Inefficient Cross-Presentation Limits the CD8$^+$ T Cell Response to a Subdominant Tumor Antigen Epitope[1]

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CD8$^+$ T lymphocytes (T_{CD8}) responding to subdominant epitopes provide alternate targets for the immunotherapy of cancer, particularly when self-tolerance limits the response to immunodominant epitopes. However, the mechanisms that promote T_{CD8} subdominance to tumor Ags remain obscure. We investigated the basis for the lack of priming against a subdominant tumor epitope following immunization of C57BL/6 (B6) mice with SV40 large tumor Ag (T Ag)-transformed cells. Immunization of B6 mice with wild-type T Ag-transformed cells primes T_{CD8} specific for three immunodominant T Ag epitopes (epitopes I, II/III, and IV) but fails to induce T_{CD8} specific for the subdominant T Ag epitope V. Using adoptively transferred T_{CD8} from epitope V-specific TCR transgenic mice and immunization with T Ag-transformed cells, we demonstrate that the subdominant epitope V is weakly cross-presented relative to immunodominant epitopes derived from the same protein Ag. Priming of naive epitope V-specific TCR transgenic T_{CD8} in B6 mice required cross-presentation by host APC. However, robust expansion of these T_{CD8} required additional direct presentation of the subdominant epitope by T Ag-transformed cells and was only significant following immunization with T Ag-expressing cells lacking the immunodominant epitopes. These results indicate that limited cross-presentation coupled with competition by immunodominant epitope-specific T_{CD8} contributes to the subdominant nature of a tumor-specific epitope. This finding has implications for vaccination strategies targeting T_{CD8} responses to cancer. ***The Journal of Immunology,*** **2005, 175: 700–712.**

The response of CD8$^+$ T lymphocytes (T_{CD8})[5] to microorganisms, tumors, and tissue grafts is typically focused toward multiple epitopes with one or a few epitopes predominant (1, 2). These epitopes have been grouped into two broad categories, dominant and subdominant, based on the relative frequency of T_{CD8} that respond following Ag exposure. Defining the basis for this hierarchical response will provide insight for monitoring natural T_{CD8} immunity and developing vaccines to cancer and infectious diseases. Multiple mechanisms can contribute to immunodominance following immunization with complex Ags. These include the efficiency of processes involved in Ag presentation such as peptide liberation from protein substrates (3–5), TAP-dependent peptide transport into the endoplasmic reticulum (6), transport of peptide-MHC complexes to the cell surface (7, 8), as well as the stability of peptide-MHC class I complexes (9, 10). In addition, T cell precursor frequency (1, 11, 12), TCR avidity (13, 14), the nature of the APC (15), and T_{CD8} competition for the APC (16–18) have been implicated. Thus, the position of an epitope within the immunodominance hierarchy derives from the interplay of multiple factors important for initiation of T_{CD8} responses. Because no general rules have been established to this point, the basis for subdominance of a particular epitope must be defined empirically.

Professional APC, such as dendritic cells, are capable of activating naive T_{CD8} following engagement of the TCR with MHC class I/peptide complexes in addition to the provision of costimulatory signals such as B7/BB1 engagement with CD28 on the T_{CD8} (19). The antigenic peptides presented by MHC class I molecules on professional APC can be either derived from de novo synthesized proteins within the APC (direct presentation) or from cell-associated Ags via the mechanism of cross-presentation (20–22). Cross-presentation is particularly important for priming T_{CD8} responses to tumor Ags because most tumor cells lack the expression of costimulatory molecules (23–25).

The contribution of cross-presentation in establishing T_{CD8} immunodominance to tumor-specific epitopes remains unknown. Cross-presentation can be influenced by the dose of Ag, as some studies have shown that high levels of Ag are more efficiently cross-presented than low levels of the same Ag (26–29). Whether variability in the T_{CD8} response to multiple epitopes within the same protein can be attributed to differences in the efficiency of cross-presentation has not been investigated. In this study, we assessed the mechanisms that contribute to the subdominant nature of the H-2D^b-resticted epitope V (residues 489–497) from SV40 T Ag. The tumor-specific T_{CD8} response to SV40 T Ag in C57BL/6 (B6) mice is targeted against three dominant epitopes (designated epitopes I, II/III, and IV) and one subdominant epitope (designated epitope V)

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[5] Abbreviations used in this paper: T_{CD8}, CD8$^+$ T lymphocyte; T Ag, SV40 large tumor Ag; TAP1$^{-/-}$, TAP 1 knockout; wt, wild type; TCR-V, epitope V-specific TCR transgenic; V-only, epitope V-only; TCR-I, epitope I-specific TCR transgenic.

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(30). The T_{CD8} response to the H-2D^b-restricted epitope V is undetectable following immunization with wild type (wt) T Ag-transformed cells, SV40, or even a recombinant vaccinia virus expressing full-length T Ag (31). This strict immunodomination is relieved by deletion of the three immunodominant epitopes from T Ag (32) or following immunization with a recombinant vaccinia virus expressing epitope V as a minigene (33). Although epitope V-specific T_{CD8} are not induced following immunization with wt T Ag, this subdominant epitope is efficiently presented by T Ag-transformed cells in vitro (32). One potential mechanism contributing to the subdominant nature of epitope V in vivo is the relative instability of the epitope V/Db complexes compared with the dominant T Ag epitopes (30, 33), particularly under conditions where a fixed amount of Ag is cross-presented. A recent study by Chen et al. (34) demonstrated that the subdominant nature of epitope V is maintained under conditions where T Ag is exclusively cross-presented.

In the present study, we investigated the mechanism of epitope V subdominance by measuring the response of epitope V-specific TCR transgenic (TCR-V) T cells to immunization with syngeneic or TAP1 knockout (TAP1$^{-/-}$) T Ag-transformed cells. The results indicate that epitope V is inefficiently cross-presented, resulting in limited T cell priming and expansion. The additive effect of competition by immunodominant epitope-specific T_{CD8} further inhibits the response to epitope V following immunization with wt T Ag.

Materials and Methods

Mice

C57BL/6 (H-2^b), B6.129S2-Tap1^{tm1Arp} (TAP1$^{-/-}$), and B6.SJL-Ptprc^{a}Pep3^b/BoyJ (B6.SJL) mice were purchased from The Jackson Laboratory and used between the ages of 8 and 16 wk. TCR transgenic mice specific for the T Ag epitope I (TCR-I mice) are on a B6 background and were described previously (35). All mice were maintained at the animal facility of the Milton S. Hershey Medical Center. All animal studies were performed in accordance with guidelines established by the Pennsylvania State University College of Medicine Institutional Animal Care and Use Committee under an approved protocol.

Cloning of epitope V-specific TCR subunits from the CTL clone Y-5

TCR sequences corresponding to both the α- and β-chains expressed by the SV40 T Ag epitope V-specific CTL clone Y-5 (36) were derived as described previously (35). The TCRα-chain combining region was amplified by PCR from clone Y-5-derived cDNA using an antisense 3′-constant region oligonucleotide (5′-CGAGGATCTTTTAACTGGTA-3′) and a 5′-variable region Vα4 chain sense oligo (5′-GCCACCTCCTTCCACTTG CAGAAAG-3′), whereas the TCR Vβ-chain combining region was amplified using the 3′-antisense constant region oligonucleotide (5′-CTT GGGTGGAGTCACATTTCT-3′) and a 5′-sense Vβ7 oligonucleotide (5′-AAGAAGCGGGAGCATTTCTTC-3′) (37–39). The Y-5 TCRα and TCRβ PCR products were subcloned into pUC19, and sequence analysis revealed Jα18 and Jβ1.3 usage, respectively. Sequencing of additional 5′-extended Vα4 cDNA clones obtained by 5′-RACE (Invitrogen Life Technologies) from total Y-5 RNA was conducted to confirm the identity of the variable region as Vα4.1. Accordingly, 3′-antisense genomic primers corresponding to intron sequences downstream of the respective TCRα and TCRβ joining regions (Jα18, 5′-TGCGGCCGCAAATTTTATACT TACTGGGCTTGATAGATAAC-3′; Jβ1.3, 5′-CACTGCAACCGCG GCACCTCAGAGAGAA-3′) (38, 40) were used in combination, respectively, with sense primers corresponding to sequences located upstream of the Vα4.1 (5′-CTTCCCGGGCTCAAAATATTTGTATTCACACACTCCA-3′) or Vβ7 (5′-CACACTTTCCTCGAGACCACCATGAGAGTTAGG-3′ (37, 39)) coding regions to amplify the corresponding genomic sequences from CTL clone Y-5 nuclear DNA and incorporate restriction endonuclease cleavage sites at the ends of each product (α, 5′-*Xma*I, 3′-*Not*I; β, 5′-*Xho*I, and 3′-*Kpn*I/*Sac*II). The nucleotide sequences of the subcloned genomic V(D)J fragments were verified and liberated from the cloning vector by endonuclease digestion and ligated into the appropriately digested TCRα or β expression cassette plasmids (pTαcass and pTβcass, respectively, obtained from Dr. D. Mathis (Harvard Medical School, Boston, MA) (41)). The full-length Y-5α- and β-chain TCR expression cassette fragments were released by endonuclease digestion as previously described (35) and eluted from unstained agarose gel slices directly into microinjection buffer.

Generation of SV40 T Ag TCR-V mice

Purified Y-5 TCRα- and β-chain expression cassettes were combined before injection. Microinjection of fertilized embryos from B6 mice was performed as described previously (42). The presence of the α and β transgenes in weanlings was determined at 4 wk of age by PCR analysis of tail-derived DNA using the following primer pairs: Vα4.1 chain sense, 5′-GAAGCCACCTCCTTCCACTTGCAG-3′; Jα18 chain antisense, 5′-TGCGGCCGCAAATTTTATACTTACTGGGCTTGATAGATAAC-3′; Vβ7 chain sense, 5′-AAGAAGCGGGAGCATTTCTCC-3′; and Jβ1.3 chain antisense, 5′-CACTGCAACCGCGGCACCTCAGAGAGAA-3′. Amplification of the corresponding 160- and 200-bp fragments from genomic DNA was diagnostic for the presence of the respective transgenes. Expression of the transgene products was confirmed by staining lymphocytes from various lymphoid tissues with a TCR Vβ7-specific mAb and a Db/V tetramer (31). The founder line, line 459, was maintained by backcrossing transgene-positive males with B6 females, and progeny were screened for the presence of both α and β TCR transgenes by PCR analysis (35). A preliminary characterization of TCR-V transgenic mice was reported recently (43). Greater than 90% of T_{CD8} among PBL of TCR-V mice are specific for epitope V, as indicated by positive staining with Db/V tetramer (Fig. 1*A*). Importantly, the surface expression of CD44 on Db/V tetramer$^+$ T cells from TCR-V mice is low, which is consistent with a naive phenotype for TCR-V T cells (Fig. 1*A*).

Cell lines and media

Cell lines used in this study are summarized in Table I. B6/T116A1 cells (B6/V-only T Ag) express a T Ag variant in which epitopes I (residues 207–215) and II/III (residues 223–231) are deleted, and epitope IV is inactivated by alanine substitution of residues 406, 408, and 411 but in which epitope V remains intact (31). B6/T5Aa (B6/wt T Ag) (30) and B6/K-0 (44) cells express wt T Ag. The cell line B6/122B1 (ΔI, II/III, IV, V) expresses a T Ag derivative in which all four CTL determinants were inactivated by substitution of critical MHC class I anchor residues (N210A, N227A, F408A, and N493A) (31). The Ag loss variant B6/K-1,4 was derived by sequential in vitro coculture of B6/K-0 cells with T Ag-specific CTL clones, which resulted in the selection of a clone expressing a T Ag variant in which epitopes I and II/III are deleted, and epitope IV contains an inactivating point mutation (45, 46). The cell line B6/K-1,4-SV was derived previously from the B6/K-1,4 cells by supertransfection with a plasmid encoding the wt T Ag (45). TAP1$^{-/-}$ cells expressing either wt T Ag or V-only T Ag were generated by transfection of B6.129S2-Tap1^{tm1Arp} mouse primary kidney cells with plasmid pPVU0 (47) encoding wt T Ag and pSLM361-11 (31) encoding epitope V-only T Ag, respectively. To ensure that the TAP1$^{-/-}$ cells had the expected phenotype, we determined their ability to activate *LacZ*-inducible T cell hybridomas specific for T Ag dominant (I and IV) and subdominant (V) epitopes (Fig. 1*B*). Coincubation of T cell hybridomas with B6-derived T Ag-transformed cells expressing either wt or V-only T Ag resulted in β-galactosidase production by the epitope V-specific T cell hybridoma. In contrast, T cell hybridomas specific for epitopes I or IV were activated only following coincubation with wt T Ag-expressing cells. T Ag-transformed cell lines on the TAP1$^{-/-}$ background expressing either wt or V-only T Ag failed to activate T cell hybridomas. T Ag expression was confirmed by immunofluorescent staining with T Ag-specific mAbs (data not shown). Thus, the T Ag-transformed cell lines used here have the expected phenotypes and support previous findings that epitope V is efficiently presented from wt T Ag in vitro (30, 33). All cell lines were maintained in DMEM and supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml kanamycin, 2 mM L-glutamine, 10 mM HEPES, 0.075% (w/v) $NaHCO_3$, and 5–10% FBS.

Viruses and synthetic peptides

The recombinant vaccinia virus expressing human TAP1 and TAP2 proteins (VV-TAP(1 + 2) (48)) was obtained from Drs. J. R. Bennink and J. W. Yewdell (National Institutes of Health, Bethesda, MD). The VV-SC vaccinia virus contains only empty vector. Viruses were propagated and titrated in HuTK$^-$143 cells essentially as described previously (33). Peptides were synthesized at the Macromolecular Core Facility of the Milton S. Hershey Medical Center by Fmoc chemistry using an automated peptide synthesizer (9050 MiliGen PepSynthesizer; Millipore). Peptides were solubilized in DMSO and diluted to the appropriate concentration with RPMI 1640 medium. Peptides used in these experiments correspond to the SV40 T Ag epitope I (SAINNYAQKL; peptide I), epitope V (QGINNLDNL; peptide V), and influenza virus nucleoprotein 366–374 (ASNENMETM; peptide Flu).

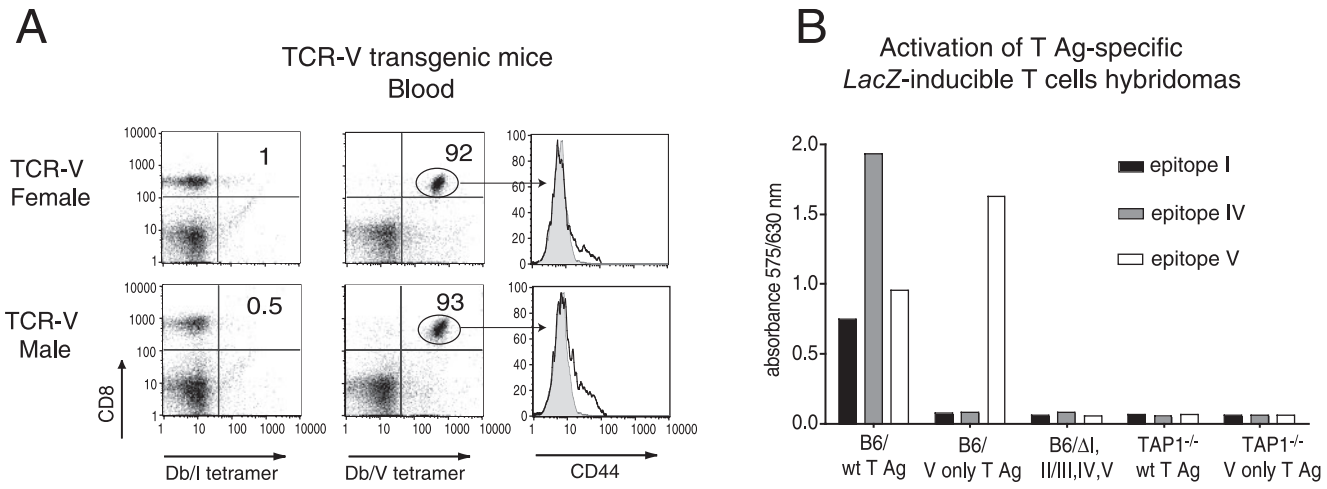

FIGURE 1. Characterization of TCR-V transgenic mice and T Ag-transformed cell lines used for immunization. *A*, Characterization of T Ag epitope V-specific TCR transgenic mice. PBL from TCR-V male and female mice were triple stained with anti-CD8α Ab, the indicated tetramer, and anti-CD44 Ab. Numbers in the *upper right quadrant* indicate the percentage of epitope-specific T_{CD8}. Open histograms indicate the surface expression of CD44 on Db/V tetramer$^+$ T cells. The background fluorescence shown in closed histograms was determined on unstained cells. *B*, Recognition of T Ag-transformed cells by epitope-specific *LacZ* T cell hybridomas. A total of 3×10^4 hybridoma cells specific for T Ag epitope I, IV, or V was incubated overnight with an equal number of B6/wt T Ag, B6/V-only T Ag, B6/Δ I, II/III, IV, and V cells lacking all H-2^b CTL epitopes, TAP1$^{-/-}$ wt T Ag, and TAP1$^{-/-}$ V-only T Ag cells. Accumulation of *LacZ* in activated cells was detected by incubating culture lysates with the *LacZ* substrate, chlorophenol red-β-D-galactopyranoside, and measuring the absorbance of the product at 575 nm using 630 nm as a reference wavelength.

Flow cytometric analysis

Ex vivo staining of T_{CD8} lymphocytes with MHC tetramers and primary-conjugated Abs was performed on single-cell suspension prepared from spleens as described previously (31). Cells were then fixed with 2% paraformaldehyde in PBS and analyzed using a FACScan flow cytometer (BD Biosciences). Routinely, at least 50,000 events were recorded. Data were analyzed and prepared using FlowJo software (Tree Star). Production and characterization of the MHC class I tetramers specific for the H-2D^b/T Ag epitope I (Db/I), H-2D^b/T Ag epitope V (Db/V), H-2K^b/T Ag epitope IV (Kb/IV), and H-2D^b/influenza virus nucleoprotein epitope 366–374 (Db/Flu) was described previously (31). The following Abs were purchased from BD Pharmingen: PE- and cychrome-labeled anti-mouse CD8a (clone 53-6-7), FITC-labeled anti-mouse TCR Vβ7 (clone TR310), FITC-labeled anti-mouse CD44 (clone IM7), FITC-labeled anti-mouse CD62L (clone MEL-14), and FITC-labeled anti-mouse CD122 (clone TM-β1). PE-labeled anti-mouse CD45.1 (clone A20). The percentage of T_{CD8} cells that stained positive for T Ag-specific tetramer was determined by subtracting the percentage of cells that stained positive for Db/Flu tetramer within the same population.

In vivo proliferation assay

RBC-depleted lymphocytes derived from spleens and lymph nodes of TCR-V transgenic mice were resuspended at 1×10^7/ml in PBS/0.2% BSA and labeled with 5 μM 5- and 6-CFSE (Molecular Probes) for 10 min at 37°C. Cells were than washed three times with PBS, resuspended in HBSS, and injected i.v. at a dose of 5×10^6 clonotypic TCR-V T cells/B6 mouse. The mice were then immunized i.p. the following day with T Ag-expressing cells. After 4 days, the dilution of the CFSE label was determined by tetramer staining of splenic lymphocytes.

SV40-specific CTL clones and T Ag-specific LacZ-inducible T cell hybridomas

SV40 epitope V specific CTL line 96 (T. D. Schell, unpublished results) was derived from line SV11 mice by rVV-ES-V immunization followed by booster with B6/WT-19 cells (49). Epitope I-specific CTL were obtained by in vitro activation of spleen cells from line TCR-I mice using gamma-irradiated T Ag-transformed stimulator cells. *LacZ*-inducible T cell hybridomas specific for the T Ag epitopes I, IV, and V were generated by fusing T Ag-specific CTL clones K-11 (epitope I (50)), Y-4 (epitope IV (45)), and H-1 (epitope V (30)) with BWZ.36.1/CD8 cells (kindly provided by Dr. N. Shastri, University of California, Berkeley, CA), using an approach detailed elsewhere (51). After in vitro selection in the presence of hypoxanthine/aminopterin/thymidine and hygromycin, the hybridoma clones responding to peptides specific for epitopes I, IV, and V were identified and further cloned by limiting dilution.

In vitro and in vivo cytotoxicity assays

In vitro cytotoxicity assays were performed as described previously (30). T Ag-transformed cell lines were treated with γ-IFN (40 U/ml) for 48 h followed by labeling overnight with 1 mCi of ^{51}Cr per T-75 flask. The cells were then trypsinized, washed once with PBS/0.1% BSA, resuspended at 5×10^6 cells/ml, and infected with the indicated vaccinia viruses at multiplicity of infection of 10 for 1 h at 37°C with occasional agitation. Cells were then diluted with 10 ml of complete RPMI 1640 medium-10% FBS and rocked at 37°C for an additional 4 h. After centrifugation, target cells were resuspended in complete RPMI 1640 medium-10% FBS and added in 0.1-ml aliquots to 96-well V-bottom plates to yield 1×10^4 cells/well. Effector cells were added to targets in 0.1-ml aliquots to give the E:T ratio of 15 for CTL clone 96 and 10 for in vitro-activated TCR-I cells. Plates were incubated for 4 h at 37°C in 5% CO_2, and cells were then pelleted by centrifugation ($200 \times g$ for 2 min). A total of 0.1 ml of supernatant was transferred to glass tubes, and the radioactivity was counted in a Packard Cobra model 5005 gamma counter. Percent-specific lysis was calculated as follows: percent-specific lysis = ((experimental − spontaneous)/(maximum − spontaneous)) × 100, where spontaneous is the counts per minute released from target cells incubated with medium alone, while maximum is the counts per minute released from target cells incubated in the presence of 2.5% SDS. All data represent the means of triplicate samples.

For in vivo cytotoxicity assays, targets were prepared from sex-matched B6.SJL (CD45.1$^+$) spleen cells incubated in the presence of the indicated peptides (1 μM) in RPMI 1640 medium/10% FBS at 37°C for 90 min and washed three times. Targets were then labeled with different concentrations of CFSE (5 μM/peptide IV; 0.5 μM/peptide I; 0.025 μM/peptide Flu) for 10 min at 37°C in PBS/0.1% BSA, washed twice, and 2×10^6 cells/target (6×10^6 total cells) were injected i.v. into the tail vein in 0.2 ml of HBSS. The elimination of CFSE-labeled targets was assessed the next day by staining splenic cells with PE-labeled anti-CD45.1 mAb. The following formula was used to determine the percentage of specific killing: percentage = (1 − (ratio unprimed/ratio primed) × 100), where ratio = (percentage of CFSE ^{low}/percentage of CFSE$^{high\ or\ medium}$).

^{35}S-metabolic labeling and pulse-chase immunoprecipitation of SV40 T Ag

T Ag-expressing cells grown in T-75 flasks were starved for 1 h at 37°C in methionine-free DMEM supplemented with 2% dialyzed FBS and then pulsed for 1 h at 37°C with 400 μCi of [^{35}S]methionine in 1 ml of medium. After washing the cells three times with cold PBS, the label was chased for

Table I. *SV40 T Ag-transformed cell lines used in this study*

Cell Designation	Cell Type	Transforming Agent	T Ag Construct	H-2^b CTL Epitopes Present	References
B6/T5Aa	C57BL/6 embryo fibroblasts	pLM234	WT	I, II/III, IV, V	30
B6/T116A1	C57BL/6 embryo fibroblasts	pSLM361–11	Δ207–215, Δ223–231, Y406A, F408A, C411A (V-only T Ag)	V	31
B6/K-0	C57BL/6 kidney	pPVU0	WT	I, II/III, IV, V	45
B6/K-1,4[a]	C57BL/6 kidney	Derived from K-0	Δ134–263, V405L	V	45
B6/K-1,4-SV[b]	C57BL/6 kidney	Derived from K-1,4 + pSV2neo-SV40	Δ134–263, V405L T Ag + WT	I, II/III, IV, V	45
B6/122B1 (B6 ΔI, II/III, IV, V T Ag)	C57BL/6 embryo fibroblasts	PLMTS364–1	N210A, N227A, F408A, N493A (no CTL epitopes)	None	31
TAP1$^{-/-}$ wt	B6.129S2-Tap1^{tm1Arp} kidney	pPVU0	WT	I, II/III, IV, V	This study
TAP1$^{-/-}$ 361–11	B6.129S2-Tap1^{tm1Arp} kidney	pSLM361–11	Δ207–215, Δ223–231, Y406A, F408A, C411A (V-only T Ag)	V	This study

[a] K-0 epitope loss variant selected by coculture with T Ag-specific CTL clones in vitro.
[b] K-1,4 cells transfected with pSV2neo-SV40 encoding WT T Ag.

the indicated time in the presence of 100-fold molar excess of cold methionine. Subsequently, cells were washed three times with cold PBS, scraped into tubes, and centrifuged. The cell pellet was lysed in 1 ml of lysis buffer (50 mM Tris-HCl (pH 8.5), 120 mM NaCl, 0.5% Nonidet P-40, and protease inhibitors 1:100 (Sigma-Aldrich)) for 20 min on ice followed by centrifuged at 12,000 × *g* for 5 min. Each cell lysate was precleared with protein A-Sepharose beads conjugated with 2 mg/ml BSA for 2 h at 4°C. Precleared samples were immunoprecipitated overnight with Pab 901 (47) directed to the C-terminal of T Ag and a control Ab to herpes simplex virus glycoprotein D (52). The immune complexes were collected on protein A-Sepharose beads, washed three times with radioimmunoprecipitation assay buffer (10 mM Tris-HCl (pH 8.0), 140 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS), once with SNNTE (50 mM Tris, 5 mM EDTA, 0.5 M NaCl, 5% sucrose, and 1% Nonidet P-40) buffer, and denatured for 5 min at 95°C in 30 μl of 2× sample buffer (100 mM Tris-HCl, 4% SDS, 20% glycerol, 2% 2-ME, and 0.01% bromphenol blue). Proteins were separated on 7.5% SDS polyacrylamide gel under reducing conditions. Gels were fixed in methanol, acetic acid, and water and were treated with Amplify (Amersham Biosciences) and dried. Autoradiography was performed at −80°C using Kodak X-omat LS film.

Results

Naive TCR-V T cells recognize wt T Ag in vivo but only a subset of cells proliferate

One explanation for the subdominant phenotype of epitope V is that the precursor frequency in naive B6 mice might be limiting (1, 11, 12). To determine whether an increase in the precursor frequency of epitope V-specific T_{CD8} could overcome subdominance following immunization with wt T Ag-expressing cells, we developed transgenic mice that express a TCR specific for epitope V to provide a source of naive epitope V-specific T_{CD8}. Line TCR-V mice express the TCRα- and β-chains from the epitope V-specific CTL clone Y-5 on the B6 background (see *Materials and Methods*). Lymphocytes from TCR-V mice were transferred into B6 mice to determine their responsiveness to immunization with T Ag-transformed B6 cells expressing either wt or V-only T Ag. Groups of naive B6 mice received two different doses of TCR-V T cells (1×10^6 or 1×10^7) followed by i.p. immunization with wt or V-only T Ag-transformed cells the next day. Seven days postimmunization, CD8$^+$,Db/V tetramer$^+$ spleen cells were quantitated. TCR-V T cells expanded dramatically in mice immunized with B6/V-only T Ag-transformed cells (Fig. 2*A*). In mice that received 1×10^6 donor cells, TCR-V T cells expanded to 24% of T_{CD8} following immunization with B6/V-only T Ag-transformed cells. Limited expansion of TCR-V T cells, representing 3.4% of T_{CD8}, was observed in mice immunized with B6/wt T Ag-transformed cells (Fig. 2*A*, *left middle panel*). A 10-fold increase in the initial TCR-V donor population failed to result in further increases in the percentage of T_{CD8} specific for epitope V following immunization (Fig. 2*A*, *right panels*). Likewise, a 5-fold increase in the immunizing dose of B6/wt T Ag cells failed to result in increased expansion of TCR-V T cells (data not shown). The absolute number of TCR-V T cells per spleen also was calculated to ensure that the magnitude of the response was not biased by varying numbers of total spleen cells among individual mice. The results are consistent with the data presented as percentages of T_{CD8} (Fig. 2*A*). Thus, only limited accumulation of TCR-V T cells was observed following immunization with wt T Ag, despite the presence of large numbers of naive TCR-V T cells.

This result might be explained by a quantitatively small proportion of naive TCR-V T cells recognizing epitope V in vivo, despite the large Ag dose used (1×10^7 B6/wt T Ag cells). Examination of the cell surface phenotype of TCR-V T cells following immunization revealed that CD44 surface expression was up-regulated on the majority of cells following immunization with either V-only T Ag or wt T Ag-transformed cells (Fig. 2*B*). In contrast, only a subset of TCR-V T cells down-regulated the CD62L lymph node homing receptor or up-regulated the CD122 IL-2β-chain receptor following immunization with wt T Ag. Taken together, these results suggest that only a fraction of the TCR-V T cells are fully activated following exposure to wt T Ag, consistent with limited T cell expansion.

Because suboptimal T cell activation might fail to result in cell division (53), we monitored the proliferation of CFSE-labeled TCR-V T cells 4 days after transfer into B6 mice immunized with either wt or V-only T Ag-transformed cells. Immunization with V-only T Ag induced robust proliferation of TCR-V T cells, representing 71–81% of splenic CD8$^+$ Db/V tetramer$^+$ cells (Fig. 2*C*). In contrast, immunization with wt T Ag-transformed cells induced proliferation of only a small proportion of TCR-V T cells, representing 17–19% of recovered TCR-V T cells. No proliferation was detected after immunization with cells expressing a T Ag variant that lacks all defined T_{CD8} epitopes (data not shown).

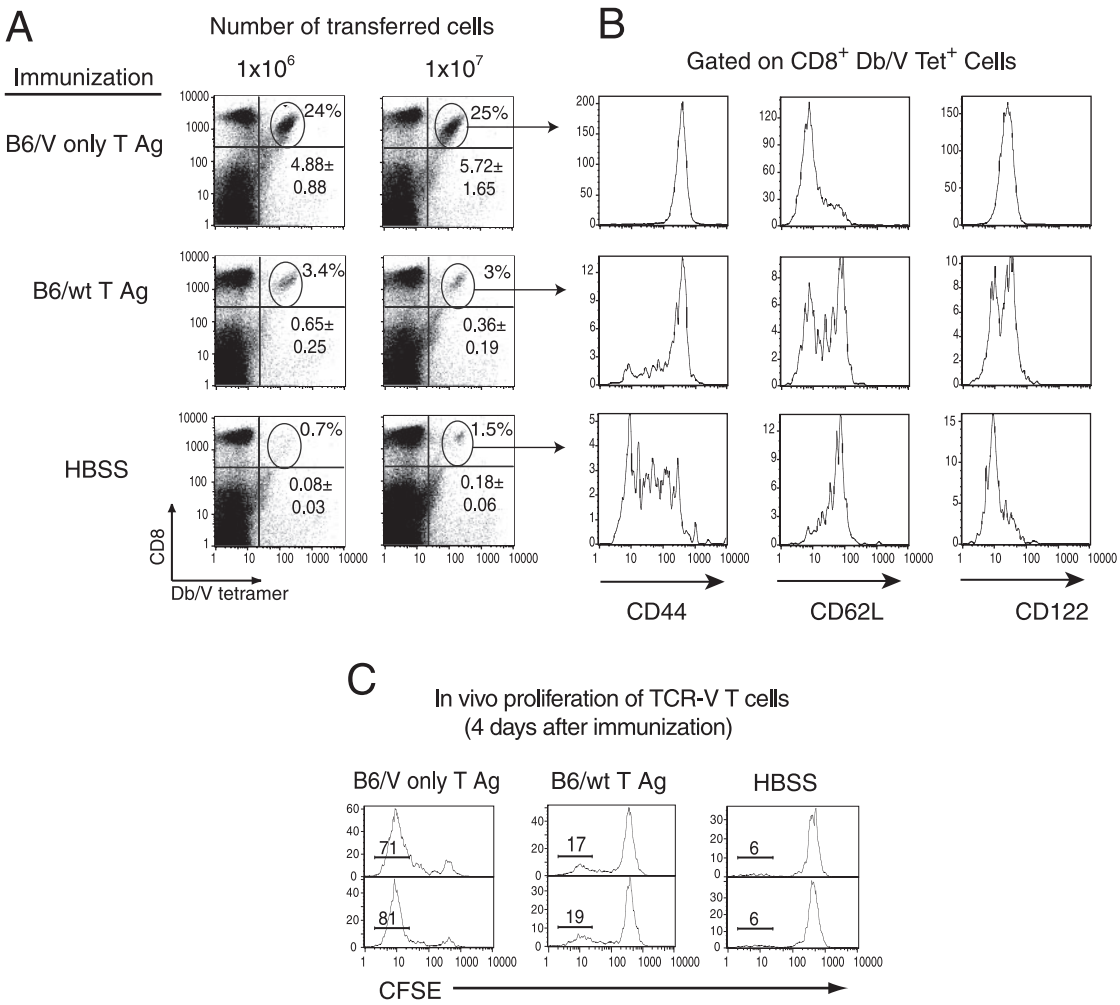

FIGURE 2. Activation and proliferation of TCR-V T cells in response to T Ag immunization. *A*, TCR-V expansion following immunization with T Ag-transformed cells. B6 mice were adoptively transferred with either of two doses of TCR-V T cells (1×10^6 or 1×10^7) followed by immunization the next day with 1×10^7 B6/V-only T Ag or B6/wt T Ag cells or remained unimmunized. Seven days postimmunization, spleen cells were triple stained for CD8α, Db/V tetramer, and cell surface markers CD44, CD62L, or CD122. The *top value* in each dot plot indicates the percentage of splenic T_{CD8} cells positive for Db/V tetramer in one individual mouse, and the bottom value indicates the total number of TCR-V T cells per spleen $\times 10^6 \pm$ SD ($n = 2$ mice/group). *B*, Flow cytometric analysis of T cell activation markers. Histograms show the level of expression of the indicated cell surface markers on the gated population of Db/V tetramer$^+$,T_{CD8} cells shown in *A*. *C*, Immunization with wt T Ag-expressing cells induces extensive proliferation in a small proportion of naive TCR-V T cells. A total of 5×10^6 CFSE-labeled TCR-V T cells was adoptively transferred into naive B6 mice; the next day, recipients were immunized with 1×10^7 B6/V-only T Ag or B6/wt T Ag cells or remained unimmunized. Four days postimmunization, spleen cells were stained for CD8α and Db/V tetramer to reveal the intensity of CFSE fluorescence on TCR-V T cells. Two individual mice per group are shown.

These experiments indicate that under conditions where T cell precursor frequency is not limiting, the subdominant phenotype of epitope V is maintained.

Coexpression of wt T Ag inhibits the immunogenicity of the epitope V-only T Ag variant

The finding that TCR-V T cells expanded dramatically following immunization with V-only T Ag but not wt T Ag indicates that T_{CD8} responding to the dominant epitopes inhibit the T_{CD8} response to the subdominant epitope V. To exclude the possibility that the V-only T Ag is inherently more immunogenic than the wt T Ag due to factors other than the lack of the dominant T Ag epitopes, we determined whether wt T Ag would affect the immunogenicity of V-only T Ag when coexpressed in the same cell. To perform this set of experiments, we used a panel of cell lines derived from the wt T Ag-transformed cell line B6/K-0. In a previous study (44), sequential in vitro selection of B6/K-0 cells with CTL clones specific for epitopes I and IV resulted in the isolation of cells expressing a T Ag variant in which residues 134–263 are deleted, which removes epitopes I (206–215) and II/III (223–231), and an additional mutation at residue 405 (V→L) inactivates epitope IV (404–411) (30, 46). This cell line, called B6/K-1,4, was subsequently supertransfected with a plasmid encoding wt T Ag (32) such that both wt and V-only T Ag constructs are expressed in the same cell. Coexpression of the two T Ags in B6/K-1,4-SV cells was demonstrated previously by immunoprecipitation of the two different-sized T Ag proteins (45). The expression of wt T Ag in B6/K-1,4-SV cells restores presentation of all T Ag epitopes in vitro, as shown by reactivity with epitope-specific CTL clones (36, 45).

TCR-V T cells were transferred into B6 mice followed 1 day later by immunization with 1) B6/K-0 (wt T Ag) cells, 2) B6/K-1,4 (V-only T Ag) cells, or 3) B6/K-1,4-SV (V-only + wt T Ags) cells. Seven days after immunization, mice were sacrificed, and the T_{CD8} response was evaluated by MHC tetramer staining (Fig. 3*A*). Consistent with the data in Fig. 2, immunization with B6/K-0 (wt

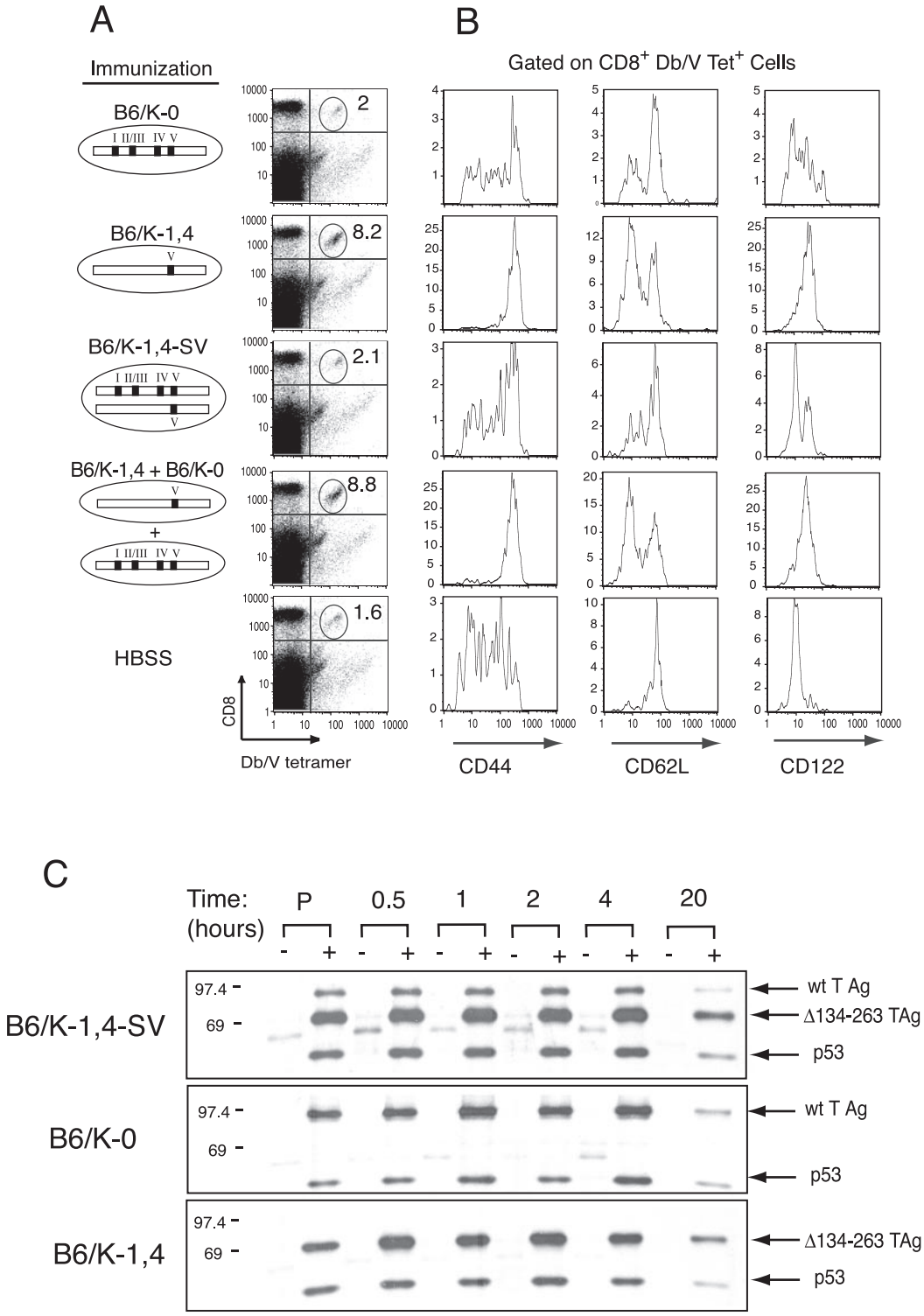

FIGURE 3. Coexpression of wt T Ag and V-only T Ag inhibits TCR-V T cell expansion. *A*, Quantitation of TCR-V T cells following immunization with cells coexpressing wt and V-only T Ags. B6 mice were adoptively transferred with 5×10^6 TCR-V T cells and immunized the next day with 5×10^7 B6/K-0 (wt T Ag), B6/K-1,4 (V-only T Ag), or B6/K-1,4-SV (expresses simultaneously wt T Ag and V-only T Ag) cells. Some mice received 5×10^7 B6/K-0 cells mixed with 5×10^7 B6/K-1,4 cells or remained unimmunized. Seven days later, splenic T_{CD8} specific for epitope V were quantitated by flow cytometry following staining with Db/V tetramer and anti-CD8 Ab. The values shown in the *upper right quadrant* indicate the percentage of splenic T_{CD8} cells positive for Db/V tetramer. *B*, Flow cytometric analysis of activation markers on TCR-V T cells. Histograms show the level of expression of the indicated cell surface marker on the population of Db/V tetramer$^+$ T_{CD8} cells shown within the gate in *A*. *C*, Pulse-chase immunoprecipitation of T Ag from T Ag-transformed cell lines. Cell lines, as indicated on the *left*, were metabolically labeled with [^{35}S]methionine followed by chase in medium containing unlabeled methionine for the indicated times (P; pulse, without chase). After preclearing with BSA-conjugated Sepharose 4B beads, lysates were immunoprecipitated using Sepharose 4B beads conjugated with PAb901 Ab (+) or with negative control Ab anti-HSV gD (−). The beads were then washed, denatured in sample buffer, and proteins were separated by SDS/7.5% PAGE. Size markers in kDa are shown on the *left*. The location of immunoprecipitated proteins is indicated on the *right*.

T Ag) cells led to only a weak expansion of naive TCR-V T cells and the activation of a subset of cells as measured by modulation of CD44, CD62L, and CD122 on the cell surface (Fig. 3*B*). In contrast, immunization with B6/K-1,4 (V-only) cells led to a 5-fold expansion of TCR-V T cells (8.2% of T_{CD8} vs 1.6% of T_{CD8} in the HBSS group) and the induction of an activated phenotype on the majority of cells. Importantly, immunization with B6/K-1,4-SV cells (coexpressing wt and V-only T Ags) mimicked the results obtained with cells expressing wt T Ag alone. In this group, only partial activation and minimal expansion of TCR-V T cells occurred (2.1% of T_{CD8}). Thus, the potent immunogenicity of cells expressing the V-only T Ag is inhibited by coexpression of wt T Ag in the same cells.

To determine whether this inhibitory effect required that wt and V-only T Ags be expressed in the same cells, a fourth group of mice was immunized with B6/K-0 (wt T Ag) cells mixed with an equal number of B6/K-1,4 (V-only T Ag) cells. TCR-V T cells isolated from mice immunized with this cell mixture were fully activated and expanded to the same level as in mice immunized with B6/K-1,4 cells alone (Fig. 3, *A* and *B*). These results indicate that the epitope V-specific T_{CD8} response is inhibited when T_{CD8} specific for the immunodominant epitopes can recognize the same but not different cells. Whether this competition affects the T_{CD8} response to epitope V at the level of APC that are cross-presenting T Ag or by preventing access to the tumor cells themselves remains to be determined.

Coexpression of wt T Ag does not alter the stability of epitope V-only T Ag

Recent studies have suggested that cross-presented Ag is derived from long-lived proteins found in the donor cell (54–56). Thus, a change in V-only T Ag stability might alter its immunogenicity. To determine whether coexpression of wt T Ag alters the stability of V-only T Ag, we performed pulse-chase immunoprecipitation of the T Ags in B6/K-0, B6/K-1,4, and B6/K-1,4-SV cells (Fig. 3*C*). The T Ags were detected as two prominent bands: 94 kDa corresponding to wt T Ag and 75 kDa corresponding to the epitope V-only variant (Δ134-263 T Ag; Fig. 3*C*). The stability of V-only T Ag was similar in both B6/K-1,4-SV cells and the parental B6/K-1,4 cells. This finding demonstrates that wt T Ag does not adversely affect the stability of the V-only T Ag variant when coexpressed in the same cell, although it dramatically reduces the epitope V immunogenicity of these cells. Therefore, the inability of K-1,4-SV cells to induce significant expansion of naive TCR-V T cells does not correlate with differences in protein stability within the transformed cells.

Cross-presentation of T Ag leads to inefficient expansion of TCR-V cells

The initiation of T_{CD8} responses to cell-associated Ags has been shown to depend on cross-presentation by professional APC in several experimental systems (57–60). Thus, one possible explanation for the weak activation of TCR-V T cells following immunization with wt T Ag-transformed cells is that epitope V might be poorly cross-presented compared with the immunodominant epitopes. To examine the role of cross-presentation in the activation and the expansion of TCR-V T cells, we used $TAP1^{-/-}$ cells transformed with either wt or V-only T Ag for immunization. $TAP1^{-/-}$ cells are defective in the transport of cytosolic antigenic peptides into the endoplasmic reticulum due to the absence of the TAP1 component of the peptide transporter (61). Thus, these cells are defective in the presentation of most endogenous Ags by MHC class I molecules but are capable of donating Ag for cross-priming in vivo (62).

To ensure that T Ag epitopes are not directly presented by T Ag-transformed $TAP1^{-/-}$ cells and that no other defects in these cells could inhibit T cell recognition, we reconstituted the TAP1 protein by infecting each $TAP1^{-/-}$ cell line with a recombinant vaccinia virus expressing the TAP1 and TAP2 proteins ((VV-TAP1 + 2); Ref. 48). $TAP1^{-/-}$ wt T Ag cells were efficiently recognized by in vitro-activated T cells derived from TCR-I mice after infection with VV-TAP(1 + 2) but not when infected with vaccinia recombinant VV-SC expressing an empty vector (Fig. 4*A*). Likewise, $TAP1^{-/-}$ V-only T Ag cells were lysed by a CTL clone specific for epitope V after infection with VV-TAP(1 + 2) but not after infection with empty vector VV-SC. Thus, the expected T Ag epitopes are presented by $TAP1^{-/-}$ cells following restoration of functional TAP. No CTL lysis was detected following infection of T Ag epitope null cells (B6/122B1) with VV-TAP(1 + 2) or with VV-SC. In addition, T Ag epitope-specific *LacZ* T cell hybridomas failed to recognize the T Ag-transformed $TAP1^{-/-}$ cells (see *Materials and Methods*). These results demonstrate that $TAP1^{-/-}$ cells expressing wt or V-only T Ag do not directly present T Ag epitopes for T cell recognition unless functional TAP is restored. In the absence of direct presentation of epitopes on the surface of T Ag-transformed cells, the T_{CD8} response induced by immunization with $TAP1^{-/-}$ cells can be attributed solely to the cross-presentation of T Ag by host APC.

To determine the efficiency of epitope V cross-presentation in vivo, we measured the activation, proliferation, and accumulation of TCR-V T cells in B6 mice immunized with $TAP1^{-/-}$ cells. These results were compared with cross-presentation of a dominant T Ag epitope, using TCR transgenic T cells specific for the immunodominant T Ag epitope I (TCR-I cells; Ref. 35). Mice were adoptively transferred with naive TCR-I (Fig. 4*B*) or TCR-V (Fig. 4*C*) T cells. The following day, mice that received TCR-I T cells were immunized with B6/wt T Ag or $TAP1^{-/-}$ wt T Ag-transformed cells or remained unimmunized. Similarly, TCR-V T cell recipients were immunized with B6/V-only T Ag or $TAP1^{-/-}$V-only T Ag-transformed cells or remained unimmunized. After 7 days, spleen cells were analyzed. Naive TCR-I T cells expanded to 49 and 12% of splenic T_{CD8} cells, respectively, following priming with the B6- or $TAP1^{-/-}$-derived wt T Ag-expressing cells, (Fig. 4*B*). TCR-I T cells recovered from both sets of mice expressed high levels of CD44. Likewise, TCR-V T cells expanded to 32% of splenic T_{CD8} cells and up-regulated CD44 after priming with B6/V-only T Ag-expressing cells (Fig. 4*C*) but failed to expand significantly after priming with $TAP1^{-/-}$ V-only T Ag-expressing cells. In addition, only a small population of TCR-V T cells showed up-regulation of CD44. This result suggests that only a small fraction of naive TCR-V T cells detected the presence of cross-presented epitope V following immunization with $TAP1^{-/-}$ V-only T Ag-expressing cells and failed to expand significantly.

Because no apparent increase in TCR-V frequency was observed 7 days postimmunization with $TAP1^{-/-}$ V-only T Ag-expressing cells, we asked whether any TCR-V cells were induced to proliferate early after immunization. Thus, the experiment shown in Fig. 4, *B* and *C*, was repeated using CFSE-labeled TCR-I and TCR-V donor cells. Three days after immunization, spleen cells were analyzed to determine the extent of TCR-I and TCR-V T cell proliferation. The data in Fig. 4*D* show that TCR-I T cells proliferated extensively after immunization with B6/wt T Ag or $TAP1^{-/-}$ wt T Ag cells. The TCR-V T cells similarly proliferated after immunization with B6/V-only T Ag. Importantly, immunization with $TAP1^{-/-}$ V-only T Ag cells triggered proliferation in only a subset of TCR-V T cells (28% CFSE negative). This result demonstrates that cross-presentation of epitope V results in proliferation of only a fraction of naive TCR-V T cells and implies

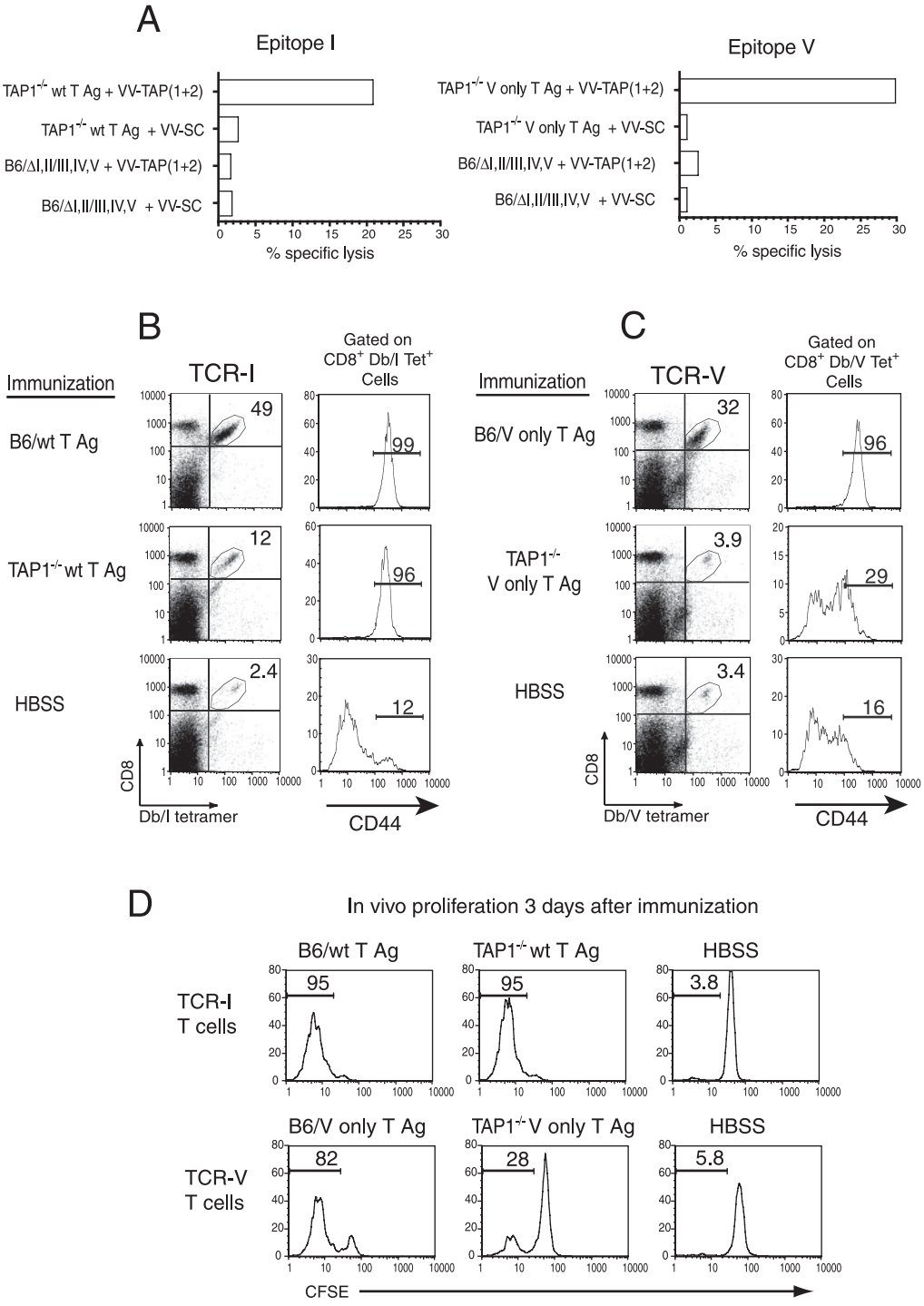

FIGURE 4. Cross-presentation of epitope V leads to proliferation of only a subset of naive TCR-V T cells. *A*, CTL recognition of T Ag-expressing TAP1$^{-/-}$ cells. ^{51}Cr-labeled T Ag-transformed cells were infected at multiplicity of infection = 10 with vaccinia viruses expressing TAP 1 + 2 (VV-TAP(1 + 2)) or VV-SC containing no insert. Following 5 h of infection, target cells were combined with a CTL clone specific for T Ag epitope V at an effector-to-target cell ratio (E:T) of 15:1 and with in vitro-activated TCR-I T cells at an E:T of 10:1 for 4 h. B6/T122B1 cells express a T Ag variant lacking all H-2^b CTL epitopes (Δ I, II/III, IV, V). *B*, Flow cytometric analysis of TCR-I T cell response to immunization with TAP1$^{-/-}$ cells. Naive B6 mice were adoptively transferred with 1×10^6 TCR-I T cells followed by immunization with 5×10^7 B6/wt T Ag (TAP$^{+/+}$) cells, TAP1$^{-/-}$ wt T Ag cells, or with vehicle (HBSS). Seven days postimmunization, spleen cells were triple stained with anti-CD8α Ab, the indicated tetramer and anti-CD44 Ab. The values in *upper right quadrants* indicate the percentage of splenic T_{CD8} that are positive for either Db/I tetramer or Db/V tetramer. Histograms show the level of surface expression of CD44 on the gated population of tetramer$^+$,T_{CD8}. *C*, Flow cytometric analysis of TCR-V T cell response to immunization with TAP1$^{-/-}$ cells. Naive B6 mice were adoptively transferred with 1×10^6 TCR-V T cells followed by immunization with 5×10^7 B6/V-only T Ag (TAP$^{+/+}$) cells, TAP1$^{-/-}$V-only T Ag cells, or with vehicle (HBSS). Cells were analyzed as in *B*. *D*, In vivo proliferation of CFSE-labeled TCR transgenic T cells. Mice were adoptively transferred with CFSE-labeled TCR-I (*top*) or TCR-V (*bottom*) T cells and immunized the next day with the indicated B6 or TAP1$^{-/-}$ cells or left unimmunized (HBSS). Three days after immunization, spleen cells were stained for CD8α and Db/I or Db/V tetramer, and the intensity of CFSE fluorescence on TCR-I and TCR-V T cells was determined by flow cytometry.

that additional direct presentation of epitope V by the B6/V-only T Ag-transformed cells also is required for maximal expansion of these activated T_{CD8}. By comparison, direct presentation of epitope I by wt T Ag-transformed cells was shown to be dispensable for inducing proliferation of TCR-I T cells by day 3 postimmunization. However, a role for direct presentation of epitope I by wt T Ag-transformed cells in obtaining maximal TCR-I T cell expansion is suggested because TCR-I T cells accumulated to higher levels by day 7 postimmunization with B6 vs TAP1$^{-/-}$ wt T Ag cells (Fig. 4*B*).

Direct presentation alone by T Ag-transformed cells is not sufficient to prime naive TCR-V T cells

To address the possibility that T Ag-transformed cells can prime naive TCR-V T cells directly and initiate an immune response in the absence of costimulatory signals provided by professional APC cross-presenting T Ag, we compared the response of adoptively transferred TCR-V T cells to immunization in B6 vs TAP1$^{-/-}$ hosts. To ensure that only naive TCR-V T cells were transferred, T_{CD8} expressing low amounts of cell surface CD44 were sorted by flow cytometry before adoptive transfer (Fig. 5*B*). B6 and TAP1$^{-/-}$ mice were adoptively transferred with TCR-V T cells and immunized on the same day with B6/V-only T Ag-expressing cells. Seven days later, mice were sacrificed, and TCR-V T cell expansion was evaluated.

Consistent with our previous results, naive TCR-V T cells expanded dramatically in B6 mice immunized with B6/V-only T Ag (Fig. 5*A*). In contrast, immunization of TAP1$^{-/-}$ mice with B6/V-only T Ag-expressing cells did not lead to detectable expansion of TCR-V T cells. To ensure that TCR-V T cells could respond to specific immunization in TAP1$^{-/-}$ hosts, one group of TAP1$^{-/-}$ mice was immunized with a vaccinia virus recombinant expressing epitope V as a minigene preceded with an endoplasmic reticulum-targeting sequence (rVV-ES-V) in addition to B6/V-only T Ag cells. The use of rVV-ES-V bypasses any requirements for TAP in the presentation of epitope V (33). TCR-V T cells expanded to ~18% of T_{CD8} in these mice, confirming the ability of the sorted TCR-V T cells to expand in TAP1$^{-/-}$ mice. Thus, direct presentation of epitope V by B6/V-only T Ag-transformed cells is not sufficient to induce the extensive accumulation of TCR-V T cells observed in B6 mice. This finding is consistent with the results of others who demonstrated that cross-presentation of Ag by host APC is required for activation of naive T_{CD8} (60, 63). Taken together, the results in Figs. 4 and 5 suggest that limited cross-presentation of epitope V is needed to activate a few naive TCR-V T cells in B6 mice, but direct presentation of Db/V complexes by T Ag-transformed cells drives the more extensive proliferation observed following immunization with V-only T Ag-transformed cells.

Cross-presentation of epitope V inefficiently boosts memory T_{CD8}

We next determined the extent to which Ag experienced T_{CD8} specific for epitope V were reactivated following cross-presentation of epitope V. For this experiment, we used the endogenous epitope V-specific T_{CD8} established in B6 mice following primary immunization with B6/V-only T Ag-transformed cells. At days 14 and 21 postimmunization, primed mice were boosted with either B6 or TAP1$^{-/-}$ cells expressing wt or V-only T Ag. Seven days later, the immune response to T Ag was analyzed using both MHC tetramer staining and the in vivo cytotoxicity assay (Fig. 6). Mice that received only primary immunization 28 days earlier with B6/V-only T Ag cells had low levels of epitope V-specific T_{CD8} (HBSS; 0.2%) and failed to show any significant elimination of peptide V-coated targets in the in vivo cytotoxicity assay. Epitope V-specific in vivo cytotoxicity was observed at earlier times after immunization (data not shown). Epitope V-specific T cells increased 30-fold following boosters with B6/V-only cells, representing 6% of splenic $T_{CD8,}$ and this resulted in the elimination of 79% of peptide V-pulsed target cells. As expected, there was no elimination of peptide I-pulsed target cells in these mice. Boosting with B6/wt T Ag cells led to a 10-fold increase in the percentage of T_{CD8} specific for epitope V (B6/wt; 2%) compared with unboosted mice and resulted in a detectable population of epitope I- and IV-specific T cells (2 and 12%, respectively). Accordingly, the in vivo cytotoxicity assay revealed killing of both peptide I- and V-coated targets (82 and 63% elimination, respectively) following boosting with B6/wt T Ag cells.

In contrast to boosting with B6-derived T Ag-transformed cells, TAP1$^{-/-}$ cells expressing either epitope V-only T Ag or wt T Ag failed to induce significant expansion of the epitope V-specific memory T cells. However, some reactivation of epitope V-specific memory T cells was indicated by increased levels of killing against peptide V-pulsed target cells following boosting with TAP1$^{-/-}$ V-only T Ag cells (20% elimination) and TAP1$^{-/-}$ wt T Ag cells (8% elimination). In contrast, immunization with TAP1$^{-/-}$ wt T Ag cells induced detectable responses against epitopes I and IV (1 and 2% of

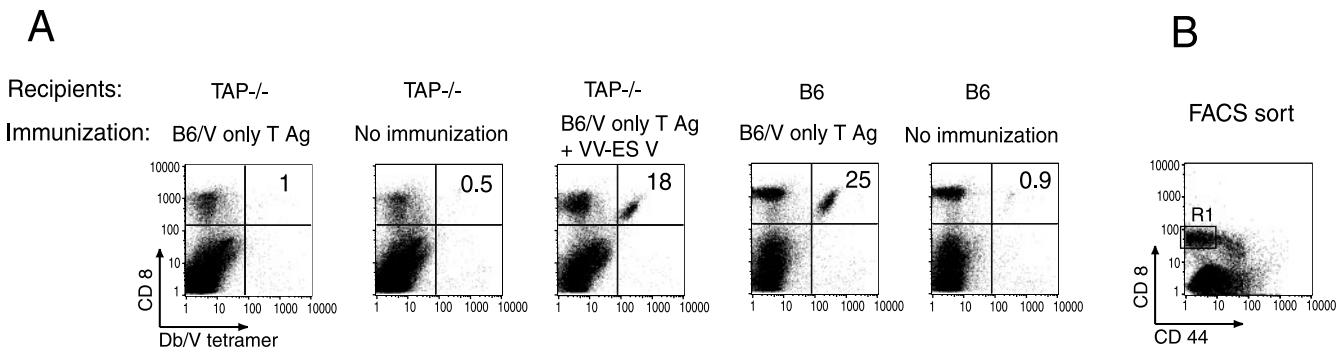

FIGURE 5. Direct priming by T Ag-transformed cells in the absence of cross-presentation is not sufficient to expand TCR-V T cells. *A*, Analysis of TCR-V T cell expansion in TAP1$^{-/-}$ mice. FACS-sorted naive TCR-V T cells (1×10^6) were adoptively transferred into gamma-irradiated (400 rad) TAP1$^{-/-}$ mice or nonirradiated B6 control mice. Recipient mice were immunized with 5×10^7 B6/V-only T Ag (TAP$^{+/+}$) on the same day or received HBSS as a control. One group of TAP1$^{-/-}$ mice was immunized with 5×10^7 B6/V-only T Ag cells plus i.p. immunization with 1×10^7 PFU of VV-ES-V. The values show the percentage of Db/V tetramer$^+$ T cells of splenic T_{CD8}. This experiment was repeated twice with similar results. *B*, Phenotype of donor TCR-V T cells. CD44^{low}-expressing CD8$^+$ T cells from naive TCR-V mice were sorted by flow cytometry before transfer into TAP1$^{-/-}$ mice.

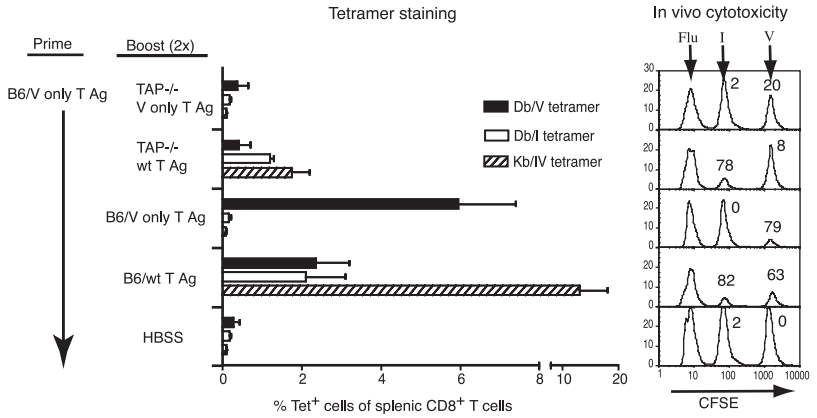

FIGURE 6. Epitope V memory T_{CD8} preferentially expand in response to direct presentation by T Ag-transformed cells. Groups of two or three B6 mice were immunized with 5×10^7 B6/V-only T Ag cells. Two and 3 wk later, mice were boosted with the same dose of B6/V-only T Ag, B6/wt T Ag, TAP1$^{-/-}$ V-only T Ag, or TAP1$^{-/-}$ wt T Ag cells or received no boost (HBSS). One week later, spleen cells from individual mice were analyzed for the presence of T_{CD8} specific for epitopes I, IV, and V by costaining with MHC class I tetramers. The data are presented as a percentage of splenic T_{CD8} that stained specifically with the indicated tetramer, and the background staining on naive B6 mice was subtracted and did not exceed 1% of T_{CD8}. Mice were simultaneously analyzed for the presence of epitope I- and V-specific effector T cells by in vivo cytotoxicity assay. B6.SJL spleen cell targets were incubated with peptides V, I, or Flu control peptide. Peptide-pulsed cells were then differentially labeled with CFSE (5, 0.5, and 0.025 μM, respectively), and 2×10^6 of each target population were injected 18 h before analysis of CD45.1$^+$ cells reisolated from spleens by flow cytometry. Histograms for representative mice are shown. The values indicate the percentage of specific elimination of CD45.1$^+$ targets (see *Materials and Methods*).

splenic T_{CD8} cells, respectively) and efficient killing of peptide I-coated targets (78% elimination). These results suggest that cross-presentation of epitope V alone is unable to expand epitope V-specific memory T_{CD8}. Efficient expansion required direct presentation of epitope V/Db complexes by B6-derived V-only or wt T Ag-transformed cells. By comparison, cross-presentation of wt T Ag led to expansion of the endogenous epitope I- and IV-specific T_{CD8}, although the frequencies achieved were reduced compared with immunization with B6/wt T Ag cells.

Discussion

Transfer of Ag from tumor cells to professional APC provides a viable mechanism to present tumor Ag epitopes for activation of naive T_{CD8} in cases where tumor cells lack the necessary costimulatory molecules. In support of this mechanism, cross-presentation of tumor Ags has been documented in multiple tumor systems (28, 58, 64–67). In this article, we demonstrate that the efficiency of cross-presentation can vary for epitopes within the same tumor Ag. Our results indicate that the subdominant epitope V is only weakly cross-presented in vivo from T Ag-transformed cells. Inefficient cross-presentation led to priming of only a subset of naive TCR-V T cells and also failed to significantly expand epitope V-specific memory T_{CD8}. However, under cross-priming conditions, all naive immunodominant TCR-I T cells were induced to proliferate and expanded to substantial levels. In addition, immunization of B6 mice with TAP1$^{-/-}$ wt T Ag cells primed endogenous T_{CD8} specific for epitopes I and IV. These data indicate that the subdominant epitope V is poorly cross-presented, whereas the immunodominant T Ag epitopes are more efficiently cross-presented under the same conditions. Thus, limited cross-presentation in vivo contributes to the subdominant nature of T Ag epitope V following immunization with T Ag-transformed cells.

Previous investigations of epitope V revealed that this antigenic peptide forms relatively short-lived complexes with H-2D^b compared with the dominant epitopes of T Ag (30, 33). Thus, in a system where only a limited amount of epitope is available, such as might occur during cross-presentation of cell-associated Ags, epitopes that are generated less efficiently or form more labile complexes with MHC class I molecules would be at a disadvantage regarding T cell priming by the APC (68). Cross-presentation involves the transfer of cell-associated Ag from a donor cell to a host professional APC (60). Although the nature of the cross-presented substrate has not been clearly identified, recent reports (54–56) suggest that the substrate for cross-presented Ags are native proteins or larger protein fragments. Our data indicate that cross-presentation of the epitope V is severely compromised even though cross-presentation of immunodominant T Ag epitopes within the same protein is maintained. Thus, one possible explanation is that the relatively short half-life of epitope V/D^b complexes generated in the APC from a fixed amount of transferred T Ag might provide limited opportunity for cross-priming to occur before epitope V/Db complexes fall below detectable levels. Such a mechanism has been proposed to explain immunodominance to some minor histocompatibility Ags (69). Furthermore, the instability of peptide/MHC complexes has been shown to limit the immunogenicity of an epitope derived from the gp100 tumor-associated Ag (70) and may explain the subdominant nature of a *Listeria monocytogenes*-derived epitope from the p60 protein (71). Recent studies investigating T cell activation in vivo have suggested that naive T cells require only a brief encounter with the Ag (4–8 h) to result in the modulation of cell surface receptors such as CD44 and CD69 but require more extended Ag exposure to enter productive proliferation and acquire effector function (72, 73). It should be noted that these studies were performed using immunodominant epitope-specific T cells and have yet to be confirmed using T cells specific for a subdominant epitope. Given the need for such time periods and the lability of epitope V/Db complexes, the opportunity for extended Ag engagement with APC cross-presenting epitope V in vivo might be limited, resulting in proliferation of only a small number of epitope V-specific T cells. Pamer and colleagues (74) previously demonstrated that premature termination of *L. monocytogenes* infection at 12 h by antibiotic treatment resulted in a dramatic decrease in the number of subdominant epitope-specific TCR transgenic T_{CD8} that proliferate, whereas the expansion of dominant epitope-specific TCR transgenic T_{CD8} was reduced only 2-fold. As in the model used here, this difference is consistent with the relative low stability of the subdominant epitope/

MHC complexes. Thus, the rapid loss of peptide/MHC complexes correlates with triggering of relatively fewer naive T_{CD8}.

Alternatively, epitope V may be inefficiently processed and presented by professional APC compared with the immunodominant epitopes as has been reported for some subdominant epitopes (75). We have shown previously that epitope V-specific CTL efficiently recognize IFN-γ-treated wt T Ag-transformed fibroblast cells, suggesting that induction of immunoproteasomes in general does not preclude presentation of epitope V in vitro (33). However, whether epitope V may be generated less efficiently by APC in vivo is unknown.

The ability of tumor cells to directly prime T_{CD8} responses in vivo has been implicated under conditions in which the tumor cells are able to migrate to the draining lymph nodes (25). The finding that cross-presentation of epitope V alone induces inefficient expansion of naive TCR-V T cells prompted us to investigate whether direct presentation by the B6/V-only T Ag cells was sufficient to result in priming and expansion of TCR-V T cells. However, lack of TCR-V T cell expansion in TAP1$^{-/-}$ hosts following immunization with B6/V-only T Ag cells indicated that direct priming alone was not sufficient to activate and expand naive TCR-V T cells. Why then do TCR-V T cells expand so dramatically in B6 mice following immunization with B6/V-only T Ag cells? We suggest that TCR-V T cell activation requires initial cross-presentation by host APC, but more extensive expansion occurs only after direct presentation by the T Ag-transformed cells themselves. This scenario is supported by the finding that a small population of TCR-V T cells are initially induced to proliferate following immunization with TAP1$^{-/-}$ V-only T Ag cells but fail to expand significantly. B6/V-only T Ag immunization could provide substrate for both cross-presentation to activate a few naive TCR-V T cells and direct presentation by the tumor cells themselves to drive further expansion. In this manner, constitutive direct presentation by the tumor cells could supplement the limited number of labile complexes found on the APC. Whether this interaction might happen simultaneously or serially remains to be determined but could be explained by the formation of three cell complexes in the lymphoid organs such that host APC cross-presenting epitope V, tumor cells directly presenting epitope V and TCR-V T cells interact simultaneously.

The participation of direct presentation by the tumor cells in driving T_{CD8} expansion in this model is also implied for the immunodominant epitopes. In this case, we found that efficient priming of naive TCR-I T cells was similar 3 days following immunization with TAP1$^{-/-}$ wt T Ag cells or B6/wt T Ag cells. However, total accumulation by day 7 was significantly higher following immunization with B6/wt T Ag cells, suggesting that additional direct presentation by the tumor cells led to enhanced expansion of T_{CD8} cells. This scenario is also supported by the finding that immunization of B6 mice with TAP1$^{-/-}$ wt T Ag cells induced fewer endogenous epitope I and IV-specific T_{CD8} than the B6-derived cells. Thus, the combination of cross-presentation of T Ag epitopes for activation of naive T_{CD8} plus additional direct presentation of T Ag epitopes by the tumor cells might lead to more efficient expansion of activated T_{CD8}. However, this effect might be more dramatic for weaker T_{CD8} responses.

In addition to inefficient cross-presentation of epitope V, our results indicate that the presence of T_{CD8} responding to the dominant T Ag epitopes contribute to the subdominant nature of epitope V. This is most evident in experiments in which immunization with B6/V-only T Ag cells led to extensive expansion of naive TCR-V T cells, while B6/wt T Ag cells led to only minimal expansion. This was demonstrated using two different epitope V-only T Ag constructs, indicating that this effect is due to the absence of immunodominant T Ag epitopes and not other intrinsic factors of the particular cell line. The frequency of epitope-specific T cell precursors has been shown to alter the immunodominance hierarchy in several Ag systems (1, 11, 12). Our results demonstrate that epitope V remains subdominant, even in the presence of supraphysiological numbers of naive TCR-V T cells and pre-existing endogenous memory T cells specific for epitope V. The inability of TCR-V T cells to expand significantly following immunization with B6/wt T Ag-expressing cells is likely due to the combination of inefficient cross-presentation of epitope V coupled with competition by the immunodominant T Ag epitope-specific T_{CD8}.

Previous studies on T cell competition revealed that presentation of dominant and subdominant epitopes by the same APC is required for maintenance of the immunologic hierarchy (11, 16–18, 76, 77). Kedl et al. (17) suggested that T cell interaction with specific peptide-MHC complexes on the surface of the APC induced the loss of that particular complex, thereby preventing activation of lower-affinity T_{CD8} specific for the same epitope. However, this mechanism does not explain domination of one epitope over another. Experiments using minor histocompatibility Ags demonstrated that T_{CD8} cells can compete for different epitopes if presented by the same APC (18, 78, 79). The nature of this competition was proposed to be of either a steric nature, competition for cytokines in the local environment, inactivation of the APC via cell killing or another unknown mechanism. Although this phenomenon, called cross-competition (76), was reported to be far less efficient than the competition with T_{CD8} cells of the same specificity, we reasoned that it might play a significant role under certain conditions, particularly if Ag is limiting as is suggested for epitope V.

Although our results indicate that T_{CD8} responding to the immunodominant T Ag epitopes inhibit expansion of epitope V-specific T_{CD8}, the mechanism remains unknown. We suggest that competition may occur at the level of the tumor cells themselves. The finding that epitope V memory T_{CD8} are less efficiently expanded by B6/wt T Ag cells than B6/V-only T Ag cells suggests that competition for the tumor cells following priming of endogenous T_{CD8} specific for the immunodominant epitopes limits the expansion of epitope V-specific memory T_{CD8}. Conversely, there was minimal expansion of epitope V-specific memory T_{CD8} following immunization with TAP1$^{-/-}$ wt or V-only T Ag-expressing cells, suggesting that the endogenous epitope V-specific memory T_{CD8} are responding preferentially to direct presentation by the tumor cells. The additional finding that coimmunization with a mixture of wt and V-only T Ag-transformed B6 cells led to efficient expansion of naive TCR-V T cells suggests that immunodomination requires copresentation of the dominant and subdominant epitopes by the same cell. Our results do not rule out the possibility that T_{CD8} responding to the dominant epitopes also can act at the level of the cross-presenting APC. In fact, this mechanism might be more important for inhibiting priming of the endogenous epitope V-specific T_{CD8} response because fewer precursor T_{CD8} are available in the normal repertoire of B6 mice than in the experiments using adoptively transferred TCR-V T cells.

The results presented in this article suggest that when multiple T_{CD8} epitopes derive from the same antigenic protein, epitope-specific factors that affect cross-presentation can limit T_{CD8} immunity. Thus, even if an epitope is directly presented on tumor cells that constitutively express the antigenic protein, transfer of a fixed amount of tumor Ag to the APC might result in subthreshold levels of peptide/MHC complexes to activate a significant number of T_{CD8}. One potential benefit of this effect for the tumor-specific T_{CD8} repertoire is that T_{CD8} specific for poorly cross-presented

self-tumor epitopes might be less susceptible to tolerance induction due to limited presentation of epitopes in the steady state. We have shown previously that epitope V-specific T_{CD8} are less susceptible to both central and peripheral tolerance mechanisms than the immunodominant T Ag epitopes in T Ag transgenic mice developing spontaneous tumors (43, 49, 80). Thus, T cells specific for epitopes that are poorly cross-presented might represent good vaccine candidates for cancer as they may be less susceptible to tolerance yet capable of responding to specific immunization approaches. Our results, and those of others (11, 16), also suggest that immunization with individual epitopes, as opposed to multisubunit vaccines, would reduce the development of immunological hierarchies for epitopes that are limited by cross-presentation, allowing efficient priming of both dominant and subdominant epitope-specific T_{CD8}. Thus, future vaccination approaches that require cross-presentation should consider the epitope-specific factors that affect the efficiency of T_{CD8} responses.

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Disclosures

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References

1. Chen, W., L. C. Anton, J. R. Bennink, and J. W. Yewdell. 2000. Dissecting the multifactorial causes of immunodominance in class I-restricted T cell responses to viruses. *Immunity* 12: 83–93.
2. Vijh, S., and E. G. Pamer. 1997. Immunodominant and subdominant CTL responses to *Listeria monocytogenes* infection. *J. Immunol.* 158: 3366–3371.
3. Niedermann, G., S. Butz, H. G. Ihlenfeldt, R. Grimm, M. Lucchiari, H. Hoschutzky, G. Jung, B. Maier, and K. Eichmann. 1995. Contribution of proteasome-mediated proteolysis to the hierarchy of epitopes presented by major histocompatibility complex class I molecules. *Immunity* 2: 289–299.
4. Deng, Y., J. W. Yewdell, L. C. Eisenlohr, and J. R. Bennink. 1997. MHC affinity, peptide liberation, T cell repertoire, and immunodominance all contribute to the paucity of MHC class I-restricted peptides recognized by antiviral CTL. *J. Immunol.* 158: 1507–1515.
5. Mo, A. X., S. F. van Lelyveld, A. Craiu, and K. L. Rock. 2000. Sequences that flank subdominant and cryptic epitopes influence the proteolytic generation of MHC class I-presented peptides. *J. Immunol.* 164: 4003–4010.
6. Neisig, A., J. Roelse, A. J. Sijts, F. Ossendorp, M. C. W. Feltkamp, W. M. Kast, C. J. M. Melief, and J. J. Neefjes. 1995. Major differences in transporter associated with antigen processing (TAP)-dependent translocation of MHC class I-presentable peptides and the effect of flanking sequences. *J. Immunol.* 154: 1273–1279.
7. Cox, J. H., J. W. Yewdell, L. C. Eisenlohr, P. R. Johnson, and J. R. Bennink. 1990. Antigen presentation requires transport of MHC class I molecules from the endoplasmic reticulum. *Science* 247: 715–718.
8. Jackson, M. R., M. F. Cohen-Doyle, P. A. Peterson, and D. B. Williams. 1994. Regulation of MHC class I transport by the molecular chaperone, calnexin (p88, IP90). *Science* 263: 384–387.
9. van der Burg, S. H., M. J. W. Visseren, R. M. P. Brandt, W. M. Kast, and C. J. M. Melief. 1996. Immunogenicity of peptides bound to MHC class I molecules depends on the MHC-peptide complex stability. *J. Immunol.* 156: 3308–3314.
10. Sette, A., A. Vitiello, B. Reherman, P. Fowler, R. Nayersina, W. M. Kast, C. J. M. Melief, C. Oseroff, L. Yuan, J. Ruppert, et al. 1994. The relationship between class I binding affinity and immunogenicity of potential cytotoxic T cell epitopes. *J. Immunol.* 153: 5586–5592.
11. Palmowski, M. J., E. M. Choi, I. F. Hermans, S. C. Gilbert, J. L. Chen, U. Gileadi, M. Salio, A. Van Pel, S. Man, E. Bonin, et al. 2002. Competition between CTL narrows the immune response induced by prime-boost vaccination protocols. *J. Immunol.* 168: 4391–4398.
12. Choi, E. Y., G. J. Christianson, Y. Yoshimura, T. J. Sproule, N. Jung, S. Joyce, and D. C. Roopenian. 2002. Immunodominance of H60 is caused by an abnormally high precursor T cell pool directed against its unique minor histocompatibility antigen peptide. *Immunity* 17: 593–603.
13. Rodriguez, F., S. Harkins, M. K. Slifka, and J. L. Whitton. 2002. Immunodominance in virus-induced $CD8^+$ T cell responses is dramatically modified by DNA immunization and is regulated by γ interferon. *J. Virol.* 76: 4251–4259.
14. Busch, D. H., and E. G. Pamer. 1999. T cell affinity maturation by selective expansion during infection. *J. Exp. Med.* 189: 701–710.
15. Crowe, S. R., S. J. Turner, S. C. Miller, A. D. Roberts, R. A. Rappolo, P. C. Doherty, K. H. Ely, and D. L. Woodland. 2003. Differential antigen presentation regulates the changing patterns of $CD8^+$ T cell immunodominance in primary and secondary influenza virus infections. *J. Exp. Med.* 198: 399–410.
16. Kedl, R. M., W. A. Rees, D. A. Hildeman, B. Schaefer, T. Mitchell, J. Kappler, and P. Marrack. 2000. T cells compete for access to antigen-bearing antigen-presenting cells. *J. Exp. Med.* 192: 1105–1113.
17. Kedl, R. M., B. C. Schaefer, J. W. Kappler, and P. Marrack. 2002. T cells down-modulate peptide-MHC complexes on APCs in vivo. *Nat. Immunol.* 3: 27–32.
18. Grufman, P., E. Z. Wolpert, J. K. Sandberg, and K. Karre. 1999. T cell competition for the antigen-presenting cell as a model for immunodominance in the cytotoxic T lymphocyte response against minor histocompatibility antigens. *Eur. J. Immunol.* 29: 2197–2204.
19. Mueller, D. L., M. K. Jenkins, and R. H. Schwartz. 1989. Clonal expansion versus functional clonal inactivation: a costimulatory signalling pathway determines the outcome of T cell antigen receptor occupancy. *Annu. Rev. Immunol.* 7: 445–480.
20. Moore, M. W., F. R. Carbone, and M. J. Bevan. 1988. Introduction of soluble protein into the class I pathway of antigen processing and presentation. *Cell* 54: 777–785.
21. Bevan, M. J. 1976. Cross-priming for a secondary cytotoxic response to minor H antigens with H-2 congenic cells which do not cross-react in the cytotoxic assay. *J. Exp. Med.* 143: 1283–1288.
22. Belz, G. T., G. M. N. Behrens, C. M. Smith, J. F. A. P. Miller, C. Jones, K. Lejon, C. G. Fathman, S. N. Mueller, K. Shortman, F. R. Carbone, and W. R. Heath. 2002. The $CD8\alpha^+$ dendritic cell is responsible for inducing peripheral self-tolerance to tissue-associated antigens. *J. Exp. Med.* 196: 1099–1104.
23. Chen, L., S. Ashe, W. A. Brady, I. Hellstrom, K. E. Hellstrom, J. A. Ledbetter, P. McGowan, and P. S. Linsley. 1992. Costimulation of antitumor immunity by the B7 counterreceptor for the T lymphocyte molecules CD28 and CTLA-4. *Cell* 71: 1093–1102.
24. Denfeld, R. W., A. Dietrich, C. Wuttig, E. Tanczos, J. M. Weiss, W. Vanscheidt, E. Schopf, and J. C. Simon. 1995. In situ expression of B7 and CD28 receptor families in human malignant melanoma: relevance for T cell-mediated antitumor immunity. *Int. J. Cancer* 62: 259–265.
25. Ochsenbein, A. F., S. Sierro, B. Odermatt, M. Pericin, U. Karrer, J. Hermans, S. Hemmi, H. Hengartner, and R. M. Zinkernagel. 2001. Roles of tumour localization, second signals and cross priming in cytotoxic T cell induction. [Published erratum appears in *2001 Nature* 413: 183.] *Nature* 411: 1058–1064.
26. Kurts, C., J. F. Miller, R. M. Subramaniam, F. R. Carbone, and W. R. Heath. 1998. Major histocompatibility complex class I-restricted cross-presentation is biased towards high dose antigens and those released during cellular destruction. *J. Exp. Med.* 188: 409–414.
27. Morgan, D. J., H. T. Kreuwel, and L. A. Sherman. 1999. Antigen concentration and precursor frequency determine the rate of $CD8^+$ T cell tolerance to peripherally expressed antigens. *J. Immunol.* 163: 723–727.
28. Spiotto, M. T., P. Yu, D. A. Rowley, M. I. Nishimura, S. C. Meredith, T. F. Gajewski, Y. X. Fu, and H. Schreiber. 2002. Increasing tumor antigen expression overcomes "ignorance" to solid tumors via cross-presentation by bone marrow-derived stromal cells. *Immunity* 17: 737–747.
29. Nelson, D., C. Bundell, and B. Robinson. 2000. In vivo cross-presentation of a soluble protein antigen: kinetics, distribution, and generation of effector CTL recognizing dominant and subdominant epitopes. *J. Immunol.* 165: 6123–6132.
30. Mylin, L. M., R. H. Bonneau, J. D. Lippolis, and S. S. Tevethia. 1995. Hierarchy among multiple $H\text{-}2^b$-restricted cytotoxic T lymphocyte epitopes within simian virus 40 T antigen. *J. Virol.* 69: 6665–6677.
31. Mylin, L. M., T. D. Schell, D. Roberts, M. Epler, A. Boesteanu, E. J. Collins, J. A. Frelinger, S. Joyce, and S. S. Tevethia. 2000. Quantitation of $CD8^+$ T lymphocyte responses to multiple epitopes from simian virus 40 (SV40) large T antigen in C57BL/6 mice immunized with SV40, SV40 T-antigen-transformed cells, or vaccinia virus recombinants expressing full-length T antigen or epitope minigenes. *J. Virol.* 74: 6922–6934.
32. Tanaka, Y., R. W. Anderson, W. L. Maloy, and S. S. Tevethia. 1989. Localization of an immunorecessive epitope on SV40 T antigen by $H\text{-}2D^b$-restricted cytotoxic T lymphocyte clones and a synthetic peptide. *Virology* 171: 205–213.
33. Fu, T.-M., L. M. Mylin, T. D. Schell, I. Bacik, G. Russ, J. W. Yewdell, J. R. Bennink, and S. S. Tevethia. 1998. An endoplasmic reticulum-targeting signal sequence enhances the immunogenicity of an immunorecessive simian virus 40 large T antigen cytotoxic T lymphocyte epitope. *J. Virol.* 72: 1469–1481.
34. Chen, W., K. A. Masterman, S. Basta, S. M. Haeryfar, N. Dimopoulos, B. Knowles, J. R. Bennink, and J. W. Yewdell. 2004. Cross-priming of $CD8^+$ T cells by viral and tumor antigens is a robust phenomenon. *Eur. J. Immunol.* 34: 194–199.
35. Staveley-O'Carroll, K., T. D. Schell, M. Jimenez, L. M. Mylin, M. J. Tevethia, S. P. Schoenberger, and S. S. Tevethia. 2003. In vivo ligation of CD40 enhances priming against the endogenous tumor antigen and promotes $CD8^+$ T cell effector function in SV40 T antigen transgenic mice. *J. Immunol.* 171: 697–707.
36. Tanaka, Y., and S. S. Tevethia. 1990. Loss of immunorecessive cytotoxic T lymphocyte determinant V on SV40 T antigen following cocultivation with site-specific cytotoxic T lymphocyte clone Y-5. *Intervirology* 31: 197–202.
37. Saito, H., D. M. Kranz, Y. Takagaki, A. C. Hayday, H. N. Eisen, and S. Tonegawa. 1984. A third rearranged and expressed gene in a clone of cytotoxic T lymphocytes. *Nature* 312: 36–40.

38. Gascoigne, N. R., Y. Chien, D. M. Becker, J. Kavaler, and M. M. Davis. 1984. Genomic organization and sequence of T cell receptor β-chain constant- and joining-region genes. *Nature* 310: 387–391.
39. Chen, F., L. Rowen, L. Hood, and E. V. Rothenberg. 2001. Differential transcriptional regulation of individual TCR Vβ segments before gene rearrangement. *J. Immunol.* 166: 1771–1780.
40. Koop, B. F., R. K. Wilson, K. Wang, B. Vernooij, D. Zallwer, C. L. Kuo, D. Seto, M. Toda, and L. Hood. 1992. Organization, structure, and function of 95 kb of DNA spanning the murine T cell receptor C α/Cδ region. *Genomics* 13: 1209–1230.
41. Kouskoff, V., K. Signorelli, C. Benoist, and D. Mathis. 1995. Cassette vectors directing expression of T cell receptor genes in transgenic mice. *J. Immunol. Methods* 180: 273–280.
42. Tevethia, M. J., R. H. Bonneau, J. W. Griffith, and L. Mylin. 1997. A simian virus 40 large T-antigen segment containing amino acids 1 to 127 and expressed under the control of the rat elastase-1 promoter produces pancreatic acinar carcinomas in transgenic mice. *J. Virol.* 71: 8157–8166.
43. Schell, T. D. 2004. In vivo expansion of the residual tumor antigen-specific $CD8^+$ T lymphocytes that survive negative selection in simian virus 40 T-antigen-transgenic mice. *J. Virol.* 78: 1751–1762.
44. Tanaka, Y., M. J. Tevethia, D. Kalderon, A. E. Smith, and S. S. Tevethia. 1988. Clustering of antigenic sites recognized by cytotoxic T lymphocyte clones in the amino-terminal half of SV40 T antigen. *Virology* 162: 427–436.
45. Tanaka, Y., and S. S. Tevethia. 1988. In vitro selection of SV40 T antigen epitope loss variants by site-specific cytotoxic T lymphocyte clones. *J. Immunol.* 140: 4348–4354.
46. Lill, N. L., M. J. Tevethia, W. G. Hendrickson, and S. S. Tevethia. 1992. Cytotoxic T lymphocytes (CTL) against a transforming gene product select for transformed cells with point mutations within sequences encoding CTL recognition epitopes. *J. Exp. Med.* 176: 449–457.
47. Cavender, J. F., A. Conn, M. Epler, H. Lacko, and M. J. Tevethia. 1995. Simian virus 40 large T antigen contains two independent activities that cooperate with a ras oncogene to transform rat embryo fibroblasts. *J. Virol.* 69: 923–934.
48. Russ, G., F. Esquivel, J. W. Yewdell, P. Cresswell, T. Spies, and J. R. Bennink. 1995. Assembly, intracellular localization, and nucleotide binding properties of the human peptide transporters TAP1 and TAP2 expressed by recombinant vaccinia viruses. *J. Biol. Chem.* 270: 21312–21318.
49. Schell, T. D., L. M. Mylin, I. Georgoff, A. K. Teresky, A. J. Levine, and S. S. Tevethia. 1999. Cytotoxic T lymphocyte epitope immunodominance in the control of choroid plexus tumors in simian virus 40 large T antigen transgenic mice. *J. Virol.* 73: 5981–5993.
50. Campbell, A. E., F. L. Foley, and S. S. Tevethia. 1983. Demonstration of multiple antigenic sites of the SV40 transplantation rejection antigen by using cytotoxic T lymphocyte clones. *J. Immunol.* 130: 490–492.
51. Sanderson, S., and N. Shastri. 1994. LacZ inducible, antigen/MHC-specific T cell hybrids. *Int. Immunol.* 6: 369–376.
52. Johnson, D. C., and P. G. Spear. 1982. Monensin inhibits the processing of herpes simplex virus glycoproteins, their transport to the cell surface, and the egress of virions from infected cells. *J. Virol.* 43: 1102–1112.
53. van Stipdonk, M. J., G. Hardenberg, M. S. Bijker, E. E. Lemmens, N. M. Droin, D. R. Green, and S. P. Schoenberger. 2003. Dynamic programming of $CD8^+$ T lymphocyte responses. *Nat. Immunol.* 4: 361–365.
54. Norbury, C. C., S. Basta, K. B. Donohue, D. C. Tscharke, M. F. Princiotta, P. Berglund, J. Gibbs, J. R. Bennink, and J. W. Yewdell. 2004. $CD8^+$ T cell cross-priming via transfer of proteasome substrates. *Science* 304: 1318–1321.
55. Wolkers, M. C., N. Brouwenstijn, A. H. Bakker, M. Toebes, and T. N. Schumacher. 2004. Antigen bias in T cell cross-priming. [Published erratum appears in *2004 Science* 305: 1912.] *Science* 304: 1314–1317.
56. Shen, L., and K. L. Rock. 2004. Cellular protein is the source of cross-priming antigen in vivo. *Proc. Natl. Acad. Sci. USA* 101: 3035–3040.
57. Sigal, L. J., S. Crotty, R. Andino, and K. L. Rock. 1999. Cytotoxic T cell immunity to virus-infected non-haematopoietic cells requires presentation of exogenous antigen. *Nature* 398: 77–80.
58. Huang, A. Y., P. Golumbek, M. Ahmadzadeh, E. Jaffee, D. Pardoll, and H. Levitsky. 1994. Role of bone marrow-derived cells in presenting MHC class I-restricted tumor antigens. *Science* 264: 961–965.
59. den Haan, J. M. M., S. M. Lehar, and M. J. Bevan. 2000. $CD8^+$ but not $CD8^-$ dendritic cells cross-prime cytotoxic T cells in vivo. *J. Exp. Med.* 192: 1685–1696.
60. Heath, W. R., and F. R. Carbone. 2001. Cross-presentation, dendritic cells, tolerance and immunity. *Annu. Rev. Immunol.* 19: 47–64.
61. Van Kaer, L., P. G. Ashton-Rickardt, H. L. Ploegh, and S. Tonegawa. 1992. TAP1 mutant mice are deficient in antigen presentation, surface class I molecules, and $CD4^-8^+$ T cells. *Cell* 71: 1205–1214.
62. Schoenberger, S. P., E. I. van der Voort, G. M. Krietemeijer, R. Offringa, C. J. Melief, and R. E. Toes. 1998. Cross-priming of CTL responses in vivo does not require antigenic peptides in the endoplasmic reticulum of immunizing cells. *J. Immunol.* 161: 3808–3812.
63. Jung, S., D. Unutmaz, P. Wong, G. Sano, K. De los Santos, T. Sparwasser, S. Wu, S. Vuthoori, K. Ko, F. Zavala, et al. 2002. In vivo depletion of $CD11c^+$ dendritic cells abrogates priming of $CD8^+$ T cells by exogenous cell-associated antigens. *Immunity* 17: 211–220.
64. Nguyen, L. T., A. R. Elford, K. Murakami, K. M. Garza, S. P. Schoenberger, B. Odermatt, D. E. Speiser, and P. S. Ohashi. 2002. Tumor growth enhances cross-presentation leading to limited T cell activation without tolerance. *J. Exp. Med.* 195: 423–435.
65. Wolkers, M. C., G. Stoetter, F. A. Vyth-Dreese, and T. N. Schumacher. 2001. Redundancy of direct priming and cross-priming in tumor-specific $CD8^+$ T cell responses. *J. Immunol.* 167: 3577–3584.
66. van Mierlo, G. J., Z. F. Boonman, H. M. Dumortier, A. T. den Boer, M. F. Fransen, J. Nouta, E. I. van der Voort, R. Offringa, R. E. Toes, and C. J. Melief. 2004. Activation of dendritic cells that cross-present tumor-derived antigen licenses $CD8^+$ CTL to cause tumor eradication. *J. Immunol.* 173: 6753–6759.
67. Nowak, A. K., R. A. Lake, A. L. Marzo, B. Scott, W. R. Heath, E. J. Collins, J. A. Frelinger, and B. W. Robinson. 2003. Induction of tumor cell apoptosis in vivo increases tumor antigen cross-presentation, cross-priming rather than cross-tolerizing host tumor-specific CD8 T cells. *J. Immunol.* 170: 4905–4913.
68. Schreiber, H., T. H. Wu, J. Nachman, and W. M. Kast. 2002. Immunodominance and tumor escape. *Semin. Cancer Biol.* 12: 25–31.
69. Yoshimura, Y., R. Yadav, G. J. Christianson, W. U. Ajayi, D. C. Roopenian, and S. Joyce. 2004. Duration of alloantigen presentation and avidity of T cell antigen recognition correlate with immunodominance of CTL response to minor histocompatibility antigens. *J. Immunol.* 172: 6666–6674.
70. Yu, Z., M. R. Theoret, C. E. Touloukian, D. R. Surman, S. C. Garman, L. Feigenbaum, T. K. Baxter, B. M. Baker, and N. P. Restifo. 2004. Poor immunogenicity of a self/tumor antigen derives from peptide-MHC-I instability and is independent of tolerance. *J. Clin. Invest.* 114: 551–559.
71. Sijts, A. J., and E. G. Pamer. 1997. Enhanced intracellular dissociation of major histocompatibility complex class I-associated peptides: a mechanism for optimizing the spectrum of cell surface-presented cytotoxic T lymphocyte epitopes. *J. Exp. Med.* 185: 1403–1411.
72. van Stipdonk, M. J., E. E. Lemmens, and S. P. Schoenberger. 2001. Naive CTLs require a single brief period of antigenic stimulation for clonal expansion and differentiation. *Nat. Immunol.* 2: 423–429.
73. Mempel, T. R., S. E. Henrickson, and U. H. Von Andrian. 2004. T cell priming by dendritic cells in lymph nodes occurs in three distinct phases. *Nature* 427: 154–159.
74. Mercado, R., S. Vijh, S. E. Allen, K. Kerksiek, I. M. Pilip, and E. G. Pamer. 2000. Early programming of T cell populations responding to bacterial infection. *J. Immunol.* 165: 6833–6839.
75. Butz, E. A., and M. J. Bevan. 1998. Differential presentation of the same MHC class I epitopes by fibroblasts and dendritic cells. *J. Immunol.* 160: 2139–2144.
76. Kedl, R. M., J. W. Kappler, and P. Marrack. 2003. Epitope dominance, competition and T cell affinity maturation. *Curr. Opin. Immunol.* 15: 120–127.
77. Probst, H. C., T. Dumrese, and M. F. van den Broek. 2002. Cutting edge: competition for APC by CTLs of different specificities is not functionally important during induction of antiviral responses. *J. Immunol.* 168: 5387–5391.
78. Grufman, P., J. K. Sandberg, E. Z. Wolpert, and K. Karre. 1999. Immunization with dendritic cells breaks immunodominance in CTL responses against minor histocompatibility and synthetic peptide antigens. *J. Leukocyte Biol.* 66: 268–271.
79. Wolpert, E. Z., P. Grufman, J. K. Sandberg, A. Tegnesjo, and K. Karre. 1998. Immunodominance in the CTL response against minor histocompatibility antigens: interference between responding T cells, rather than with presentation of epitopes. *J. Immunol.* 161: 4499–4505.
80. Schell, T. D., B. B. Knowles, and S. S. Tevethia. 2000. Sequential loss of cytotoxic T lymphocyte responses to simian virus 40 large T antigen epitopes in T antigen transgenic mice developing osteosarcomas. *Cancer Res.* 60: 3002–3012.