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A Spontaneously Arising Pancreatic Tumor Does Not Promote the Differentiation of Naive CD8+ T Lymphocytes into Effector CTL

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In this report, we address whether a growing tumor provides sufficient inflammatory signals to promote activation, clonal expansion, and acquisition of effector functions by naive tumor-specific CD8+ T lymphocytes. CD8+ T lymphocytes obtained from hemagglutinin (HA)-specific clone 4 TCR-transgenic mice were injected into recipient mice that spontaneously develop pancreatic tumors expressing HA as a tumor-associated Ag (RIP-Tag2-HA mice). When 3 x 10^6 clone 4 CD8+ T cells were transferred into tumor-bearing mice, the cells became activated in the pancreatic lymph nodes where they proliferated and acquired effector functions such as cytolytic activity and IFN-γ production. Surprisingly, reducing the number of adoptively transferred CD8+ T cells led to a parallel reduction in the proportion of the activated cells that exhibited effector functions, suggesting that CTL differentiation was induced by the large numbers of activated CD8+ T cells and not the tumor environment. Provision of tumor-specific CD4+ helper cells provides the signals required to promote both the development of CTL effector functions and increased clonal expansion, resulting in tumor eradication. Considering that only small numbers of tumor-specific CD8+ T cells would be present in a conventional T cell repertoire, these data suggest that tumor growth alone may not provide the inflammatory signals necessary to support the development of CD8+ T cell effector functions. The Journal of Immunology, 2004, 172: 6558–6567.
Materials and Methods

Mice

InSNA-transgenic mice on the B10.D2 background have been previously described (22). Clone 4 TCR (23), RIP-Tag2 (24), and SFE TCR (25) mice were each backcrossed with B10.D2 mice for at least eight generations. Clone 4 TCR mice express a TCR specific for the HA111-119 epitope restricted by MHC class I E-E. The RIP-Tag2-HA line was generated by crossing RIP-Tag2 mice with InSNA mice. All RIP-Tag2-HA mice used in these experiments were Tag2/2 and HA+/+, and all InSNA mice were HA+/+, Ly5.1/− (also referred to as CD45.1/− or Ptpre/−) animals on the C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME) and backcrossed to B10.D2 for at least four generations before mating to clone 4 TCR mice. All animals were housed at The Scripps Research Institute’s animal facility, and all procedures were performed according to the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Cell lines

The SV40-transformed H-2d cell line B10.D2 was originally obtained from Dr. B. Knowles, (University of Pennsylvania, Philadelphia, PA). B10.D2 cells were maintained in complete RPMI medium−medium 1640 (Life Technologies, Gaithersburg, MD) supplemented with 10% (v/v) heat-inactivated FCS (Gemini Biological Products, Calabasas, CA), 2 mM glutamine (Life Technologies), 5 × 10^−5 M 2-ME (Sigma−Aldrich, St. Louis, MO), and 50 mg/ml gentamicin (Gemini Bio-Products, Woodland, CA). Cells were cultured in a humidified incubator at 37°C with 5% (v/v) CO₂ and were used as targets in CTL assays.

In vitro cytotoxicity assays

Mice were infected i.p. with 1200 HA units of influenza virus A/PR8/34 H1N1 (PR8). After 3 wk, splenocytes from infected mice were restimulated with irradiated syngeneic spleenocytes (3000 rad) pulsed with the K⁺−restricted influenza nucleoprotein (NP) epitope, (ITYQRTRLV), or K⁺-HA (IYSTVASSL) peptide for 6 days in complete RPMI. Effector cells were then harvested and counted, and cytolytic activity against peptide-loaded (1 μM or 1 nM) or unloading target cells was measured in a 5-h ⁵¹Cr release assay. Target cells were prepared by incubating B10.D2 cells at 37°C with 200 μg/ml sodium ¹⁹Cr (New England Nuclear, Boston, MA) for 1 h in the presence or the absence of K⁺-NP or K⁺-HA peptide as indicated. Target cells were washed four times, resuspended in complete RPMI, and seeded into 96-well round-bottom plates at 1 × 10⁴ cells/well in 100 μl. Effector CTL were seeded into duplicate wells containing the target cells at various E:T cell ratios, making a final volume of 200 μl. Plates were incubated at 37°C in a humidified incubator with 5% (v/v) CO₂ for 5 h. Plates were centrifuged, and 100 μl of supernatant was removed from each well to assess isotope release using an ICN Isomedic beta radiation counter (WWR Scientific, San Diego, CA). Percent specific lysis was calculated with the following formula: 100 × (sample release − spontaneous release)/ (maximum release − spontaneous release).

Preparation and adoptive transfer of naive TCR-transgenic T cells

CD8+ T cells were isolated from the LNs of clone 4 TCR mice (6–8 wk of age) by negative selection using the MACS CD8+ T cell isolation kit (Miltenyi Biotec, Auburn, CA). 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 4 CD4+ T cells or SFE CD4+ T cells were labeled with CFSE (Molecular Probes, Eugene, OR). 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.

Preparation of target cells for in vivo CTL assays

In vivo CTL activity was measured as previously described (3, 26). Briefly, B10.D2 splenocytes were isolated and divided into two populations, one of which was pulsed with 1 μg/ml K⁺-HA peptide (IYSTVASSL) for 60 min at 37°C. After two washes in HBSS, HA peptide-pulsed cells were labeled with 5 μM CFSE (CFSE½pul) for 15 min at 37°C. Unpulsed cells were labeled with 0.5 μM CFSE (CFSE½un). Cells were washed and resuspended at a 1:1 ratio in HBSS at a total cell concentration of 3–5 × 10⁵ cells/ml. Two hundred microliters of the target cell suspension was injected through the tail vein. In all experiments, cells were recovered from the spleen and/or LNs 16–18 h after transfer and analyzed by FACS to detect and quantify CFSE-labeled cells.

Intracellular cytokine staining

To assess the ex vivo production of IFN-γ in response to Ag, pancreatic lymph nodes (pLNs) were dissociated and cells were incubated in complete medium with 1 μg/ml K⁺-HA peptide and 1 μM GolgiPlug solution (BD Pharmingen, San Diego, CA) for 5 h at 37°C. Cells were then incubated in 100 μl of 2.4G2 hybridoma supernatant (American Type Culture Collection, Manassas, VA) for 10 min at 4°C to block FcRs. PerCP-conjugated anti-CD8 and PE-conjugated anti-Thy-1.2 (or anti-Ly-5.1) 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/Cytoperp Plus kit (BD Pharmingen) and allophycocyanin-conjugated rat anti-mouse IFN-γ. Cells were analyzed on a BD Biosciences FACSsort 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.

Ab treatment

For transient depletion of endogenous CD4⁺ cells, mice were treated once i.p. with 100 μl of ascites fluid from GK1.5 cells (American Type Culture Collection) 5 days before adoptive transfer of clone 4 cells. Depletion of CD4⁺ cells was determined to be 100% by FACS analysis of peripheral blood at the time of adoptive transfer.

Results

RIP-Tag2-HA mice are tolerant of HA expressed as a TAA

RIP-Tag2-transgenic mice express the SV40 large T Ag (Tag) under the control of the rat insulin promoter (24) and develop progressive hypoglycemia starting at 10–12 wk of age due to uncontrolled insulin production by growing β cell tumors. These mice are tolerant to Tag, and do not mount a spontaneous immune response to the growing tumors (27). To generate mice that express HA as a tumor Ag, RIP-Tag2 mice were mated and backcrossed with InSNA mice to obtain mice that were Tag2/− and HA/− (22). Mice homozygous for the HA transgene are profoundly tolerant due to both thymic and peripheral tolerance mechanisms (28, 29). To determine whether the expression of HA as a TAA could break tolerance and impede tumor growth, blood glucose was monitored weekly in RIP-Tag2-HA animals to assess tumor progression. Although control InSNA mice maintained normal glucose levels over time, RIP-Tag2-HA mice consistently became hypoglycemic between 10 and 12 wk of age (Fig. 1A). Also, development of hypoglycemia (tumor growth) occurred with identical kinetics in RIP-Tag2 and RIP-Tag2-HA mice (data not shown). Thus, expression of HA on growing β cell tumors did not appear to trigger a spontaneous, protective antitumor response. No infiltration was observed in hyperplastic islets of these mice (data not shown), further supporting a lack of tumor-specific immunity.

Tolerance to HA was also maintained after the mice were challenged with influenza virus (PR8 strain). As shown in Fig. 1B, RIP-Tag2-HA mice were unable to respond to HA as assessed by CTL activity, but these animals responded normally to the influenza NP epitope when compared with B10.D2 control mice. Therefore, like InSNA mice (Fig. 1B), RIP-Tag2-HA mice are tolerant to HA. In addition, PR8 infection of RIP-Tag2-HA mice did not delay the onset of hypoglycemia (data not shown), demonstrating that RIP-Tag2-HA mice do not have a HA-specific CD8⁺ T cell repertoire capable of impeding the growth of the RIP-Tag2-transformed cells.
Our previous studies revealed that clone 4 CD8+ T cells correlate with tumor burden with the HA peptide, followed by intracellular staining for IFN-γ (Fig. 2A). As previously observed, clone 4 cells transferred into InsHA mice showed little proliferation and minimal IFN-γ production (4.8% of dividing clone 4 cells). In contrast, clone 4 T cells from both 6- and 10-wk-old RIP-Tag2-HA recipients showed increased proliferation and a significantly higher proportion of IFN-γ-producing cells. Furthermore, the levels of proliferation and IFN-γ production appeared to increase with increasing tumor burden (48.3% IFN-γ+ in 10-wk-old mice vs 23.6% IFN-γ+ in 6-wk-old mice with smaller tumors). As expected, clone 4 cells transferred into control B10.D2 mice did not proliferate. It should be noted that we consistently observe low levels of IFN-γ production by uninduced clone 4 cells upon ex vivo stimulation (see Figs. 2A and 3). The basis for IFN-γ secretion by uninduced cells is not known and the use of RAG-2/-/- clone 4 CD8+ T cells or CD44low sorted clone 4 cells did not diminish this IFN-γ secretion (data not shown).

In vivo cytolytic activity of clone 4 cells was also augmented in tumor-bearing RIP-Tag2-HA mice as compared with InsHA mice, with greater killing observed in older recipient mice bearing larger tumors (14 wk of age vs 7 wk, Fig. 2B). Thus, development of effector function by clone 4 CD8+ T cells correlated directly with tumor burden.

Clone 4 differentiation into effector cells is not dependent on endogenous CD4+ help, but is dependent on the number of adoptively transferred clone 4 cells

Development of effector function by Ag-specific T cells is thought to require Ag presentation by an activated DC that can provide signal 1 (TCR stimulation), signal 2 (costimulation), and a third signal in the form of IL-12 or other inflammatory cytokines (9, 30). Quiescent DCs, on the other hand, provide signal 1 in the absence of sufficient costimulation and other accessory signals and do not efficiently promote T cell effector function (13, 14). The observed differentiation of clone 4 CD8+ T cells into effector cells in RIP-Tag2-HA mice suggested that the cross-presenting DCs responsible for the presentation of HA may have been activated by an inflammatory environment, presumably established by an innate immune response to tumor growth. However, another possible explanation for the observed development of effector function by the clone 4 cells in RIP-Tag2-HA animals was that the mice may contain residual endogenous tumor-specific CD4+ T cells. In previous studies we showed that when cotransferred into InsHA recipients with clone 4 CD8+ T cells, activated HA-specific CD4+ helper cells could promote the induction of clone 4 effector function (11). To test this possibility, endogenous CD4+ T cells were depleted from RIP-Tag2-HA recipients before clone 4 transfer and proliferating clone 4 CD8+ cells were analyzed for IFN-γ production. As shown in Fig. 3A, depletion of CD4+ T cells did not lead to diminished clone 4 effector function. Surprisingly, we observed increased proliferation and IFN-γ production in the absence of the endogenous CD4+ T cells.

Along these same lines, it had been reported previously that large numbers of CD8+ T cells can provide their own “help,” both in vitro and in vivo (31–34). These studies have shown that the interaction of DCs with large numbers of cognate CD8+ T cells can provide signals that activate the maturation of the DCs, leading to efficient CTL priming. Therefore, we next considered the possibility that the activation of large numbers of clone 4 CD8+ cells, as occurs in RIP-Tag2-HA animals with considerable tumor burden, overcomes the need for a preactivated DC to promote development of effector function. To address this possibility, we asked whether variation in the number of adoptively transferred clone 4 cells influenced their ability to acquire effector function. Naive clone 4 CD8+ cells (0.38, 1.5, or 6.0 × 10⁶ cells) were transferred into age-matched RIP-Tag2-HA recipients, and 4 days later trans-

**FIGURE 1.** RIP-Tag2-HA mice are tolerant to HA expressed as a TAA. A, Blood glucose was monitored weekly in InsHA and RIP-Tag2-HA mice to assess tumor development. Glucose readings shown are averages of four mice per group. It should be noted that most RIP-Tag2-HA animals do not survive past 16 wk, and only one mouse survived for 22 wk. The averages shown are for surviving mice. This experiment has been repeated multiple times with >20 RIP-Tag2-HA animals with similar results. B, Mice (two per group) were infected i.p. with 1200 HA units of influenza virus (PR8). After 3 wk, spleens were restimulated with irradiated splenocytes pulsed with NP or HA peptide for 6 days. Cytolytic activity against unloaded target cells or against cells loaded with the stimulating peptide (1 μM or 1 nM) was measured in a 5-h ⁵¹Cr release assay.

**Development of effector function by CD8+ clone 4 T cells correlates with tumor burden**

Our previous studies revealed that clone 4 CD8+ T cells undergoing tolerance in the pLNs of InsHA recipients do not develop effector function (8). To determine whether clone 4 cells develop effector function in response to HA expressed by tumor cells, 3 × 10⁶ clone 4 CD8+ T cells were labeled with CFSE and transferred into B10.D2 mice, InsHA mice, or RIP-Tag2-HA mice with small (6 wk of age) or large (10 wk of age) tumor burdens. Four days later, pLN cells were harvested and stimulated in vitro for 5–6 h with the HA peptide, followed by intracellular staining for IFN-γ (Fig. 2A). As previously observed, clone 4 cells transferred into InsHA mice showed little proliferation and minimal IFN-γ production (4.8% of dividing clone 4 cells). In contrast, clone 4 T cells from both 6- and 10-wk-old RIP-Tag2-HA recipients showed increased proliferation and a significantly higher proportion of IFN-γ-producing cells. Furthermore, the levels of proliferation and IFN-γ production appeared to increase with increasing tumor burden (48.3% IFN-γ+ in 10-wk-old mice vs 23.6% IFN-γ+ in 6-wk-old mice with smaller tumors). As expected, clone 4 cells transferred into control B10.D2 mice did not proliferate. It should be noted that we consistently observe low levels of IFN-γ production by uninduced clone 4 cells upon ex vivo stimulation (see Figs. 2A and 3). The basis for IFN-γ secretion by uninduced cells is not known and the use of RAG-2/-/- clone 4 CD8+ T cells or CD44low sorted clone 4 cells did not diminish this IFN-γ secretion (data not shown).

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ferred cells were assessed for their ability to produce IFN-γ. Surpris-
ingly, dilution of the number of transferred T cells resulted in a progressive decrease in the overall percentage of dividing cells capable of producing IFN-γ (Fig. 3B). Also, when cells that had undergone the same number of divisions were compared between the three different groups, the percentage of IFN-γ-producing clone 4 cells progressively increased in RIP-Tag2-HA recipients that received higher numbers of transferred cells (see Fig. 3 legend). Furthermore, in subsequent experiments where even lower numbers of clone 4 cells (≤1 × 10^6) were transferred, IFN-γ production was even further diminished to 0.8–5.1% of proliferating cells (see Fig. 6A and data not shown). Thus, naive clone 4 CD8^+ T cells develop into cytokine-producing effectors in response to cross-presented tumor Ag only when large numbers are transferred. These data suggest that, in the RIP-Tag2 model, tumor growth does not provide the inflammatory environment required to activate resident DCs and promote development of CD8^+ T cell effector functions, as we would expect both high and low numbers of CD8^+ clone 4 cells to develop effector function in such an inflammatory setting.

**Adoptively transferred HA-specific clone 4 T cells are tolerized in tumor-bearing RIP-Tag2-HA mice**

Cross-presentation of self-Ags in a noninflammatory environment results in the tolerance of Ag-specific CD8^+ T cells, either via deletion or functional inactivation (10). Since the growing tumors did not appear to induce inflammatory signals in the RIP-Tag2-HA mice, we hypothesized that clone 4 T cells should become tolerized in the tumor-bearing recipients as previously observed in InsHA recipients (7). To test for tolerance, clone 4 CD8^+ T cells
were adoptively transferred into RIP-Tag2-HA mice, tumor-free InsHA mice, or B10.D2 control mice. Tolerance induction was then assessed over time by determining whether the mice retained clone 4 cells that could respond to secondary Ag challenge in the form of influenza PR8 infection. In the first experiment, RIP-Tag2-HA recipients received 2.5 × 10^6 clone 4 CD8^+ T cells and were infected with PR8 at 1, 3, or 5 wk posttransfer to expand any remaining (nontolerized) clone 4 cells. Five days after virus infection, in vivo CTL activity against the Kd-HA peptide was assessed (Fig. 4A). As expected based on our previous studies, T cells were completely tolerized after 5 wk in InsHA mice, as indicated by a lack of in vivo CTL activity against the injected HA peptide (and by CFSE) compared with equivalent numbers of unloaded cells (CFSElow). Furthermore, clone 4 T cells were also tolerized in RIP-Tag2-HA mice (Fig. 4A). In fact, tolerance occurred more rapidly in these tumor-bearing recipients, since HA-specific CTL activity was eliminated by 3 wk posttransfer. This is consistent with our previous observation that the rate of tolerance is directly proportional to the amount of available cross-presented Ag (7). It should be noted that clone 4 T cells were not tolerized in control B10.D2 mice, since high levels of lytic activity against HA-loaded targets were observed after virus infection at all time points (data not shown).

We were concerned that the observed lytic activity against HA-loaded targets in recipient B10.D2 control mice may be due to endogenous HA-specific CTL rather than transferred clone 4 cells, since unlike the other recipients, B10.D2 mice would not be tolerant to HA. To address this issue and to more directly assess the presence of responsive clone 4 T cells after in vivo exposure to tumor-associated HA, the experiment was repeated using a slight variation in protocol. Instead of using in vivo CTL activity against HA-loaded target cells to measure the degree of tolerance of clone 4 cells, we used Ly5.1 clone 4 CD8^+ T cells for our adoptive transfer experiments so that we could directly detect the presence of remaining clone 4 cells by flow cytometry. Approximately 6 × 10^6 Ly5.1 clone 4 CD8^+ T cells were transferred and recipient mice were infected with PR8 at 2 or 5 wk posttransfer. Seven days after PR8 infection, splenocytes were examined for the presence of expanded Ly5.1 clone 4 cells (Fig. 4B). As expected, Ly5.1 cells were easily detectable at both time points after transfer into B10.D2 control mice. In InsHA recipients, Ly5.1 clone 4 cells were detected at 2 wk (8.0% of total CD8^+ cells), but not at 5 wk. Ly5.1 donor cells were also detectable at 2 wk in RIP-Tag2-HA mice, but at lower levels (3.7% of total CD8^+ cells) than in InsHA recipients at this same time point. The Ly5.1 clone 4 cells were no longer detectable by 5 wk after transfer into RIP-Tag2-HA mice. Thus, both experimental protocols represented in Fig. 2...
show that clone 4 CD8+ T cells are tolerized in tumor-bearing RIP-Tag2-HA mice. Clone 4 cells were transferred into B10.D2, InsHA, or RIP-Tag2-HA mice (8–9 wk of age), and recipients were infected i.p. with influenza virus (PR8) at various time points posttransfer (1, 3, or 5 wk) to expand remaining clone 4 cells. A. In vivo CTL activity against Kd-HA peptide-loaded (CFSEhigh) or unloaded (CFSElow) splenocyte targets was monitored 5 days after PR8 infection by flow cytometric analysis of pLN cells. Similar results were observed in nondraining LNs and spleen. No in vivo CTL activity was observed in PR8-infected InsHA or RIP-Tag2-HA mice that did not receive clone 4 cells. B. Recipient mice were analyzed at the indicated time points (2 or 5 wk) after transfer for the presence of Ly5.1+ clone 4 cells in the spleen 7 days after PR8 infection. Only CD8+ events are shown, and values shown represent the percentage of Ly5.1+ cells (M1) among total CD8+ cells. The background M1 value for mice receiving no Ly5.1+ cells was 0.1% (data not shown). Data shown are for one mouse per group and are representative of three to four mice per group.

**FIGURE 4.** Adoptively transferred clone 4 CD8+ T cells become tolerized in tumor-bearing RIP-Tag2-HA mice. Clone 4 cells were transferred into B10.D2, InsHA, or RIP-Tag2-HA mice (8–9 wk of age), and recipients were infected i.p. with influenza virus (PR8) at various time points posttransfer (1, 3, or 5 wk) to expand remaining clone 4 cells. A. In vivo CTL activity against Kd-HA peptide-loaded (CFSEhigh) or unloaded (CFSElow) splenocyte targets was monitored 5 days after PR8 infection by flow cytometric analysis of pLN cells. Similar results were observed in nondraining LNs and spleen. No in vivo CTL activity was observed in PR8-infected InsHA or RIP-Tag2-HA mice that did not receive clone 4 cells. B. Recipient mice were analyzed at the indicated time points (2 or 5 wk) after transfer for the presence of Ly5.1+ clone 4 cells in the spleen 7 days after PR8 infection. Only CD8+ events are shown, and values shown represent the percentage of Ly5.1+ cells (M1) among total CD8+ cells. The background M1 value for mice receiving no Ly5.1+ cells was 0.1% (data not shown). Data shown are for one mouse per group and are representative of three to four mice per group.

**Clone 4 CD8+ T cells cannot protect mice from tumor progression (hypoglycemia) without cotransfer of tumor Ag-specific CD4+ helper T cells**

Since the transfer of larger numbers of clone 4 cells successfully led to the development of effector CTL, it was of interest to determine whether these CTL could protect RIP-Tag2-HA animals from tumor development. Therefore, 3 x 10^6 clone 4 cells were transferred into RIP-Tag2-HA recipients at 8–9 wk of age and mice were monitored weekly for tumor growth by assessing blood glucose levels (Fig. 5B). Although some mice became transiently hyperglycemic, indicating antitumor activity by activated clone 4 cells, all animals eventually developed severe hypoglycemia with kinetics similar to those of untreated RIP-Tag2-HA mice (Fig. 5, A and B). Transfer of up to 1 x 10^7 clone 4 CD8+ cells was also unable to provide lasting protection against β cell tumor growth (data not shown). Thus, despite development of effector function, clone 4 CD8+ T cells alone were unable to eradicate tumors.

To see whether provision of CD4 help could enhance the antitumor activity of clone 4 CD8+ T cells, naive CD4+ T cells (1 x
and SFE CD4⁺ T cells became hyperglycemic by 1 wk posttransfer and all animals succumbed to diabetes within 2–3 wk (Fig. 5D). This antitumor activity required both CD4⁺ and CD8⁺ T cells, since transfer of 3 × 10⁶ SFE CD4⁺ T cells alone did not affect tumor growth (Fig. 5C).

**CD4⁺ T cell help leads to a significant increase in tumor-specific clone 4 effector function and accumulation**

Considering that the provision of CD4 help in the form of naive SFE cells had such a dramatic impact on the ability of clone 4 CD8⁺ T cells to eliminate growing tumors, we thought it likely that SFE cells were either enhancing the development of effector function by clone 4 cells and/or promoting the accumulation of activated clone 4 cells. To address this issue, clone 4 cells were transferred into RIP-Tag2-HA recipients in the presence or absence of SFE helper cells and assessed for their ability to produce IFN-γ ex vivo 4 days later (Fig. 6A). As described earlier, transfer of a low number (1 × 10⁶) of clone 4 CD8⁺ cells alone resulted in proliferation, yet only ~5% of the proliferating cells produced IFN-γ. In contrast, cotransfer of 3 × 10⁶ SFE helper cells led to a dramatic increase in effector function development, as nearly 60% of proliferating cells produced IFN-γ upon short-term in vitro stimulation. In addition, roughly 3-fold more Ly5.1⁺ clone 4 cells were recovered from the draining LN of mice receiving SFE CD4⁺ T cell help (Fig. 6B). Thus, provision of help increased both the accumulation and the effector function of clone 4 CD8⁺ T cells in response to the cross-presented HA tumor Ag. As expected, development of clone 4 effector function correlated with the number of SFE helper cells cotransferred (Fig. 6A).

**Discussion**

In this report, we describe the consequences of the interaction between naïve CD8⁺ T cells and DCs cross-presenting TAA obtained from a pancreatic tumor. Our previous studies on the mechanism of tolerance of HA-specific clone 4 CD8⁺ T cells in response to cross-presented Ag in tumor-free InsHA mice indicated that tolerance occurs without the development of cytolytic activity or IFN-γ production by the activated T cells. In the current study, we wished to determine whether the tumor environment provided the type of inflammatory milieu that was necessary to activate the Ag-presenting DCs and thereby promote development of TAA-specific CD8⁺ T cell effector function. Initially, our experiments suggested that the tumor environment in the RIP-Tag2-HA mice was sufficient to promote an immune response, as adoptively transferred clone 4 CD8⁺ T cells demonstrated considerable production of IFN-γ and developed into CTL (Fig. 2) capable of significant levels of islet cell destruction (Fig. 5). Clone 4 effector function was not dependent on endogenous CD4⁺ T cell help, as elimination of all CD4⁺ cells actually enhanced the activation and IFN-γ production by clone 4 CD8⁺ T cells in RIP-Tag2-HA mice. This suggests that the mice may harbor a population of CD4⁺ cells that can inhibit the development of CD8⁺ T cell effector functions. Several candidate suppressor cell populations have already been described in the literature, including CD4⁺ NKT cells (36) and CD4⁺CD25⁺ regulatory T cells (37–39), both of which have been shown to inhibit tumor-specific T cell activity. Alternatively, depletion of CD4⁺ T cells in the RIP-Tag2-HA recipient mice could allow for the enhanced clone 4 activation due to increased expansion under lymphopenic conditions.

Having ruled out the possibility that tumor-specific CD4⁺ T cells were responsible for the development of effector function by the clone 4 CD8⁺ T cells, we next considered the possibility that the CD8⁺ T cells themselves may be responsible for promoting the

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**Figure 5.** Clone 4 CD8⁺ T cells require exogenous CD4⁺ T cell help to eradicate β cell tumors in RIP-Tag2-HA recipients. RIP-Tag2-HA mice (8–9 wk of age) were monitored weekly for tumor eradication (hyperglycemia) or tumor growth (hypoglycemia) after receiving no cells (A), clone 4 CD8⁺ T cells alone (B), SFE CD4⁺ T cells alone (C), or clone 4 and SFE cells (D). For the in vivo activation assay shown in the inset of C, SFE CD4⁺ T cells were isolated and labeled with CFSE as indicated in Materials and Methods. Two million cells were transferred into RIP-Tag2-HA recipients (8–9 wk) and pLNs were harvested 4 days later for FACS analysis to assess CFSE dilution.
development of their effector function. Several reports in the literature have shown that large numbers of activated CD8 T cells can provide their own help by directly activating DCs (31, 34). Such activation was dependent on the amount of Ag and the numbers of cognate CD8 T cells (34). To determine whether this was responsible for the development of effector function in RIP-Tag2-HA mice, we tested whether the ability to produce IFN-γ was dependent on the number of injected CD8 T cells. Surprisingly, we observed a direct correlation between the number of adoptively transferred clone 4 cells and the proportion of cells that could produce IFN-γ. This implies that it was not the tumor environment, but rather the activated CD8 T cells that were responsible for the development of CTL effector function. Based on these data, we conclude that in the RIP-Tag2 model the growing tumor does not induce the type of inflammatory environment that is necessary to activate DCs and promote a productive immune response.

We next addressed the question of whether the adoptively transferred clone 4 CD8 T cells are eliminated in RIP-Tag2-HA mice. We previously devised an assay that monitors tolerance of clone 4 CD8 T cells in InsHA recipients by following the rate of elimination of HA-responsive cells in InsHA recipients. Small numbers of clone 4 CD8 T cells were transferred into groups of InsHA mice that were subsequently infected with influenza at progressive time points to determine how long it takes for elimination of responsive clone 4 cells. The absence of cells that can respond to influenza virus is taken as an indication of their tolerance and deletion. However, we do not rule out the possibility that small numbers of tolerant CD8 T cells may persist that are unable to clonally respond to influenza and therefore may be below our level of detection. Two different methods were used to detect residual, influenza HA-responsive clone 4 CD8 T cells, an in vivo CTL assay and direct detection by flow cytometry. Both assays indicated that elimination of virus-responsive cells occurred in both InsHA and RIP-Tag2-HA mice. In fact, it appeared that elimination of responsive cells occurred somewhat more rapidly in tumor-bearing RIP-Tag2-HA recipient mice than in InsHA recipients. This was not unexpected since there is more Ag available for cross-tolerance in tumor-bearing mice, and we showed previously that the rate at which cells were eliminated correlated directly with the expression level of the cross-presented Ag (7).

Our findings that CD8 T cells do not develop effector function and are eliminated by tolerance in tumor-bearing mice contrasts
with the results of Ohashi and coworkers (5, 40) who also used RIP-Tag2 mice to study the fate of CD8$^+$ T cells in tumor-bearing mice. They used the lymphocytic choriomeningitis virus glycoprotein (GP) as a TAA and GP-specific P14 TCR-transgenic cells as the tumor-specific CD8$^+$ T cell population. They concluded from their studies that P14 T cells develop effector function and are not tolerated in RIP(GP × Tag2) recipients. The different conclusions drawn from their study is likely due to several differences between their experiments and ours. First, they used very large numbers (5 × $10^5$) of adoptively transferred T cells, a number that we too find leads to development of CTL and tumor destruction. The basis for their observed lack of tolerance and development of effector function could also be due to a fundamental difference between the GP and HA models. In both the RIP-GP and the RIP(GP × Tag2) mice, there remain substantial numbers of endogenous T cells that are responsive to GP (40, 41). This contrasts with InsHA and RIP-Tag2-HA mice, both of which are profoundly tolerant to HA. The reasons for the differences between the two models are not known, but the remaining endogenous GP-specific CD4$^+$ and/or CD8$^+$ T cells could prevent the tolerance of P14 T cells in their tumor model.

The fact that low numbers of transferred clone 4 CD8$^+$ T cells undergo activation but do not differentiate into effector CTL (Fig. 6A) suggests that, at least in the RIP-Tag2-HA model, tumor growth does not promote the activation of the DCs that cross-present tumor Agrs. However, we show that this activation signal can be exogenously provided by SFE CD4$^+$ helper T cells or by large numbers of clone 4 CD8$^+$ T cells. Although DC activation by CD4$^+$ T cells is thought to occur through CD40-CD40 ligand interactions, the mechanism for CD8$^+$ T cell-mediated activation of DCs is not known (31, 34). In both situations though, T-DC interaction leads to the up-regulation of costimulatory molecules and cytokines by the cross-presenting DC (34), which is likely critical for CTL differentiation in the absence of other inflammatory signals. Indeed, we have observed that when CD28-deficient clone 4 CD8$^+$ T cells are transferred into RIP-Tag2-HA recipients, even in large numbers ($3 \times 10^5$), they do not exhibit effector function (M. Lyman, unpublished observation).

The transfer of large numbers of clone 4 CD8$^+$ T cells into RIP-Tag2-HA mice led to the development of CTL effector function and detectable in vivo antitumor activity (transient hyperglycemia), but was ultimately unable to prevent tumor outgrowth. In contrast, cotransfer of HA-specific CD4$^+$ T cells promoted rapid tumor eradication. It is often assumed that CD8$^+$ T cells are efficiently primed by growing tumors, but are unable to survive due to Ag-induced cell death or “exhaustion” (42). Previous studies have demonstrated an important role for CD4$^+$ T cells in preventing such CTL exhaustion (43, 44). In addition, recent studies have revealed a requirement for CD4 help in the establishment of CD8$^+$ memory T cells (45–48). Thus, it has been suggested that a critical role of CD4 help in antitumor responses is enhancement of CTL survival and maintenance of CTL effector function in the face of chronic exposure to tumor Agrs (3, 49). We show here that in addition to their proposed role in CTL maintenance, activated tumor-specific CD4$^+$ T cells are critical for the early accumulation and development of effector function by responding CTL, especially when the pool of responding CD8$^+$ T cells is limiting and there are no other inflammatory signals available to activate the DCs. These helper effects may be exerted by the cross-presenting DC and through CD4$^+$→CD8$^+$ T cell interactions (50). It is also possible that the adoptively transferred SFE helper cells contribute directly to tumor eradication through their production of effector cytokines (51).

In conclusion, our data present evidence for a model in which tumor growth leads to efficient tolerance of tumor-specific CD8$^+$ T cells without the development of CD8$^+$ T cell effector function. The simplest explanation for these results is that the tumor environment does not promote the activation of the cross-presenting DCs. In support of this, we find evidence for the development of effector function when large numbers of CD8$^+$ T cells simultaneously undergo activation, a situation in which it has been shown that CD8$^+$ T cells can promote DC activation and, accordingly, provide their own help. However, we hesitate to conclude that our results imply the absence of immune surveillance until we learn more about the early events that occur during tumor establishment. A successful tumor may be responsible for the induction of anti-inflammatory molecules that inhibit DC activation (21). Indeed, the observation that removal of CD4$^+$ T cells from tumor-bearing mice results in enhanced activation and effector function by the CD8$^+$ T cells hints at the layers of complexity that are involved.

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