

Loss of CTL Function among High-Avidity Tumor-Specific CD8⁺ T Cells following Tumor Infiltration

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Abstract

A major problem in generating effective antitumor CTL responses is that most tumors express self-antigens to which the immune system is rendered unresponsive due to mechanisms of self-tolerance induction. CTL precursors (CTLp) expressing high-affinity T-cell receptors (TCR) are often functionally deleted from the repertoire, leaving a residual repertoire of CTLp having only low-affinity TCR. Furthermore, even when unique antigens are expressed, their presentation by dendritic cells (DC) may predispose to peripheral tolerance induction rather than the establishment of CTL responses that kill tumor cells. In this study, we examined both high-avidity (CL4) and low-avidity (CL1) CD8⁺ T-cell responses to a murine renal carcinoma expressing, as a neoantigen, high and low levels of the hemagglutinin (HA) protein from influenza virus A/PR/8 H1N1 (PR8; RencaHA^{high} and RencaHA^{low}). Our data show that, following encounter with K^dHA epitopes cross-presented by bone marrow-derived DC, low-avidity CL1 cells become tolerized within tumor-draining lymph nodes (TDLN), and in mice bearing either RencaHA^{high} or RencaHA^{low} tumors, very few form tumor-infiltrating lymphocytes (TIL). In marked contrast, high-avidity CL4 cells differentiate into effector CTL within the TDLN of mice bearing either RencaHA^{high} or RencaHA^{low} tumors, and although they form TIL in both tumors, they lose CTL effector function. Critically, these results show that anticancer therapies involving either adoptive transfer of high-avidity tumor-specific CTL populations or targeting of preexisting tumor antigen-specific memory CD8⁺ T cells could fail due to the fact that CTL effector function is lost following tumor infiltration. [Cancer Res 2008;68(8):2993–3000]

Introduction

Naive CD8⁺ T-cell activation by tumor-specific antigens can occur primarily via two major mechanisms. The first involves direct presentation of tumor-derived epitopes to naive T cells. This may only occur if the tumor cell has the ability both to metastasize to lymph nodes (1) and to provide sufficient costimulatory signals to activate naive T cells. The second mechanism is cross-presentation, which relies on professional antigen-presenting cells (APC), of which dendritic cells (DC) are thought to play a primary role, as they are able to acquire tumor cell-derived antigens either at the

site of tumor growth or from dying tumor cells via lymph, and then travel to lymph nodes where they present peptide epitopes to naive CD8⁺ T cells (2–4). Despite these processes, in cancer, tumor growth is often incompletely restricted due to the lack of effector CTL responses against tumor antigens.

Many tumor antigens are peptides derived from proteins ubiquitously expressed by most somatic cells (5). Due to mechanisms of self-tolerance induction in the thymus (5–7), and periphery (5), many potentially self-reactive T cells with high avidity for self-peptides are often eliminated from the T-cell repertoire. This renders the immune system incapable of eliciting productive immune responses against such tumor cell-expressed self-antigens as only residual CTL precursors (CTLp), having low avidity for these, exist within the repertoire. This low-affinity repertoire may not be activated to form effective antitumor CTL unless they are previously primed with another external stimulus, such as a vaccine (8).

Under conditions of tumor-specific antigen expression, tumor-specific CTLp in the peripheral T-cell repertoire can have high and low avidity for tumor-specific antigen. Under these conditions, some tumors can still escape host immune responses using various evasion strategies (9–12). For example, one mechanism involves lowering the level of expression of tumor-specific antigen to below the threshold required to prime tumor antigen-specific CTLp (13–15). This may result in a lack of tumor-specific effector CTL responses or, depending on the TCR affinity, induction of peripheral tolerance.

Consideration of the effect of tumor responses on effector T cells is vital when designing potential antitumor vaccines because many strategies rely on inducing proliferation or expansion of preexisting tumor antigen-specific memory T cells (7). If these cells have become tolerant to the tumor, their response to these vaccines is likely to be limited, reducing the success rate of such therapies. To study the consequences following naive CD8⁺ T-cell interaction with tumor-specific antigens *in vivo*, we used a murine Renca model (16) where influenza virus hemagglutinin (HA) antigen is expressed as a tumor-specific antigen (RencaHA; ref. 17). Transgenic CL4 and CL1 cells expressing TCRs, with high and low affinity, respectively, for immunodominant K^d-restricted epitope of HA protein from PR8 (IYSTVASSL_{518–526}) were also used (8, 18). We hypothesized that, in response to RencaHA^{high} tumor *in vivo*, high-avidity CL4 cells would differentiate into effector CTL, whereas low-avidity CL1 cells would be tolerized. Our findings support this hypothesis and also show that, as a result of tolerance induction, very few CL1 cells are able to form tumor-infiltrating lymphocytes (TIL). Furthermore, our data also indicate that, following infiltration, CL4 cells become incapable of producing IFN γ *in vitro* in response to restimulation with K^dHA peptide. These results have critical implications for vaccination studies that target either naive high-avidity tumor-specific T cells or preexisting tumor antigen-specific memory T cells.

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Materials and Methods

Mice. BALB/k (H-2^k), BALB/c (H-2^d), BALB/c CL1 TCR transgenic (18), and BALB/c CL4 TCR transgenic mice (8) were bred and housed under specific pathogen-free conditions within University of Bristol Animal Services Unit. All experiments were conducted in accordance with UK Home Office Guidelines.

Cell lines. The RencaNT cell line was single cell cloned from a population of Renca cells (16). Cells were maintained in complete medium (RPMI 1640, 10% FCS, 2 mmol/L glutamine, 50 units/mL penicillin/streptomycin, and 5×10^{-5} mol/L 2-mercaptoethanol). RencaHA^{high} and RencaHA^{low} cell lines were previously obtained by transfection of RencaNT cell line with HA from PR8 (17). These were grown in complete medium supplemented with 0.1 mg/mL geneticin (Invitrogen). The mastocytoma line P815 was used as a target in ⁵¹Cr release CTL assays.

Enrichment of CD8⁺ T cells. Single-cell suspensions were generated from lymph nodes and spleens from CL1 or CL4 TCR transgenic mice. CD8⁺ T cells were enriched by positive magnetic-activated cell sorting (MACS) using anti-CD8 MACS microbeads together with LS separation columns and Midi-MACS magnets (Miltenyi Biotec) according to the manufacturers' instructions. In some experiments, CL1 and CL4 cells were labeled with 5 μmol/L carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Inc.) in accordance with described protocols (19).

Flow cytometry. Single-cell suspensions were incubated with fluorochrome-conjugated monoclonal antibodies (mAb) against cell surface markers [e.g., anti-Thy1.1-PE (Becton Dickinson) and supernatant from anti-FcγRIIR mAb-secreting 2.4G2 cell line]. Intracellular IFNγ was detected using a Cytofix/Cytoperm Plus kit with GolgiPlug (Becton Dickinson) according to the manufacturers' instructions and staining with APC-conjugated anti-IFNγ mAb (Becton Dickinson). Cells were acquired on a FACSCalibur flow cytometer with CellQuest software (BD Cytometry Systems).

CD107a was used to measure CD8⁺ T-cell cytotoxicity by flow cytometric analysis of degranulation (20). Briefly, cells were harvested from lymph nodes of recipient mice and incubated in complete medium with K^dHA₍₅₁₈₋₅₂₆₎ peptide (1 μg/mL) and anti-CD107a-APC mAb (Southern Biotech) at 37°C for 30 min. GolgiPlug (1 μL/mL) was added and cells were incubated for a further 2.5 h, after which they were stained with anti-Thy1.1-PE mAb and analyzed. All flow cytometric analyses were performed using WinMDI software (available online).³

Adoptive transfers. BALB/c mice were given a s.c. injection into the left shoulder of 1×10^6 Renca cells in 100 μL PBS followed by an i.v. injection of either CFSE-labeled, purified, naive CL1 or CL4 cells in 200 μL PBS. BALB/c mice receiving only CFSE-labeled CL1 or CL4 cells were given PR8 i.p. as controls. At various time points after transfer, tumors and lymph nodes were excised and cell suspensions were stained with anti-Thy1.1-PE mAb for flow cytometric analyses.

Bone marrow chimeras. H-2^dBALB/c or H-2^kBALB/k bone marrow (BM) was harvested into 5% FCS supplemented with HBSS. Cell suspensions were passed through 40-μm cell strainers and centrifuged. To remove mature T cells, BM cells were resuspended in anti-Thy1.2 antibody J1/J20 for 45 min on ice. Cells were centrifuged and resuspended in Rabbit complement (Cedarlane Labs) before incubation at 37°C for 45 min. They were passed through a 40-μm cell strainer before being washed in PBS. Cells (10^7) in 200 μL PBS were injected i.v. into recipient BALB/c mice irradiated 24 h earlier with 930 rads.

CD8⁺ T-cell proliferation assay. Purified CL1 or CL4 cells (1×10^4) were cultured with 1×10^5 irradiated BALB/c splenocytes in the presence of decreasing concentrations of K^dHA₍₅₁₈₋₅₂₆₎ peptide. Control wells contained splenocytes, naive CL1 or CL4 cells, or both in the absence of peptide. After 40 h, cells were incubated with 0.5 μCi/well of [³H]thymidine at 37°C for 8 h before freezing. After thawing, cells were transferred to filters, added to sample bags containing 3 mL Betaplate scintillant (Perkin-Elmer), and read

on a 1450 Microbeta liquid scintillation counter with Microbeta for Windows 2.7 (Wallac Oy).

IFNγ ELISA. Supernatants, taken from proliferation assay cocultures before addition of [³H]thymidine, were analyzed for IFNγ by ELISA. Briefly, MaxiSorp plates were coated with purified anti-IFNγ mAb (Becton Dickinson) and blocked with 1% bovine serum albumin in PBS and supernatants or titrated recombinant IFNγ standards were added and plates were incubated at 37°C for 3 h. After addition of biotinylated IFNγ mAb (Becton Dickinson), bound IFNγ was detected using ExtrAvidin-horseradish peroxidase (Sigma-Aldrich) and tetramethylbenzidine substrate (Sigma-Aldrich) was added for color development. The reaction was stopped with H₂SO₄ before plates were read at 450 nm with a 595-nm reference.

Cytotoxicity assay. Cytotoxicity was measured by standard chromium release assay. Target P815 cells were labeled by incubation in complete RPMI 1640 containing 3.7 MBq/10⁶ cells of sodium [⁵¹Cr]chromate (Amersham) at 37°C for 1 h. CL1 or CL4 cells were harvested from coculture setup 48 h before the assay containing 5×10^6 irradiated BALB/c splenocytes and 1×10^6 purified naive CL1 or CL4 cells with 1 μg/mL K^dHA₍₅₁₈₋₅₂₆₎ peptide and washed. They were added to P815 target cells at an E:T ratio of 10:1 in the presence of decreasing concentrations of K^dHA₍₅₁₈₋₅₂₆₎. Plates were incubated at 37°C in 5% CO₂ for 18 h. Supernatant (50 μL) was removed and mixed with 150 μL of Ultima Gold scintillation fluid (Perkin-Elmer), and scintillation was recorded using a 1450 Microbeta liquid scintillation counter. Specific lysis, expressed as a percentage, was determined using the following formula:

$$\frac{(\text{sample release} - \text{spontaneous release})}{(\text{maximum release} - \text{spontaneous release})} \times 100$$

Maximum lysis was determined from mixing labeled target cells with 100 μL of 1% SDS.

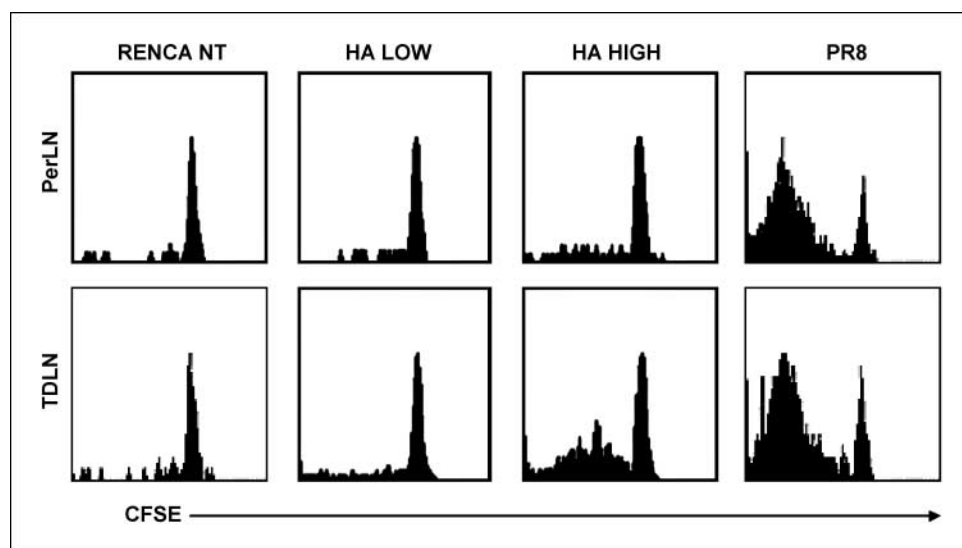
Isolation of tumor-infiltrating lymphocytes. Tumors were excised and teased apart in serum-free medium before the cell suspension was passed through a 40-μm cell strainer. It was layered over 15 mL of Nycoprep (Axis Shield) and spun at 1,800 rpm for 25 min. Cells on the meniscus were harvested, washed, and prepared for flow cytometric analyses.

Results

Activation of high-avidity CL4 cells in tumor-draining lymph nodes relies on cross-presentation of RencaHA-derived K^dHA epitopes by DC. To establish whether naive CL4 cells are primed by RencaHA tumor cells *in vivo*, Thy1.2⁺ BALB/c mice were given RencaHA cells followed 11 days later by CFSE-labeled, purified, naive Thy1.1⁺ CL4 cells. As negative controls, groups of BALB/c mice were given RencaNT and CL4 cells. As positive controls, BALB/c mice were given only CL4 cells together with PR8 for T-cell priming. On day 16 of tumor growth, all mice were sacrificed and various lymph nodes were removed and levels of CFSE among Thy1.1⁺ CL4 cells were examined (Fig. 1). Cervical and axillary lymph nodes were tumor-draining lymph nodes (TDLN), and non-tumor-draining peripheral lymph nodes (PerLN) consisted of inguinal and popliteal lymph nodes. Flow cytometric analyses of lymph nodes taken from positive controls showed high levels of CL4 cell proliferation on day 16 in both TDLN as well as PerLN, as evidenced by detection of highly divided CFSE low cells. In mice given RencaHA, CL4 cell proliferation occurred in TDLN but only slightly in PerLN, suggesting that T-cell priming was occurring in TDLN. In negative controls, given RencaNT cells plus CL4 cells, T-cell proliferation was not detected in TDLN nor in PerLN, confirming that CL4 cell responses to RencaHA cells within TDLN were K^dHA specific.

³ <http://facs.scripps.edu>

Figure 1. CL4 cell priming occurs only in the TDLN of mice bearing RencaHA tumors. Thy1.2⁺ BALB/c mice were injected s.c. with RencaNT or RencaHA tumor cells. On day 11, mice were given 3.75×10^6 CFSE-labeled, purified, naive Thy1.1⁺ CL4 cells i.v., and on day 16, cells obtained from the PerLN and TDLN were stained with antibodies against Thy1.1. BALB/c mice that had received only CFSE-labeled, purified, naive CL4 cells plus, on day 13, 1,200 HA units of PR8 i.p. were used as positive controls. Histograms show levels of CFSE among Thy1.1⁺ CL4 cells. Data are representative of four animals per group.



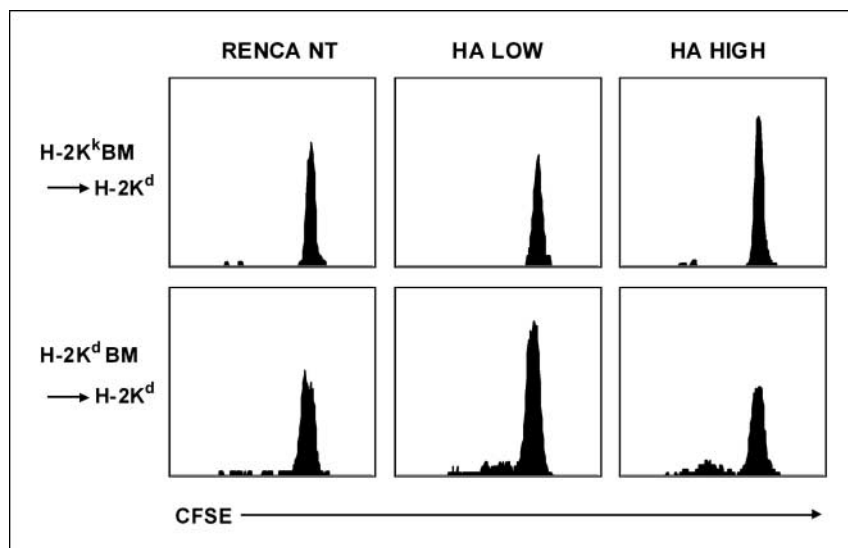
Previous studies *in vitro* showed that, although RencaHA tumor cells do not express CD80 or CD86, their expression of ICAM-1 circumvents the requirement for CD80/CD86-mediated costimulation and, through interaction with LFA-1 expressed by naive CL4 cells (8), induces both effector and memory antitumor CTL (17). It is possible that the activated CL4 cells we observed in our experiments resulted either from direct presentation of K^dHA epitopes by tumor cells or from cross-presentation by DC in TDLN.

To determine whether cross-presentation of Renca-derived K^dHA epitopes to naive K^dHA-specific T cells was occurring, BM chimeras were generated lacking H-2^d expression among BM-derived APC. Irradiated BALB/c (H-2K^d) mice were given either BALB/k (H-2K^k) BM or, as a control, BALB/c (H-2K^d) BM. To allow full BM reconstitution within the APC compartment, all mice were maintained for a period of 4 months before being given either RencaNT or RencaHA tumor cells. Ten days later, all mice were given CFSE-labeled, purified, naive CL4 cells (Thy1.1). After a further 4 days, TDLNs were isolated and cell suspensions were stained with anti-Thy1.1 antibodies. Figure 2 clearly shows that, in the absence of HA expression, CL4 cells failed to divide in TDLN of all RencaNT tumor-bearing mice. However, in control BALB/c

BM→BALB/c chimeras given RencaHA cells, CL4 cells divided in TDLN, confirming that the presence of the HA protein in BALB/c BM→BALB/c chimeras resulted in CL4 cell proliferation. Importantly, in TDLN of BALB/k BM→BALB/c chimeras, CL4 cells remained undivided, confirming that proliferation of CL4 cells in TDLN relies on cross-presentation of RencaHA-derived K^dHA epitopes by BM-derived APC.

High- and low-avidity CD8⁺ T-cell responses to cognate tumor antigen. Mechanisms of self-tolerance serve to purge the immune system of self-reactivity among CD8⁺ T cells, leaving a residual repertoire of T cells exhibiting low avidity for self-epitopes. Whereas high-avidity tumor-specific CD8⁺ T-cell responses may be generated against tumor-specific antigens, failure to make effective antitumor-specific CTL responses may occur among low-avidity CTLp. Our earlier studies showed that, when activated by vaccination with PR8, residual low-avidity K^dHA-specific CTLp were able to differentiate into effector CTL, possibly preventing RencaHA tumor growth *in vivo* (8). Injection of RencaHA cells into mice in the absence of PR8 infection resulted in normal tumor growth, suggesting that either low-avidity CTLps fail to respond directly to tumor antigen or they were undergoing tolerance induction.

Figure 2. Activation of CL4 cells in the TDLN requires cross-presentation of Renca cell-derived K^dHA₍₅₁₈₋₅₂₆₎ epitopes. BALB/c (H-2K^d) mice were lethally irradiated with 930 rads and, 24 h later, reconstituted with BM from either Thy1.2⁺ BALB/k (H-2K^k) or Thy1.2⁺ BALB/c (H-2K^d) mice. After 16 wk, BM chimeric mice were injected s.c. with RencaNT or RencaHA tumor cells. Ten days later, mice were given $\sim 4 \times 10^6$ CFSE-labeled, purified, naive Thy1.1⁺ CL4 cells i.v., and then 4 d later, mice were sacrificed and the cells obtained from the TDLN were stained with antibodies against Thy1.1. Histograms show levels of CFSE among Thy1.1⁺ CL4 cells. Data are representative of five animals per group.



Establishing the fate of both high- and low-avidity CD8⁺ T cells following interaction with tumor cell-derived antigens is crucial to designing effective immunotherapeutic strategies to combat cancer. Thus, to determine the fate of high- and low-avidity tumor-specific T cells, we carried out a series of experiments using CL4 and CL1 cells. To evaluate their relative avidity for K^dHA as well as their ability to respond to cognate peptides, CL1 or CL4 cells were cocultured with limiting amounts of cognate K^dHA-pulsed splenocytes for 48 h. Relative avidities were evaluated by measuring T-cell proliferation, IFN γ production, and cytotoxicity against ⁵¹Cr-labeled K^dHA-pulsed target cells. Figure 3A shows that, whereas CL4 cell proliferation was measured in response to 10 ng/mL of K^dHA, CL1 cell proliferation could only be measured above a threshold concentration of K^dHA of 100 ng/mL. Similarly, there was a 10-fold difference in threshold level of K^dHA required to elaborate IFN γ into coculture supernatants (Fig. 3B). Furthermore, comparison of CTL activity

between CL4 and CL1 effector cells revealed that CL1 CTL generated in the presence of APC pulsed with a high concentration of K^dHA required target cells to be pulsed with 10-fold more K^dHA peptide than CL4 CTL to generate equivalent target cell lysis (Fig. 3C).

IFN γ release and CD107a cell surface expression by CL1 and CL4 cells following activation by tumor-derived cognate epitopes *in vivo*. IFN γ production following CD8⁺ T-cell priming in peripheral lymphoid tissues is often associated with induction of CTL effector function and productive immune responses (21–23), whereas lack of IFN γ production following priming is often associated with abortive activation and peripheral tolerance induction (23). Expression of the marker CD107a, which is located within membranes of cytotoxic granules, is also used to define effector activity in CD8⁺ T cells using flow cytometry (20). Thus, to determine responses made by both CL1 and CL4 cells following cross-presentation of tumor-derived K^dHA epitopes in TDLN of

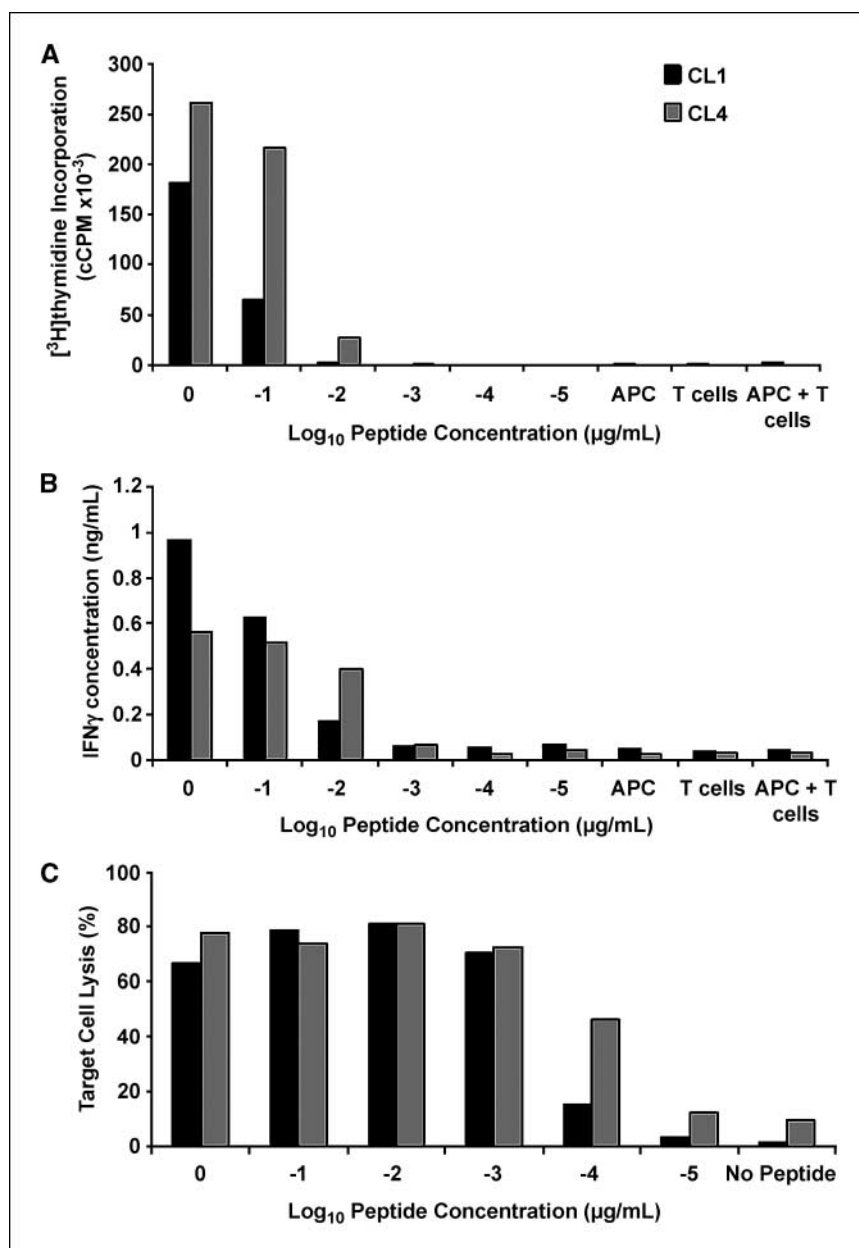
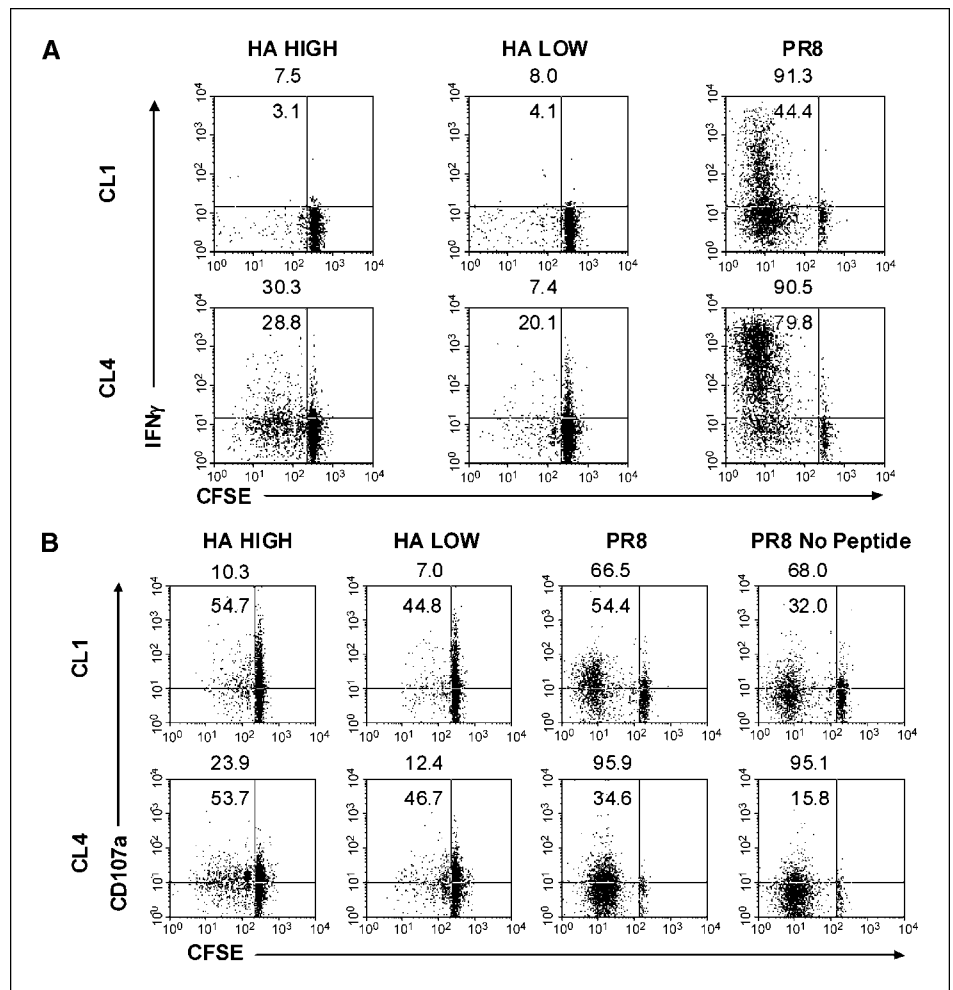


Figure 3. CL1 and CL4 cell responses to cognate tumor peptide K^dHA₍₅₁₈₋₅₂₆₎ *in vitro*. Purified naive CL1 or CL4 cells were cocultured for 48 h with peptide-pulsed irradiated syngeneic BALB/c splenocytes as APC.K^dHA₍₅₁₈₋₅₂₆₎ (1 µg/mL). A, CL4 cell proliferation was evaluated by measuring the incorporation of [³H]thymidine during the last 8 h of coculture. B, release of IFN γ into the tissue culture supernatant was measured by ELISA. C, CTL activity of cocultured CL1 and CL4 cells against H-2^d P815 target cells that were unpulsed or pulsed with decreasing concentrations of K^dHA₍₅₁₈₋₅₂₆₎ peptide was measured using a standard ⁵¹Cr release assay. Data are representative of two separate experiments.

Figure 4. IFN γ production and CD107a expression by CL1 and CL4 cells in response to K^dHA₍₅₁₈₋₅₂₆₎ peptide cross-presented in the TDLN of tumor-bearing mice. Thy1.2⁺ BALB/c mice were injected s.c. with RencaHA^{high} or RencaHA^{low} tumor cells. On day 9/10, mice were given 3×10^5 CFSE-labeled, purified, naive Thy1.1⁺ CL1 or Thy1.1⁺ CL4 cells i.v., and on day 15, the cells in the TDLN were harvested and stained with antibodies against Thy1.1 and for intracellular IFN γ production or CD107a expression. TDLNs from Thy1.2⁺ BALB/c mice that had received only CFSE-labeled, purified, naive Thy1.1⁺ CL1 or Thy1.1⁺ CL4 cells plus, on day 11, 1,200 HA units of PR8 i.p. were used as positive controls. Dot plots are gated on Thy1.1 expression and show the level of intracellular IFN γ (A) or CD107a expression among dividing cells (as shown by loss of CFSE; B). The percentage of divided cells is given above each plot and the percentage of divided cells that are IFN γ ⁺ or CD107a⁺, respectively, is given in the top left-hand quadrant. Data are representative of two separate experiments containing at least three animals per group.



tumor-bearing mice, flow cytometric analyses were carried out to evaluate IFN γ production and CD107a expression.

Thy1.2⁺ BALB/c mice were injected with RencaHA^{high} and RencaHA^{low} tumor cell lines. Ten days later, mice were given CFSE-labeled, purified, naive Thy1.1⁺ CL1 or CL4 cells. After a further 5 days, TDLN cells were stained with antibodies against Thy1.1 and for intracellular IFN γ (Fig. 4A) or cell surface CD107a (Fig. 4B). Expression of IFN γ or CD107a versus CFSE among Thy1.1⁺ cells was examined using flow cytometry. BALB/c mice, receiving only CL1 or CL4 cells on day 10 followed by PR8 on day 11, were used as positive controls for productive activation of naive CD8⁺ T cells (23).

Examination of TDLN cells of mice given RencaHA^{high} cells revealed that naive CL4 cells underwent several rounds of division, as evidenced by a reduction in the level of CFSE in ~30% of Thy1.1⁺ cells analyzed (Fig. 4A). Importantly, dividing CL4 cells in mice given RencaHA^{high} or RencaHA^{low} cells produced IFN γ on restimulation *in vitro* with cognate K^dHA, suggesting that, like CL4 cells activated in control mice immunized with PR8, they were undergoing productive activation to form effector cells. In contrast, CL1 cell proliferation was considerably less, and of those dividing cells, very few produced IFN γ on K^dHA restimulation *in vitro*. Such lack of responsiveness was not due to an intrinsic inability of naive CL1 cells to respond to K^dHA epitopes, as in control mice, immunized with PR8, naive CL1 cells isolated from TDLN underwent high levels of proliferation and IFN γ production. As

approximately twice as many CL4 cells produce IFN γ following peptide restimulation *in vitro* compared with CL1 cells in controls, it shows that affinity of the TCR for cognate peptide could influence quantitatively certain CTL effector functions *in vivo*.

Although, compared with CL4, fewer CL1 cells were dividing in TDLN of mice given RencaHA^{high} or RencaHA^{low} cells, of these similar proportions expressed CD107a following peptide restimulation *in vitro* (Fig. 4B). These results suggest that, within TDLN of RencaHA-bearing mice, whereas more CL4 cells seem to undergo several rounds of division and differentiate into effector CTL, CL1 cell division is less and even those that divide gain only a partial effector phenotype. Under these conditions, whereas CL4 cells seemed to undergo productive activation, CL1 cells may either be unresponsive or undergo an abortive activation response associated with the induction of peripheral tolerance (24).

Lack of IFN γ release by CL4 TIL. It was unclear as to the fate of CL1 and CL4 cells following priming with cross-presented RencaHA-derived K^dHA epitopes within TDLN. It is possible that proliferation may result in apoptosis *in situ* through activation-induced cell death. However, priming could result in migration from the TDLN into the tumor, forming TIL. Flow cytometric analyses of tumors taken from mice given CFSE-labeled CL1 or CL4 CD8⁺ T cells revealed that CL1 cells did not form TIL 7 days after injection, whereas CL4 TILs were present within the RencaHA^{high} tumor (Fig. 5A).

These data suggested that either low-avidity CL1 cells proliferating within the TDLN of RencaHA^{high} tumor-bearing mice are unable to form TIL or the fact that proliferation is less among CL1 cells than among CL4 cells within the TDLN, formation of CL1 TIL may take longer. Therefore, it was necessary to compare the fate of CL1 cells with that of CL4 cells following priming within the TDLN at a later time point. Another factor that can influence the formation of TIL is the level of tumor-specific antigen expression. RencaHA^{high} tumors express considerable amounts of cell surface HA protein, whereas HA expression by RencaHA^{low} represents a more physiologically relevant level of tumor-specific antigen. To address these issues, Thy1.2⁺ BALB/c mice were injected s.c. with RencaHA^{low} tumor cells followed by CFSE-labeled, purified, naive Thy1.1⁺ CL1 or Thy1.1⁺ CL4 cells 9 days later. On day 28, mice were culled and the TDLN and tumors were excised and stained with anti-Thy1.1 antibodies and for intracellular IFN γ . Our results show that PR8 immunization of control BALB/c mice given only Thy1.1⁺ CL4 or CL1 cells results in widespread proliferation of both CL4

and CL1 cells within all peripheral lymphoid tissues and the formation of highly divided effector CTL, as evidenced by loss of CFSE, cell surface expression of CD107a, and intracellular staining of IFN γ . Flow cytometric analyses of TDLN cells from RencaHA^{low} tumor-bearing BALB/c mice given either Thy1.1⁺ CL1 or CL4 cells revealed the presence of roughly equal numbers of highly divided CL1 and CL4 cells, as evidenced by their low level of CFSE (Fig. 5B). Importantly, even after ~3 weeks of *in vivo* exposure to RencaHA^{low} tumor, whereas highly divided CL4 cells were able to elaborate IFN γ following restimulation with K^dHA peptide *in vitro*, CL1 cells failed to produce IFN γ under these circumstances.

Analyses of tumors revealed the presence of large numbers of Thy1.1⁺ CL4 TILs, which have lower levels of CFSE than CL4 cells within the TDLN, thus indicating that they had migrated from the TDLN to form TIL. Importantly, CL4 TILs were unable to produce IFN γ following peptide restimulation *in vitro*, suggesting that TIL formation was associated with a loss of CTL effector function. In marked contrast, despite similar proliferation rates within the

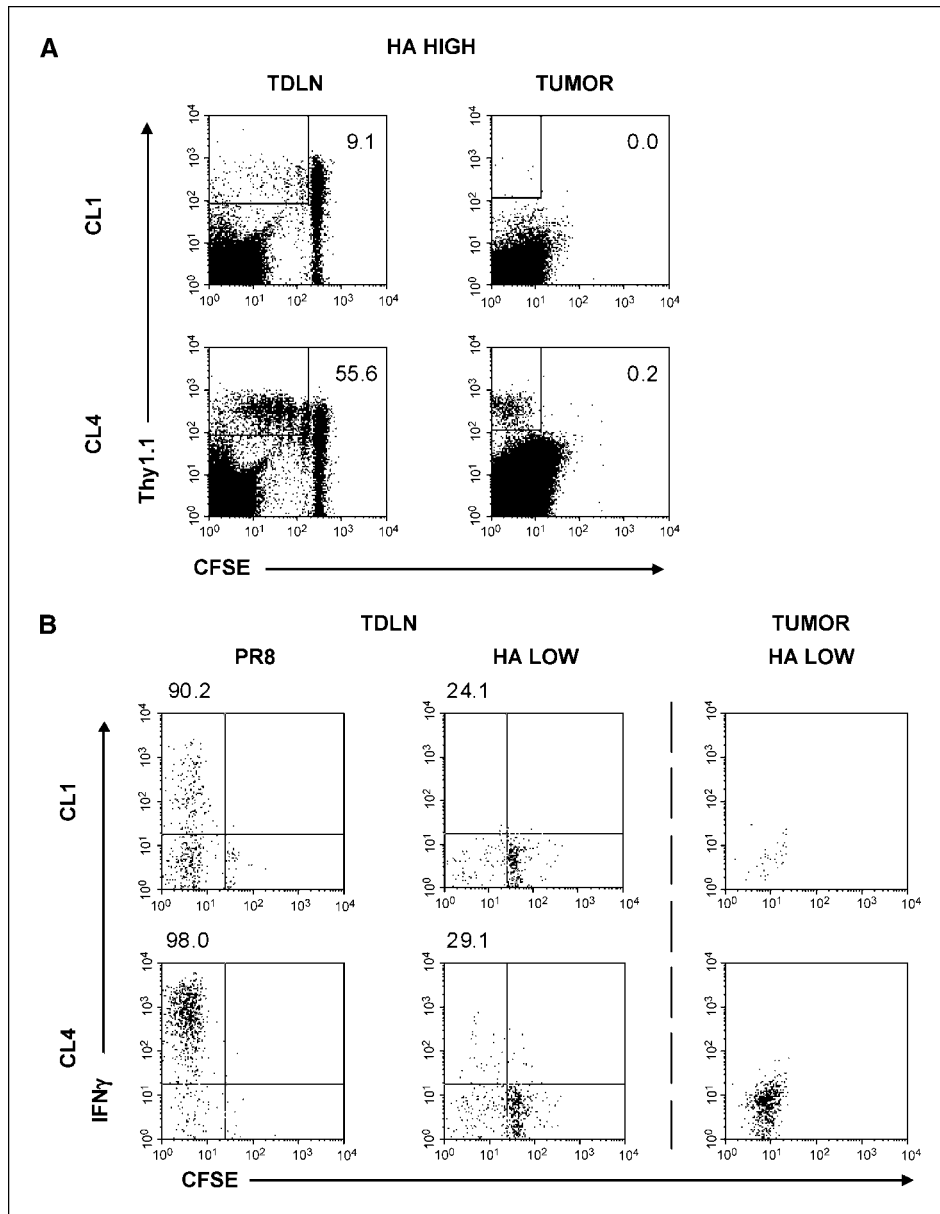


Figure 5. IFN γ production by tumor-infiltrating CL1 and CL4 cells in response to K^dHA₍₅₁₈₋₅₂₆₎ peptide in RencaHA^{low} tumor-bearing mice. **A**, Thy1.2⁺ BALB/c mice were injected s.c. with RencaHA^{high} tumor cells. On day 9 or 10, mice were given 3×10^6 CFSE-labeled, purified, naive Thy1.1⁺ CL1 or Thy1.1⁺ CL4 cells i.v., and on day 17, lymphoid cells from the TDLN and the tumor were stained with antibodies against Thy1.1. Dot plots show proliferation as shown by loss of CFSE among Thy1.1⁺ cells. For the TDLN, the numbers indicate the percentage of Thy1.1⁺ cells that have divided, and for the tumor, the numbers show the percentage of TILs that are Thy1.1⁺, respectively. Data are representative of between four and six mice per group. **B**, Thy1.2⁺ BALB/c mice were injected s.c. with RencaHA^{low} tumor cells. On day 9, mice were given 3×10^6 CFSE-labeled, purified, naive Thy1.1⁺ CL1 or Thy1.1⁺ CL4 cells i.v., and on day 28, lymphoid cells from the TDLN and the tumor were stained with antibodies against Thy1.1 and for intracellular IFN γ production. Dot plots show IFN γ production versus proliferation as shown by loss of CFSE among Thy1.1⁺ cells. TDLN plots from RencaHA^{low} tumor-bearing mice show the percentage of divided cells. Tumor plots show IFN γ versus CFSE expression by Thy1.1⁺ TIL when equal numbers of TIL were collected. TDLN cells from Thy1.2⁺ BALB/c mice that had received only CFSE-labeled, purified, naive Thy1.1⁺ CL1 or Thy1.1⁺ CL4 cells plus 1,200 HA units of PR8 i.p. were used as positive controls. Data are representative of 10 mice per group.

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TDLN, very few CL1 TILs could be observed within the RencaHA^{low} tumor. Taken together, these data indicate that low-avidity tumor-specific CD8⁺ T cells are unable to form effector cells and as a consequence are unable to form TIL.

Discussion

Whether direct or cross-presentation of tumor-specific antigens to the host immune system is the more efficient mechanism for eliciting a CD8⁺ effector response against tumors is unknown. Evidence exists supporting involvement of both mechanisms in tumor recognition, but usage depends on the nature of the tumor tested and the milieu surrounding the tumor (1, 25, 26). In our system, we showed that the mechanism involved in priming effector CTL responses among naive CL4 cells in BALB/c mice bearing RencaHA tumors is cross-presentation, as tumor antigen recognition occurs only in chimeric mice reconstituted with BALB/c BM and not BALB/k BM. We reason that cross-presentation is also likely to be the mechanism of presentation for recognition of K^dHA Renca-derived epitopes by CL1 cells because the only difference is T-cell avidity.

Clearly, there is a difference in the threshold level of cognate K^dHA required to elicit effector CTL responses among CL1 and CL4 cells. Recognition of HA *in vitro* by CL1 cells occurs at a peptide concentration 10-fold higher than for CL4 cells, although for initiation of CTL killing of targets by CL1 and CL4 cells the peptide concentration required is reduced by 100-fold in each case. This can be attributed to the level of TCR occupancy required to elicit different cellular responses, as CTL lysis *in vitro* requires triggering of very few TCRs due to response localization, whereas to elaborate IFN γ or proliferate, 20% to 50% of TCR must encounter cognate peptide (27). Such observations may also explain why CL1 cells, adoptively transferred into BALB/c mice, bearing RencaHA tumors recruit CD107a (an indicator of cytotoxic activity) to their cell surface, whereas production of IFN γ is undetectable (20).

CL4 cells become effectors within TDLN in BALB/c mice bearing RencaHA tumors as shown by release of IFN γ and recruitment of CD107a to their cell surface in response to restimulation *in vitro*. This response is not unexpected because these cells were derived from a conventional BALB/c mouse that had been immunized with PR8 (8). If these CL4 cells existed within the repertoire of the InSHA mouse (which expresses the HA protein as a neo-self-antigen on pancreatic islet β cells), they would have been functionally tolerized following their abortive activation in the pancreatic lymph nodes due to their high avidity for HA (28). However, CL1 cells were derived from an InSHA mouse and had previously escaped tolerance due to their low avidity for HA (18), so it is unsurprising that they do not release IFN γ in response to K^dHA as there maybe insufficient tumor-expressed K^dHA to induce priming leading to abortive activation through anergy or possibly deletion (24).

Although abortive activation of CL1 cells seems to occur in our system, a small proportion of these cells are still able to infiltrate the RencaHA^{low} tumor in BALB/c mice. This suggests that perhaps it is the small number of CL1 cells that become partially activated in TDLN, shown by expression of CD107a on their cell surface, which enter the tumor. Therefore, influx of CD8⁺ T cells into the tumor is dependent on their activation in TDLN, which is determined by the overall affinity of the TCR for cognate tumor peptide. It remains to be determined whether, on infiltration, these cells have any effect on limiting tumor growth.

It has been shown that effective immune responses leading to eradication of tumors can be achieved in initial stages of tumor expansion (9). However, after prolonged exposure, effectiveness of responses is substantially reduced, as tumors develop strategies to allow their escape from T-cell surveillance and the host immune system becomes accustomed to their presence (9–12). CL1 RencaHA^{low} TIL from BALB/c mice failed to produce IFN γ in response to restimulation with K^dHA peptide *in vitro*. This result is consistent with the lack of IFN γ production by these cells within TDLN in these mice. Thus, it is unlikely that these cells will be able to limit tumor expansion, as they seem to have undergone abortive activation resulting in suppression of effector CTL activity early on in tumor growth. Indeed, this T cell effector function cannot be regained *in vitro* even on addition of peptide-pulsed APCs, suggesting that it has been permanently switched off.⁴ CL4 cells become effectors in TDLN in BALB/c mice as shown by expression of IFN γ and migration of CD107a to the cell surface, so it might be expected that these cells would become potent cytotoxic TIL; however, CL4 TILs lose their effector function. So under prolonged tumor exposure, these cells seem to have become defective in cytokine secretion possibly due to an inability to transmit TCR-mediated signals beyond p56^{lck} engagement to the cell interior (29). This loss of cytokine secretion may explain why these cells are incapable of mediating tumor regression, as therapeutically effective TILs specifically secrete IFN γ *in vitro* (30). Additionally, loss of T-cell effector function and inability to promote tumor rejection may be due to accumulation within the tumor of CD4⁺CD25⁺ regulatory T cells (Treg), which suppress tumor-specific T-cell responses, enhancing tumor spread (31). Other tumor escape responses involve production of immunosuppressive factors, such as interleukin-10, transforming growth factor- β , and vascular endothelial growth factor (10), and expansion of the Treg population perhaps through conversion of CD4⁺CD25⁻ T cells into CD4⁺CD25⁺ Treg cells (11).

In conclusion, we have shown that, in the presence of RencaHA^{high} tumors, high-avidity CL4 cells undergo productive activation and differentiate into effector CTL, whereas low-avidity CL1 cells undergo abortive activation. Despite differences in activation, both CL4 and CL1 cells are still able to infiltrate the RencaHA^{low} tumor to a greater or lesser extent, and critically on infiltration, CL4 cells become defective in their IFN γ production. These results support the view that residual low-avidity self-antigen-specific T cells remaining in the T-cell repertoire are unable to counteract tumor growth because they are abortively activated before tumor infiltration. These data have profound implications on therapies involving high-avidity T cells because although large numbers of these cells could be cultured *in vitro* following injection, their IFN γ -producing capability would be lost once they formed TIL. Moreover, for both CL1 and CL4 TILs, lack of IFN γ production could not be reversed by the addition of APC;⁴ thus, vaccine strategies may be unsuccessful unless they are coupled with a means of counteracting the suppressive influence caused by tumor cells. Removal of Treg cells would prove a major complication for therapy as these cells perform a vital function in reducing susceptibility to autoimmune diseases by suppressing peripheral autoreactive T cells. Effective cancer therapies will need to break this tumor-induced tolerance

⁴ C.N. Janicki and D.J. Morgan, unpublished data.

perhaps through mechanisms that prevent release of immunosuppressive factors by tumors, as this would help to limit the influence of Treg cells by reducing CD25⁺ conversion without severely compromising autoimmune suppression. Large numbers of high-avidity T cells may then be introduced to reduce tumor expansion.

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