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The Fate of Low Affinity Tumor-Specific CD8⁺ T Cells in Tumor-Bearing Mice

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A major challenge in tumor immunology is how best to activate the relatively low avidity self-specific and tumor-specific T cells that are available in the self-tolerant repertoire. To address this issue, we produced a TCR transgenic mouse expressing a class I-restricted hemagglutinin (HA)-specific TCR (clone 1 TCR) derived from a mouse that expressed HA as a self-Ag in the insulin-producing β cells of the pancreatic islets (InsHA) mice. Upon transfer of clone 1 TCR CD8⁺ T cells into InsHA mice, very few cells were activated by cross-presented HA, indicating that the cells were retained in InsHA mice because they ignored the presence of Ag, and not because they were functionally inactivated by anergy or tuning. Upon transfer into recipient mice in which HA is expressed at high concentrations as a tumor-associated Ag in spontaneously arising insulinomas (RIP-Tag2-HA mice), a high proportion of clone 1 cells were activated when they encountered cross-presented tumor Ag in the pancreatic lymph nodes. However, the activated cells exhibited very weak effector function and were soon tolerized. The few activated cells that did migrate to the tumor were unable to delay tumor progression. However, when HA-specific CD4 helper cells were cotransferred with clone 1 cells into RIP-Tag2-HA recipients and the mice were vaccinated with influenza, clone 1 cells were found to exert a significant value to learn how best to optimize the effector capabilities of low avidity tumor-specific T cells.

The net effect of central and peripheral tolerance mechanisms is the deletion or functional inactivation of T cells with low avidity for self-Ags (1, 2). Thus, only T cells with low avidity for self remain functional in the tolerant T cell repertoire (3–6). Although this is an important safeguard that helps prevent autoimmunity, it also has implications for antitumor immunity because many tumor Ags are also expressed as self-Ags on normal tissue (7, 8). CD8⁺ T cells expressing TCRs with high avidity for tumor Ags are most effective in tumor elimination (9–13). However, because high avidity T cells specific for tumor Ags are often deleted or inactivated through central or peripheral tolerance mechanisms, either before or soon after tumor growth begins (14–17), it has proven difficult to obtain such high avidity T cells from a tumor-bearing host. It would therefore be of great value to learn how best to optimize the effector capabilities of low avidity tumor-specific T cells, because these will be the T cells available in the tolerant repertoire to deal with growing tumors. The goal of this study was to develop a tumor model that could be used to optimize conditions for tumor eradication by low avidity tumor-specific T cells.

Previous work from our laboratory demonstrated that transgenic mice expressing influenza hemagglutinin (HA)⁵ as a self-Ag in the insulin-producing β cells of the pancreatic islets (InsHA mice) possess autoreactive HA-specific CD8⁺ T cells that could be expanded upon infection with influenza virus (4, 18). However, the CD8⁺ T cells from InsHA mice have a lower functional avidity for HA than do CD8⁺ T cells from conventional B10.D2 mice that do not express the HA transgene. Low avidity HA-specific CD8⁺ T cells obtained from HA-tolerant InsHA mice differed both quantitatively and qualitatively from the higher avidity T cells from nontolerant mice. In vitro, they required higher concentrations of Ag to achieve target cell lysis and to produce effector cytokines. Yet even at high Ag concentrations, the response to HA resulted in only partial phosphorylation of CD3ζ and poor phosphorylation of ZAP70, hallmarks of stimulation by a partial agonist or antagonist, rather than a strong agonist (19). Upon transfer into InsHA hosts, even large numbers of low avidity T cells were unable to promote tissue destruction (4). It was further suggested by tetramer binding assays that a major difference between CD8⁺ T cells isolated from tolerant InsHA mice and those from nontolerant B10.D2 mice was the expression of T cell receptors with lower affinity for HA in the tolerant mice. In this study we report the generation of a TCR transgenic mouse that expresses a TCR obtained from a low avidity HA-specific CD8⁺ T cell clone (clone 1) that was originally expanded from the tolerant repertoire of an InsHA mouse. We show that low avidity clone 1 T cells tend to ignore the presence of HA self-Ag that is cross-presented in the pancreatic lymph nodes of InsHA mice. However, when transferred into recipient mice that spontaneously develop pancreatic β cells tumors expressing HA as a tumor-associated Ag (RIP-Tag2-HA mice), thus increasing the amount of cross-presented HA, the clone 1 cells become responsive. Although such activation by tumor Ag leads to tolerance, we describe stimulatory conditions that can override

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⁵ Abbreviation used in this paper: HA, hemagglutinin.
tolerance and result in significant tumor destruction by these low avidity T cells.

Materials and Methods

Generation of clone 1 TCR transgenic mice

B10.D2 clone 1 TCR mice express a TCR specific for the influenza virus HA (K^b- HA) restricted by MHC class I H-2K^d. Functional characterization of the CTL clone (clone 1) and molecular typing of the clone 1 TCR were previously described (4). The α-chain (Vα1/βα11) and the β-chain (Vβ8/2/DBJβ2.4) sequences were amplified from genomic DNA and subsequently cloned into the TCR cassette expression vectors pCRII and pβcas, originally described by Kouskoff et al. (20). These vectors harbor the genomic promoter and enhancer regions for the respective α and β TCR loci. For the TCR-α construct, the 5′ primer TCCCCCGGG GAGGAGA-GGAGGAGAATGAAATTCCTTATTCCTTA was constructed to harbor an NcoI site 17 bp before the ATG leader translation initiation codon. The 3′ primer TGGTGATCGCGCGGCG CGCGTCGTTCTCATGGGCACGTT was constructed to harbor a NotI site 21 bp downstream from the Jα111 encoding region within the intron. For the TCR-β construct, the 5′ primer GAACTGCTCGAGTCGAC GATGGCTCTACGGCTTTC was constructed to harbor an XhoI site 8 bp upstream from the Vβ8.2 leader translation initiation codon. The 3′ primer CCAGAACCCACCGCGGAAACTATACCCCAGCT was constructed to harbor a NcoI site 20 bp downstream from the Jβ2.4 encoding region within the intron. Plasmid sequences were verified by PCR DNA sequencing and restriction enzyme digestion. Constructs were then purified and submitted for injection directly onto the B10.D2 background, as conducted by The Scripps Research Institute Transgenic core facility. Five founder pups were identified by PCR detection using the primers just described. Founders were set up for breeding with transgene-negative B10.D2 mice, and offspring were analyzed for transgenic TCR (Vβ8.2) expression by flow cytometry. One founder line was chosen for expansion and further characterization.

Mice

InsHA transgenic mice on the B10.D2 background have been previously described (21). Clone 4 TCR (22), RIP-Tag2 (23), and SFE TCR (24) mice were each backcrossed with B10.D2 mice for greater than eight generations. Clone 4 TCR mice express a TCR specific for the HA (K^b- HA) restricted by MHC class I H-2K^d, SFE TCR mouse expresses a TCR specific for the HA111-119 epitope restricted by MHC class II I-E^d. The TCR expressed by clone 4 TCR transgenic mice came from the repertoire of InsHA mice infected with the A/PR/8/32 strain of influenza virus (PR8). Clone 4 TCR transgenic mice were generated by crossing B10.M mice with clone 1 TCR transgenic C57BL/6 mice. The TCR expressed by clone 4 TCR transgenic mouse (Fig. 1A). Preparation and adoptive transfer of naive TCR transgenic T cells

CD8^+ T cells were isolated from the lymph nodes of clone 1 or clone 4 TCR mice (6–8 wk of age) by negative selection using the MACS CD8^+ T cell isolation kit (Miltenyi Biotec). T cell purity was >85% with no contaminating CD4^+ cells. SFE transgenic CD4^+ T cells were similarly isolated with the MACS CD4^+ T cell isolation kit (Miltenyi Biotec) with >95% purity and no contaminating CD8^+ cells. Where indicated, purified clone 1 or clone 4 CD8^+ T cells were labeled with CFSE (Molecular Probes). Briefly, cells were incubated in 5 μM CFSE in HBSS for 10 min at 37°C and then washed in cold HBSS before adoptive transfer. For adoptive transfer experiments, the indicated number of cells was injected through the tail vein in a volume of 200 μl of HBSS.

Intracellular cytokine staining

To assess the ex vivo production of IFN-γ in response to Ag, pancreatic lymph nodes were dissociated and cells were incubated in complete media with 1 μg/ml of the K^b- HA peptide (1Y5T5VSSL) and 1 μl/ml GolgiPlug solution (BD Pharmingen) for 5 h at 37°C. Cells were then incubated in 100 μl of 2.4G2 hybridoma supernatant (American Type Culture Collection) for 10 min at 4°C to block FcRs. PerCP-conjugated anti-CD8 and PE-conjugated anti-CD4 were then added, and cells were incubated for an additional 30 min at 4°C. After two washes, intracellular IFN-γ staining was performed according to the manufacturer’s instructions using the Cytofix/Cytoperm Plus kit (BD Pharmingen) and allophycocyanin-conjugated rat anti-mouse IFN-γ. Cells were analyzed on a BD Biosciences FACSort flow cytometer, and data were analyzed using CellQuest software. All Abs for flow cytometry were purchased from BD Pharmingen.

Assessment of tumor growth/eradication by blood glucose monitoring

Mice were assessed for tumor growth (hypoglycemia) or tumor destruction (hyperglycemia) by weekly monitoring of blood glucose. Mice were considered hypoglycemic when blood glucose levels were below 70 mg/dl, and were euthanized after consecutive blood glucose readings below 40 mg/dl.

Proliferation assay

Equivalent numbers of CD8^+ spleen cells from either clone 1 or clone 4 mice were cocultured with irradiated APC pulsed with titrated amounts of HA peptide for 72 h before pulsing with 1 μCi/well of [3H]Thy111 for 8 h. Plates were then frozen and thawed before proliferation analysis.

Tetramer binding assay

For tetramer binding studies, 1 × 10^5 lymph node cells from naïve clone 1 TCR or naïve clone 4 TCR transgenic mice were incubated at room temperature for 45 min with FITC-conjugated anti-CD8 (BD Pharmingen) and the indicated dilutions of PE-conjugated K^b- HA tetramer. Samples were then washed twice and immediately analyzed. K^b- HA tetramers were produced as previously described (4).

Histology

Pancreatic tissue was embedded and frozen in Tissue-Tek OCT Compound (Sakura Finetek) and samples were stored at −70°C. Sections (5 μm) were cut and allowed to dry at room temperature. Sections were then fixed in cold 1% paraformaldehyde for 10 min and washed with PBS. Blocking was performed with the avidin-biotin blocking kit (Vector Laboratories) according to the manufacturer’s protocol. Sections were then incubated at room temperature for 90 min in purified primary Ab to mouse CD8 (clone 53.6.7, BD Biosciences) or CD4 (clone RM4-5, BD Biosciences) at a concentration of 1 μg/ml. After washing in PBS, biotin-SP-Affinipure F(ab’)_2 mouse anti- rat IgG secondary Ab (Jackson ImmunoResearch Laboratories) was added at a 1/500 dilution for 1 h at room temperature. After washing in PBS, sections were incubated in a 1/500 dilution peroxidase-conjugated streptavidin (Jackson ImmunoResearch Laboratories) for 45 min, and then developed with 3-amino-9-ethylcarbazole (A.E.C.) Peroxidase Substrate kit (Vector Laboratories). Sections were counterstained with hematoxylin.

Statistics

To determine whether differences in survival of RIP-Tag2-HA mice receiving different forms of immunotherapy were significant, p values were determined by the logrank test of Kaplan-Meier plots.

Results

Functional characteristics of CD8^+ T cells from clone 1 TCR transgenic mice

Our previous studies showed that, as a result of exposure to HA as a self-Ag expressed in the pancreatic β cells, CD8^+ T cells obtained from the repertoire of InsHA mice infected with the A/PR/8/32 strain of influenza virus (PR8) are of lower avidity than CD8^+ T cells isolated from similarly infected B10.D2 mice (4). The HA-specific TCR from one of these low avidity clones, clone 1, was used to produce clone 1 TCR transgenic mice, as described in Materials and Methods. Expression of CD4 and CD8 by thymocytes and lymph node cells in clone 1 mice showed skewing toward the CD8^+ T cell compartment, as expected for a CD8-derived TCR transgenic mouse (Fig. 1A).

The TCR expressed by clone 4 TCR transgenic mice came from a PR8 immunized B10.D2 mouse (22), and exhibits relatively high avidity for the same HA peptide epitope (HA 518–526) seen by the clone 1 TCR. A variety of assays were used to compare CD8^+ T cells from these two different transgenic lines for their ability to respond to HA Ag. First, to compare the avidity of naïve CD8^+ T cells, clone 1 and clone 4 cells were isolated from the spleens of naïve TCR transgenic mice and stimulated in vitro with titrated amounts of the HA peptide. As shown in the titration curve in Fig.

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FIGURE 1. Clone 1 TCR transgenic T cell have low avidity for HA. A, Thybus and lymph node cells from clone 1 TCR transgenic mice were analyzed for surface expression of CD4 and CD8. B, Avidity comparison of naive clone 1 and clone 4 T cells. Equivalent numbers of naive V$$\beta$$8/CD8+ spleen cells from either clone 1 or clone 4 mice were assessed for proliferation in response to titrated amounts of HA peptide. C, K$$^d$$-HA tetramer binding by naive clones 1 and 4 T cells. One million lymph node cells from naive clone 1 or clone 4 TCR transgenic mice were incubated with the indicated dilutions of PE-conjugated K$$^d$$-HA tetramer and were analyzed by flow cytometry. Y-axis values represent the mean fluorescence intensity (MFI) for each dilution. D, Functional comparison of effector clone 1 and clone 4 T cells. A total of 2 x 10$$^6$$ CFSE-labeled Thy1.1+ clone 1 or clone 4 cells were adoptively transferred into B10.D2 host mice followed by immediate i.p. immunization with PR8. Three days later, transferred cells were assessed for IFN-$$\gamma$$ production in response to varying concentrations of HA peptide. Only activated Thy1.1+ CD8+ cells that had diluted CFSE were included in the analysis. Data are represented as the percentage of maximal stimulation at each peptide dilution. E, CD8+ T cells were isolated from clone 1 or clone 4 TCR transgenic mice, and equal numbers of cells were transferred into InsHA+/+ recipients at the indicated cell numbers. Recipient mice were immediately immunized with PR8 and monitored weekly for the development of diabetes. Mice were considered diabetic when blood glucose levels reached 300 mg/dl for two consecutive weeks. The data shown represent the percentage of animals (eight animals per group) that developed diabetes after immunization for each group.

1B, T cells from clone 1 mice required 10- to 100-fold more peptide to induce a level of proliferation comparable to that of clone 4 T cells. In addition, when naive CD8+ T cells from clone 1 and clone 4 mice were compared with respect to their ability to bind tetramers of K$$^d$$ containing the HA peptide, we observed weaker binding by the clone 1 CD8+ cells (Fig. 1C). When optimal tetramer concentrations were used, 87-90% of both clone 1 and clone 4 CD8+ T cells were able to bind tetramer (data not shown), indicating that similar frequencies of the CD8+ cells from the different TCR transgenic lines were specific for HA. Thus, any functional differences observed between the T cells from these two lines are not due to differences in the frequency of HA-specific T cells. Also, the decreased responsiveness to HA and decreased K$$^d$$-HA tetramer binding by clone 1 T cells were not due to lower levels of CD8 or TCR expression because both clone 1 and clone 4 T cells expressed comparable levels of both molecules (data not shown).

To compare the functional capabilities of clone 1 and clone 4 T cells in vivo, equal numbers (2 x 10$$^6$$) of CFSE-labeled Thy1.1+ clone 1 or clone 4 cells were adoptively transferred into B10.D2 host mice and activated in vivo by immunization with PR8. Three days later, mice were sacrificed and CD8+ T cells from clone 1 and clone 4 mice were estimated by flow cytometry. When optimal tetramer concentrations were used, 87-90% of both clone 1 and clone 4 CD8+ T cells were able to bind tetramer (data not shown), indicating that similar frequencies of the CD8+ cells from the different TCR transgenic lines were specific for HA. Thus, any functional differences observed between the T cells from these two lines are not due to differences in the frequency of HA-specific T cells. Also, the decreased responsiveness to HA and decreased K$$^d$$-HA tetramer binding by clone 1 T cells were not due to lower levels of CD8 or TCR expression because both clone 1 and clone 4 T cells expressed comparable levels of both molecules (data not shown).

Avidity comparison of naive clone 1 and clone 4 T cells. Equivalent numbers of naive V$$\beta$$8/CD8+ spleen cells from either clone 1 or clone 4 mice were assessed for proliferation in response to titrated amounts of HA peptide. Only activated Thy1.1+ CD8+ cells that had diluted CFSE were included in the analysis. Data are represented as the percentage of maximal stimulation at each peptide dilution. E, CD8+ T cells were isolated from clone 1 or clone 4 TCR transgenic mice, and equal numbers of cells were transferred into InsHA+/+ recipients at the indicated cell numbers. Recipient mice were immediately immunized with PR8 and monitored weekly for the development of diabetes. Mice were considered diabetic when blood glucose levels reached 300 mg/dl for two consecutive weeks. The data shown represent the percentage of animals (eight animals per group) that developed diabetes after immunization for each group.

In vivo response by clone 1 T cells to cross-presented Ag in InsHA mice

In previous studies we demonstrated that, upon transfer into InsHA mice, clone 4 CD8+ T cells undergo an abortive form of activation and are deleted (25, 26). We proposed that the reason we could obtain low avidity CD8+ T cells, such as clone 1, from immunized InsHA mice was because low avidity T cells were not activated by the relatively small amount of HA Ag that is cross-presented in the draining pancreatic lymph nodes. That is, they remained ignorant of the presence of HA (27, 28). To test this hypothesis, we adoptively transferred CFSE-labeled transgenic clone 4 and clone 1 T cells into InsHA mice that were either homozygous (InsHA$$^+/+$$) or heterozygous (InsHA$$^+/−$$) for the HA transgene to compare their activation in the pancreatic draining lymph nodes, the site where the cross-presented HA Ag is first encountered (Fig. 1D). It should be noted that the clone 1 TCR was originally obtained from an InsHA$$^+/−$$ mouse that was immunized with influenza. After 4 days in the InsHA$$^+/−$$ host, a smaller percentage of the transferred clone 1 cells proliferated as compared with clone 4 (compare Fig. 2, A
lymph nodes were harvested from each group (two to three mice per group)
cells (3/H11003/percentage of Thy1.1
are shown, and values shown represent the mean per-
cells were transferred into 10-wk-old InsHA
Ag is expressed in the islets and cross-presented in the draining
One representative plot is shown for each group.

FIGURE 3. Low avidity tumor-specific clone 1 T cells are tolerized in response to tumors expressing HA
We previously reported that clone 4 CD8+ T cells are effectively tolerated in tumor-bearing mice (17). However, it is possible that a certain threshold may exist for tolerance induction, and low avidity T cells such as clone 1 may not receive a sufficient signal to program their deletion or inactivation. To address this issue, we transferred Thy1.1+ clone 1 CD8+ T cells into RIP-Tag2-HA recipients (heterozygous for the HA transgene) that spontaneously developed pancreatic β cell tumors (23). As shown in Fig. 2, expression of HA as a tumor-associated Ag significantly enhanced the activation of clone 1 T cells in the pancreatic draining lymph nodes (compare Fig. 2, F with D). Thus, tumor growth led to the activation of these low avidity HA-specific CD8+ T cells that responded poorly to the HA self-Ag in the absence of tumor.

Low avidity clone 1 T cells are tolerated in response to tumors expressing HA

Although clone 1 cells remained largely ignorant of HA in InsHA+/− mice, it was anticipated that presence of an HA-expressing tumor would increase the amount of HA available for cross-presentation, and would result in a sufficient level of Ag to activate these cells. Indeed we recently reported that high avidity clone 4 CD8+ T cells proliferate significantly more in response to HA when it is expressed as a tumor-associated Ag than when it is expressed as a normal self-Ag (Fig. 2C) (17). To determine whether the expression of HA as a tumor-associated Ag would also lead to enhanced activation of low avidity clone 1 T cells, transgenic clone 1 CD8+ cells were transferred into RIP-Tag2-HA recipients (heterozygous for the HA transgene) that spontaneously developed pancreatic β cell tumors (23). As shown in Fig. 2, expression of HA as a tumor-associated Ag significantly enhanced the activation of clone 1 T cells in the pancreatic draining lymph nodes (compare Fig. 2, F with D). Thus, tumor growth led to the activation of these low avidity HA-specific CD8+ T cells that responded poorly to the HA self-Ag in the absence of tumor.

Activation of clone 1 T cells in RIP-Tag2-HA recipients

We previously reported that clone 4 CD8+ T cells are effectively tolerated in tumor-bearing mice (17). However, it is possible that a certain threshold may exist for tolerance induction, and low avidity T cells such as clone 1 may not receive a sufficient signal to program their deletion or inactivation. To address this issue, we transferred Thy1.1+ clone 1 CD8+ T cells into RIP-Tag2-HA recipients. At various time points posttransfer, the recipient mice were immunized with PR8 virus to expand any nontolerized Thy1.1+ clone 1 cells. As expected, clone 1 T cells were not tolerated in B10.D2 recipient mice that do not express the HA transgene, as evidenced by the fact that similar numbers of clone 1 cells were detected at the different time points following immunization (Fig. 3). Tolerance was also incomplete in InsHA+/− hosts, as many clone 1 cells could still be expanded by PR8 immunization even as late as 7 wk posttransfer. This result was consistent with

`# FIGURE 2. Comparison of clones 1 and 4 activation in response to cross-presented HA in InsHA and tumor-bearing RIP-Tag2-HA recipients. Two million CFSE-labeled Thy1.1 CD8+ clone 4 (A–C) or clone 1 (D–F) cells were transferred into 10-wk-old InsHA+/− (A and D), InsHA+/− (C and E), or RIP-Tag2-HA+/− (C and F) recipients. After 72 h, pancreatic lymph nodes were harvested from each group (two to three mice per group) and Thy1.1+CD8+ cells were assessed for proliferation as demonstrated by the dilution of CFSE. The background proliferation (M1) for clones 1 and 4 proliferation were observed for clone 4 and clone 1 cells (Fig. 2, B and E). These data suggest that, as compared with clone 4 cells, a greater number of the lower avidity clone 1 cells remain ignorant of cross-presented HA in InsHA+/− mice. However, when more Ag is expressed in the islets and cross-presented in the draining

# FIGURE 3. Low avidity tumor-specific clone 1 T cells are tolerized RIP-Tag2-HA mice. Thy1.1+ clone 1 cells (3 × 10⁶) were transferred into B10.D2, InsHA+/− or RIP-Tag2-HA+/− mice (8–9 wk of age), and recipients were infected i.p. with PR8 at various time points posttransfer to expand any remaining nontolerized clone 1 cells. Recipient mice were then analyzed for the presence of Thy1.1+ clone 1 cells in the spleen 6 days after PR8 infection. Only CD8+ events are shown, and values shown represent the mean percentage of Thy1.1+ cells (M1) among total CD8+ cells for each group (three to four mice per group). The background mean percentage (M1) value for mice receiving no Thy1.1+ cells was 0.08 ± 0.02% (data not shown). One representative plot is shown for each group.

- **Recipient**
  - B10.D2
  - InsHA+/−
  - RIP-Tag2-HA+/−

- **Time Points**
  - 2 wks
  - 4 wks
  - 7 wks

- **Events**
  - 3.0 × 10⁶ Thy1.1+ Clone 1 T cells
  - 2, 4, 7 wks
  - PR8 infection
  - FACs to detect Thy1.1+ Clone 1 cells

- **Results**
  - 2 wks: 1.9 ± 0.6
  - 4 wks: 1.7 ± 0.7
  - 7 wks: 1.4 ± 0.5

- **Comments**
  - Tolerance was also incomplete in InsHA+/− hosts, as many clone 1 cells could still be expanded by PR8 immunization even as late as 7 wk posttransfer. This result was consistent with…`
FIGURE 4. Development of effector function by clone 1 T cells in tumor-bearing mice in the presence and absence of CD4 T cell help. One million CFSE-labeled Thy1.1+ clone 1 CD8+ T cells were transferred into RIP-Tag2-HA recipients (11 wk of age) in the presence (top) or absence (middle) of 1 x 10^6 SFE CD4+ Th cells. Four days later, pancreatic lymph nodes were harvested and stimulated ex vivo for 5 h with HA peptide. IFN-γ production was assessed by intracellular cytokine staining. As a positive control for IFN-γ production, 1 x 10^6 clone 1 T cells were transferred into B10.D2 mice, and mice were immediately immunized i.p. with PR8 virus (bottom). Three days later, transferred cells were analyzed for IFN-γ production. Only Thy1.1+CD8+ events are shown.

FIGURE 5. Certain in vivo stimulatory conditions lead to transient antitumor immunity by clone 1 T cells. Two million clone 1 T cells were transferred into 11-wk-old tumor-bearing RIP-Tag2-HA recipients in the presence of 1 x 10^6 SFE CD4+ helper cells and/or PR8 immunization as indicated. In mice that received only SFE cells with PR8 immunization, 2 x 10^6 SFE cells were transferred. After T cell transfer, mice were assessed for tumor growth (hypoglycemia) or tumor destruction (hyperglycemia) by measuring blood glucose levels at weekly intervals.

the fact that clone 1 T cells remain largely ignorant of cross-presented HA in these hosts (Fig. 2D). In contrast, clone 1 cells were effectively tolerized in tumor-bearing RIP-Tag2-HA recipients (Fig. 3). By 4 wk posttransfer, significantly fewer Thy1.1+ clone 1 cells were detectable after PR8 infection, and almost no clone 1 cells were detectable above background levels by 7 wk posttransfer. Thus, despite their low avidity for the tumor Ag, clone 1 T cells were still efficiently tolerized by the growing tumors. It should be noted, however, that it took longer to tolerize equivalent numbers of low avidity clone 1 tumor-specific T cells (~7 wk) than it did to tolerize the higher avidity clone 4 T cells (<4 wk) (Ref. 17 and data not shown). This is consistent with the relatively reduced rate of activation of clone 1 as compared with clone 4, even in the RIP-Tag2-HA recipient (Fig. 2, C and F).
Low avidity clone 1 T cells can be "helped" to develop into effector CTL

We observed previously that during tolerance induction in either InsHA or RIP-Tag2-HA recipients, clone 4 CD8⁺ T cells activated in response to cross-presented HA Ag did not develop effector functions such as IFN-γ production or cytolytic activity (26, 29). However, the cotransfer of HA-specific CD4⁺ Th cells along with the clone 4 CD8⁺ T cells led to the differentiation of clone 4 into potent effector CTL capable of IFN-γ production and, more importantly, tumor eradication in RIP-Tag2-HA mice (17). Therefore we assessed whether CD4⁺ T cell help could also promote the differentiation of low avidity clone 1 CD8⁺ T cells into effector CTL. One million clone 1 CD8⁺ cells were transferred into tumor-bearing RIP-Tag2-HA recipient mice in the presence or absence of 1 × 10⁶ SFE TCR transgenic HA-specific CD4⁺ helper cells. Four days later, tumor draining pancreatic lymph nodes were harvested to determine whether clone 1 cells that were activated in the presence of CD4 help had developed into effector CTL capable of producing IFN-γ (Fig. 4). Transfer of clone 1 CD8⁺ T cells in the absence of CD4 help led to the proliferation of clone 1 cells without development of effector function (<1% IFN-γ⁺). The provision of help in the form of SFE CD4⁺ Th cells led to a significant increase in the number of activated clone 1 T cells that were capable of producing effector cytokines (23.9% IFN-γ⁺). However, this increase in effector function by the CD8⁺ clone 1 T cells was not as dramatic as we had previously observed for high avidity clone 4 T cells (17). The relative decrease in cytokine production by helped clone 1 as compared with helped clone 4 was not due to an overall inability of the clone 1 T cells to respond to the HA Ag because immunization with PR8 led to a very potent clone 1 effector response (Fig. 4, bottom panels).

Tumor destruction by clone 1 CD8⁺ T cells

RIP-Tag2-HA mice spontaneously develop β cell tumors over time, and exhibit a drop in blood sugar (hypoglycemia) due to the increased amount of insulin that is produced as the tumors grow larger (23). Untreated RIP-Tag2-HA animals develop tumors starting at ~6 wk of age and become hypoglycemic at 10–13 wk of age (17). To assess the ability of clone 1 cells to delay tumor progression, clone 1 T cells were transferred into RIP-Tag2-HA recipients at ~10 wk of age. Transfer of clone 1 cells, either with (Fig. 5B) or without (data not shown) cotransfer of SFE CD4⁺ Th cells, did

**FIGURE 6.** Infiltration of RIP-Tag2-HA tumors after clone 1 transfer. Pancreata were harvested from RIP-Tag2-HA mice (10–11 wk of age) that received no T cells (A and B), 3 × 10⁶ clone 1 cells (C and D), 2 × 10⁶ clone 1 cells + 1 × 10⁶ SFE cells (E and F), 2 × 10⁶ clone 1 cells + PR8 (G and H), or 2 × 10⁶ clone 1 cells + 1 × 10⁶ SFE cells + PR8 (I and J). Tissues were taken at day 6 after adoptive transfer. Frozen pancreatic sections were stained with Abs against CD8 (A, C, E, G, and I) or CD4 (B, D, F, H, and J).
not prevent tumor growth, and mice became hypoglycemic with similar kinetics to untreated RIP-Tag2-HA mice. Histologic examination of the islets revealed a low level of lymphocyte infiltration of the tumors by both CD8$^+$ and CD4$^+$ cells when clone 1 cells were transferred alone (Fig. 6, C and D), or when mice received both clone 1 and SFE (Fig. 6, E and F). Such infiltration was dependent upon T cell transfer, as no infiltrates were observed in tumor-bearing mice that did not receive adoptively transferred cells (Fig. 6, A and B).

As the tumor alone was inadequate to activate T cells for tumor destruction, we next assessed the ability of influenza virus (PR8) to induce antitumor activity. When the transfer of clone 1 cells was followed by immediate vaccination of the mice with PR8 (Fig. 5C), a transient antitumor response was observed in three of nine animals, as demonstrated by a brief period of hyperglycemia that accompanied a loss of β cell mass and reduced insulin production. As expected, PR8 vaccination led to a significant increase in the expansion of the transferred clone 1 cells in RIP-Tag2-HA recipients as compared with uninfected clone 1 recipients (Fig. 7). In addition, increased lymphocyte infiltration of tumors by CD8$^+$ T cells was clearly evident in the PR8-infected mice receiving clone 1 cells (Fig. 6, G and H). However, all mice eventually became hypoglycemic due to tumor outgrowth, and the mean survival time of these mice (61 ± 18 days posttransfer) was not significantly different from that of untreated RIP-Tag2-HA (57 ± 13 days).

We next assessed the ability of PR8 vaccination to initiate tumor destruction when both clone 1 CD8$^+$ cells and SFE CD4$^+$ cells were cotransferred into the tumor-bearing recipients. Under these conditions, significantly greater tumor infiltration by both CD8$^+$ and CD4$^+$ cells was observed (Fig. 6, I and J). This correlated with an increased expansion of clone 1 cells because roughly 2- to 3-fold more clone 1 CD8$^+$ T cells were recovered from the spleen and lymph nodes of PR8-infected mice that received SFE cells (Fig. 7). More importantly, significant antitumor activity was observed, as all mice became diabetic by 2 wk posttransfer and remained hyperglycemic or euglycemic for an average of 4–5 wk longer than control animals (Fig. 5D). In addition, mice survived an average of 18 days longer than untreated control RIP-Tag2-HA mice (75 ± 15 days, p = 0.0265). This antitumor activity was not due solely to the CD4$^+$ Th cell response because transfer of SFE CD4$^+$ T cells followed by PR8 vaccination (in the absence of clone 1 CD8$^+$ cells) had no effect on tumor progression (Fig. 5E). Despite the antitumor activity promoted by the presence of both CD4 help and PR8 vaccination, clone 1 CD8$^+$ T cells were ultimately unable to fully eradicate tumors because all recipient mice eventually became hypoglycemic and succumbed to tumor growth.

**Discussion**

It has been frequently reported that high avidity T cells are distinctively superior to low avidity cells in antiviral and antitumor immunity (9–13, 30, 31). Most studies that have examined the consequence of interaction between TCR transgenic T cells and tumor have used cells expressing TCRs with high affinity for a tumor Ag. Although such high avidity T cells would be most desirable for immunotherapy, self-tolerance may preclude their availability. Clone 1 TCR transgenic mice were produced to study the fate of low avidity self-specific and tumor-specific CD8$^+$ T cells, the type of cells most likely to be available within the normal T cell repertoire.

The TCR expressed by T cells from clone 1 transgenic mice was obtained from the repertoire of an InsHA$^{-/-}$ mouse that was tolerant to the HA self-Ag. We showed previously, using the original cloned T cell line from which the clone 1 TCR was derived, that cells expressing this TCR were of low avidity. However, we could not be sure this reflected the presence of a low affinity TCR. The expression of HA as a self-Ag may have been instrumental in anerogizing or “tuning” the cells to deliver a weaker signal (32–34), such that they were less responsive to Ag. An important new piece of information obtained in the current study was that when clone 1 TCR transgenic CD8$^+$ T cells developed in an environment in which HA was not expressed, they exhibited low avidity for HA and were poorly activated in InsHA$^{+/-}$ recipients (Fig. 2). This supports the hypothesis that clone 1 cells were retained within the T cell repertoire of InsHA mice because their TCR was of sufficiently low affinity that they ignored the HA Ag, and not because they were anergized or tuned. As we have only examined a single example of an HA-specific transgenic TCR obtained from InsHA mice, we cannot rule out the possibility that there also exist within the InsHA repertoire other HA-specific T cells that may have been anergized or tuned. However, we have found that clone 4 T cells, which are tolerized after transfer to InsHA mice, undergo peripheral deletion rather than anergy.

We next analyzed the fate of clone 1 cells as they encountered the HA Ag cross-presented at high concentrations in tumor-bearing RIP-Tag2-HA mice. As seen in Figs. 2 and 4, the clone 1 cells underwent vigorous proliferation in the draining pancreatic lymph nodes of RIP-Tag2-HA recipients. This suggests that the increased availability of Ag that accompanies tumor progression promotes the activation of low avidity self-specific and tumor-specific T cells that had been previously retained within the repertoire. Indeed, even a 2-fold increase in Ag availability, as experienced upon transfer of clone 1 cells into InsHA$^{-/-}$ mice homozygous for the HA transgene, was sufficient to result in a significant increase in activation (Fig. 2E).

As shown in a number of different studies, activation by self-Ags can result in tolerance. We and others have shown that high avidity T cells are effectively tolerized in both the CD4 and CD8 compartments during tumor progression (15–17, 35). However, we thought it possible that there may exist an avidity threshold below which cells may be activated, but spared from tolerance due to the fact that they do not receive a strong enough signal through their TCR to undergo programmed cell death or anergy. This could explain why some tumor-specific T cells are present in the tolerant repertoire and can be stimulated to exert antitumor activity upon

**FIGURE 7.** Clone 1 T cell expansion under different stimulatory conditions. Thy1.1$^+$ clone 1 CD8$^+$ T cells (1.5 × 10$^6$) were transferred into RIP-Tag2-HA recipient mice (11 wk of age) in the presence or absence of SFE CD4$^+$ helper cells (1 × 10$^6$) and/or PR8 vaccination as indicated. Spleens and lymph nodes (axillary and inguinal) were harvested 6 days posttransfer, and the number of Thy1.1$^+$ CD8$^+$ clone 1 T cells was assessed for each group (two mice pooled per group) by flow cytometry. Data shown represent the total number of Thy1.1$^+$ CD8$^+$ cells per animal for the indicated lymphoid organ.
vaccination with tumor Ags in an immunostimulatory setting (18, 36–38). This has obvious implications for the potential of T cell immunotherapy as an effective treatment for cancer. To determine whether tumor growth would lead to the induction of tolerance in low avidity clone 1 T cells, the T cells were transferred into tumor-bearing RIP-Tag2-HA mice, and tolerance was assessed over time by immunizing with influenza virus to expand any remaining responsive clone 1 cells. We found that the number of responsive clone 1 cells waned over time in RIP-Tag2-HA recipients, but persisted in mice that did not express HA (B10.D2) or expressed HA at low levels (InsHA^{+/+}). Thus, the increased Ag concentration due to tumor growth led to effective tolerance of low avidity T cells that would otherwise ignore the HA self-Ag. We have not yet determined the underlying mechanisms responsible for tolerance of clone 1 cells in RIP-Tag2-HA recipients, which may be either deletion or anergy. Importantly, tolerance prevents responsiveness to influenza virus, a very potent activating stimulus.

These results do not necessarily argue that all potentially tumor-reactive T cells are deleted or inactivated before, or during, tumor growth. It is interesting to note that it took longer for the lower avidity clone 1 CD8{sup} T cells to become tolerated in RIP-Tag2-HA recipients than it did for the higher avidity clone 4 cells (17). Thus, despite their eventual tolerance, low avidity clone 1 cells persist longer than higher avidity clone 4 cells in the available tumor-specific T cell repertoire. Therefore, it is possible that T cells that possess relatively low avidity for self-Ag and tumor Ag would be available for activation by immunotherapy at the early stages of tumor growth. However, it may be inevitable that, in the absence of inflammation or intentional immunization, as the tumor progresses, increasing numbers of low avidity T cells would become activated and tolerated, thereby further decreasing the avidity of the repertoire available for recognition of tumor Ags. It has been suggested that immune surveillance of cancer may lead to the “immunoediting” of tumors over time (39). Our data suggest that, in parallel, progressive T cell tolerance due to tumor growth “edits” the T cell repertoire that is available for tumor recognition and immunotherapy. A potential exception to this scenario would be any self-Ags or tumor-expressed Ags that may be poorly cross-presented or cross-presented at extremely low levels, and the repertoire may retain relatively high avidity T cells with specificities for these cryptic Ags (12, 18, 38, 40–43).

For successful immunotherapy, tumor-specific T cells must be activated in a way that promotes their differentiation into effector CTL and facilitates the trafficking of the activated cells to the site of tumor growth. We found that despite the fact that many clone 1 cells were activated in tumor-bearing mice, the low level of expression of IFN-γ exhibited by the activated cells indicated weak effector function. This is supported by the observation that despite the fact that we could detect activated clone 1 cells in transformed islets, they had no apparent effect on islet destruction or tumor progression. In our previous study, in which high avidity clone 4 T cells were used, we found that cotransfer of SFE CD4{sup} Th cells to achieve optimal survival and effector function at the tumor site. We show in this study that provision of CD4 help also enhanced the early expansion/accumulation of PR8-activated clone 1 cells in the peripheral lymphoid tissue at day 6 posttransfer. This result suggests that even when clone 1 cells are activated by a potent vaccine, CD4 help promotes additional expansion/accumulation. It has been shown recently that provision of exogenous IL-2 leads to an increase in both number and effector function of tumor-specific CD8{sup} T cells at the site of the tumor (48). It will be of interest to determine how CD4{sup} Th cells assist tumor-specific CD8{sup} cells at each stage of CD8{sup} T cell differentiation and function, and whether IL-2 production by the CD4{sup} helper cells is a critical component.

The basis for the inability of low avidity clone 1 T cells to completely eradicate tumors in our model is unclear and is the focus of our current studies. The priming of tumor-specific T cells is only the first step in establishing a productive antitumor response. Successfully activated effector T cells must then travel to the site of tumor growth and exert their effector functions in response to the amount of tumor Ag expressed by the tumor cells. It is of interest that, as compared with clone 4 cells, 3- to 10-fold more activated clone 1 effector cells were required to produce diabetes in InsHA recipients (Fig. 1). In addition, effector clone 1 CTL required more Ag for the induction of their effector function. Thus, even after optimal activation, low avidity clone 1 cells are at a disadvantage compared with higher avidity T cells such as clone 4 due to the requirement for greater Ag expression by the tumor to achieve optimal effector function. This may represent an important
obstacle to antitumor immunity for low avidity T cells. Future experiments will explore conditions that may sustain the activity of these effector cells at the tumor site. Recent reports suggest that treatments removing regulatory cells may be of value (50–52), as well as delivery of Toll receptor agonists such as CpG nucleotides, that also help to sustain immunity and assist in lymphocyte infiltration of tumors (53, 54). In addition, provision of exogenous cytokines such as IL-2 or IL-15 may also significantly enhance the in vivo antitumor activity of adoptively transferred tumor-reactive CD8+ T cells (48, 55).

In conclusion, we describe a model that should prove of great value in optimizing the function of low avidity tumor-specific T cells. Regardless of which step is found to be rate limiting in tumor eradication by clone 1, this model will assist in identifying therapies that perpetuate survival and effector function by these low avidity T cells to make them more successful in an immunotherapy setting.

Disclosure
The authors have no financial conflict of interest.

References


