Characterization of circulating T cells specific for tumor-associated antigens in melanoma patients

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We identified circulating CD8⁺ T-cell populations specific for the tumor-associated antigens (TAAs) MART-1 (27-35) or tyrosinase (368-376) in six of eleven patients with metastatic melanoma using peptide/HLA-A*0201 tetramers. These TAA-specific populations were of two phenotypically distinct types: one, typical for memory/effector T cells; the other, a previously undescribed phenotype expressing both naive and effector cell markers. This latter type represented more than 2% of the total CD8⁺ T cells in one patient, permitting detailed phenotypic and functional analysis. Although these cells have many of the hallmarks of effector T cells, they were functionally unresponsive, unable to directly lyse melanoma target cells or produce cytokines in response to mitogens. In contrast, CD8⁺ T cells from the same patient were able to lyse EBV-pulsed target cells and showed robust allogeneic responses. Thus, the clonally expanded TAA-specific population seems to have been selectively rendered anergic *in vivo*. Peptide stimulation of the TAA-specific T-cell populations in other patients failed to induce substantial upregulation of CD69 expression, indicating that these cells may also have functional defects, leading to blunted activation responses. These data demonstrate that systemic TAA-specific T-cell responses can develop *de novo* in cancer patients, but that antigen-specific unresponsiveness may explain why such cells are unable to control tumor growth.

The nature of the immune system's ability, or inability, to respond to cancer remains unclear. As cancer cells are of 'self' origin, it has been suggested that they often are ignored by the immune system. However, several lines of evidence now indicate that an endogenous immune response against cancer develops in some patients. Tumor-infiltrating lymphocytes (TILs) can be isolated and expanded *in vitro* with IL-2 from a variety of human cancers[1], and some TILs have specific cytolytic activity for fresh autologous cancer cells[2]. These data indicate that local immune responses may develop within tumors, and these may represent specific anti-tumor responses rather than nonspecific inflammatory responses. Furthermore, tumor-associated antigen (TAA)-specific T cells have been detected in some tumor-infiltrated lymph nodes[3]. Nonetheless, the biological importance of TAA-specific T cells in cancer patients remains unclear, as they are generally detected in the setting of metastatic disease. The *in situ* biology and function of these cells have eluded definition mainly because exposure to repeated antigen stimulation and exogenous cytokines, which could alter the native functional and phenotypic states of cells, is needed to expand TILs to sufficient numbers for specificity analysis. If TAA-specific T cells indeed exist in cancer patients, whether they modulate cancer progression and why they are not protective for the host remain important unresolved questions.

To evaluate whether or not circulating TAA-specific T cells exist in patients with metastatic melanoma as evidence for systemic immune response to cancer, and to study the native biological properties of such cells, we used MHC/peptide tetramers to stain T cells in an antigen-specific manner[4]. This method has been proven useful for identifying T cell responses to viral infections[5] and metastatic lymph nodes in cancer[3], without the need for prior *in vitro* expansion. For melanoma, the peptide targets of a number of tumor-reactive T cell clones have been determined. Many of these peptides are restricted by HLA-A*0201, and are processed from

Table 1 HLA-A2-restricted peptides used in tetramer production

Peptide	Sequence
MART 27-35	AAGIGILTV
gp100 154-162	KTWGQYWQV
Tyrosinase 368-376[a]	YMDGTMSQV
EBV BMLF1	GLCTLVAML
CMV pp65	NLVPMVATV

[a]With the post-translational modification of N to D at position 370.

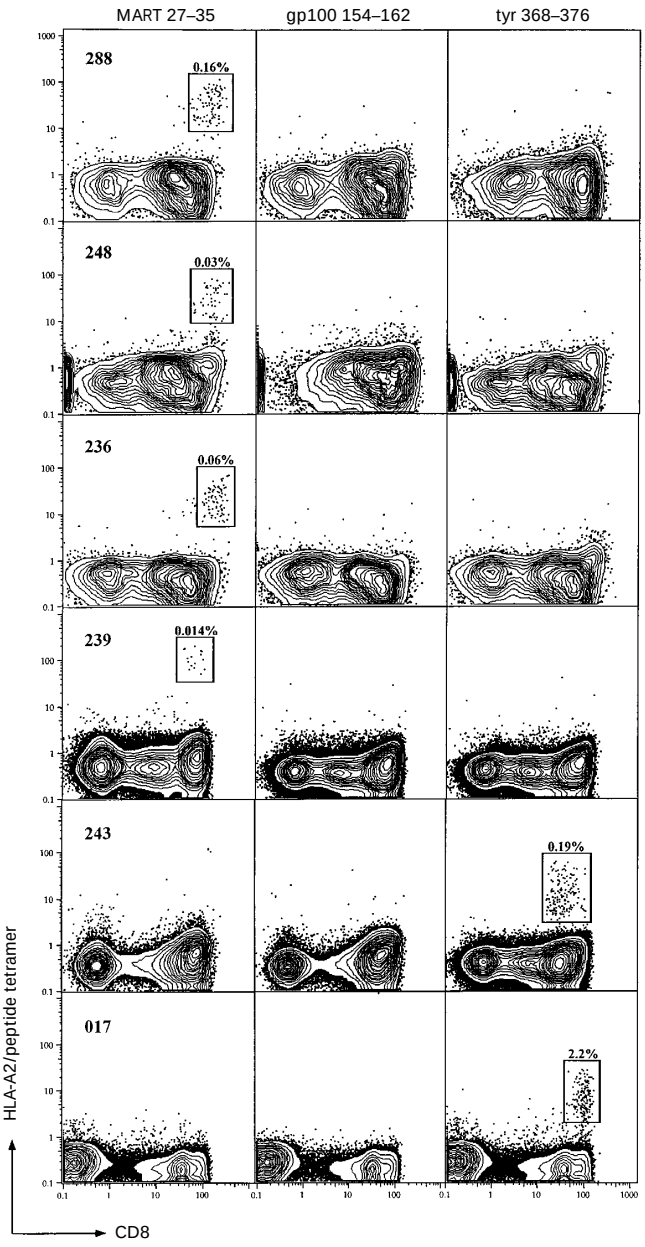


Fig. 1 Identification of TAA-specific CD8⁺ T cells by tetramer staining from PBMCs of six patients with metastatic melanoma. Cells were progressively gated by forward- and side-scatter for lymphocytes, CD3+ for T cells, and negatively for the expression of CD4, CD14, and CD19. Gated populations are plotted as CD8-staining (horizontal axis) versus tetramer staining (vertical axis). In these probability contour plots with outlier events, a different number of total events were collected for each sample. FACS analyses were done at different times with different instruments, thus they have variations in the background and signal intensities. Results represent six (of eleven) patients with detectable tetramer staining; each column shows the staining for a different peptide-loaded HLA-A*0201 tetramer. Boxed populations, CD8⁺ tetramer-positive cells (frequencies within total CD8⁺ T cells above each box). Upper left-hand corners, patient identifiers. The limit of detection is below 0.01% of CD8 T cells; even at 0.014%, the MART27-35-specific population from patient 239 is easily distinguishable from background.

nonmutated, 'self' proteins[6–8]. For this study, we made a panel of HLA-A*0201 tetrameric complexes using immunogenic peptides from MART-1, gp100 and tyrosinase (Table 1) to identify TAA-specific T cells in the blood of melanoma patients by flow cytometric analysis. Using these reagents, we detected TAA-specific CD8⁺ T cells in the peripheral blood from six of eleven patients with metastatic melanoma. Phenotypic and functional analyses of these responses indicate that although TAA-specific T-cell populations can arise in the cancer patient, they may be subject to inactivation *in vivo*. This has important implications both for host–tumor interactions and for tumor vaccine protocols.

Identification and characterization of TAA-specific T cells

We analyzed peripheral blood mononuclear cells (PBMCs) from 11 HLA-A2⁺ melanoma patients for TAA-specific CD8⁺ T cells using a panel of melanoma tetramers. These patients all have metastatic melanoma, either in regional lymph nodes (AJCC American Joint Committee on Cancer, stage III) or at distant sites (AJCC stage IV). PBMC samples were obtained before treatment began. Of the eleven patients, six (55%) had tetramer-staining CD8⁺ T cells (Fig. 1): four specific for MART27-35/A*0201 and two specific for tyr368-376/A*0201. The CD8⁺ T-cell populations specific for MART in patients 236, 239, 248 and 288 were relatively rare, representing 0.014–0.16% of total CD8⁺ T cells, as was the tyrosinase-specific population from patient 243, at 0.19%. In contrast, the tyrosinase-specific population in patient 017 represented 2.2% of total CD8⁺ T cells. None of these patients had evidence of vitiligo (depigmented patches of skin), indicating that there was no concurrent antimelanocyte activity[9,10]. PBMCs from ten healthy HLA-A2⁺ individuals served as negative controls and did not stain more than background with any of the melanoma tetramers (data not shown).

To assess the phenotypic characteristics of these TAA-specific CD8⁺ T cells, we stained PBMCs from these six patients with the appropriate melanoma tetramers plus antibodies against a panel of surface markers using ten-color flow cytometric analysis. The surface marker expression of these cells segregated into two distinct phenotypes (Table 2). The MART-specific populations in patients 236, 239 and 288 and the tyrosinase-specific population from patient 243 were CD8β^{hi}, CD16^{-}, CD27^{+}, CD28^{+}, CD44 bright, CD45RO^{+} and CD45RA^{-}. This phenotype is characteristic of most memory effector T cells[11]. In contrast, the tyrosinase-specific population in patient 017 was very different: CD8β^{lo}, CD16^{+}, CD38^{+}, CD44 dull, CD45RA^{+}, CD45RO^{-} and CD57^{+}. The MART-specific T-cell population in patient 248 had cells of both phenotypes. The consistent staining patterns of these tetramer-positive populations with many different markers indicate that these cells represent homogeneous populations of antigen-specific cells, not random cells that bind tetramers nonspecifically.

The paradox of TAA-specific T cells in cancer patients

An essential question is what part these TAA-specific T cells play *in vivo*, as they exist in patients with metastatic disease, and thus have not successfully contained tumor progression. This question was particularly perplexing for patient 017, in whom the tyrosinase-specific population represented nearly 1 in 40 circulating CD8⁺ T cells. The high frequency of these cells allowed for further analysis and isolation by sorting for cytotoxicity assays.

One mechanism of immune escape by tumor cells is downregulation of their target epitopes for cytotoxic T lymphocytes (CTLs); for example, HLA class I molecules[12] or antigen[13]. The tumor biopsy obtained from patient 017 at presentation was positive for both tyrosinase and HLA-A2 (data not shown). Thus, the inability of the large tyrosinase-specific T-cell population in patient 017 at the time of diagnosis to eliminate tumor could not

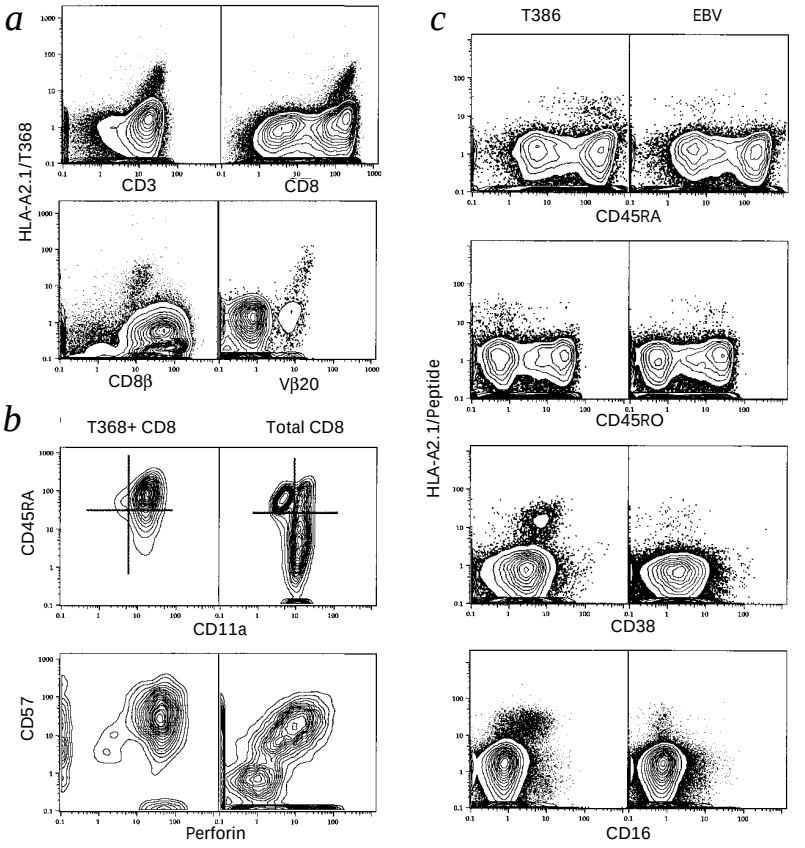


Fig. 2 Phenotypic analyses from ten-color FACS of PBMCs from patient 017. Cells were stained with combinations of antibodies to determine phenotypes of tyrosinase-specific or EBV BMLF1-specific T cells. Most stains included a minimum of the tetramer reagent, and antibodies against CD3 and CD8 (positive gate), and single-color combination of antibodies against CD4, CD14 and CD19 (negative gate). Some experiments used unseparated PBMCs; others used CD4-, CD14- and CD20-depleted PBMCs. In general, 0.5×10^6–2×10^6 events were collected for each analysis. ***a***, Upper, total $CD3^+$ T cells, demonstrating normal expression of CD3 and CD8 on the tyrosinase-specific T cells. Lower, gated for $CD3^+CD8^+$ T cells. ***b***, Probability contour plots comparing the phenotypes of total $CD8^+$ T cells (right) and tyrosinase-specific $CD8^+$ T cells (left). ***c***, Comparison of surface markers expressed by $CD8^+$ T cells. The tyrosinase-specific (left; 2.2% of total $CD8^+$ T cells) and EBV-specific (right; 0.14% of total $CD8^+$ T cells) populations from patient 017 had substantially different expressions of CD45RA (top), CD45RO (second), CD38 (third) and CD16 (bottom).

be explained by the lack of target proteins in the tumor cells.

Antigen-specific T-cell populations in patient 017

To assess whether or not the tyrosinase-specific population was enriched for particular T-cell receptors, we stained PBMCs from patient 017 with the T368 tetramer and a panel of antibodies against human Vβ. More than 95% of the T368 tetramer-staining cells stained with a single antibody against Vβ, Vβ20 (Fig. 2*a*), consistent with this being an antigen-specific response using a highly restricted, possibly monoclonal, T-cell receptor repertoire.

We further characterized the native phenotypic state of the tyrosinase-specific CD8 cells in patient 017 by measuring the expression of 26 different surface and intracellular markers using ten-color flow cytometric analysis. An HLA-A*0201/EBV (BMLF1; ref. 14) tetramer stained 0.14% of total $CD8^+$ T cells in the same patient. This population serves as an internal control. Although the tyrosinase-specific cells expressed normal levels of total CD8, they expressed only low levels of CD8β (as determined by staining with an antibody specific for the β chain of CD8; most antibodies against CD8 recognize mainly the α chain) and thus expressed mainly CD8αα homodimers (Fig. 2*a*). They also have an unusual phenotype for $CD8^+$ T cells in the peripheral blood, and are considerably different from that of the EBV-specific population in the same patient (Fig. 2*c*). In particular, the tyrosinase-specific T cells expressed high levels of CD45RA (and no CD45RO), and relatively low levels of CD44 (Fig. 2*c*). Although these phenotypic markers are normally associated with naive T cells, this population also expressed high levels of CD11a, CD57 and intracellular perforin, and low levels of CD27, and did not express CD28, all of which are consistent with highly differentiated effector CTLs (ref. 15). Indeed, the tyrosinase-specific cells expressed the highest intracellular perforin levels of all the $CD8^+$ T cells. In contrast, the EBV-specific T cells in this patient showed a more classical phenotype for $CD8^+$ effectors: $CD8\beta^{hi}$, $CD28^+$, $CD44^+$, $CD45RO^+$ and $CD45RA^-$. The tyrosinase-specific population also expressed low levels of the NK marker

Table 2 Phenotypic analysis of TAA-specific T-cell populations identified from six patients with metastatic melanoma

Antigen	A2/Tyr368-376		A2/MART27-35				A2/Tyr368-376
Patient ID	243	236	239	288	248		017
% of CD8	0.19	0.057	0.014	0.16	0.018	0.012	2.2
Subset type			A			B	
Subset-distinguishing markers							
CD8β	Bright	Bright	Bright	Bright	Bright	Dull	Dull
CD16	–	–	–	–	–	+	+
CD28	+	+	+	+	+	+/–	+/–
CD45RA	–	–	–	–	–	+	+
CD45RO	+	+	+	+	+	–	–
CD38	–	–	–	–	–	+	+
CD57	–	–	–	–	–	+	+
Other markers							
CD11a	Bright	Bright	Dull	Bright	Bright	Bright	Bright
CD27	+	+	+	+	+	+	–
CD44	Bright	Bright	Bright	Bright	Bright	Bright	Dull

Marker expression: +, on the tumor antigen-specific cells; +/–, heterogeneous; –, none. For patients 236, 239, 248, and 288, only MART-specific T cells were found; for patients 243 and 017, only tyrosinase-specific T cells were found (Fig. 1). % of CD8, percentage of total $CD8^+$ T cells that bound the specific tetramer (more than one log above background). Two phenotypically-distinct types of specific $CD8^+$ T cells (A and B) were identified based on the expression pattern of seven surface markers. MART-specific T cells of each type were identified in patient 248; these are shown in separate columns. For CD8β, CD11a and CD44, most or all $CD8^+$ T cells express these markers; subsets expressing higher (Bright) or lower (Dull) levels of these markers can be easily discerned.

CD16, but not CD56 (Table 3), whereas the EBV-specific population was CD16⁻ and CD56⁻.

Tyrosinase-specific T cells in patient 017 are non-cytolytic

The high frequency of tyrosinase-specific T cells in patient 017 permitted direct analysis of the cytolytic function of these cells after cell sorting on the basis of tetramer staining; such an approach was not technically feasible for the other patients, whose TAA-specific populations were present at much lower frequencies. As the cytolytic activity of sorted cells could be tested without further *in vitro* manipulation, this is a good representation of the *in vivo* state of these cells. CD8⁺ T368 tetramer-positive cells sorted from patient 017 failed to lyse either target cells pulsed with T368 (Fig. 3*a*) or HLA-A2⁺ tyrosinase-positive melanoma cell lines. As a control, a population of CMV-specific CD8⁺ T cells was identified from a CMV-immune individual (donor 06) using an HLA-A*0201/CMV (pp65) tetramer, which represented 1.6% of total CD8⁺ T cells. These CD8⁺ CMV tetramer-positive cells from donor 06, stained and sorted in an identical manner, demonstrated direct specific lysis of CMV pp65-pulsed targets, with no lysis of unpulsed or T368-pulsed targets (Fig. 3*a*). Thus, the absence of cytolytic activity observed in the tyrosinase-specific T cells in patient 017 indicates that these cells were non-cytolytic *in vivo*.

In some HIV-infected subjects, freshly isolated virus-specific CD8⁺ T cells have also been found to be non-cytolytic, but regained activity after brief *in vitro* culture with IL-2 (ref. 16). To assess whether the cytolytic activity of the tyrosinase-specific T cells in patient 017 could be similarly rescued, we sorted CD8⁺ T368 tetramer-positive cells and cultured them for 48 hours in the presence of 50 IU/ml of IL-2. This treatment did not restore cytolytic activity in these cells (Fig. 3*b*).

Because patient 017 was found to also have an EBV-specific CD8 population by tetramer staining, we did cytotoxicity assays with CD8-enriched PBMCs; these demonstrated specific cytolytic activity against EBV-pulsed targets (Fig. 3*c*). We also did allogeneic mixed lymphocyte reactions and proliferation assays against recall antigens to assess the general integrity of the immune system of patient 017. PBMCs from patient 017 showed robust proliferation against all three allogeneic PBMC populations as well as to influenza and tetanus toxoid antigens (Table 4). These results are consistent with an antigen-specific T-cell dysfunction in patient 017, restricted to the tyrosinase-specific population.

Table 3 Expanded phenotypic analysis of total CD8 and antigen-specific CD8 T-cell populations from patient 017 and donor 06

	Patient 017			Donor 06		
Antigen	Total	T368	EBV	Total	CMV	EBV
Lineage markers						
CD5	++	–	++	+++	+++	+++
CD8 beta	++++	+	+++	+++	++++	++++
CD16	+/–	+++	+/–	–	–	–
CD56	–	+/–	–	+/–	–	–
Differentiation markers						
CD7	+++	++++	++++	++++	++++	ND
CD11a	++	++++	+++	++	++++	++++
CD26	+	–	–	+/–	–	–
CD27	+	–	–	+++	++	++++
CD28	+++	+/–	+++	+++	++	++
CD44	++	+/–	++	+++	++++	++++
CD45RA	++	++++	+/–	++	+	+
CD45RO	++	+/–	++++	+++	+++	++++
CD49d	++	+++	++	+	+	ND
CD57	++	++++	+	+/–	++	–
CDw60	+	–	–	+	+	+
Activation markers						
HLA-DR	+/–	–	+	–	–	+/–
CD38	+	++	+	+/–	+/–	+
CD69	+/–	+/–	+	–	+/–	+/–
Functional markers						
Perforin	+	++++	+	+/–	++	+/–
CD3 zeta	++++	++++	++++	++++	++++	++++

Relative expression of markers for total CD8, or for CD8⁺ T cells binding T368 tetramer (patient 017), CMV tetramer (donor 06; healthy control), or EBV tetramer (patient 017 and donor 06). Fraction of cells expressing a given marker: –, 0–10%; +/–, 10–20%; +, 20–40%, ++, 40–60%; +++, 60–80%; ++++, >80%. ND, not determined. Expression of CD95, CD95L, CD152, CD154 and CD71 and binding of Annexin V were also determined and found on less than 10% of all subsets.

Tyrosinase-specific T cells are unable to produce cytokines

To further explore the functional unresponsiveness of the tyrosinase-specific CD8⁺ T cells in patient 017, we studied the cytokine profile of this population by intracellular cytokine staining. The

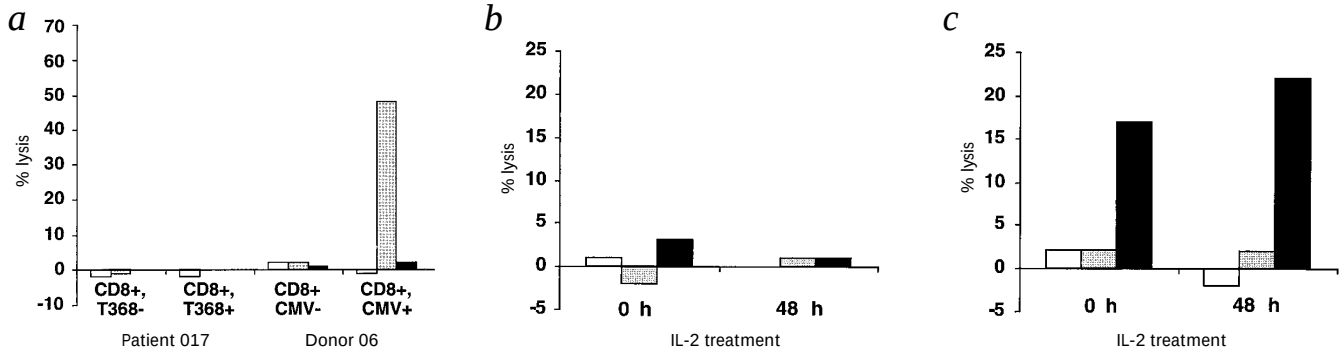


Fig. 3 Cytolytic activity of directly sorted and CD8-enriched T cells. Despite the high expression of intracellular perforin by the TAA-specific T cells, they have no cytolytic activity *in vitro*. EBV-specific cells from the same patient, however, have normal cytolytic activity. ***a***, CD8⁺ T368⁺ and CD8⁺ T368⁻ cells from PBMCs from patient 017 and CD8⁺ CMV⁺ and CD8⁺ CMV⁻ cells from PBMCs from donor 06 were sorted and tested directly against the following targets (E:T ratio, 10:1): unpulsed A221-A2 (□); A221-A2 pulsed with 10 μg/ml of T368 (■); and A221-A2 pulsed with 10 μg/ml of CMV pp65 (▒). ***b***, CD8⁺ T368⁺ cells were sorted from PMBCs from patient 017 and either tested directly against targets as in *a* (left) or cultured with 50 IU/ml of rhIL-2 for 48 h and then tested (right). ***c***, CD8-enriched T cells from patient 017 were tested for cytolytic activity either directly (left) or after 48-hour exposure to 50 IU/ml of rhIL-2 (right). Targets (E:T, 100:1) were unpulsed A375 (HLA-A*0201 cell line; □), T368-pulsed A375 (▒) and EBV BMLF1-pulsed A375 (■). A375 cells were used as targets in these experiments because they are not EBV-transformed, whereas A221 is an EBV-transformed cell line.

combination of tetramer and intracellular cytokine staining allowed us to analyze the cytokine profile of an antigen-specific T-cell population without further *in vitro* manipulation, which may otherwise alter the functional state of the cells. We analyzed the tyrosinase-specific CD8$^+$ T cells from patient 017 and the CMV-specific CD8$^+$ T cells from donor 06 for the expression of IL-2, IL-10, IFN-γ and TNF-α after stimulation with the phorbol ester PMA and calcium ionophore Ionomycin. Most of the CMV-specific T cells from donor 06 stained positive for IFN-γ and TNF-α (63% and 53%, respectively), whereas little or no expression of these cytokines could be detected in the tyrosinase-specific T cells from patient 017 (2% and 9%, respectively; Fig. 4). These results were also confirmed by ELISPOT assays, and ELISA of supernatants of PBMCs from patient 017 and donor 06, incubated with the appropriate peptide-pulsed APC (data not shown).

Deficient activation in other TAA-specific T-cell populations

To assess the functional capacities of the smaller TAA-specific populations in patients 288, 248, 236 and 243, we measured CD69 expression by these cells after peptide stimulation. CMV- or EBV-specific populations from two healthy donors served as controls. In addition, patients 288 and 236 were also found to have CMV-specific populations by tetramer staining, representing 0.91% and 0.64% of total CD8$^+$ T cells, which served as internal controls. The viral-specific populations from donor 06, donor 11, patients 288 and 236 all had substantial upregulation of CD69 expression after stimulation with the appropriate peptide (Fig. 5). This expression was specific, as irrelevant peptides did not upregulate CD69 expression over background. The MART-specific populations from patients 288, 248 and 236 and the tyrosinase-specific population from patient 243 showed minimal or no upregulation of CD69 expression after peptide stimulation. These data show that the TAA-specific T-cell populations in these patients have blunted activation responses to peptide and, in patients 288 and 236, this defect is antigen-specific, as the CMV-specific populations in these patients showed CD69 upregulation equivalent to that of the healthy controls.

Disappearance of tyrosinase-specific T cells after chemotherapy

Patient 017 received a combination of biologic and chemotherapy as treatment for her metastatic disease. Her treatment regimen was a modification of the Dartmouth protocol, with the addition of alpha interferon (IFN-α) and IL-2: 150 mg/m^2 BCNU, 660 mg/m^2 DTIC and 75 mg/m^2 CDDP on day 1 of each cycle; 5 mU/m^2 IFN-α on days 3, 5, 7 and 9; 3 mU/m^2 IL-2 on days 3–9; for a total of four cycles. In addition, she received 20 mg daily of Tamoxifen. As part of our analysis of this patient, we analyzed blood samples at various times before, during and after treatment. The tyrosinase-specific CD8 population was relatively stable before chemotherapy: 2.2–2.3% of total CD8$^+$ T cells (Fig. 6).

Table 4 Proliferation against allogeneic PBMCs (mixed lymphocyte reactions) and recall antigens.

	Patient 017	Donor 06
No stimulator	222 ± 143	369 ± 1,234
Allogeneic PBMCs 1	14,387 ± 2,603	8,046 ± 1,403
Allogeneic PBMCs 2	9,542 ± 2,128	9,613 ± 507
Allogeneic PBMCs 3	12,925 ± 4,117	6703 ± 3,038
Flu 1,600:1	3,910 ± 303	3,489 ± 455
TT 1.25 LFU/ml	7,420 ± 429	6,973 ± 697
PMA & ionomycin	7,402 ± 901	8,151 ± 1,790

PBMCs from patient 017 or donor 06 and irradiated (3,000 rads) allogeneic PBMCs from three different donors were added at 5 x 10^5 cells/well (of each population) in the mixed lymphocyte reactions. For reactivity to recall antigens influenza (Flu) and tetanus toxoid (TT) and mitogens (100 ng/ml PMA and 1 μg/ml Ionomycin), 1 x 10^6 cells/well were added with the antigens at the indicated dilution or concentration. Cells were pulsed on day 6 with ^{3}H-thymidine and collected on day 7. Values expressed are counts per minute ± s.d. LFU, lytic-forming units.

This population decreased precipitously to less than 0.2% shortly after the first cycle of chemotherapy, and remained persistently low (less than 0.2% of total CD8) throughout the remainder of the treatment period and beyond. In contrast, the EBV-specific T cells in this patient remained stable at approximately 0.14% of total CD8$^+$ T cells both before and after treatment (Fig. 6). The combination chemotherapy given may have caused the disappearance of the tyrosinase-specific T cells, although it is also possible that an unrecognized intervening event such as a viral infection may have contributed to this. Because the chemotherapeutic agents used are cell cycle-dependent, this may mean that the tyrosinase-specific cells were slowly dividing *in vivo*, like the oligoclonal CD8$^+$ T cells studied in aged mice[17], and thus were sensitive to the effects of anti-proliferative chemotherapeutic agents. The fact that the EBV-specific cells were not affected could be because they are non-dividing memory cells.

Discussion

Studies of tumor immunology have largely focused on how tumor cells may evade immune recognition: absence of unique 'tumor' antigens, downregulation of target antigens and/or HLA expression[12,13], absence of co-stimulatory and/or adhesion molecules expression[18], or secretion of immunosuppressive cytokines[19]. The data presented here show that despite these

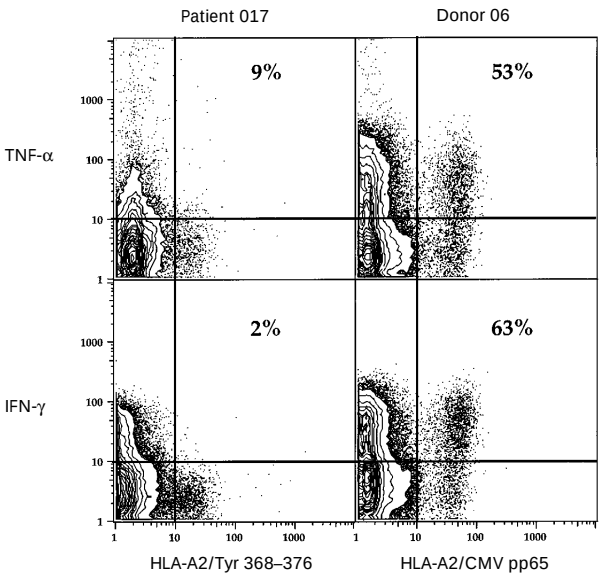


Fig. 4 Intracellular cytokine staining of antigen-specific T cells. Tyrosinase-specific CD8$^+$ T cells from patient 017 and CMV-specific CD8$^+$ T cells from donor 06 were stained for the expression of TNF-α and IFN-γ after stimulation with PMA and ionomycin for 6 h (last 3 h with Brefeldin A). Cells were gated for lymphocytes (resting and activated) by FSC and SSC, CD8$^+$ and negatively gated for CD4, CD14 and CD19. Gated populations are plotted as tetramer staining (horizontal axis) versus cytokine staining (vertical axis). Staining with irrelevant tetramers (HPV E7 12-20/HLA-A*0201) allowed setting of the threshold for tetramer-positive cells, and staining with IgG isotype controls allowed setting of the threshold for cytokine-positive cells (data not shown). Upper right corners, percent tetramer-positive cells that are cytokine-positive.

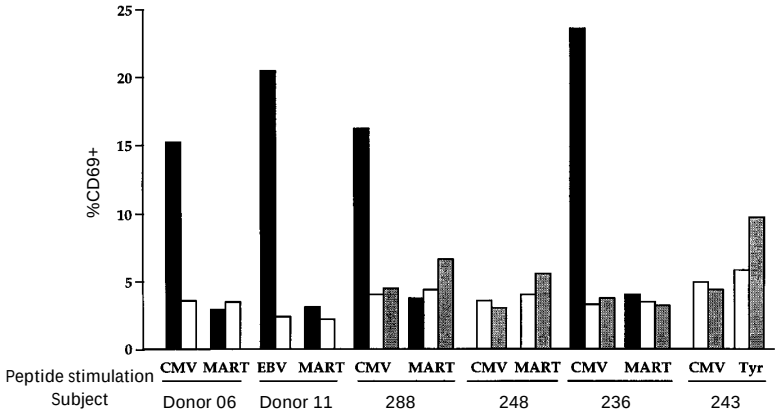
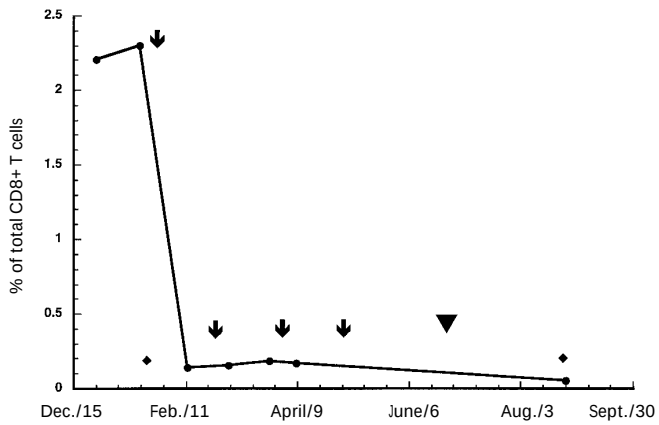


Fig. 5 Expression of CD69 by antigen-specific T cells after peptide stimulation. PBMCs from subjects were incubated with peptides (listed below graph; 1 μg/ml final concentration) for 4 h at 37 °C. Data represent fraction of total cells expressing CD69 after peptide stimulation: □, $CD8^+$ T cells; ▒, MART- or tyrosinase-specific $CD8^+$ T cells; ■, CMV- or EBV-specific $CD8^+$ T cells.

potential mechanisms of immune evasion, TAA-specific T-cell responses nonetheless develop in many patients with metastatic melanoma, representing as much as more than 2% of total $CD8^+$ T cells. This adds substantially to the previous findings that TAA-specific T cells can be detected in tumors[1] and tumor-infiltrated lymph nodes, including studies using MHC/peptide tetramers[3]. These data lend strong support to the premise that the immune system can recognize and respond to endogenous cancer cells. The fact that most of the TAA-specific T cells identified were specific for MART 27-35 is also consistent with previous observations of the immunodominance of this antigen in melanoma[20].

However, the presence of TAA-specific T cells in patients with metastatic disease indicate that these responses are unable to contain tumor progression. Analysis of the functional state of the TAA-specific $CD8^+$ T cells in most melanoma patients is limited by the low frequencies of these populations. This has led to the need to expand such cells *in vitro* before specificity or functional testing, which could otherwise alter the cells and thus not reflect their underlying state *in vivo*. MHC/peptide tetramers allow the direct sorting and analysis of antigen-specific T cells without *in vitro* stimulation and expansion. This has enabled us to address the native functional state of the tyrosinase-specific population in patient 017, demonstrating that these cells were non-cytolytic *in vivo*. Moreover, these cells failed to produce cytokines even after PMA and ionomycin stimulation. These characteristics fit well with the definition of T-cell anergy.

Although anergy in $CD8^+$ T cells has been described, most studies have been in mice, and the phenotypes vary widely. Some regard $CD8^+$ T cells as 'anergic' if they are non-cytolytic *in vitro*[21]; others, if they do not secrete IL-2 or TNF-α (ref. 22); and still others, on the basis of differences in certain phenotypic markers, such as lower levels of CD11a expression[21]. The tyrosinase-specific $CD8^+$ T-cell population from patient 017 showed many of these defects, which could not be reversed with IL-2 incubation for 48 hours. Thus, this population may represent an extreme form of anergy. Although global T-cell dysfunction has been described in tumor-bearing mice[23,24] and humans[25,26], anergy has not previously been shown in a TAA-specific population *ex vivo*.



Assessing the native functional capacities of the TAA-specific populations in the other melanoma patients was more difficult because of their much lower frequencies. Given the constraints of the amount of blood or PBMCs one can obtain from a patient, it is not feasible to isolate sufficient cells by sorting for cytotoxicity assays from populations representing less than 0.5% of total $CD8^+$ T cells. Thus, we assessed the ability of such cells to become activated after peptide stimulation as an indication of their functional potential. The combination of tetramer staining with peptide stimulation of CD69 expression represents a sensitive method of detecting early activation of an antigen-specific population[27]. The TAA-specific T-cell populations in patients 288, 248, 236 and 243 demonstrated little or no upregulation of CD69 expression after peptide stimulation. In contrast, CMV-specific T cells from patients 288 and 236, and CMV- or EBV-specific T cells from two healthy controls, showed substantial upregulation of CD69 expression after peptide stimulation. These data show that the TAA-specific T cells have blunted activation responses, and that these defects are antigen-specific. However, these data do not address the cytolytic or effector potential of such cells, and therefore can only be regarded as suggestive of functional defects in these small TAA-specific populations.

Together, these data indicate functional inactivation of potentially tumor-reactive T cells as an important mechanism of immune evasion in cancer. Previous studies of TAA-specific T cells in humans have relied on functional and therefore could not have demonstrated the functional defects in such cells. It is likely that the frequency of TAA-specific T cells in cancer patients has been grossly underestimated because only cells that retained their functional capacities *in vivo* and *in vitro* were detected. This has been seen in studies of viral immunity, in which the frequencies of antigen-specific cells were found to be 10-fold to 50-fold lower by limiting dilution analysis than by tetramer staining[5].

The high frequency of the tyrosinase-specific T cells in patient 017 must be the result of extensive clonal expansion *in vivo*. Thus, the situation is different from classical peripheral tolerance in which antigen-specific precursors encounter ligand without the appropriate costimulatory signals, causing the cells to become an-

Fig. 6 Changes in antigen-specific $CD8^+$ T-cell populations with chemotherapy in patient 017. ➔, dates of chemotherapy; ▼, date of a new biopsy-proven melanoma relapse. ◆, CD8 T cells specific for EBV before and after chemotherapy; ●, $CD8^+$ T cells specific for Tyr368 before, during and after chemotherapy, including after relapse of the melanoma.

ergic without further clonal expansion[28,29]. One possible explanation for the existence of an expanded population of anergic TAA-specific T cells in this patient is that MHC I-restricted antigens were transferred from tumor cells to bone marrow-derived antigen presenting cells *in vivo*[30,31], thereby eliciting TAA-specific T cells through 'cross-priming'. This could lead to an expanded population generated at a nontumor site such as a lymph node, which may then become tolerized after encountering tumor cells in the immunosuppressive milieu of a tumor[32,33]. Alternatively, a recent study demonstrated the persistence of viral-specific $CD8^+$ T cells that were functionally silenced in chronically infected mice in conditions of $CD4^+$ T-cell deficiency[34], demonstrating the importance of $CD4^+$ T cells for maintaining $CD8^+$ T-cell activity. Although patient 017 had normal numbers of $CD4^+$ T cells that showed robust activity in mixed lymphocyte reactions and response to recall antigens, it is possible that TAA-specific CD4 help was lost during tumor progression, leading to functional silencing of the TAA-specific CD8 population.

Phenotypic analysis of the tyrosinase-specific T cells in patient 017 showed many unique characteristics of this population. Although these cells seemed to be terminally differentiated effectors, expressing high levels of perforin, they did not express common activation markers such as CD25 or CD69. The low level of CD44 expression found on these T cells is normally found only on naive T cells. This unusual phenotype may have an important effect on the *in vivo* migration of this subset, as CD44 is the principal molecule initiating extravasation[35]. Thus, the low level of expression of CD44 on the tyrosinase-specific T cells may indicate a defective homing potential of these cells, and perhaps explains their prevalence in the peripheral blood. They expressed unusual markers such as CD16 and were $CD8\beta^{lo}$; thus, these cells might have an intraepithelial origin and NK-like properties. Consistent with this designation, these cells also expressed CD38 (but not other activation markers such as HLA-DR, CD25, CD69, or CD71), and low levels of CD5 (ref. 36). In studies with mice, a proportion of intraepithelial lymphocytes expressing the $CD8\alpha\alpha$ homodimer were thought to be of extrathymic origin[36,37] and thus have a higher propensity for reacting against 'self' antigens. Dendritic epidermal T cells in mice may be involved in tumor surveillance in a non-MHC-restricted manner[38]. In humans, skin intraepithelial lymphocytes are mainly in the dermis and express the $\alpha\beta$ T-cell receptor[39]. Perhaps cells of this type might be involved in surveillance against melanoma, especially as many of the melanoma-associated antigens identified have been nonmutated, 'self' proteins. Half of the MART-specific $CD8^+$ T cells in patient 248 also share this phenotypic pattern, indicating that this type of T-cell response to cancer may not be anomalous.

How these patterns of phenotype marker expression correlate with *in vivo* function remains unresolved. The phenotypic pattern of the tyrosinase-specific cells in patient 017 may be that of a terminally anergized cell. Perhaps as responding cells become progressively anergized *in vivo*, they undergo a phenotypic transition from that of normal effectors (Table 2, subset A) to that of the tyrosinase-specific population in patient 017 (Table 2, subset B). In patient 248, the coexistence of both phenotypes may be evidence of such a transition occurring *in vivo*.

These findings have profound implications for cancer immunotherapy. The heterogeneity of T-cell responses in different patients (some have T cells specific for MART-1 and some, for tyrosinase; others have none) may be important in stratifying patients into different treatment groups. Studies have shown increases in precursor frequencies of TAA-specific CTLs after vaccination without evidence of clinical regression in some patients[40–42]. Our results indicate one possible explanation for this discordance, as TAA-specific T cells may be subject to being selectively rendered anergic *in vivo*. Successful vaccination protocols may therefore require not only TAA-specific CTLs to substantial numbers, but also strategies to prevent such cells from becoming anergized *in vivo*.

Methods

Peptides and cell lines. Peptides were produced by Multiple Peptide Systems (San Diego, California) and/or the Stanford PAN Facility. The 221-A2 cell line is an mutant human B lymphoblastoid cell line null for HLA-A, -B and -C, transfected with the HLA-A2.1 gene[43] (a gift from D. Gerahty). The tumor cell lines A375 (a gift from Y. Kawakami) and SK29.1 (a gift from P. Coulie) are human melanoma cell lines that have been described[20,44]. Both lines express HLA-A2, but only SK29.1 also expresses tyrosinase.

Patient samples. Patients were from the melanoma and surgical oncology clinics at Stanford, the Fred Hutchinson Cancer Center and the USC Norris Cancer Center. The patients all have metastatic melanoma, as defined by the presence of disease in regional lymph nodes (AJCC stage III) or at distant sites (AJCC stage IV). These patients had leukapheresis for other purposes, and a small portion of these cells were obtained for our analysis. Frozen samples were thawed and cultured overnight in IMDM supplemented with 10% pooled AB serum (Stanford Blood Center, Stanford, California). Before staining, dead cells and debris were excluded by centrifugation with ficoll-hypaque (Pharmacia) and samples were washed extensively.

Production of MHC/peptide tetramers. Production of MHC/peptide tetramers has been described in detail[4]. A 15-amino-acid substrate peptide (BSP) for BirA-dependent biotinylation has been genetically fused onto the C terminus of HLA-A2. The A2–BSP fusion protein and human β_2-microglobulin (β_2m) were expressed in *Escherichia coli*, and were folded *in vitro* with the specific peptide ligand. The properly folded MHC–peptide complexes were extensively purified using FPLC and anion exchange and were biotinylated on a single lysine within the BSP using the BirA enzyme (Avidity, Denver, Colorado). Tetramers were produced by mixing the biotinylated MHC–peptide complexes with either PE- (PharMingen, San Diego, California) or APC-conjugated avidin (Prozyme, San Leandro, California) at a molar ratio of 4:1.

The specificity of each melanoma tetramers was confirmed by staining against a CTL line or clone specific for HLA-A2 in association with the peptide of interest. EBV and CMV tetramers were confirmed by staining with PBMCs from immune individuals. Specificity was demonstrated by the lack of staining of irrelevant CTLs. Each tetramer reagent was titered individually and used at the lowest concentration that still gave a clearly discernable positive population, generally 10–50 μg/ml, to minimize background staining. We consider cells positive for CD8 and tetramer staining only when populations clustered and that are at least 1 log above the negative population The limit of detection of each tetramer is determined by titrating known antigen-specific T cells into normal PBMCs. By collecting 1×10^6 or more events per sample to increase the number of potentially tetramer-positive events to observe clustering, we established the limit of detection to be 0.01% of $CD8^+$ T cells.

FACS analysis and sorting. Three-color FACS analyses used a Becton-Dickinson FACScan (Mountain View, California). Ten-color FACS analyses used a 12-parameter FACS instrument developed by the Stanford Shared FACS Facility. Sorting was done on a Vantage Cell Sorter (Becton-Dickinson, Mountain View, California). Data were compensated, analyzed and presented using FlowJo (Tree Star, San Carlos, California). All fluorescein- or phycoerythrin-conjugated as well as unconjugated monoclonal antibodies were obtained from PharMingen (San Diego, California), except for the following: anti-CD8–FITC, anti-CD4–Cy5PE, anti-CD13–Cy5PE and anti-CD19–Cy5PE were purchased from Caltag (South San Francisco, California); antibodies against CD5 were purified in the Herzenberg laboratory; Cy5PE anti-CD56 was from C. Prussin; anti-CD8β was from E. Reinherz; and anti-perforin was from E. Podack.

Conjugations to the ten fluorochromes used here were done as described (Roederer, M., http://www.drmr.com/abcon/). The fluorochromes used include fluorescein (FITC), phycoerythrin (PE), Cy5PE, Cy5.5PE, and Cy7PE, all excited by a 488-nm laser; Texas Red or Alexa 595, allophycocyanin (APC), Cy5.5APC, Cy7APC, excited by a 600-nm (dye) laser; and Cascade Blue (CB), excited by a 406-nm (ultraviolet) laser. Singly stained samples were collected in each experiment for compensation, which was done using FlowJo (Tree Star, San Carlos, California).

Antibody kits for discriminating V-β families were obtained from Immunotech (A. Necker, Immunotech, Marseille, France). In some experiments, CD8 enrichment was done before staining, using a depletion 'cocktail' of hapten-bound antibodies consisting of antibodies against CD4, CD19, CD14, CD16 and CD56 to which anti-hapten microbeads were added to enable magnetic separation by passage through a midiMACs column (Miltenyi Biotec, Auburn, California). Enrichment of the $CD8^+$ population to 60–75% of the total PBMCs was achieved. For staining of intracellular antigens, after surface staining, cells were washed and fixed with 4% formaldehyde, then permeabilized with 10% saponin (Sigma). Staining was done in saponin, as were the first two washes after staining.

Cytotoxicity analysis. CTLs were assayed for specific lysis of CMV pp65, EBV BMLF1, or tyrosinase peptide-pulsed targets using a 4-hour chromium release assay. Target A221-A2 cells were pulsed with 0.01, 0.1 or 10 μM of CMV-pp65 or T368 peptide, for at least 4 h in the presence of 100 μCi of ^{51}Cr. The labeled target cells were washed three times in RPMI and plated in triplicate wells at a minimum of 1,000 targets per well. Effector cells were incubated with the target cells at effector-to-target ratio of 10:1, unless otherwise stated. After 4 h, 100 μl of supernatant was collected from each well and the ^{51}Cr released was measured using a gamma counter. Spontaneous release of chromium, maximum release, and the percentage of cells killed (specific lysis) were calculated as described[45,46].

Intracellular cytokine staining. PBMCs were activated with 1 μg/ml phorbol 12-myristidate 13-acetate (PMA; Sigma) and 0.25 μg/ml ionomycin (Sigma) for 6 h at 37 °C, 5% CO_2. After the first 3 h, 10 μg/ml Brefeldin A (Epicentre Technologies, Madison, Wisconsin) was added to the culture. At the end of this incubation cells were treated with Fc-Block (Sigma) for 20 min at 4 °C to block the Fc receptors. Samples consisting of 1×10^6–3×10^6 cells were resuspended in FACS buffer (2% FCS/DPBS), and the relevant peptide-MHC tetramer–PE was added and stained at room temperature. After 30 min, the cells were stained for an additional 30 min at 0 °C with a 'cocktail' of antibodies consisting of anti-CD4–FITC, anti-CD14–FITC, anti-CD19–FITC and anti-CD8–Cy5PE to identify and gate out nonspecific tetramer-staining $CD8^-$ PBMCs. The cells were then fixed for 20 min at 4 °C with 2% paraformaldehyde in PBS followed by an permeabilization step for 10 min at room temperature using 0.5% saponin in PBS. Cells were stained in permeabilization buffer (0.5% saponin in PBS) for 20 min at 0 °C with predetermined optimal concentrations of allophycocyanin (APC)-conjugated antibodies against IL-2, IL-10, IFNγ or TNFα (PharMingen, San Diego, California). As a negative staining control, cells were stained with an isotype-matched control antibody of irrelevant specificity (APC-conjugated rat IgG_{2a}, mouse IgG_1; PharMingen, San Diego, California) at the same concentration as the antibody against cytokine. Cells were washed twice in permeabilization buffer and once in FACS buffer and then analyzed by four-color flow cytometry on a Becton Dickinson FACSCalibur cytometer (Mountain View, California).

Peptide stimulation. Frozen PBMCs were thawed and cultured overnight in IMDM supplemented with 10% pooled human AB serum (Stanford Blood Center, Stanford, California). Live cells were isolated by centrifugation with ficoll-hypaque (Pharmacia), then washed three times. In sterile, 96-well plates, 2×10^6 PBMCs were added to each well with peptide to a final concentration of 1 μg/ml, and incubated at 37 °C for 4 h. The cells were washed three times, then stained with MHC/peptide tetramers (PE- or APC-conjugated), anti-CD69–FITC, anti-CD4/14/19–Cy5PE, anti-CD3–CB and anti-CD8–-Cy7PE antibodies at room temperature for 30 min before flow cytometric analysis. Various peptide concentrations and stimulation times were tested to determine the optimal stimulation conditions for previously frozen PBMCs. The threshold for CD69 positivity is set by staining PBMCs stimulated with PMA and ionomycin in each experiment.

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