Modification of a Tumor Antigen Determinant To Improve Peptide/MHC Stability Is Associated with Increased Immunogenicity and Cross-Priming a Larger Fraction of CD8+ T Cells

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Modification of a Tumor Antigen Determinant To Improve Peptide/MHC Stability Is Associated with Increased Immunogenicity and Cross-Priming a Larger Fraction of CD8+ T Cells

Alan M. Watson,* Lawrence M. Mylin,† Megan M. Thompson,†,2 and Todd D. Schell*

Altered peptide ligands (APLs) with enhanced binding to MHC class I can increase the CD8+ T cell response to native Ags, including tumor Ags. In this study, we investigate the influence of peptide–MHC (pMHC) stability on recruitment of tumor Ag-specific CD8+ T cells through cross-priming. Among the four known H-2b–restricted CD8+ T cell determinants within SV40 large tumor Ag (TAG), the site V determinant (ApqQG1NLDNAPQ) forms relatively low-stability pMHC and is characteristically immunorecessive. Absence of detectable site V–specific CD8+ T cells following immunization with wild-type TAG is due in part to inefficient cross-priming. We mutated nonanchor residues within the TAG site V determinant that increased pMHC stability but preserved recognition by both TCR–transgenic and polyclonal endogenous T cells. Using a novel approach to quantify the fraction of naive T cells triggered through cross-priming in vivo, we show that immunization with TAG variants expressing higher-stability determinants increased the fraction of site V–specific T cells cross-primed and effectively overcame the immunorecessive phenotype. In addition, using MHC class I tetramer–based enrichment, we demonstrate for the first time, to our knowledge, that endogenous site V–specific T cells are primed following wild-type TAG immunization despite their low initial frequency, but that the magnitude of T cell accumulation is enhanced following immunization with a site V variant TAG. Our results demonstrate that site V APLs cross-prime a higher fraction of available T cells, providing a potential mechanism for high-stability APLs to enhance immunogenicity and accumulation of T cells specific for the native determinant. The Journal of Immunology, 2012, 189: 000–000.

The T cell response to viral and tumor Ags is typically skewed toward a subset of immunodominant determinants. Such determinants have been the main focus of research for vaccine development and immunotherapy. However, T cells targeting subdominant determinants, which induce fewer responder T cells, can also play a role in the control of tumors (1, 2) and virus infections (3–5). Limited immunological tolerance toward subdominant determinants found in self-tumor–associated Ags in mice (6–8) and humans (9) makes them attractive targets for CD8+ T cell–based immunotherapy approaches. Understanding the mechanisms contributing to the subdominant phenotype may facilitate inclusion of T cells targeting a broader repertoire of determinants for vaccination and immunotherapy.

Expression of the SV40 large TAg (TAG) oncoprotein in primary mouse fibroblasts leads to cell immortalization and transformation in vitro and also serves as a tumor rejection Ag when these cells are injected into immunocompetent mice (10). The CD8+ T cell response to SV40 TAG in C57BL/6 mice is characterized by a reproducible immunodominance hierarchy directed toward four determinants, designated sites I, II/III, and IV and V (11). T cells specific for sites I, II/III, and IV are detected following immunization with wild-type TAG (wt-TAG), whereas the endogenous site V–specific T cell response is detected only following immunization with TAG variants lacking sites I, II/III, and IV or following immunization with a site V minigene-based vaccine (12, 13). We previously found that injection of site V–specific TCR–transgenic T cells (TCR-V) into C57BL/6 mice was not sufficient to overcome the weak response to site V in the context of wt-TAG immunization, indicating that precursor frequency is not the sole limiting factor (14). Rather, we found that site V is weakly cross-presented in vivo compared with the more dominant determinants from TAG. Based on previous data showing a kinetic difference in the stability of site V/H-2Dbeta complexes relative to peptide–MHC (pMHC) formed with the other known TAG determinants (13), we proposed that unstable pMHC interactions may contribute to limited cross-presentation and the immunorecessive phenotype of site V.

Amino acid substitutions within weakly immunogenic T cell determinants can lead to improved immunogenicity. Such altered peptide ligands (APLs) have been shown to induce higher magnitude CD8+ T cell responses, particularly in the setting of self-tolerance, and can increase CD8+ T cell–mediated antitumor...
immunity (15–18). APLs with engineered substitutions at TCR contact residues can directly enhance T cell activation by increasing TCR signal transduction (17). A second class of APLs effects residues that improve peptide affinity for MHC class I (MHC-I) and/or increase stability of pMHC. The basis for improved immunogenicity of this latter class of APLs may result from structural changes in the pMHC complex that improves TCR–pMHC affinity (19). Alternatively, changes in peptide affinity for MHC-I may promote higher levels of Ag presentation that could lead to more durable T cell–APC interactions and more efficient T cell activation (18, 20). Although evidence suggests that a minimal affinity for MHC-I is necessary for a peptide to be immunogenic (21, 22), multiple studies additionally show a strong correlation between pMHC stability (17) and immunogenicity (23–25). In fact, a recent extensive study by Harndahl and colleagues (26) provides strong support that pMHC stability can accurately predict immunogenicity. Thus, APLs containing amino acid substitutions that increase pMHC stability are likely to be more immunogenic, although no consensus has been reached regarding the basis for this effect.

Cell-associated Ags, such as tumor Ags, can induce CD8+ T cell responses by accessing the cross-presentation pathway that is active in professional APCs (pAPCs) (27, 28). Thus, pMHC stability could potentially impact the efficiency of cross-priming by influencing the level and/or duration of pMHC cross-presentation. We evaluated the extent to which site V APLs promote increased immunogenicity following expression of variant TAgS in C57BL/6 (B6) fibroblasts. Using newly defined APLs and a unique approach to monitor the initial recruitment of naive TCR–transgenic T cells, we find that APLs with enhanced pMHC stability increase the fraction of naive TCR–transgenic T cells recruited by cross-presentation and overcome the immunorecessive nature of site V by the endogenous T cells. In addition, using MHC tetramer enrichment, we show that wt-TAg immunization induces limited accumulation of endogenous site V–specific T cells and that this response is dramatically enhanced by immunization with APL-expressing TAg.

Materials and Methods

Mice

B6, B6.129S2-Tap11m1Ap, and B6.PL-Thy1a/CyJ (Thy1.1) mice were purchased from The Jackson Laboratory and bred on site. TCR-V (14) mice and site I–specific TCR–transgenic (TCR-I) (29) mice were described previously. TCR-V mice were bred onto the B6.SJL-Ptprc-PepckC.B6 (Pep-V), site I (SAINNYAQRKL, Pep-I), and a control H-2Dq–binding peptide corresponding to influenza virus nucleoprotein 366–374 (ASNNEMTEM; Pep-Flu). The following site V variant peptides were used: Q489A (AGINNADNL), G490A (QAINNADNL), 1491F (QGIFNADNL), N492A (QGIFANADLN), L494A (QGIFNADNL), D495A (QGIFNADNL), and N496A (QGIFNADNL).

Site-directed mutagenesis

Tag constructs expressing the Q489A mutation were produced via Quikchange II XL (Stratagene) site-directed mutagenesis using oligonucleotides 5’-TTGCT TCA GGT GCT GGA ATT AAT AAC CTC GAC-3’ (sense) and 5’-GTC CAG GAT TT AAT TCC AGC ACC TGA AGG CAA-3’ (antisense). G490A substitutions were introduced into SV40 TAg by the Altered Sites II mutagenesis procedure (11) (Promega) alone or in combination with MHC anchor residue alanine substitutions inactivating sites I, II, III, and IV. The mutagenic oligonucleotide encoding G490A was 5’-TTG CCT TCA GGT GCT GGA ATT AAT AAC CTC GAC-3’; and F408A, 5’-TCA GTG GTG TAT GAC GCT TTA AAA TGC ATG-3’; and F408A, 5’-TCA GTG GTG TAT GAC GCT TTA AAA TGC ATG-3’.

Synthetic peptides

Peptides were synthesized at the Penn State Hershey Macromolecular Core Facility using Fmoc chemistry. Peptides were dissolved in DMSO, then diluted to the appropriate concentration in RPMI 1640 medium with Glutamax (Invitrogen), and stored at −80°C. Peptides correspond to the SV40 TAg site V (QGIFNADNL; Pep-V), site I (SAINNYAQRKL, Pep-I), and a control H-2Dq–binding peptide corresponding to influenza virus nucleoprotein 366–374 (ASNNEMTEM; Pep-Flu). The following site V variant peptides were used: Q489A (AGINNADNL), G490A (QAINNADNL), 1491F (QGIFNADNL), N492A (QGIFANADLN), L494A (QGIFNADNL), D495A (QGIFNADNL), and N496A (QGIFNADNL).

Table I. Cell lines used in this study

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<th>Reference No.</th>
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Measurement of relative affinity and H-2D\(^d\) complex stability

Assays to estimate relative affinity have been previously described (36, 37). RMAs cells (38) were grown overnight at 26°C, 5% CO\(_2\), and then 5 \(\times\) 10\(^7\) cells were plated per well of a 96-well round-bottom plate and the indicated concentrations of peptide added. Cultures were incubated at 37°C, 5% CO\(_2\), for 4 h and then stained with 28-14-8s anti-\(\beta\)-mAb for 30 min at 4°C. Bound primary Ab was detected by addition of goat anti-mouse IgG-FITC (MP Biomedicalals) for 30 min at 4°C. Samples were collected on a FACS Canto instrument (BD Biosciences) and the geometric mean of FITC staining determined using FlowJo Software (Tree Star). To calculate the percent maximum mean fluorescence intensity (MFI), the following formula was used: 
\[
\text{MFI}_{\text{probe}} = \frac{\text{MFI}_{\text{probe}} - \text{MFI}_{\text{no peptide}}}{\text{MFI}_{\text{no peptide}}} 
\]

Centrifugation to pellet cells and debris. A total of 100 \(\mu\)l supernatant was determined, and a ratio of wt-TAg or Q489A cells, and at least 1 d prior to the experiment, mice received 10\(^5\) naive TCR-V cells i.v. Immunization plus adoptive transfer of TCR-V cells in positive control mice yielded detectable populations of both site I– and site V–specific T cells to facilitate identification of tetramer-positive cells during gating. A variation of previously published protocols (41–43) was used for tetramer enrichment. Briefly, on the day of enrichment, the spleen and superficial cervical, brachial, inguinal, lumbar, and mesenteric lymph nodes were harvested. Organs were mechanically disrupted, and the resulting suspension was incubated in RPMI 1640 supplemented with 150 U/ml collagenase (17018-029; Life Technologies) and 100 U/ml DNase I (04 536 282 001; Roche) at 37°C for 30 min with rocking. Tissues were passed through a 60-mesh screen, RBCs were lysed using Tris-ammonium-chloride, and the cells were washed once with FACS buffer. Cells were resuspended in 3 ml MACS buffer and passed over an LS column (Miltenyi Biotec) at 4°C. Columns were washed three times with 3 ml MACS buffer, and cells bound to the column were eluted twice with 5 ml MACS buffer. Eluted cells were washed with MACS buffer.

Eluted cells were stained in a 96-well plate with the following Abs: unlabeled anti-CD16/32, FITC DUMP (Abs to CD19, MHC class II, F4-80, CD11b, and CD4), anti-CD8-PerCP Cy5.5, anti-Thy-1.2-PE, FITC-V–PE, and Tet-I–allophycocyanin for 15 min at RT. Cells were washed three times with FACS buffer and resuspended in 2% paraformaldehyde. All samples were run on a BD FACS Canto II or LSRII (BD Biosciences). The entire eluted cell fraction was acquired. First, live cells were determined by FSC/A-SSC-A gating followed by gating on FITC-negative/CD8\(^+\) cells and then Thy-1.2\(^+\) cells. Dot plots were displayed as Tet-I versus Tet-V– cells. Tetramer-positive populations from positive control mice were used as a guide to set up the tetramer\(^\ast\) gates.

Statistics

All data are displayed as mean ± SD. The \(p\) values were evaluated using an unpaired Student \(t\) test unless otherwise noted.

Results

Identification of site V analogs with increased MH C stability

Immunogenicity requires that a peptide determinant have a minimum affinity for the presenting MHC molecule (21, 22). In ad-
dition, several studies suggest that the stability of pMHC complexes is predictive of the immunogenicity of CD8+ T cell determinants (23–26) and has been directly linked to the establishment of immunodominance hierarchies for CD4+ T cells (44). We sought to identify site V APLs that could form pMHC with increased stability. Despite the formation of relatively unstable pMHC complexes, the wt site V determinant, encompassing TAg residues 489–497 (QGINNLDNL), harbors the canonical D6 anchor residues at position (P) 5-N and 9-L (Fig. 1A). Thus, to stabilize pMHC interactions, we considered the role of nonanchor residues to enhance pMHC binding without disrupting TCR recognition (36, 45, 46). P3-I and P8-N were previously shown to be critical for recognition by site V–specific CTL (47) and, as demonstrated in other systems, P3, P4, P6, P7, and P8 can protrude from the MHC binding cleft and/or serve as TCR contact residues (48, 49). Thus, we identified positions P1 and P2 for substitution with alanine residues. The resulting analogs are designated Q489A and G490A, respectively. We evaluated both the relative binding affinity of the Q489A and G490A peptides for H-2Db (Fig. 1B) as well as the stability of H-2Db molecules formed with each peptide (Fig. 1C). To determine the relative affinity of peptide binding to H-2Db, TAP2 mutant RMA/s cells were incubated overnight at 26˚C and then aliquots of cells mixed with increasing concentrations of each peptide. After 4 h at 37˚C, cells were stained for H-2Db surface expression and the relative affinity determined (Fig. 1B). The results indicate that peptide Q489A has an increased relative affinity for D6 (4-fold), whereas that of G490A is similar to wt site V. Thus, only the Q489A analog showed an increased relative affinity, which was similar to the dominant site I peptide.

The stability of peptide-loaded D6 complexes was estimated by measuring the decay of pMHC from RMA/s cells following removal of excess peptide. Complexes formed with the Q489A, G490A, and site V peptides had t1/2 of ~4, 2.75, and 2 h, respectively (Fig. 1C). The site I peptide was the most stable, with a t1/2 >4 h. These results indicate that both Q489A and G490A formed more stable pMHC than the wt site V peptide, although only Q489A peptide showed a measurable change in relative affinity for H-2Db.

We next evaluated cross-recognition of Q489A and G490A by site V–specific T cells. T cell lines derived from TCR-V (Fig. 1D, 1E) or polyclonal site V–specific T cells isolated from immunized mice (Fig. 1F, 1G) were stimulated with decreasing concentrations of site V, Q489A, G490A, or control Flu (NP366–374) peptide. At the highest concentration, site V, Q489A, and G490A peptides stimulated a similar percentage of cells to produce IFN-γ for both

**FIGURE 1.** Site V substitution mutants Q489A and G490A enhance pMHC stability and conserve recognition by site V–specific T cells. (A) Sequence of synthetic peptides representing the Q489A and G490A mutants. (B) Relative affinity of synthetic peptides bound to H-2Db was determined by incubation of RMA/s cells overnight at 26˚C followed by addition of the indicated concentrations of each peptide to aliquots of RMA/s cells and incubation at 37˚C for 4 h. Cells were stained for H-2D6 expression and the data expressed as the percent maximum MFI of D6 expression for each peptide. Relative affinity (Kd) was determined as described in the Materials and Methods using nonlinear regression analysis. (C) RMA/s cells were incubated with the indicated peptides overnight at 29˚C, excess peptide was removed by washing, and the cells were incubated at 37˚C for the indicated times. Cells were held on ice prior to staining for H-2Db. Data are presented as percent of maximum arbitrary fluorescence units (AFU) for each peptide. Values represent the percent of CD8+ T cells producing IFN-γ (D, F) or the percent of maximum CD8+ IFN-γ+ cells for each peptide (E, G). All results are representative of at least two independent experiments in which similar results were obtained.
TCR–transgenic and polyclonal T cells (Fig. 1D, 1F). The wt site V and Q489A peptides demonstrated similar functional avidity for clonal TCR-V (Fig. 1E) and polyclonal (Fig. 1G) T cell lines at all peptide dilutions, indicating no discernable difference in T cell recognition. Functional avidity for G490A was reduced between 5- and 10-fold for TCR-V and polyclonal T cells, respectively. Overall, these results demonstrate that compared with wt site V, Q489A binds to Dβ with a higher relative affinity, forms a more stable pMHC, and is recognized by site V-specific T cells with a similar efficiency. The G490A analog forms pMHC with moderately increased stability compared with wt site V, but has a similar relative affinity for Dβ and provides a less efficient target for site V–specific T cells in vitro, indicating that the functional avidity of site V–specific T cells for the G490A variant is reduced. Thus, two site V APLs were identified with increased pMHC stability, but which varied in the other parameters measured.

Q489A and G490A TAg variants are processed and presented for T cell recognition

We produced full-length TAg variants containing the Q489A or G490A substitutions in the context of both wt and site V-only TAg. Primary mouse B6 and tap1 knockout primary kidney cells were transfected with plasmid DNA, and immortalized cell lines were derived for each of the constructs except Q489A V-only TAg (Table I), which was unable to immortalize primary kidney cells. Relative expression of TAg in each cell line was verified by immunoprecipitation/Western blot and did not vary by >39% (A.M. Watson and T.D. Schell, unpublished observations). Incorporation of the particular site V variant in each cell line was verified by sequencing of PCR-amplified genomic DNA (A.M. Watson and T.D. Schell, unpublished observations). We confirmed that the TAg determinants were processed and presented by the Q489A and G490A TAg variants using in vitro recognition by site I and site V–specific CTL lines. T cells produced IFN-γ in response to B6-derived, but not tap1 knockout–derived, cell lines (Fig. 2), demonstrating direct presentation of the expected determinants by the B6-derived cell lines. Furthermore, CTL killing of Q489A or G490A B6-derived cell lines was similar to that of wt-TAg-expressing cells, and the TAg variants did not interfere with target cell killing by site I–, II/III–, and IV–specific T cells (Supplemental Fig. 1). Thus, Q489A and G490A are processed and presented in a TAP-dependent manner, and the B6-derived cells present the expected array of TAg determinants.

Immunization with Q489A or G490A induces endogenous site V–specific T cell responses

Endogenous site V–specific T cell responses have not previously been detected following immunization with TAg-expressing cells that encode both site V and dominant TAg determinants. To test the immunogenicity of the Q489A and G490A TAgS, mice were immunized with wt-TAg, V-only, Q489A, G490A, or G490A V-only B6-derived cell lines (Fig. 3A, left panels). Discernable tetramer V*CD8+ T cells were observed for one of three mice and two of three mice immunized with Q489A and G490A, respectively. Two of three mice had detectable site V–specific CD8+ T cell responses following immunization with V-only cells. Although previous studies had used pooled lymphocytes from V-only–immunized mice (12), we consistently found using individual mice that an average of one in three mice could mount a detectable ex vivo response to site V (data not shown). In contrast, immunization with G490A V-only cells produced strong ex vivo responses in all mice ranging from 1.18–2.75% of CD8+ T cells (Fig. 3A, left panels), indicating that the combination of the G490A analog and the absence of the dominant determinants was optimal for the induction of site V cross-reactive CD8+ T cells. No significant accumulation of site V–specific T cells was detected in wt-TAg–immunized or unimmunized mice. We observed no discernable relationship between the magnitude of the site V–specific T cell response and the site I– or IV–specific T cell response in individual mice (Fig. 3B, 3C, respectively), indicating that the site V variants did not compromise the response to the dominant determinants and likewise the detection of site V–specific T cells was not facilitated by reduced responses to the dominant determinants.

To discern whether low numbers of site V–specific T cells were primed in some immunized mice, splenocytes were restimulated in vitro with K-3,1,4 cells, which express a TAg variant lacking sites I, II/III, and IV. In general, we found that mice with detectable ex vivo Tet-V+ cells demonstrated marked expansion of T cells in culture (Fig. 3A, right panels), with the exception of mouse 1 immunized with V-only TAg. Additionally, mouse 2 from the Q489A-immunized group showed accumulation of a prominent population of Tet-V+ cells despite no detection ex vivo. Consistent with previous studies, site V–specific T cells did not expand from wt-TAg–immunized mice or from unimmunized mice following in vitro culture, indicating that the expansion of site V–specific T cells was not due to in vitro priming. These data demonstrate that Q489A and G490A are immunogenic when coexpressed with the dominant TAg determinants, overcoming the immunorecessive phenotype of site V. Even so, the site V–specific response remained quantitatively subdominant to both sites I and IV.

Site V–reactive T cells primed by Q489A and G490A have a similar phenotype to those primed by wt site V

The increased immunogenicity of Q489A and G490A could be explained by recruitment of additional T cell clones. To investigate whether an overall change in the site V–reactive population occurs following immunization with Q489A and G490A, we evaluated both the fine specificity of the responding site V–reactive T cells and the TCR β-chain usage by these T cells. Because only low levels of T cells can be detected ex vivo after primary immunization with Q489A and G490A cells (Fig. 3), we used a prime/boost approach in which mice were first immunized with Q489A or G490A cells followed by booster immunizations with V-only cells to expand any site V cross-reactive T cells. Some mice received prime/boost with either V-only cells or G490A V-only cells for comparison. Ex vivo analysis of responding cells was performed at day 8 postboost, prior to the time when CD8+ T cell

**FIGURE 2.** Direct presentation of Q489A and G490A determinants by B6-derived cells. B6-derived and tap1 knockout–derived cell lines expressing wt-TAg or TAg variants expressing the Q489A or G490A substitutions were tested for their ability to stimulate IFN-γ production by either site I–specific CTL clone K-11 or bulk polyclonal site V–specific CTL. K-1,4,5 cells lack expression of the four known H-2d restricted determinants. Cells were stained for intracellular IFN-γ production and analyzed by flow cytometry.
responses to primary site V-only immunization can be detected. CD8+ T cell responses were tested against a panel of site V variant peptides containing single amino acid substitutions at all positions except for N493 and L497, which serve as the primary anchor residues and for which substitutions interrupt peptide binding. CD8+ T cells from each of the four groups of mice showed similar fine specificities (Fig. 4), with substitutions at Q489 and G490 having the least impact, residue L494 having the greatest impact, and the remaining four residues having intermediate impacts on T cell recognition. For the G490A and G490A V-only–immunized groups, there was a trend toward greater impact for substitutions at D495 and N496, but these changes were not statistically different from the V-only–immunized group. These results indicate that the fine specificity of site V–reactive T cells is maintained following priming with the Q489A and G490A variants.

We also broadly determined the TCR β-chain usage of site V–reactive T cells derived from mice primed with wt site V or site V variants. We first compared the profiles of site V–reactive CD8+ T cells that accumulate following a prime and boost with either V-only cells or G490A V-only cells, because these immunizations induce higher levels of T cells facilitating direct ex vivo analysis. All mice showed dominant accumulation of Vβ7+ T cells, with some mice in both groups also using Vβ2 (Supplemental Fig. 2A). One mouse in the G490A V-only group had a minor population of Vβ17+ cells, which was not observed in the V-only–immunized group. We also evaluated TCRβ expression by site V–reactive CD8+ T cells expanded in vitro from mice initially primed with V-only or Q489A cells (Supplemental Fig. 2B). In vitro expansion was necessary due to the limited accumulation of cells following immunization with Q489A. This analysis also revealed predominant accumulation of Vβ7+ T cells in the V-only and Q489A

FIGURE 3. Q489A and G490A prime endogenous site V–specific T cell responses in the presence of the dominant TAg determinants. (A–C) B6 mice were immunized with the indicated B6-derived cell lines. Ten days following immunization, spleens were harvested, and cells were stained with anti-CD8 and site V MHC tetramer. The percentage of endogenous site V–specific CD8+ T cells was assessed ex vivo or following two rounds of in vitro restimulation with K-3,1,4 cells. (A) Values indicate the percent of CD8+ T cells that are site V MHC tetramer positive. For comparison purposes, the data for site I (B) and site IV (C) are plotted along with the CD8"Tet-V" T cell response to site V. Each data point represents an individual mouse, and the open and closed paired symbols represent data from the same animal. Results are representative of at least two independent experiments with three mice per group.

FIGURE 4. Fine specificity of CD8+ T cells primed with Q489A and G490A is similar to those primed against wt site V. Groups of three C57BL/6 mice were immunized i.p. with the indicated B6-derived cell lines and rested for 3 wk. Mice received booster vaccinations with either V-only cells or G490A V-only cells. On day 8, splenocyte responses to site V wt and variant peptides containing single amino acid substitutions were tested ex vivo using intracellular staining for IFN-γ production by CD8+ cells. Data are presented as the percentage of response to the wt site V peptide. All mice were tested individually, and the error bars represent SD. No statistically significant differences were observed among the immunization groups. The data are representative of two independent experiments in which similar results were obtained.
groups. Taken together, the fine specificity and TCR β-chain analyses suggest that the responsive repertoire of T cells is similar following priming with wt site V compared with the site V variants. Although we cannot exclude CDR3 differences among the responding clones, the results indicate that site V–reactive TCRs use a restricted set of TCR β-chain families.

**Q489A and G490A variants enhance cross-priming of TCR-V T cells**

Previous evaluation of mechanisms regulating the immunogenicity of site V indicated that the wt determinant is inefficiently cross-presented, resulting in proliferation of only a fraction of adoptively transferred naive TCR-V T cells (14). To determine whether the site V variants would promote more efficient triggering of TCR-V T cells through cross-priming, we developed an assay to quantify the fraction of cells remaining undivided following immunization. In this approach, naive CFSE-labeled TCR-V cells are cotransferred with CFSE-labeled spleen cells from naive Thy1.1+ mice prior to immunization (Fig. 5A). Because all mice received the same ratio of TCR-V to CD8+Thy1.1+ cells, and the Thy1.1+ cells did not divide following immunization (Fig. 5A, bottom right panel), the CFSE-hi CD8+Thy1.1+ population provides an internal reference to evaluate changes in the undivided TCR-V population. In this way, the frequency of cells that initially proliferate can be quantified independent of the expansion and survival of the divided cells. Data are represented as the number of undivided CFSE-hi TCR-V cells divided by the number of CFSE-hi Thy1.1+CD8+ cells such that a reduced ratio indicates more naive cells are triggered.

**FIGURE 5.** A greater fraction of naive TCR-V cells undergoes proliferation in response to Q489A or G490A. (A–D) Total CFSE-labeled splenocytes from TCR-V mice and Thy1.1-congenic mice were mixed together (Thy1.1-spike), and aliquots containing 1 × 10^6 TCR-V T cells were transferred into B6 mice. The following day, mice were immunized with the indicated cell lines (A–C) or with the indicated peptide or rVV expressing endoplasmic reticulum–targeted minigene products (D). Three days following immunization, splenocytes were stained with Tet-V, anti-CD8, and anti-Thy1.1. The ratio of CFSE-hi UD TCR-V/CD8+Thy1.1+ cells was calculated as in the example shown in (A). (B) Results show the fraction of undivided TCR-V cells in each immunization group. The control group consists of unimmunized mice or mice immunized with null cells. (C) The percent of naive T cells that divided was calculated by the equation 100 × (1 – [I/U]), where I is the mean ratio from a group of immunized mice, and U is the mean ratio from the group of unimmunized mice. (B and C) Results from two independent experiments were normalized and combined. The p values were determined by unpaired t tests. (D) Data are presented as in (B) and the percentages shown as in (C). For mice immunized with peptide, U is the mean ratio from peptide I–immunized mice. For mice immunized with rVV-ES-V, U is the mean ratio from rVV-ES-I–immunized mice. The data are representative of two independent experiments with groups of three mice.
To restrict the T cell response to cross-presented Ag, we immunized mice with TAPI−/− cell lines TAP-wt, TAP-Q489A, and TAP-G490A. As expected (14), immunization with TAP-wt cells triggered a small fraction of TCR-V cells to divide (Fig. 5B). Immunization with TAP-Q489A or TAP-G490A cells resulted in significantly more TCR-V cells dividing compared with TAP-WT immunization. In summary, 33.4% of TCR-V cells divided following immunization with TAP-wt, whereas 69.9 and 69.4% of TCR-V cells divided following immunization with TAP-Q489A and TAP-G490A, respectively (Fig. 5C). These results demonstrate that Q489A and G490A cross-prime a higher fraction of TCR-V cells than the wt-TAg.

In contrast to these results, we previously found that almost all TCR-I cells divided in response to cross-presented Ag following immunization with wt-TAg cells (14), raising the possibility that a subset of TCR-V cells may be incapable of responding to the Ag in vivo. To examine this question, we asked whether bypassing cross-presentation would initiate priming of a higher fraction of the TCR-V donor cells. Mice were immunized with synthetic peptides (40) or rVV expressing an endoplasmic reticulum–targeted minigene (e.g., rVV-ES-V) (13). These approaches bypass the need for Ag processing and cross-presentation of Ag from donor cells. The results revealed that almost all of the TCR-V cells underwent division following immunization with peptide V and peptide Q489A or rVV-ES-V but not with control site I immunizations (Fig. 5D). These results rule out the possibility of an unresponsive subpopulation of TCR-V T cells and support the conclusion that cross-priming constitutes a major limitation in the response to TAg site V. Taken together, these data reveal that enhanced cross-presentation of site V is associated with increased pMHC stability, but that methods of immunization that bypass cross-presentation are most efficient in activation of TCR-V cells in vivo.

**TCR-V precursor number does not influence the efficiency of cross-priming**

Incomplete cross-priming of TCR-V cells following immunization with TAg-transformed cells could result from transfer of large numbers of TCR–transgenic T cells because T cells of the same specificity can compete for available pMHC during priming (50). Thus, we transferred decreasing numbers (106–104) of TCR-V cells plus Thy1.1+ cells into mice prior to immunization with TAP-wt cells. Three days following immunization, CD8+ T cells were enriched from splenocytes, and the fraction of undivided TCR-V T cells was measured. The results indicate that a similar fraction of naive TCR-V cells divided regardless of the number of transferred TCR-V cells (Fig. 6A). This result is not due to altered T cell seeding efficiencies because the percentage of CD8+ Thy1.1+ cells detected at each dilution was reduced proportionately (Fig. 6B). These data indicate that the fraction of naive TCR-V cells that is cross-primed remains the same when the number of precursors is between 106 and 104 per mouse and suggest that competition among responding TCR-V T cells does not contribute toward the incomplete response. Extrapolating these results suggests that as naive T cells approach more physiologic numbers [103 and below (41–43)], incomplete cross-presentation of available site V–specific T cells remains a limiting factor.

**Endogenous site V–specific T cells are primed following immunization with wt-TAg**

Although an endogenous site V–specific T cell response has not been detected following wt-TAg immunization, our results using transferred TCR-V–transgenic cells suggest that limited priming might occur. Lack of detection could be explained by a combination of inefficient cross-priming and low precursor number. We determined the number of site V–specific precursor T cells available in naive mice using MHC-tetramer–based magnetic enrichment (41–43). Site I–specific T cells were coenriched from each mouse so that relative levels of site I– and site V–specific T cells could be compared in individual mice. Cells isolated from spleens and lymph nodes of individual mice were stained and gated as described in the Materials and Methods and depicted in Fig. 7A. The values reported represent the number of cells directly detected per murine following analysis of the entire cell population. On average, 6.4 site V–specific precursor T cells and 19.2 site I–specific T cells were detected per naive mouse (Fig. 7C). Thus, naive mice contained ~3:1 Tet-I/Tet-V–specific precursor T cells, indicating that naive site V–specific T cells are initially present at lower numbers than site I–specific T cells.

The ability to detect low numbers of endogenous site V–specific T cells provided an opportunity to readdress whether endogenous site V–specific T cells are primed following wt-TAg immunization and to evaluate the early kinetics of response to the site V analogs. Mice were immunized with wt-TAg cells or Q489A cells and isolated at day 7 postimmunization. We found that endogenous site V–specific T cells accumulated in mice following wt-TAg immunization (Fig. 7B). Our cumulative results demonstrate a 22-fold expansion (141.4 cells/mouse; Fig. 7C) of endogenous site V–specific T cells compared with naive mice. By comparison, site I–specific T cells expanded 184-fold (3524 cells/mouse) following immunization with wt-TAg, suggesting that site I–specific T cells accumulate at a higher rate over the 7-d period. Immunization with Q489A cells resulted in endogenous site V–specific T cells expanding to 2868 cells/mouse, a 450-fold expansion of the precursor population, representing an ~20 fold (2868/141.4) greater expansion of site V–specific T cells relative to naive mice.
to wt-TAg immunization. We observed that Q489A immunization also promoted accumulation of higher numbers of site I–specific T cells (22,159 cells per mouse), suggesting that the Q489A cells may be inherently more immunogenic than wt-TAg cells. Because site I– and site V–specific T cells were coisolated from individual mice, we were able to directly compare the ratio of site I– and site V–specific CD8+ T cells in naive mice versus that obtained from mice immunized with wt-TAg cells and transfer of TCR-V cells prior to the day of the experiment. The negative controls were naive mice that received no tetramer staining prior to magnetic enrichment. (B) Representative plots of TAg-specific T cells obtained from mice 7 d following immunization with wt-TAg or Q489A cells. (C) Data compiled from all experiments. Values indicate the number of cells in each gate (A, B) or the mean value of all Tet-V+ or Tet-I+ cells in all experiments (C). The data are representative of two (B) or three (A) experiments comprising at least three mice per group. (D) The ratio of Tet I+ cells to Tet V+ cells from (C) is plotted for individual mice. Bars indicate mean values. The p values were calculated using the Mann–Whitney U test.

FIGURE 7. Endogenous site V–specific T cells proliferate following immunization with wt-TAg cells. (A–C) Total splenocytes and lymphocytes were stained with Tet-I–APC and Tet-V–PE and isolated by magnetic enrichment. (A) The flow cytometric gating scheme was based on FSC-A versus SSC-A followed by cells positive for CD8 and negative for the DUMP stain and finally CD8+Thyl.2+ cells (A, top panel). Representative dot plots from positive and negative control mice and naive mice are displayed (A, bottom panel). Positive control mice received immunization with wt-TAg cells and transfer of TCR-V cells prior to the day of the experiment. The negative controls were naive mice that received no tetramer staining prior to magnetic enrichment. (B) Representative plots of TAg-specific T cells obtained from mice 7 d following immunization with wt-TAg or Q489A cells. (C) Data compiled from all experiments. Values indicate the number of cells in each gate (A, B) or the mean value of all Tet-V+ or Tet-I+ cells in all experiments (C). The data are representative of two (B) or three (A) experiments comprising at least three mice per group. (D) The ratio of Tet I+ cells to Tet V+ cells from (C) is plotted for individual mice. Bars indicate mean values. The p values were calculated using the Mann–Whitney U test.

Discussion
Identification of APLs that form stable pMHC-I complexes provides an effective way to enhance determinant-specific immunization for cancer or infectious diseases (18, 20, 51). Although the mechanisms promoting increased immunogenicity of APLs may vary, previous studies indicate that agonist peptides can modify the molecular contacts between the TCR and pMHC, resulting in more effective triggering through the TCR (18, 52). Alternatively, quantitative increases in pMHC on APCs may enhance priming of naive CD8+ T cells by allowing prolonged T cell–APC interactions (53). The resulting augmented accumulation of T cells is potentially explained by more complete recruitment of the available naive T cells or through greater expansion and increased survival of primed T cells. In the current study, we provide evidence that APLs with increased pMHC stability both enhance endogenous T cell accumulation and cross-prime a higher fraction of available naive TCR–transgenic CD8+ T cells specific for an immunorecessive determinant. This mechanism may contribute to the overall increase in T cell numbers following immunization, thereby overcoming the immunorecessive phenotype.

A potential consequence of the increased pMHC stability of the site V variants is higher or more durable pMHC presentation on APCs. High-stability peptides are typically presented in greater numbers than low-stability peptides (25) due to preferential loading of high-stability peptides by the MHC-I Ag presentation machinery (54). The consequences of increased pMHC presentation for T cell activation have been revealed by intravital microscopy studies showing that the number of pMHC presented by dendritic cells (DCs) inversely correlated with the speed of T cell transition through the three phases of T cell–APC interaction leading to T cell activation (53). Those T cells unable to form prolonged contacts with DCs due to encountering subthreshold Ag levels were unable to transition to the
latter stages of T cell activation. Relevant to the current study, the number of pMHCs presented by DCs in vivo was dependent on pMHC stability. Thus, site V analogs that form stable pMHC may trigger a higher proportion of site V–reactive CD8+ T cells due to sustained cross-presentation of increased pMHC complexes.

Differences in pMHC stability can potentially influence both direct presentation by the TAg-transformed cells and cross-presentation by APCs. However, the impact on cross-presentation may be more dramatic because Ag capture by DCs is downregulated following initial activation and licensing (55, 56), suggesting that DCs take up a fixed amount of Ag for cross-presentation. This scenario differs from the constant supply of directly presented determinants generated in cells that synthesize the Ag de novo. Shortly following acquisition of Ag, DCs may present a broad range of determinants that later becomes biased toward high-stability pMHC due to rapid loss of low-stability pMHC. Indeed, Yu and colleagues (51) found that pMHC $t_{1/2}$ on DCs was directly related to the increased immunogenicity of a self/tumor Ag determinant. Although the short $t_{1/2}$ of site V–pMHC complexes on the cell surface (between 2 and 3 h) may be sufficient for immunogenicity, it may limit the proportion of T cells that can achieve sustained signaling through the TCR required for T cell activation (57, 58). This may explain why a subpopulation of naive TCR-V T cells never divides in response to cross-presented TAg.

Changes in pMHC stability or epitope production may influence the number of pMHC complexes available for T cell priming; however, increased immunogenicity of the site V variants may additionally be explained by a direct change in TCR–pMHC contacts, leading to more efficient triggering through the TCR. For some APLs, amino acid substitutions that increase pMHC stability and immunogenicity do not influence the overall structure of the pMHC complex, indicating that increased immunogenicity was primarily due to formation of more stable pMHC (18, 20). This mechanism may best explain the increased immunogenicity of the Q489A variant, which showed the most dramatic increase in pMHC stability and relative affinity. Alternatively, some APLs with increased pMHC stability alter TCR contacts, resulting in increased affinity of the TCR for the pMHC complex (19). Such a mechanism may explain the increased immunogenicity of the G490A variant that showed a modest increase in pMHC stability without a net increase in the relative affinity for MHC. However, we note that the functional avidity of site V–specific polyclonal T cells and TCR–transgenic T cells was not increased for either the Q489A or G490A variant peptides in vitro, suggesting that the variant pMHC do not inherently increase the efficiency of TCR signaling among site V–specific T cells. Further discernment of the contribution of pMHC stability and pMHC conformation toward improved immunogenicity of the site V variants would require structural analysis of the pMHC and pMHC–TCR complex by x-ray crystallography (18, 19).

Increased immunogenicity of the site V variants for the endogenous site V–specific CD8+ T cells could be explained by activation of new clonotypes (59, 60). We found only minor differences in TCR-V β-chain family usage among site V–reactive CD8+ T cells induced by wt and Q489A or G490A TAgS, suggesting that the repertoire is generally restricted to the dominant TCR-V β-chains. However, our results leave open the possibility of increased recruitment of T cells using TCRs with unique CDR3 regions within the dominant TCR-Vβ families. Our data demonstrating that the site V variants prime a higher fraction of naive TCR–transgenic T cells are consistent with the idea that additional site V T cells in the naive polyclonal repertoire could be recruited.

We previously considered that T cell competition for pAPCs contributes to the immunorecessive phenotype of TAg site V (14). Mice coimmunized with wt-TAg and V-only TAg expressed in separate cells developed T cells specific for both site V and the immunodominant determinants. In contrast, site V–specific T cells were undetectable following immunization with cells coexpressing wt-TAg and V-only Tag. These results suggested that T cells specific for the immunodominant determinants limit priming or accumulation of site V–specific T cells as has been observed in several different systems (61–63). A recent study by Galea and coworkers (64) provides evidence that T cells specific for the dominant TAg site I or site IV determinants compete with T cells specific for site V at the early stages of T cell priming. In addition, they found that modulation of the site IV determinant to reduce the $t_{1/2}$ of pMHC complexes alleviated immunodomination over site V when the determinants were coexpressed in tandem in a DNA vaccine. Our observation that enhanced site V pMHC stability is associated with relieving the immunorecessive phenotype may be explained in part by decreased competition from CD8+ T cells responding to the immunodominant TAg determinants, resulting in more efficient priming or expansion of site V–specific T cells. However, optimal site V–specific responses were best obtained when the G490A variant was expressed in the absence of the dominant determinants, suggesting a continued role for T cell competition.

Although the site V variants were able to produce a detectable endogenous T cell response, site V was not raised to immunodominant status. Our data suggest that if the site V– and site I–specific naive T cell precursor numbers were equal, site V would remain subdominant due to the limited ability to cross-prime the available precursors. Thus, for site V to move up the immunodominance hierarchy, both an increase in the number of precursors and the proportion of precursors that are triggered would be required. This scenario differs from systems in which subdominant CD8+ T cells outnumber their immunodominant counterparts, such that an increase in cross-priming alone might be sufficient to increase immunodominance. For example, La Gruta and colleagues (65) found that the frequency of subdominant Flu determinant–specific CD8+ T cell precursors is significantly higher than that of their immunodominant counterparts. These authors found incomplete recruitment of the available CD8+ T cell clonotypes specific for the subdominant Flu Dβ-PB1-FluDβ determinant, which has an estimated $t_{1/2}$ of ~3 h (66). In contrast, CD8+ T cells specific for two dominant Dβ-Flu determinants with $t_{1/2}$ estimated to be ~47 (NP 366–374) and 16 h (PA 224–233) (66) underwent more efficient recruitment (65). We hypothesize that naive T cell recruitment may be nearly complete when pMHC stability is above a yet undetermined threshold and variably incomplete when pMHC stability falls below that threshold.

We also show for the first time, to our knowledge, that site V–specific CD8+ T cells are primed following immunization with wt-TAg–expressing cells. The inability to detect site V–specific T cells in previous studies is likely explained by the limited accumulation of these T cells following immunization, which remain below the limit of detection using standard flow cytometric analysis. Only through MHC tetramer enrichment was this response detected. Considering that T cells were isolated from ~1 × 10^6 total cells, the frequency of site V–specific T cells detected at 7 d postimmunization with wt-TAg cells was 1 in 1.4 × 10^6. In contrast, the frequency of site V–specific T cells detected following immunization with Q489A cells was ~5-fold higher at 1 in 2.9 × 10^5. These site V–specific T cells continue to expand over the next several days following immunization with the site V variants, whereas the response in wt-TAg-immunized mice never
reaches detectable levels (Fig. 3A). We speculate that site V-specific T cells triggered by wt-TAg may undergo abortive proliferation, whereas the site V variants induce a more characteristic extended T cell expansion in vivo.

In summary, our results suggest that more complete recruitment of the available naive T cell population can be achieved through approaches that increase pMHC stability or bypass cross-presentation. This mechanism may explain the enhanced immunogenicity of some APLs derived from similar subdominant or cryptic determinants and provide a strategy to expand the repertoire of determinants available for control of cancer and infectious diseases.

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Disclosures

The authors have no financial conflicts of interest.

References


Supplemental Figure 1: Cell lines expressing Q489A and G490A process and present the TAg determinants. MHC I presentation of site V, Q489A or G490A was assessed in each of the indicated B6 derived cell lines using a standard chromium-release assay. Each panel represents an individual target cell line and the data points indicate the percent specific lysis obtained at each effector:target ratio (E:T) using T cells specific for each of the TAg determinants. Results are representative of two independent experiments. CTL lysed the appropriate target cells demonstrating presentation of the expected determinants.
A. Ex vivo

Mouse #1

Mouse #2

Mouse #3

V-only

G490A

B. In vitro expanded

Mouse #1

Mouse #2

Mouse #3

V-only

Q489A

Supplemental Figure 2. TCRVβ chain usage among site V-reactive T cells. Groups of 3 mice were immunized i.p. with 5x10⁷ of the indicated cell lines. A. Mice received a booster immunization with the same cell line at 14 days post primary immunization and spleen cells were harvested seven days later and analyzed ex vivo for CD8+Tet V+ cells that expressed the indicated TCRVβ chain by flow cytometry. B. On day 10 post-primary immunization, spleen cells were restimulated with gamma-irradiated V-only cells for six days prior to staining for CD8+Tet V+ cells that expressed the indicated TCRVβ chain by flow cytometry.