 100-200 Mesh Wang resin as the solid support (Rapp Polymere, Tübingen, Germany). The structure of the library was FLXXXXXX; where X represents any amino acid except cysteine with the C-terminal valine residue covalently attached to the beads via an acid cleavable linker. The library complexity is calculated to be 47,045,881. On average, each bead contains ~200 pmol of a unique peptide.

**Peptide release chemistry**

Library preparation: library beads containing the peptides were suspended in dichloromethane/dimethylformamide (1:2) to form a neutral density bead suspension and arrayed in 96-well teflon filter bottom plates (Millipore, Bedford, MA) at a density of 10,000 beads/well. All solvent washes and cleavage reactions were performed in the filter plates using a vacuum manifold apparatus (Millipore). The beads in each well of the filter plate were washed two times with 100 μl dichloromethane, two times with 100 μl diethyl ether, and air dried. N-terminal Fmoc groups were cleaved with 200 μl piperidine/dimethylformamide (1:4) for 20 min and filtered away. The beads were then prepared for release of the peptides into solution and removal of the side-chain protecting groups by washing two times with 200 μl diethyl ether, two times with 200 μl dichloromethane, two times with 200 μl diethyl ether, and air dried.

**Primary screen peptide release**

Partial peptide release from the beads and the simultaneous removal of the side-chain protecting groups was achieved by incubation with 200 μl/well trifluoroacetic acid/acetonitrile/water/anisole (40:55:2:5) for 60 min at room temperature resulting in release of ~2–5% of the peptide/bead. Released peptide was captured in a 96-well polypropylene replica plate and lyophilized under high vacuum. Immediately, the beads were incubated with 200 μl acetonitrile/0.5 M Tris, pH 7.5 (1:1) for 30 min, washed once with 200 μl acetonitrile/water (1:1), once with 200 μl acetonitrile, once with 200 μl dichloromethane, once with 200 μl diethyl ether, air dried and stored at ~20°C.

**Secondary screen peptide release**

The 10,000 beads in wells of interest were suspended in dichloromethane/dimethylformamide (1:2) and arrayed evenly over a 96-well filter plate. The release chemistry was performed as before except without the piperidine incubation and the cleavage reaction was for only 30 min.

**Tertiary screen peptide release**

The ~100 beads in wells of interest on the secondary screen were arrayed as before over a new 96-well filter plate yielding ~1 bead/well. Peptide release was identical with that of the secondary screen plates.

**Peptide sequencing**

Peptide sequencing was performed by Edman degradation directly from the beads at the Harvard Microsequencing Facility (Cambridge, MA).

**Library screening**

Primary, secondary, and tertiary screens were all performed identically, using a standard 51Cr-release microcytotoxicity assay with the following modifications. A total of 2 μl released peptide was added to V-bottom 96-well plates and T2 cells were added at a density of 1000 cells/well in a total volume of 100 μl/well and incubated at 37°C/5% CO2 for 60 min. A total of 1000 T cells in 100 μl RPMI 1640/10% human AB serum were then added to each well and the plates were returned to the incubator for 4 h. Supernatant was harvested (25 μl) from each well and the amount of released 51Cr quantitated using a Wallac TriLux MicroBeta plate counter (Turku, Finland). Spontaneous 51Cr release was measured in the absence of effector T cells and total 51Cr release was measured by lysing the cells with 0.1% Triton X-100. Percentage of specific lysis was calculated according to the following formula: 100 × (experimental cpm – spontaneous cpm)/(total cpm – spontaneous cpm).

**In vitro T cell education studies**

Normal donor apheresis products were obtained from Dana-Farber Cancer Research Institute (Boston, MA). PBMC were isolated by centrifugation over Ficoll (Nycomed, Oslo, Norway). CD8+ T cells were isolated using magnetic beads (Dynal, Oslo, Norway) according to the manufacturer’s instructions. To generate autologous DCs, monocytes were isolated from the PBMC and treated with GM-CSF (Immunex, Seattle, WA) and IL-4 (PeproTech, London, U.K.) for 6 days. One day before establishing the T cell/DC cocultures, the DCs were pulsed with peptides (10 μg/ml) overnight followed by the addition of the previously isolated CD8+ T cells at a T:DC of 10:1. Cultures were restimulated with peptide-pulsed DCs. IL-2 (50 IU/ml) was added on day 8 and added every 3–4 days as needed. The bulk cultures were assayed 1 wk after the fifth restimulation. Peptide-pulsed T2 cell targets were prepared as described above and adenovirus-infected target cells were allowed to incubate with the viruses for 48 h at the indicated multiplicity of infection before being used in the CTL assay. All cultures were assayed in quadruplicate using 2E + 3 51Cr-labeled target cells at the indicated E:T for 16 h.

**Results**

**Library screening**

An overview of the SPHERE methodology is depicted in Fig. 1. The reactivity of peptides in the library is measured using a 51Cr-release microcytotoxicity assay. Parameters such as E:T ratio, tolerance to DMSO, required peptide concentration, and complexities were optimized by spiking in known solid-phase peptides under mock screening conditions (data not shown). The library deconvolution strategy involves three iterations starting with pools of 10,000 peptides for the primary screen, pools of 100 peptides for the secondary screen, and single peptides for the tertiary screen. Because high-affinity MHC binding is required for good immunogenicity, the nonamer peptide library was engineered to favor binding to a particular MHC molecule. For HLA-A2, residues 1, 2, and 9, predicted by functional studies and x-ray crystallography to contact the MHC, were fixed with amino acids F, L, and V, respectively, which have been reported to confer high-affinity MHC binding (25–27). This serves to limit the library complexity while maintaining broad diversity among the residues predicted to contact the TCR. Therefore, the structure of our dedicated HLA-A2 solid-phase library is F-L-X9-V-linker-bead, where X is any one of
19 aa (cysteine excluded) and the linker that tethers the peptides to the beads is chemically cleavable. Having a fixed amino acid (valine) coupled to the linker allows uniform kinetics for partial cleavage of all peptides, and by keeping the cleavage reagent limiting, any desired aliquot of peptide can be uniformly released from the beads in a manner that is largely independent of the amount of solid-phase peptide in the reaction (data not shown). This library has a complexity of $4.7 \times 10^7$. Because our HLA-A2-dedicated library is biased toward hydrophobic residues, all peptide and peptide mixture stock solutions were carried in 100% DMSO so as not to bias against those peptides that are poorly soluble in aqueous solution.

**Primary SPHERE screen**

Polystyrene beads representing the entire library were arrayed into 47 96-well teflon filter-bottom plates at a density of 10,000 per well. A small portion (5%/bead, ~10 pmol) of the peptide from each bead was released by acid hydrolysis and captured in standard 96-well polystyrene replica plates using a Millipore Multiscreen apparatus (Millipore) and lyophilized. The released peptides were...
resuspended with 500 μl/well DMSO and 2 μl of each 10,000 peptide mixture was pulsed onto 51Cr-labeled T2 cells and subsequently exposed to TIL1520 at an E:T of 1:1. In the interest of minimizing T cell consumption, we used 1000 target cells and 1000 effector cells per well. The effector cells used in this study were TIL1520, having specificity for the 209–217 epitope encoded in the human gp100 melanoma Ag. Under these conditions, the entire primary screen consumes only ~5 million T cells. Lytic activity was measured by 51Cr-release as described. Fig. 2 shows the frequency distribution of observed responses compiled from the entire primary screen. The vast majority of the peptide pools show little or no lysis induction; however, there are some that stimulate potent lytic activity from this T cell line (Fig. 3, a and d). The highest scoring wells were further analyzed in a secondary screen. Note that this experiment has been repeated several times with similar frequency distributions and with consistent identification of the same high-scoring peptide pools.

Secondary screen
The library beads corresponding to seven of the highest scoring peptide pools were rearrayed evenly over new 96-well filter plates and an aliquot of peptide was released into replica plates. The released peptides, now ~100-peptide mixtures, were reassayed as in the primary screen. In every case, a single pool demonstrated clear lysis above background from a single peptide pool, indicating that the peptide responsible for the observed activity in the primary screen was present in the lower complexity mixtures. Typical secondary screen results are shown in Fig. 3, b and e. The peptide pools showing activity were further analyzed in a tertiary screen.

Tertiary screen
As before, the beads corresponding to each of the positive 100-peptide pools were each rearrayed evenly over new 96-well filter plates yielding ~1 bead/well, and replica plates of solution-phase peptides prepared. These released peptides were assayed as before and in each case a single reactive well was observed. Typical tertiary screen data are shown in Fig. 3, c and f.

Upon completion of the tertiary screen, the reactive peptides were recovered as solid-phase pure populations and were sequenced directly from the beads by Edman degradation. The sequences of the seven highest scoring peptides are shown in Table I. Two of these peptides, SP-1 and SP-2, share some sequence homology with the native epitope. Analysis of the homologous sequences reveal a motif that highlights TCR contact points or structural elements required to share recognition with the native epitope. Most notably, the amino acids D, Q, and F at positions 3, 4, and 7 are dominant and appear in several of the most reactive peptides. Substitutions at other positions most likely contribute to overall MHC and TCR affinities, but differ sufficiently from the native sequence that they could not have been predicted by rational

FIGURE 3. SPHERE library deconvolution. Each graph represents the data collected for a single 96-well plate. a–c, The primary (10,000 peptide pools), secondary (100 peptide pools), and tertiary (single peptides) screen data for peptide SP-3. d–f, The same information for peptide SP-1. The library plate designation is indicated above a and d, and the wells that were deconvoluted are shown above the other graphs. The beads that were then sequenced were retrieved from wells E6 (c) and f11 (f). All data are represented as percentage of specific lysis as described in Materials and Methods.
design methods. Consistent with previous reports, we observed that the majority of the sequences had minimal or no homology to the wild-type epitope (28, 29). There appears to be a strong bias toward hydrophobic residues among the non-MHC anchor residues of these peptides. This may be related to the high density of hydrophobic side-chain residues that have been shown by x-ray crystallographic studies to line the peptide-binding groove of the HLA-A2 molecule.

**SPHERE peptides react specifically with native epitope-specific CTL.**

Some of the peptides are so divergent from the native epitope that we wished to demonstrate whether they would react specifically with TIL1520. To address this, we resynthesized and tested the reactivity of some of these peptides in a 

\[ ^{51} \text{Cr} \text{-release assay with the T cell line used in the screen (TIL1520) or an irrelevant T cell line (TIL1235)} \]

that recognizes a different epitope in another Ag. Fig. 4 shows the result of this assay and strongly supports the idea that all of these peptides were correctly identified legitimate “hits” in our screen and that they do indeed react specifically with gp100209-217-reactive TIL. Next, we sought to determine whether the SPHERE peptides would react with an independently derived gp100209-217-specific TIL population (TIL620-10). To this end, a subset of the peptides shown in Table I, chosen for their sequence diversity, were tested for reactivity with either TIL1520 or TIL620-10 in a 

\[ ^{51} \text{Cr} \text{-release assay. These results are shown in Fig. 5, and indicate that the peptides react equally well with TIL620-10, implying that even the distantly related epitope mimics are functionally similar to the native epitope in this assay.} \]

**SPHERE peptides are immunogenic.**

Having demonstrated that the identified SPHERE peptides are antigenic (i.e., they are recognized by preformed gp100209-217-specific CTL), we next sought to determine whether they were also immunogenic (i.e., could prime a de novo immune response). To this end, we compared their relative abilities to educate normal donor HLA-A2+ T cells in vitro. These in vitro T cell education studies were designed to test the ability of the SPHERE peptides to expand and sensitize T cells to lyse targets presenting the native epitope or themselves. Because most of the peptides tend to fall out of solution forming a fine precipitate when the DMSO stock solutions are added to the aqueous media, we chose to use autologous immature dendritic cells (DCs) as APC, which are better equipped to take up particulate matter than are PBMC (30). We found that the native gp100209-217 peptide is poorly immunogenic in our in vitro assay using normal donor T cells. Detection of peptide-specific reactivity routinely required five rounds of restimulation, and was best demonstrated using a 16- to 22-h incubation period with target cells in the CTL assay. Hence, we adopted these conditions for comparison of the SPHERE peptides with the wild-type epitope. We found that the SPHERE peptides were capable of eliciting responses even in those individuals that responded poorly or not at all to the wild-type peptide (Fig. 6). When these studies were extended to include a total of 20 normal donors, it was noted that none of the peptides were immunogenic in all individuals; however, there were differential responses suggesting that the T cells each peptide preferentially stimulated may represent different T cell populations, perhaps with different donor-dependent precursor frequencies. This idea was further supported by the marked increase in population coverage observed when the peptides were used in combination with one another. As shown in Table II, a mixture of three different SPHERE peptides (SP-1, SP-2, and SP-3) achieved nearly a 90% response rate in vitro. These data strongly support the utility of a SPHERE peptide mixture to overcome tolerance to a nonmutated self Ag to which the majority of individuals would be tolerant.

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### Table I. Reactive peptide sequencing results

<table>
<thead>
<tr>
<th>Peptide Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>ITDQVFSV</td>
</tr>
<tr>
<td>SP-1</td>
<td>FLDVQFSV</td>
</tr>
<tr>
<td>SP-2</td>
<td>FLDQVFSV</td>
</tr>
<tr>
<td>SP-3</td>
<td>FLDVQFSV</td>
</tr>
<tr>
<td>SP-4</td>
<td>FLRQVFSV</td>
</tr>
<tr>
<td>SP-5</td>
<td>FLDQVFSV</td>
</tr>
<tr>
<td>SP-6</td>
<td>FLDQVFSV</td>
</tr>
<tr>
<td>SP-7</td>
<td>FLDQVFSV</td>
</tr>
</tbody>
</table>

a The sequence of the native epitope is shown at the top of the table. SPHERE peptide sequences corresponding to the seven most reactive peptides based on the primary screen are shown below the native peptide and designated as SP-1 SP-7. Sequence data was generated as described in Materials and Methods.
SPHERE peptide-educated T cells demonstrate exquisite specificity for the naturally processed and presented native epitope

Given the divergence of the SPHERE peptide sequences from the native epitope, we sought to determine the specificity of the T cells educated with these peptides. To this end, we tested in vitro-educated normal donor T cells for reactivity to a human lung tumor cell line (A549), which does not express HLA-A2 or gp100 (Fig. 7). When the cell line was converted to express either HLA-A2 or gp100 wild-type protein, the SPHERE peptide-educated T cells lysed the modified A549 cells.

Table II. SPHERE peptides synergize when used in combination to increase normal donor responder population coverage in vitro

<table>
<thead>
<tr>
<th>Educating peptides</th>
<th>Educating peptides(s) (%)</th>
<th>Wild-type peptide (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>3/11 (27)</td>
<td>3/11 (27)</td>
</tr>
<tr>
<td>SP-1</td>
<td>12/17 (71)</td>
<td>9/17 (53)</td>
</tr>
<tr>
<td>SP-2</td>
<td>12/14 (86)</td>
<td>9/14 (64)</td>
</tr>
<tr>
<td>SP-3</td>
<td>10/12 (83)</td>
<td>8/12 (67)</td>
</tr>
<tr>
<td>SP-1/2/3 combination</td>
<td>8/8 (100)</td>
<td>7/8 (88)</td>
</tr>
</tbody>
</table>

In vitro T cell education experiments were carried out as described in Fig. 6 using 20 normal donors. Peptides were used either individually or in a three-peptide combination. The table shows the percentage of donors that responded to the educating peptide(s) and the reactivity to wild-type peptide-pulsed T2 cells. In order to be scored as reactive, lysis of gp100209–217-pulsed T2 cells had to be at least 20% after subtracting mock-pulsed target controls for the highest E:T tested in the experiment. Data was tabulated from the average of four replicates for each data point.

FIGURE 6. SPHERE peptides give rise to T cells that recognize the native epitope. Normal donor T cells were educated in vitro with SPHERE peptide or wild-type gp100209–217 peptide-pulsed autologous DCs as described in Materials and Methods. After five weekly stimulations, bulk T cell cultures were tested for their ability to lyse 51Cr-labeled T2 cells pulsed with the native peptide. For all assays, total peptide concentration was kept constant so that peptide combinations contained two-thirds less of each individual peptide compared with when they were used separately. All data points represent the average of four replicates and background lysis, as determined using T2 cells pulsed with equivalent amounts of DMSO containing no peptide, was subtracted out.

FIGURE 7. SPHERE peptide-educated normal donor T cells lyse targets expressing wild-type gp100 in an HLA-A2-specific manner. Normal donor T cells were educated in vitro with SPHERE peptide or wild-type gp100209–217 peptide-pulsed autologous DCs as described in Materials and Methods. After five weekly stimulations, bulk T cell cultures were tested for their ability to lyse the lung cancer cell line A549 infected with adenoviruses expressing HLA-A2 and/or gp100 wild-type protein. The cells were infected with the viruses at a multiplicity of infection of 25 for 48 h and labeled with 51Cr. The in vitro-educated bulk T cell cultures were added at an E:T of 75:1, using 2e + 3 targets. Percentage of specific lysis was calculated as described in Materials and Methods.
diversity can produce functionally indistinguishable HLA-restricted, Ag-specific responses and that naturally processed and presented peptide from the native Ag can render tumor cells susceptible to lysis by these effectors. Note that the levels of HLA-A2 and gp100 proteins expressed by infected A549 cells is lower than the endogenously expressed proteins in the HLA-A2\textsuperscript{+}/gp100\textsuperscript{+} melanoma cell line, MEL624, as measured by FACS analysis using specific Abs (data not shown).

**Discussion**

We have demonstrated the validity of the SPHERE method by screening a library of ~47 million peptides for those that react with an HLA-A2-restricted CD8\textsuperscript{+} T cell line with known specificity for the 209–217 peptide of the human melanoma Ag, gp100. We have demonstrated the capability of SPHERE to quickly and efficiently identify reactive epitope mimics that reliably give rise to T cells that cross-react well with their native epitope counterpart. This screening methodology offers several advantages over other epitope identification strategies: 1) limiting the library complexity by fixing residues that confer tight binding to the HLA-A2 molecule enables complete library screening; 2) quantitative ranking of all peptides during the screening process to readily identify peptides with the most desirable activity; 3) modest consumption of T cells (1E5 T cells per 1E6 peptides screened); 4) completion of the entire screen in just a few days; and 5) reusability of a single library (~250 times). Because our primary screen is performed at relatively low peptide complexity, it is statistically unlikely that more than one reactive peptide will be present in the same peptide pool, and this was confirmed upon deconvolution. Therefore, we obtain a quantitative ranking of all reactive peptides present in the library directly from the primary screen. Another reason that we maintain low peptide complexity is to remain statistically confident that a strongly inhibitory peptide will not mask the activity of a potent agonist.

The HLA-A2 library is biased toward hydrophobicity, because every peptide contains at least one F, L, and V. This bias inevitably leads to differences in solubility; and therefore, bioavailability in the assay. To the extent possible, we attempt to minimize these effects by using 100% DMSO to resuspend our solution-phase peptide pools and perform all peptide pulsing in at least 2% DMSO, a concentration tolerated quite well by the cells in our assay. Given the hydrophobic peptide-binding pocket of the HLA-A2 molecule, it was not entirely unexpected that the most reactive peptides identified in the screen were extremely hydrophobic, generally with a preference for hydrophobic residues throughout. We note that although this hydrophobicity complicates synthesis and purification of these peptides, it may ultimately be beneficial for in vivo vaccination. Several groups have reported that covalent modification of peptides with hydrophobic lipid moieties or noncovalent association of peptides with lipid vesicles can markedly improve their immunogenicity (31, 32).

Analyses of the sequences of the reactive epitope mimics identified reveal that the limits of antigenic mimicry are far reaching. Although some of the reactive peptides bear a strong resemblance to the native epitope, none could have been predicted by rational design methods. Other peptides, being unrelated to the native epitope although structurally related to one another, are distinctly nonobvious from a rational design perspective and challenge our current understanding of epitope structure and function. Furthermore, even the structurally divergent epitope mimics identified are reactive with an independently derived T cell clone specific for the same native epitope. We interpret this finding to mean that either these peptides share a common topology with the native epitope when bound to the MHC, or that they bind differentially to the MHC and/or the TCR.

The clinical utility of the epitope mimics identified by SPHERE is underscored by their ability to induce native epitope-specific CTL responses from normal donor T cells in vitro. This feature distinguishes SPHERE peptides from rationally designed anchor-modified epitope mimics that have been previously reported (12, 13). Structural diversity notwithstanding, it seems likely that the different SPHERE peptides preferentially engage different, yet functionally convergent, T cell populations in each normal donor. This may be the basis by which they can elicit immunologic responses from normal donors in vitro that seem unable to mount a response to the wild-type epitope. Analyzing TCR V\textsubscript{8} usage within the in vitro-educated bulk cultures of normal donor T cells by PCR analysis has further supported this hypothesis (data not shown). If the peptides are acting through different mechanisms, and considering that not every individual responded to every SPHERE peptide, we reasoned that there might be a benefit to using them in combination. We found that we could more reliably induce a wild-type peptide-specific T cell response from normal donor T cells when a combination of peptides was pulsed onto the same DC population and used to stimulate them. These responses were generally similar in magnitude to using any one peptide, even though the total peptide concentration of the mixture was the same as the individual peptide concentrations. Recently, Wang et al. (33) reported an interesting phenomenon that may account, at least in part, for these observations. Their data showed that DCs pulsed simultaneously with different peptide epitopes could prime an immune response in mice more efficiently than either peptide alone. In fact, they could do so even in the absence of CD4\textsuperscript{+} helper activity, which was required when the peptides were pulsed individually. It was concluded that different MHC class I-restricted peptides, when simultaneously present on the same DC, can provide helper function for each other in the absence of activated CD4\textsuperscript{+} T cells.

In conclusion, SPHERE can be used to rapidly identify or optimize MHC class I-restricted epitopes. It is a convenient way to reliably identify peptides that represent different “flavors” of the same native epitope which, as we have demonstrated, may allow for the formulation of vaccines capable of circumventing self tolerance or overcoming immunologic ignorance. This technology will find applications in tumor and infectious disease immunotherapy. Additionally, we note that the SPHERE screen can be modified to identify specific epitope antagonists which may find utility in the development of therapeutics for autoimmune disease or allotransplantation.

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