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Competition Between CTL Narrows the Immune Response Induced by Prime-Boost Vaccination Protocols

Michael J. Palmowski,²* Ed Man-Lik Choi,²* Ian F. Hermans,¹ Sarah C. Gilbert,³ Ji-Li Chen,¹
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Recombinant vaccines encoding strings of virus- or tumor-derived peptides and/or proteins are currently being designed for use against both cancer and infectious diseases. These vaccines aim to induce cytotoxic immune responses against several Ags simultaneously. We developed a novel tetramer-based technique, based on chimeric HLA A2/H-2Kb H chains, to directly monitor the CTL response to such vaccines in HLA-A2 transgenic mice. We found that priming and boosting with the same polyepitope construct induced immune responses that were dominated by CTL of a single specificity. When a mixture of viruses encoding single proteins was used to boost the polyepitope primed response, CTL of multiple specificities were simultaneously expanded to highly effective levels in vivo. In addition, we show that a preexisting response to one of the epitopes encoded within a polyepitope construct significantly impaired the ability of the vaccine to expand CTL of other specificities. Our findings define a novel vaccination strategy optimized for the induction of an effective polyvalent cytotoxic response. The Journal of Immunology, 2002, 168: 4391–4398.

Recent advances in our ability to detect and isolate Ag-specific CTL (1–4) have accelerated the development of new vaccines targeting both infectious disease and cancer. These vaccines aim to induce the expansion of CTL specific for virus- or tumor-derived peptides (CTL epitopes) bound to MHC class I molecules.

It has become clear that some of the most successful vaccination protocols for inducing epitope-specific CTL are the heterologous prime-boost protocols, involving sequential injections of different vectors encoding the same recombinant Ag. These protocols focus the CTL response toward peptides within the recombinant Ag, which are the only CTL epitopes shared by the different agents (5). Recent results have demonstrated that priming with plasmid DNA and boosting with recombinant modified vaccinia Ankara (MVA)⁵ generate high levels of specific immunity (6–9).

To minimize the generation of virus or tumor Ag loss variants (10–12), it would be desirable to induce CTL responses against a broad range of different epitopes, preferably encoded by distinct proteins. This rationale has led to the generation of polyvalent vaccines encoding strings of CTL epitopes (polyepitope vaccines) and/or proteins. Due to the large number of well-characterized HLA-A2 binding tumor- and virus-derived peptides, polyepitope vaccines are often designed to include peptides that can be presented by A2 to responding CTL. The availability of mice transgenic for A2 has allowed some preclinical testing of the efficacy of these vaccines. Polyepitope vaccine constructs are capable of priming multiple CTL specificities in A2 transgenic mice (13–16) and also of expanding single CTL specificities to high numbers in nonhuman primates (8, 17). However, evidence is lacking that polyepitope constructs are capable of expanding CTL of multiple different specificities to effective levels. Systematic comparison of the efficacy of different polyvalent vaccination strategies has also been hampered by the technical limitations of assays for directly monitoring CTL responses in A2 transgenic mice (18).

We reasoned that the use of polyepitope vaccines in a prime-boost strategy might lead to dominant expansion of CTL with a narrow CTL repertoire, as a result of the preferential expansion of immunodominant CTL responses. The identity of the mechanisms responsible for the expansion of immunodominant CTL responses is the subject of ongoing debate. Recent results have demonstrated that high-affinity CTL are capable of down-modulating peptide-MHC complexes on APCs (19), providing a mechanism for the preferential expansion of high-affinity CTL during a secondary T cell response.

To address our hypothesis, we developed a novel technique, based on the use of chimeric A2/H-2Kb (Kb) class I tetramers, for directly monitoring A2-restricted CTL responses in the blood of A2 transgenic mice. This technique has allowed us to accurately and rapidly monitor the frequency of CTL induced by prime-boost regimens using a polyepitope construct designed for clinical trial in melanoma patients. We compared protocols using a number of different vectors and correlated the frequency of CTL induced with their cytotoxic activity in vivo. The results suggest a new strategy for optimizing polyvalent CTL responses in human vaccine trials.
Materials and Methods

The mel3 polypeptide construct

The mel3 sequence encodes the following CTL epitopes in sequence: tyrosinase,366

Materials and Methods (MLLAVLYCL), melan-A38 (ELAGIGILTV) encoding an

analog peptide with a substitution at position 2 (A to L) (20), tyrosinase,

inserted into the tail vein. For priming or boosting with

ns250–370 (YMDDTFMSQV), linker (GS), Mage-367–175 (EVDPGILY), Mage-371–279 (FLWGPRLAV), Mage-161–169 (EADPTGHSY), linker (GS), NY-ESO-1377–163 (QLSSLLMVITQCFL), and influenza virus (flu)

nucleoprotein (NP)66–174 (ASNNMDAM).

The DNA vector pSC2, used throughout the study, was derived from

pRc/CMV (Invitrogen, Paisley, U.K.).

Generation of recombinant vaccinia virus and MVA

Recombinant MVA was made as described by cloning the mel3 polypeptide string into the vaccinia shuttle vector pSC11 (21). Vaccinia virus (WR strain) expressing mel3, full-length NY-ESO-1 (kindly provided by D. L. Panicali, Therion Biologics, Cambridge, MA), NY-ESO-1157–163 minigen encoding the NY-ESO-1 peptide SLLMWITQCFL, or tyrosinase were made by cloning each insert into the thymidine kinase gene using the vector pSC11 as previously described (22).

Generation of recombinant SFV

The mel3 polypeptide string was cloned into the transfer vector pSFV4.2-mel3 (23). RNA produced from this vector was used to construct recombinant SFV • mel3 particles. Recombinant Semliki Forest virus (SFV) stocks were made and purified as described previously (23).

Tetramer synthesis

Tetrameric A2/peptide complexes were synthesized as previously described (1). A2/Kb/peptide tetramers were synthesized using chimeric H chain with α1 and α2 domain from the A2 molecule and the α3 domain from the Kb molecule. The Kb α3 domain was amplified by PCR using forward primer 5′-AAGGAGACGCTGCAAGGACGATTCCCAAAAG GCCCATGTTACC-3′ and reverse primer 5′-CGATCCCCGCTCTCCA TCTCAGGGTGAGGCCT-3′. This PCR product was then used as reverse primer together with the forward primer 5′-GGGGGCGATTCCGGTGTTCTCA TTCTCATGAGATATTTCTTCACATCCGTG-3′ to generate the full-length A2/Kb hybrid H chain.

Inhibition of A2Kb tetramer staining by anti-CD8 Abs and A2/Kb monomers

NY-ESO-1157–163-specific CTL were incubated with 12 ng/μl A2/Kb tetramers at 22 °C for 3 h in the presence of different monomer concentrations. Cells were then incubated for 15 min on ice with CD8α Ab clone (CT; Caltag Laboratories, Silverstone, U.K.). Cells were washed twice and resuspended in PBS for FACS analysis. For CD8 blocking experiments, CD8α-specific Abs were added either before or after 20 min of tetramer staining at 37°C.

Isolation of PBL and tetramer staining

Fresh PBL were isolated from blood taken from the tail vein using red cell lysis buffer (Invitrogen). For tetramer staining, 3 × 106 cells were resuspended in 20 μl RPMI 1640 (Sigma-Aldrich, St. Louis, MO) supplemented with 10% FCS. Cells were incubated with tetramer for 15 min at 37°C. PBL were then incubated with rat anti-mouse CD8α (BD PharMingen, San Diego, CA) for 15 min at 4°C. Cells were washed twice in PBS and resuspended in PBS for FACScan (BD Biosciences, Mountain View, CA) analysis.

A2 transgenic mice

A2-Kb mice express chimeric A2 α1 and α2 domains fused with the Kb α3 domain. They express the endogenous H-2b class I molecules (24). HHD mice express a transgenic chimeric monochain class I molecule in which the C terminus of the human β2-microglobulin (β2-m) is covalently linked to the N terminus of chimeric A2 α1 and α2 domains fused with the Dα3 domain (14). HHD H-2Dd and mouse β2-m genes were disrupted by homologous recombination, resulting in complete lack of serologically detectable cell surface expression of mouse endogenous H-2b class I molecules.

Immunization protocols

Plasmid DNA for injection was purified using anion-exchange chromatography (Qiagen, Hilden, Germany) and diluted in PBS at 1 mg/ml DNA. DNA (25–50 μg) was injected into each musculus tibiaus under general anesthesia. Ten days after DNA injection, mice were boosted with 108 PFU of recombinant MVA or vaccinia viruses, which were diluted in PBS and injected i.v. into the lateral tail vein. For priming or boosting with mel3-SFV, 105 virus particles were diluted in sterile PBS and injected into the lateral tail vein. For the polyvirus boosting protocol, shown in Fig. 7, mel3-DNA mice were injected with a mixture of 108 mel3-SFV, 105 NY-ESO-1 vaccinia, and 105 tyrosinase vaccinia in a total volume of 300 μl.

For the experiment shown in Fig. 6, freshly isolated splenocytes were infected at a multiplicity of infection of 5 with mel3-VAC for 90 min in RPMI 1640 supplemented with 0.1% BSA at 37°C. To ensure that the virus was unable to replicate in vivo, mel3-VAC was UV inactivated as described previously (25). The inability of UV-inactivated virus particles to replicate was routinely confirmed using a plaque assay. Cells were then washed three times, resuspended in sterile PBS, and injected into lateral tail vein. Mice received a total of 5 × 105, 5 × 106, or 5 × 107 splenocytes. For the experiment shown in Fig. 4, mice primed with flu virus A (H17) were infected by intranasal injection (20 U of hemagglutinin per mouse). One month later mice were injected with mel3-DNA followed by mel3-MVA, as described above.

Ex vivo tetramer analysis was conducted 10 days after boosting with either recombinant viruses or infected splenocytes.

In vivo killing assay

Pools of freshly isolated splenocytes from HHD mice were separately incubated in RPMI 1640 medium with different peptides at a concentration of 10−7 M for 2 h. Each cell pool was then labeled with a different concentration of CFSE (Molecular Probes, Eugene, OR) to allow simultaneous tracking of the different populations in vivo (Ref. 26 and I. F. Hermans, J. Yang, and F. Ronchese, unpublished results). Labeled cells were pooled and injected at 105 cells/mouse into the tail vein. A control population without peptide that had been labeled with 5-(and-6-)((4-chloromethyl)benzoyl)amino)tetramethylrhodamine (CellTracker Orange; Molecular Probes) was coincubated to assess killing of peptide-pulsed targets relative to unpulsed cells. Mice were bled at the time of injection of fluorochrome-labeled targets to determine their CTL frequencies to different epitopes. Disappearance of peptide/fluorochrome-labeled cells was tracked using FACS analysis of freshly isolated PBL 5 h after the injection. The level of specific cytotoxicity was calculated relative to the unpulsed population labeled with Cell Tracker Orange using the following calculation: 100 − (100 × (percentage pulsed/percentage unpulsed)). WinMDI 2.8 software (J. Trotter, http://facs.scripps.edu) and CellQuest 3.3 software (BD Biosciences) were used to analyze the FACS data.

Results

Expansion of large numbers of Ag-specific CTL by several distinct prime-boost vaccination strategies

A string of five HLA-A2 and two HLA-A1 melanoma epitopes (mel3) was cloned into four distinct vectors: 1) naked plasmid DNA (mel3-DNA), 2) vaccinia virus (mel3-VAC), 3) MVA virus (mel3-MVA), and 4) SFV (mel3-SFV). To allow monitoring of CTL responses restricted by human as well as mouse class I molecules, we introduced an additional epitope from the flu NP restricted by H2-Db (Db) class I molecules (NP66–174, see Materials and Methods for list of epitopes in mel3).

We and others have previously demonstrated that optimal flankin residues are important to ensure expression of class I-restricted epitopes (22, 27). To assess whether mel3 peptide epitopes were properly processed, and whether competition for binding to MHC class I molecules impaired CTL recognition of lower-affinity epitopes, we infected target cells with mel3-MVA. Each of the seven epitopes contained within the polyepitope mel3 cassette was simultaneously presented to specific CTL as measured by specific lysis (Fig. 1).

Efficient presentation of the flu NP66–174 epitope by mel3-MVA-infected cells prompted us to assess in C57BL/6 (B6) mice the ability of different vaccination strategies to induce a strong flu NP66–174-specific CTL response (Fig. 2). We monitored the CTL response ex vivo by direct staining of PBL with Db tetramers to enumerate Ag-specific CTL. Frequencies of tetramer-positive cells were calculated as a percentage of total CD8-positive
cells. Heterologous prime-boost vaccination strategies (Fig. 2B) were capable of inducing vaccine-driven CTL responses, with frequencies up to 100 times greater than frequencies obtained by strategies based on a homologous Ag delivery system (Fig. 2A). Repeated injections of the same immunogen into B6 mice led to the expansion of flu NP-specific CTL ranging from 0.5 to 3% of CD8\(^+\) cells, while the injection of heterologous Ag delivery systems led to much higher frequencies ranging from 40 to 70% of CD8-positive CTL. All tetramer\(^+\) cells were CD69\(^{high}\) and CD62L\(^{low}\) (data not shown). Similar results were obtained in the spleen and blood, although we observed that the percentage of tetramer\(^+\) CD8\(^+\) cells was higher in the blood than in the spleen (data not shown), consistent with recently published results (28).

**A2/K\(^{b}\) tetramers have a higher binding affinity for A2-restricted mouse CTL than wild-type A2 tetramers**

To test the ability of the mel3 polyepitope constructs to prime A2-restricted CTL responses in vivo, A2 transgenic mice were primed by mel3-DNA and boosted by mel3-MVA or mel3-VAC. Initial experiments were conducted using transgenic mice, which express chimeric A2 molecules containing the H-2K\(^{b}\) (K\(^{b}\))/H9251 domain (A2-K\(^{b}\)), and endogenous D\(^{b}\) and K\(^{b}\) molecules (24) (A2/K\(^{b}\) mice). A2/K\(^{b}\) mice can respond to peptides presented by D\(^{b}\) and K\(^{b}\) as well as A2.

We routinely use A2 tetramers to characterize A2-restricted CTL responses in humans (2, 3, 11, 29). Previous results showed that A2 tetramers were inefficient at detecting A2-restricted CTL in A2/K\(^{b}\) mice (28). Because these findings could be accounted for by the lack of mouse CD8 binding to human A2 molecules (30), we engineered a chimeric A2/K\(^{b}\) class I molecule containing the mouse K\(^{b}\) domain and compared A2/K\(^{b}\) and A2 tetramers for their ability to stain PBL of A2/K\(^{b}\) transgenic mice immunized with a combination of DNA and vaccinia encoding the NY-ESO-1\(_{157-165}\) epitope (Fig. 3A). While wild-type A2 tetramers failed to detect Ag-specific CTL in seven of eight immunized mice, A2/K\(^{b}\) tetramers were capable of detecting Ag-specific CTL in all responding mice (Fig. 3A and data not shown). These results were

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**FIGURE 1.** Efficient processing and presentation of mel3 epitopes. B cells were infected with mel3·MVA and used as targets of CTL clones specific for each epitope contained within the mel3 construct. Specificity of each CTL clone and percentage of specific lysis are shown in each panel. Target cells were pulsed with relevant peptide (+ peptide), unpulsed (- peptide), or infected either with mel3-MVA or an irrelevant vaccinia (irr vac). Flu NP\(_{366-374}\)-specific CTL were used as effector cells against mouse MC57 fibroblasts infected with mel3-MVA. Each bar corresponds to a different E:T ratio: filled bars, 10:1; open bars, 3:1; dotted bars, 1:1.

**FIGURE 2.** Ex vivo frequency of flu NP\(_{366-374}\)-specific CTL homologous vs heterologous prime-boost vaccination. Each panel shows the percentage of flu NP\(_{366-374}\)-specific CTL out of CD8\(^{+}\) cells in PBL of B6 mice primed and boosted with the shown immunogens. Homologous (A) vs heterologous (B) vaccination protocols are shown.
of murine CD8 with the H9251 elicit for the NY-ESO-1 157-165 epitope were stained ex vivo with either A2/Kb NY-ESO-1 157-165 tetramers (left panel) or A2 NY-ESO-1 157-165 tetramers (right panel). Percentage of NY-ESO-1 157-165-specific CD8+ PBL is shown. A representative staining of a single mouse of 10 mice is shown. B, Binding of A2/Kb tetramer to A2-restricted mouse CTL is CD8 dependent. NY-ESO-1 157-165-specific CTL line derived from A2/Kb transgenic mice was stained with A2/Kb NY-ESO-1 157-165 tetramer followed by anti-CD8 Abs (left panel) or with anti-CD8 Abs followed by A2/Kb NY-ESO-1 157-165 tetramers (right panel). The percentage of NY-ESO-1 157-165-specific CD8+ cells is shown. C, A2/Kb monomers inhibit staining of A2/Kb tetramers. NY-ESO-1 157-165-specific CTL line derived from A2/Kb transgenic mice was stained with fluorochrome-labeled A2/Kb tetramer in the presence of increasing amounts of unlabeled NY-ESO-1 157-165 A2 (○) or A2/Kb (▲) monomers.

FIGURE 3. Enhanced detection of NY-ESO-1 157-165-specific CTL in A2 transgenic mice by A2/Kb tetramers. A, A2/Kb tetramers are more sensitive staining reagents than A2 tetramers. PBL from A2/Kb mice primed with DNA and boosted with vaccinia virus encoding the NY-ESO-1 157-165 epitope were stained ex vivo with either A2/Kb NY-ESO-1 157-165 tetramers or A2 NY-ESO-1 157-165 tetramers. Frequency of tetramer-positive CD8+ cells is shown in each vaccinated mouse after MVA boost. B, FLU memory CTL significantly reduce frequency of melan-A26-35-specific CTL in A2/Kb mice immunized with mel3. A2/Kb mice were preimmunized with FLU virus and subsequently injected with mel3DNA followed by mel3-MVA. Ex vivo frequency of D^bNP366-374 and A2/melan-A26-35 tetramer-positive CD8+ cells is shown in each vaccinated mouse after MVA boost. Bars indicate the frequency of tetramer+ cells in individual mice.

Priming of A2/Kb mice with mel3-DNA, followed by mel3-MVA, induced a dominant D^b-restricted flu NP366-374-specific response and a much weaker A2-restricted CTL response to melan-A26-35 (Fig. 4). The ability to detect these simultaneous CTL responses allowed us to study whether previous exposure to FLU virus would compromise the ability of prime-boost protocols to expand melan-A26-35-specific CTL in A2/Kb mice. To preimmunize against the NP366-374 epitope, A2 transgenic mice received a single injection of flu virus H17. They subsequently received mel3-DNA followed by mel3-MVA (Fig. 4). Expansion of NP366-374-specific CTL, before vaccination with mel3 polyepitope constructs, reduced the expansion of melan-A26-35-specific CTL, because 7 of 10 immunized mice failed to have melan-A26-35-specific CTL detectable by tetramer staining (Fig. 3). These results were extended to other CTL responses by demonstrating that the presence of preexisting NY-ESO-1 155-165-specific CTL was capable of inhibiting the expansion of melan-A26-35-specific CTL in mice immunized with mel3-VAC (data not shown).

Prime-boost vaccination of HHD mice induces the expansion of a narrow repertoire of melanoma-specific CTL

Because a preexisting flu NP-specific CTL response inhibited the induction of a melan-A-specific CTL response (Fig. 4), we speculated that T cell interference might compromise the induction of a broad immune response in polyvalent prime-boost regimens. Studying the interplay between A2-restricted responses proved difficult in A2/Kb mice, because we could only rarely detect priming to multiple A2-restricted epitopes in these mice (data not shown). This observation is consistent with previous reports suggesting that the presence of the endogenous mouse class I molecules significantly narrows the A2-restricted repertoire (14). Furthermore, a D^b-restricted NP366-374-specific CTL response induced by our consistent with the possibility that A2/Kb tetramers have a higher binding affinity for mouse A2-restricted CTL due to the interaction of murine CD8 with the α3 domain of the A2/Kb tetramer. To test this hypothesis, we generated a CTL line from A2 transgenic mice specific for the NY-ESO-1 157-165 epitope and demonstrated that binding of A2/Kb tetramers to the NY-ESO-1 157-165-specific CTL line was CD8 dependent, as shown by the lack of tetramer staining in the presence of anti-CD8 Abs (Fig. 3B). Furthermore, we showed that monomeric A2/Kb molecules were capable of inhibiting staining of the NY-ESO-1 157-165-specific CTL line by A2/Kb NY-ESO-1 157-165 tetramers (Fig. 3B). In contrast, A2 NY-ESO-1 157-165 monomers failed to inhibit binding of A2/Kb tetramers to the NY-ESO-1 157-165-specific CTL line (Fig. 3C).

Expansion of A2- and D^b-restricted CTL in A2/Kb transgenic mice

The creation of the A2/Kb tetramer enabled us to accurately measure ex vivo the frequency of A2-restricted CTL in comparison to the frequency of D^b-restricted flu NP366-374 epitope-specific CTL.

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polypeptide construct in these mice might interfere with the generation of A2-restricted CTL. This reasoning led us to vaccinate the HHD strain of A2 transgenic mice, which lack endogenous class I molecules. HHD mice have a much larger A2-restricted T cell repertoire than A2/Kb transgenic mice (14) because, unlike A2/Kb transgenic mice, they express chimeric human/mouse class I molecules linked to human \( \beta_2\)-m in a Db\(^{-/-}\) and mouse \( \beta_2\)-m\(^{-/-}\) background.

Priming of HHD mice with mel3-DNA led to the expansion of melan-A\(_{26-35}\) specific CTL to frequencies detectable by ex vivo tetramer staining in all vaccinated mice (Fig. 5 and data not shown). In contrast, expansion of NY-ESO-1\(_{157-165}\) and tyrosinase\(_{369-377}\) specific CTL was detectable only occasionally, while responses to the tyrosinase\(_{1-6}\) and MAGE-\(_{271-279}\) were not detected ex vivo in blood or spleen by tetramer staining (data not shown).

However, although tetramers were rarely able to detect NY-ESO-1\(_{157-165}\) and tyrosinase\(_{369-377}\) specific CTL after DNA priming, additional experiments confirmed that these responses were indeed primed by mel3-DNA injection, as shown by the significant NY-ESO-1\(_{157-165}\) and tyrosinase\(_{369-377}\) CTL responses in mel3-DNA-primed mice boosted with vaccinia virus encoding the full-length NY-ESO-1 and tyrosinase proteins. In contrast, injection of NY-ESO-1 and tyrosinase vaccinia viruses into naive mice led to a much lower frequency of NY-ESO-1\(_{157-165}\) and tyrosinase\(_{369-377}\) specific CTL (Fig. 6 and data not shown).

The observation that melan-A\(_{26-35}\) specific CTL comprised the dominant CTL response after a single DNA vaccination presented an opportunity to study the interplay between CTL specific to different vaccine encoded determinants in polyvalent prime-boost vaccination protocols. We observed that boosting of melan3-DNA-primed HHD mice with melan3-MVA (Fig. 7A), SFV-melan3 (Fig. 7B), or mel3-VAC (Fig. 7C) led to the expansion of melan-A\(_{26-35}\)-specific CTL, up to 80% of total CD8\(^+\) T cells.

### FIGURE 5

**mel3-DNA priming of HHD mice leads to the preferential expansion of melan-A\(_{26-35}\)-specific CTL.** HHD mice were immunized with mel3-DNA. Frequency of melan-A\(_{26-35}\), tyrosinase\(_{369-377}\), and NY-ESO-1\(_{157-165}\) specific responses were simultaneously measured in four mice by ex vivo tetramer staining. Percentages of tetramer-positive CD8\(^+\) PBL are shown.

### FIGURE 6

**Ability of mel3-DNA to prime NY-ESO-1\(_{157-165}\) and tyrosinase\(_{369-377}\) specific CTL response.** HHD mice were immunized either with NY-ESO-1 vaccinia and tyrosinase vaccinia (A) or with mel3-DNA followed by NY-ESO-1 vaccinia and tyrosinase vaccinia (B). NY-ESO-1\(_{157-165}\) and tyrosinase\(_{369-377}\) specific CTL responses were monitored by ex vivo tetramer staining. Percentages of tetramer-positive CD8\(^+\) PBL are shown.

### Competition of vaccine-driven CTL for mel3-expressing APCs

Because a preexisting flu memory CTL response can inhibit expansion of melan-A\(_{26-35}\)-specific CTL in A2/Kb mice (Fig. 4), we investigated whether the higher numbers of melan-A\(_{26-35}\)-specific CTL, dominating the immune response after DNA priming (Fig. 5), were capable of interfering with the expansion of NY-ESO-1\(_{157-165}\) and tyrosinase\(_{369-377}\)-specific CTL during virus boosting in HHD mice.

Competition for Ag recognition on the surface of APCs may lead to the immunodominance of higher-frequency CTL populations (31-33), and we reasoned that large numbers of melan-A\(_{26-35}\)-specific CTL after melan3-DNA priming could result in either rapid killing (34) or shielding of mel3 expressing APC during boosting in vivo. This may hamper stimulation of CTL specific to NY-ESO-1\(_{157-165}\) and tyrosinase\(_{369-377}\) epitopes expressed by the same APC population during the boosting phase.

If CTL are competing for access to APC, then the injection of increasing numbers of mel3-expressing APC should reduce CTL competition and induce stronger NY-ESO-1\(_{157-165}\) and tyrosinase\(_{369-377}\)-specific CTL responses. To address this hypothesis, mel3-DNA-primed mice were injected with increasing numbers of mel3-VAC-infected splenocytes. To ensure mel3-VAC was UV inactivated prior infection of splenocytes (see Materials and Methods). The results of these experiments demonstrated that, while the injection of \( 5 \times 10^5 \) mel3-VAC-infected splenocytes resulted in almost complete dominance of melan-A\(_{26-35}\)-specific CTL (Fig. 8A), the injection of \( 5 \times 10^4 \) and \( 5 \times 10^3 \) cells was capable of boosting a strong CTL response to both NY-ESO-1\(_{157-165}\) and tyrosinase\(_{369-377}\) epitopes (Fig. 8, B
We tested different vaccine strategies for their ability to simultaneously induce CTL of multiple different specificities. Because a great number of characterized human CTL epitopes bind to the Ag-presenting molecule A2, we developed a novel technique for rapid characterization and comparison of A2-restricted responses in A2 transgenic mice. We engineered a chimeric A2 molecule containing the murine K\(^{\text{b}}\) α3 domain to make tetramers, because wild-type A2 tetramers could not be used to reliably monitor A2-restricted CTL in these mice (Fig. 3). As predicted, the binding of murine CD8 to the K\(^{\text{b}}\) α3 domain was a critical factor in the improved affinity of the A2/K\(^{\text{b}}\) tetramers for mouse A2-restricted CTL (Fig. 3).

**Polypeptide constructs can induce narrow CTL responses in prime-boost protocols**

Comparing several vaccination strategies using ex vivo tetramer staining, we confirmed that heterologous prime-boost protocols induce higher levels of vaccine-specific immune responses than immunizations based on the injections of homologous vectors (Fig. 2). After priming, any of the viral vectors we used (i.e., vaccinia, MVA, flu, or SFV) was capable of boosting CTL frequencies to very high levels (Fig. 2 and data not shown).

When the responses to different epitopes within the polypeptide construct were compared, it was clear that prime-boost protocols skewed the immune response heavily toward a single epitope within the epitope string. Initially this immunodominance was observed in A2/K\(^{\text{b}}\) mice where prime-boost vaccination with the polypeptide resulted in a dominant response to the D\(^{\text{b}}\)-restricted flu NP\(_{366-374}\) epitope (Fig. 4A). When HHD mice were vaccinated, to eliminate presentation of the flu NP\(_{366-374}\) epitope through D\(^{\text{b}}\), heterologous prime-boost vaccination resulted in a predominant expansion of melan-A\(_{26-35}\)-specific CTL, up to 80% of total CD8\(^{+}\) T cells (Fig. 7, A and B).

**Competition for APC is responsible for the narrow immune response to polypeptide vaccination**

By dissecting the interplay between CTL responding to different epitopes in the polypeptide construct, we were able to identify a mechanism controlling the higher frequency of melan-A\(_{26-35}\) response during prime-boost regimens.

**Discussion**

We generated a polypeptide vaccine construct containing a string of melanoma-derived peptides recognized by CTL. Using this construct we tested different vaccine strategies for their ability to simultaneously induce CTL of multiple different specificities. Because a great number of characterized human CTL epitopes bind to the Ag-presenting molecule A2, we developed a novel technique for rapid characterization and comparison of A2-restricted responses in A2 transgenic mice. We engineered a chimeric A2 molecule containing the murine K\(^{\text{b}}\) α3 domain to make tetramers, because wild-type A2 tetramers could not be used to reliably monitor A2-restricted CTL in these mice (Fig. 3). As predicted, the binding of murine CD8 to the K\(^{\text{b}}\) α3 domain was a critical factor in the improved affinity of the A2/K\(^{\text{b}}\) tetramers for mouse A2-restricted CTL (Fig. 3).

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**Competition for APC is responsible for the narrow immune response to polypeptide vaccination**

By dissecting the interplay between CTL responding to different epitopes in the polypeptide construct, we were able to identify a mechanism controlling the higher frequency of melan-A\(_{26-35}\) response during prime-boost regimens.
Several factors may contribute to the immunodominance of melan-A26–35-specific CTL during the priming phase (35). The use of the melan-A26–35 peptide analog with an A→L substitution at position 2 was shown to increase its binding affinity to A2 molecules and its immunogenicity in vivo (20). We have confirmed these results by showing that the modified melan-A26–35 peptide analog has a higher binding affinity than the other mel3-encoded peptides to A2 molecules, as defined by its ability to compete for recognition of flu matrix peptide 58–66 by flu matrix-specific CTL (data not shown). Skewing of the primary response (see Fig. 5) may have allowed melan-A26–35-specific CTL to prevent CTL with subdominant specificities from making contact with mel3-expressing APC during the boost. This possibility is consistent with previously published papers showing the interplay between CTL and APC in different models (31–33).

This rationale led to the hypothesis that weakly primed CTL responses (e.g., NY-ESO-1157–165 and tyrosinase369–377) should be successfully expanded by minimizing the competition with dominant melan-A26–35-specific CTL for APC during boosting. Consistent with this hypothesis, we showed that immunodominance of melan-A26–35-specific CTL could be broken upon injection of either increasing numbers of mel3-VAC-infected splenocytes (Fig. 8) or by injecting a mixture of recombinant viruses, each encoding different antigenic determinants (Fig. 9).

These results demonstrate that polyepitope prime-boost vaccination strategies result in the expansion of a narrow CTL repertoire, due to competition of CTL for APC. The results also define an alternative boosting strategy to the polyepitope prime-boost protocol, which overcomes T cell interference and ensures the expansion of a larger CTL repertoire.

**Implications for vaccination strategies in patients**

These results are of practical importance, because several clinical trials are currently using heterologous prime-boost vaccination protocols with polyepitope and/or polyprotein constructs (8, 36). To minimize the emergence of tumor and virus Ag escape variants, it is important to ensure that polyvalent vaccine protocols are capable of expanding CTL specific for multiple epitopes.

Our results reveal several important parameters that, if confirmed in clinical trials using polyepitope constructs, would need to be taken into account in designing optimal polyvalent vaccine strategies.

First, we demonstrated that preexisting memory CTL responses significantly reduce CTL responses specific to other epitopes contained within the same construct (Fig. 4). Several groups have included immunodominant flu CTL epitopes within polyvalent vaccines for use as positive controls during immuno-monitoring (37, 38). Our results suggest that DNA- or virus-based vaccines should not encode epitopes expressed by recurrent viruses, as preexisting memory CTL response may compromise the induction of CTL responses specific to other vaccine-encoded CTL determinants.

Second, we have demonstrated that the alphavirus SFV can be used both as a priming vector in combination with MVA (Fig. 2) and for boosting in combination with DNA (Fig. 7). Alphaviruses are currently being studied extensively as vectors in vaccination protocols (39–44), and our results confirm that recombinant SFV is very attractive in prime-boost protocols, particularly because recombinant SFV expresses few viral structural proteins and the chances of generating an immune response limited to recombinant proteins may be higher than with larger viral vectors.

Finally, we demonstrated that T cell competition is likely to play a role in modifying T cell responses in prime-boost vaccination strategies. Our results strongly suggest that simultaneous presentation of different epitopes to a skewed repertoire of primed CTL leads to dominant expansion of a single CTL specificity. However, boosting the primed response with APC separately presenting the epitopes results in comparable expansion of CTL of multiple specificities to effective levels in vivo (Figs. 4, 8, and 9).

In conclusion, we have developed a novel system for dissecting the ability of different vaccination protocols to optimally induce polyvalent A2-restricted CTL responses. Future clinical trials...
aimed at inducing a broad-based CTL response should consider restricting the use of polyvalent constructs to the priming phase of the protocol and using separate vectors encoding individual epitopes or proteins for the boosting phase.

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References


