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Vaccines with Enhanced Costimulation Maintain High Avidity Memory CTL

Sixun Yang, James W. Hodge, Douglas W. Grosenbach, and Jeffrey Schlam

The avidity of Ag-specific CTL is a critical determinant for clearing viral infection and eliminating tumor. Although previous studies have demonstrated that vaccines using enhanced costimulation will enhance the level and avidity of Ag-specific T cells from naive mice, there are conflicting data about the effects of vaccines using enhanced costimulation (vector or dendritic cell based) on the survival of memory T cells. In this study we have first extended previous observations that primary vaccination with a recombinant vaccinia virus (rV-) expressing a model Ag (LacZ) and a triad of T cell costimulatory molecules (B7-1, ICAM-1, and LFA-3 (designated TRICOM)) enhances the level and avidity of T cells from naive vaccinated C57BL/6 (Thy1.2) mice. Adoptive transfer of Thy1.1 memory CD8+ T cells into naive Thy1.2 C57BL/6 mice was followed by booster vaccinations with a recombinant fowlpox (rF-) expressing LacZ (rF-LacZ) or booster vaccinations with rF-LacZ/TRICOM. Analysis of levels of β-galactosidase tetramer-positive T cells and functional assays (IFN-γ expression and lytic activity) determined that booster vaccinations with rF-LacZ/TRICOM were superior to booster vaccinations with rF-LacZ in terms of both maintenance and enhanced avidity of memory CD8+ T cells. Antitumor experiments using a self-Ag (carcinoembryonic Ag (CEA) vaccines in CEA transgenic mice bearing CEA-expressing tumors) also demonstrated that the use of booster vaccinations with vaccines bearing enhanced costimulatory capacity had superior antitumor effects. These studies thus have implications in the design of more effective vaccine strategies. The Journal of Immunology, 2005, 175: 3715–3723.

Induction of efficient long-term immune memory is the aim of all vaccination protocols. The factors required to maintain memory cell populations have been controversial. In mice, memory T cell survival does not require the persistence of cognate Ag (1–3). However, Ag-specific T cells in patients infected with HIV show a correlation between viral load and the percentage of Ag-specific cells in the blood (4). HIV-specific memory cell counts fall sharply when antiretroviral therapy is initiated, matching the fall in viral load (4). The results suggest that the size of the memory T cell pool in humans is highly dependent on the persistence of cognate Ag. In contrast, persistent infection of mice with lymphocytic choriomeningitis virus (LCMV) resulted in selective deletion or anergy of high avidity memory CTL (5). Administration of LCMV vaccines successfully induced lytic MHC-restricted CTL in the persistently virus-infected mice; however, these CTL were of low avidity and could not clear the viral infection (5).

It has previously been shown that the proliferation and activation of naive T cells are more dependent on costimulation than are those of effector/memory T cells (6,7). When APCs with decreased costimulatory capacity are used, the proliferation of naive T cells requires increasingly higher peptide concentrations compared with effector/memory T cells (6). Higher peptide concentrations usually result in apoptosis of effector/memory T cells. For the effect of costimulatory molecules on effector/memory T cells, the results are conflicting. Iezzi et al. (6) demonstrated that signaling through CD28 partially protected the effector/memory CD4 T cells (from TCR transgenic mice specific for flu hemagglutinin peptide) from apoptosis induced by high peptide concentration/prolonged peptide stimulation. In contrast, Sabzevari et al. (8) reported that effector/memory CD4 T cells from pigeon cytochrome c (PCC) TCR transgenic mice were more susceptible to apoptosis induced by APC expressing B7-1 in the presence of high affinity cognate peptide compared with naive CD4 T cells. It should be pointed out, however, that both studies (6,8) were performed in vitro, and both analyzed effector/memory CD4+ T cells from TCR transgenic mice.

We have previously shown that recombinant poxvirus vectors can be efficiently used in diversified prime and boost strategies to enhance Ag-specific murine T cell responses. Primary vaccination used the replication-competent vaccinia (rV-) and booster vaccinations used the replication-defective avipox virus (fowlpox (rF-)). Subsequent studies showed that insertion of the transgenes for a triad of T cell costimulatory molecules (B7-1, ICAM-1, and LFA-3 (TRICOM)) also enhanced the level of the CD8+ T cell response (9,10). These studies, however, did not address whether the multiple booster vaccinations were 1) simply generating more effector cells from naive T cell populations, 2) expanding memory T cell populations, or 3) both of the above. These studies also did not address the avidity of memory T cells. The present study was designed to answer these questions. We first vaccinated C57BL/6 Thy1.1 mice with an rV-LacZ/TRICOM vector. We then adoptively transferred Ag-specific memory T cells into Thy1.2 C57BL/6 mice and vaccinated those mice with either a recombinant rF-LacZ vector or a recombinant rF-LacZ/TRICOM vector. Both the level and avidity of β-galactosidase (β-gal)-specific CD8+ memory (Thy 1.1) T cells were then analyzed. The results of these studies are of importance in the design of clinical trials in

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3 Abbreviations used in this paper: LCMV, lymphocytic choriomeningitis virus; β-gal, β-galactosidase; PCC, pigeon cytochrome c; rF-, recombinant fowlpox virus; CEA, carcinoembryonic Ag; Tg, transgenic; TRICOM, triad of costimulatory molecules (B7-1, ICAM-1, and LFA-3).
which vaccines containing costimulatory molecules (vector based or dendritic cell based) are used as booster vaccinations with the purpose of maintaining and/or expanding high avidity Ag-specific memory CD8+ T cells.

**Materials and Methods**

**Mice and cell lines**

Female C57BL/6 (C57BL/6) mice were obtained from the National Cancer Institute, Frederick Cancer Research Facility. Female Thy.1.1 mice (formerly called C57BL/6.PL-Thy1+Cy) were purchased from The Jackson Laboratory. This C57BL/6 congenic strain carries the T lymphocyte-specific Thy1.1 allele. The Thy.1 and Thy.1.2 mice are genetically identical mice differing only in their expression of the Thy-1 allele. Donor T cells (Thy.1.1) can be easily distinguished from recipient T cells (Thy.1.2) by flow cytometric analysis. Mice were housed and maintained under pathogen-free conditions in microisolator cages and were used for experiments at 6–8 wk of age.

C57BL/6 mice transgenic for human carcinoembryonic Ag (CEA) (CEA-Tg) were originally obtained from a breeding pair provided by Dr. J. Thompson (Institute of Immunobiology, University of Freiburg, Freiburg, Germany). The generation and characterization of the CEA-Tg mouse were previously described (11). PCR of DNA from whole blood to detect the CEA gene was used to screen for CEA-positive mice, as previously described (12). Mice were housed and maintained under pathogen-free conditions in microisolator cages. For experiments, 8- to 12-wk-old CEA-Tg mice were used. All mouse procedures were reviewed and approved by the animal use and care committee, National Institutes of Health.

Murine colon adenocarcinoma cells expressing human CEA (MC-38-CEA) were generated by retroviral transduction of MC-38 cells with CEA cDNA (13). Before transplantation to mice, the cells were trypsinized, dispersed through a 70-μm pore size cell strainer (Falcon; BD Biosciences) and washed twice in HBSS before final suspension in HBSS. The tumor cell line EL4 (H-2d, thymoma, ATCC TIB-39) was purchased from American Type Culture Collection and was maintained in RPMI 1640 complete medium.

**Recombinant viruses**

The recombinant vaccinia virus designated rV-LacZ, rV-LacZ/B7-1, and rV-LacZ/TRICOM were constructed as previously described (14) and contained the lacZ gene encoding β-gal. The recombinant fowlpox virus LacZ containing viruses rF-LacZ and rF-LacZ/TRICOM was constructed in the similar manner.

The recombinant vaccinia virus designated rV-CEA has been described previously (15). The rV-CEA/TRICOM contains the murine B7-1, ICAM-1, and LFA-3 genes in combination with the human gene CEA, as described previously (16). The recombinant fowlpox virus rF-CEA/TRICOM contains the murine B7-1, ICAM-1, and LFA-3 genes in combination with the human gene CEA, as described previously (16). The recombinant fowlpox containing the murine GM-CSF gene (designated rF-GM-CSF) has been described previously (17). Therion Biologics provided all orthopox viruses as part of a collaborative research and development agreement with the National Cancer Institute.

**Peptide**

The H-2Kb binding peptides of β-gal, β-galase103 (DAF) and OVA epitope (SIINFEKL) were commercially synthesized (SynPep). The purity of peptides was >96%. Peptides were dissolved in DMSO and then diluted with PBS to 2 mg/ml (the final concentration of DMSO in stock solution is <5% (v/v)), filtered through a 0.2-μm pore size membrane (Millipore, Bedford, MA) and stored at −80°C.

**Abs, tetramer staining, and flow cytometric assay**

FITC-, PE-, PerCP-, or CyChrome-labeled anti-mouse CD2, CD3, CD4, CD8, CD11a, CD28, CD44, CD62L, IFN-γ, Thy1.1, Thy1.2, and all control Abs were purchased from BD Pharmingen. PE-labeled H-2Kb-β-gal tetramer was provided by the Tetramer Core Facility of the National Institutes of Health. For flow cytometric analysis of cell surface markers, 1–2 × 10^6 cells were incubated on ice with the appropriate Abs for 30–45 min, washed twice, and then analyzed on a FACSCalibur (BD Biosciences). Background staining was assessed by use of isotype control Abs. For tetramer staining, cells were stained with FITC- or PerCP-labeled CD8 or Thy1.1 and PE-labeled tetramer for 60 min on ice. For intracellular IFN-γ staining, cells were stained first with surface markers and then were permeabilized with Cytofix/Cytoperm (BD Pharmingen), followed by anti-IFN-γ staining. Data were analyzed using CellQuest software (BD Biosciences).

**Vaccinations and purification of memory T cells and adoptive transfer**

For experiments described in Fig. 1, C57BL/6 mice (Thy1.2) were vaccinated once with buffer, rV-LacZ, rV-LacZ/B7-1, or rV-LacZ/TRICOM. After 30 days, splenocytes were harvested. The β-gal-specific CD8+ T cell precursor frequency and avidity were determined immediately by intracellular IFN-γ staining as described below. All viruses were administered at 1 × 10^9 PFU/mouse. In addition, splenic T cells from rV-LacZ- and rV-LacZ/TRICOM-immunized mice were stimulated with irradiated B cells pulsed with 1 μg/ml β-gal peptide for 5 days, then CTL avidity was determined by lytic assay as described below.

For subsequent experiments, Thy1.1 mice were vaccinated with rV-LacZ/TRICOM (1 × 10^9 PFU/mouse) plus recombinant murine GM-CSF (Peprotech; 20 μg/mouse/day for 4 consecutive days) s.c. After 4 wk, pan-T cells were isolated from spleens using a pan-T kit and AutoMACS (Miltenyi Biotec), as suggested by the manufacturer. Memory T cells were negatively isolated by depletion CD62L+CD8+ cells using CD62L-labeled beads (Miltenyi Biotec) as previously described (19). Purified Thy1.1 memory T cells were washed twice, resuspended in PBS, and stained with surface markers as indicated in Fig. 2 before adoptive transfer to Thy1.2 (C57BL/6) mice. All C57BL/6 (Thy 1.2) mice received the same number of Thy1.1 memory T cells (5 × 10^6 cells/mouse) from the same source, through tail veins, and then were randomly grouped for vaccination. One week after adoptive transfer of Thy1.1 memory T cells, mice were vaccinated with PBS, rF-TRICOM, rF-LacZ, or rF-LacZ/TRICOM (each at 1 × 10^9 PFU/mouse) s.c. one to three times at 2-wk intervals. The β-gal tetramer cells were monitored as indicated using flow cytometry 5 days after each vaccination. For avidity studies, Thy1.1 memory T cell recipient C57BL/6 (Thy 1.2) mice were vaccinated with either rF-LacZ or rF-LacZ/TRICOM three times. Four weeks after the last vaccination, pan-T cells were isolated from spleens of C57BL/6 mice using a pan-T kit (Miltenyi Biotec). Thy1.1 T cells were purified from the pan-T cells by depleting Thy1.2 T cells, using Thy1.2 beads and AutoMACS (Miltenyi Biotec). Purified Thy1.1 T cells were used for CTL induction and avidity titration using the cytolytic method (20, 21).
intracellular IFN-γ staining after coculture with B cells pulsed with graded concentrations of peptide. Avidity, expressed as MC_{50} in moles, was defined as the concentration of peptide required to achieve 50% of the maximal response and was calculated using Microsoft Excel.

**Tumor therapy studies**

CEA-Tg mice were transplanted with 50,000 MC38-CEA cells to form experimental peripancreatic metastases, as previously described (22). Briefly, the spleens of anesthetized mice were exteriorized by means of a small subcostal incision. Cells were directly injected in 100 μl of HBSS using 1-ml syringes with 26-gauge, 5/8-inch needles. Splenectomy was performed ~2 min after tumor cell injection by cautereization using a high temperature cautery (Roboz). The abdominal cavity was closed in one layer using 9-mm wound autoclips. This dose of tumor cells is lethal to >80% of mice within 12 wk, with the primary tumor arising in the peripancreatic environment (22).

Fourteen days after tumor transplant, mice were vaccinated s.c. once with 1 × 10^{6} PFU of rV-CEA/TRICOM admixed with recombinant murine GM-CSF (20 μg; PeproTech) and human IL-2 (16,000 IU; Hoffmann-La Roche) i.p. GM-CSF (20 μg) was administered at the injection site for the following 3 days. Concurrently, IL-2 (16,000 IU) was administered twice a day for 3 days. This vaccination schema has been previously described (23). Seven days after the primary vaccination, mice were boosted with 1 × 10^{7} PFU of r-CEA or r-CEA/TRICOM admixed with 1 × 10^{6} PFU of GM-CSF. IL-2 (16,000 IU) was administered twice a day for 4 days. This booster vaccination regimen was repeated two additional times at 7-day intervals. Mice were monitored weekly for survival.

**Statistical analysis**

Significant differences were statistically evaluated using two-tail Student’s t test. Evaluation of survival patterns in mice bearing MC38-CEA tumors was performed by the Kaplan-Meier method.

**Results**

Vaccination with TRICOM-based vaccines induces higher levels and higher avidity of Ag-specific CTL in vivo

We first set out to determine whether direct vaccination with a TRICOM-based vector would induce increased levels of Ag-specific T cells, higher avidity Ag-specific CTL, or both in the system used in this study. We vaccinated mice with rV-LacZ, rV-LacZ/B7-1, or rV-LacZ/TRICOM and monitored β-gal-specific immune responses by both intracellular IFN-γ staining and a lytic assay. As shown in Fig. 1A, direct injection of rV-LacZ induced Ag-specific immune responses, as determined by intracellular IFN-γ staining. Including the B7-1 transgene in the vaccine (rV-LacZ/B7-1) moderately enhanced immune responses to β-gal peptide. In contrast, vaccination with rV-LacZ/TRICOM increased the number of Ag-specific IFN-γ-producing cells ~2- to 4-fold depending on the concentration of peptide (Fig. 1A). Initial studies have shown that mice vaccinated with rV-LacZ/TRICOM mount CD8+ T cell responses specific for β-gal, but those vaccinated with control vector rV-TRICOM do not (data not shown). Previous studies (9, 23, 24) have also shown that mice vaccinated with control rV-TRICOM vector or wild-type vector do not mount a T cell response against specific Ags. The cytolytic activity of splenic T cells from mice vaccinated with rV-LacZ or rV-LacZ/TRICOM was also compared. Consistent with intracellular IFN-γ staining, the cytolytic capacity of CTL generated from rV-LacZ/TRICOM-vaccinated mice was significantly enhanced compared with that of CTL from rF-LacZ-vaccinated animals (Fig. 1B). The avidity of T cells from vaccinated mice was also determined using previously defined methods (20, 21, 25). As measured using intracellular IFN-γ, T cells from mice vaccinated with rV-LacZ had an avidity of 9 × 10^{-7} M compared with 2.1 × 10^{-7} M from mice vaccinated with rV-LacZ/TRICOM (a 4-fold increase). Using the cytolytic assay, the avidities were 1.6 × 10^{-6} and 1 × 10^{-7} M for rV-LacZ- and rV-LacZ/TRICOM-vaccinated mice, respectively (a 16-fold increase). Based on the findings of this study and others (10, 17), in which stimulation of T cells with TRICOM-based vaccines was
shown to be superior to stimulation with vaccines containing B7-1, all subsequent studies used TRICOM-containing vectors for vaccination.

In vivo boost with TRICOM-based vaccines maintains memory T cell populations

Although vaccination with rV-LacZ/TRICOM increased immune responses both quantitatively and qualitatively compared with vaccination with rV-LacZ, it could be postulated that booster vaccinations with rF-LacZ/TRICOM might cause apoptosis and delete Ag-specific memory CD8\(^+\) T cells. To investigate this, we adoptively transferred memory T cells generated from Thy1.1\(^+\) mice vaccinated with rV-LacZ/TRICOM into normal C57BL/6 mice (Thy1.2\(^+\)). Memory T cells were purified from Thy1.1 mice 4 wk after vaccination with rV-LacZ/TRICOM. As shown in Fig. 2, purified memory T cells were >95% CD3\(^+\) T cells, as judged by flow cytometry; in this population, ~30% were CD8\(^+\) T cells and 65% were CD4\(^+\) T cells. Memory T cells purified from Thy1.1 mice demonstrated the phenotype of effector memory T cells (CD62L\(^{low}\)/CD44\(^{high}\); Fig. 2), Ag-specific T cells (CD8\(^+\)Tet\(^+\)), as determined by \(\beta\)-gal-MHC tetramer staining, were ~2.5–4% of CD8\(^+\) T cells (Fig. 2). In addition, the memory T cells expressed CD2, CD11a, and CD28, the ligands for TRICOM.

The function of adoptively transferred memory T cells was then investigated. Four weeks after a single boost vaccination, Thy1.1\(^+\) (transferred memory) and Thy1.2\(^+\) (endogenous) T cells were isolated using AutoMACS beads. The purified T cell populations were either not stimulated or stimulated with 1 \(\mu\)g/ml \(\beta\)-gal peptide for 24 h, and IFN-\(\gamma\) production was measured. As shown in Fig. 3A, Thy1.1\(^+\) T cells (memory T cells) from either PBS- or rF-TRICOM-boostered groups produced low levels of IFN-\(\gamma\) after peptide stimulation. Booster vaccinations with rF-LacZ increased IFN-\(\gamma\) production by Thy1.1\(^+\) T cells by 2.77-fold compared with the PBS group (Fig. 3A, left panel). IFN-\(\gamma\) production by memory T cells (Thy1.1 population) from the rF-LacZ/TRICOM-boostered group was substantially increased (5-fold) compared with that produced by memory T cells from the rF-LacZ group (Fig. 3A, left panel). As for the endogenous T cells (Thy1.2 population), a low, but marked, level of specific IFN-\(\gamma\) production was observed only in mice vaccinated with rF-LacZ/TRICOM (Fig. 3A, right panel; note difference in scale between panels in Fig. 3A). These data, taken together, support the hypothesis that vaccine boosting with high levels of costimulation increases the levels of both Ag-specific memory T cells as well as activated naïve T cells de novo.

T cells from spleens of rF-LacZ- and rF-LacZ/TRICOM-boosted groups were also stimulated in vitro with either OVA control peptide or \(\beta\)-gal peptide and then analyzed by intracellular IFN-\(\gamma\) staining (Fig. 3B). For memory CD8\(^+\) T cells (Thy1.1 population), 4.77% of Thy1.1\(^+\)/CD8\(^+\) T cells in rF-LacZ-boostered mice expressed IFN-\(\gamma\); 12.48% of Thy1.1\(^+\)/CD8\(^+\) T cells from rF-LacZ/TRICOM-boostered mice expressed IFN-\(\gamma\) after ex vivo cognate peptide stimulation, a 162% increase in IFN-\(\gamma\)-producing cells compared with the rF-LacZ group. For endogenous CD8\(^+\) T cells (Thy1.2 population), 0.18% of Thy1.2\(^+\)/CD8\(^+\) T cells in rF-LacZ-vaccinated mice expressed IFN-\(\gamma\), whereas 1.11% of Thy1.2\(^+\)/CD8\(^+\) T cells from the rF-LacZ/TRICOM group expressed IFN-\(\gamma\) after peptide stimulation, a 517% increase compared with the rF-LacZ group (Fig. 3B). The results were thus consistent with IFN-\(\gamma\) production as described above.

Thy1.1\(^+\) memory T cells were adoptively transferred into C57BL/6 mice (Thy1.2\(^+\)), and then mice were vaccinated with PBS, rF-TRICOM, rF-LacZ, or rF-LacZ/TRICOM three times at 2-wk intervals. Five days after the second and third booster vaccinations, the expansion of adopted Ag-specific memory Thy1.1\(^+\) T cell populations (CD8\(^+\)/Tet\(^+\)) in spleens was monitored.

As shown in Fig. 4, Ag-specific Thy1.1\(^+\) T cells (Thy1.1\(^+\)/CD8\(^+\)/Tet\(^+\)) in the PBS control vaccination group were ~2% of Thy1.1\(^+\) T cells after two and three boosts. Boosts with the rF-TRICOM vector devoid of the Ag transgene did not significantly change (\(p = 0.31\)) the percentage of Ag-specific memory T cells compared with the control PBS booster vaccination. Compared with the PBS group, boosts with rF-LacZ or rF-LacZ/TRICOM both significantly increased \(\beta\)-gal-specific memory T cells (\(p < 0.01\)) after two and three boosts. Moreover, boosts with rF-LacZ/TRICOM further increased the number of \(\beta\)-gal-specific memory CD8\(^+\) T cells compared with corresponding rF-LacZ groups after both two and three booster vaccinations (\(p < 0.01\)). It is unclear at this point why the percentage of CD8\(^+\)/\(\beta\)-gal tetramer\(^+\) T cells
did not increase between the second and third booster vaccinations. This will be discussed below.

Multiple boosts with TRICOM-based vaccines resulted in persistently higher avidity CD8+ T cells

After adoptive transfer of memory Thy1.1+ T cells from mice vaccinated with rF-LacZ/TRICOM into Thy1.2+ C57BL/6 mice, mice were boosted three times with either rF-LacZ or rF-LacZ/TRICOM at 2-wk intervals. Four weeks after the last booster vaccination, the number and function of Ag-specific T cells were monitored. As shown in Fig. 5A, there were 1.02 and 1.65% of memory Thy1.1+ T cells in the splenocytes from rF-LacZ and rF-LacZ/TRICOM boosted mice, respectively. Among the gated Thy1.1+ T cell populations, 7.9% were β-gal tetramer positive from mice boosted with rF-LacZ, whereas 16.6% of Thy1.1+/CD8+ T cells from mice boosted with rF-LacZ/TRICOM were β-gal tetramer positive. The results demonstrate that TRICOM-based vaccine boosts increased the number of Ag-specific memory T cells (Fig. 5A).

Thy1.1+ T cells were then purified from rF-LacZ- and rF-LacZ/TRICOM-boosted mice and stimulated in vitro for 5 days with β-gal peptide. CTL avidity was determined using a lytic assay. As shown in Fig. 5B, the dose-response curve of CTL from rF-LacZ/TRICOM-boosted mice was more sensitive to low peptide density on target cells compared with CTL from rF-LacZ-vaccinated mice. To calculate the avidity (20, 21, 25), the data shown in Fig. 5B were normalized in Fig. 5C. The avidity of CTL from rF-LacZ/TRICOM-boosted mice was 2.3 × 10−9 M, whereas that of CTL from rF-LacZ-boosted mice was 2.1 × 10−8 M, demonstrating a 10-fold increase in avidity after multiple boosts with TRICOM-based vaccines.

T cell avidity was also determined by intracellular IFN-γ staining of freshly isolated Thy1.1 T cells to rule out a possible artifact of T cell avidity measurements after a short-term in vitro peptide stimulation. Freshly isolated Thy1.1 T cells were stimulated for 6 h with autologous B cells pulsed with graded concentrations of β-gal peptide, and IFN-γ-producing cells were analyzed by intracellular IFN-γ staining. As shown in Fig. 6, T cells from the rF-LacZ/TRICOM-boosted group were much more sensitive to a lower concentration of peptide than T cells from rF-LacZ-vaccinated mice. For example, as shown in Fig. 6B, stimulation of T cells from rF-LacZ/TRICOM-boosted mice with 0.1 nM β-gal peptide elicited a substantial number of IFN-γ-producing cells, whereas stimulation of T cells from rF-LacZ-boosted mice with even 10 nM of the peptide induced barely above the background level of IFN-γ-producing cells. The avidity of T cells from rF-LacZ/TRICOM-boosted mice was calculated to be 5.0 × 10−10 M, which is 76-fold higher than the avidity of T cells (3.8 × 10−12 M) from mice boosted with rF-LacZ vaccine. Taken together, these results demonstrate that multiple boosts with a vaccine containing high levels of T cell costimulation have the ability to increase the number of high avidity Ag-specific memory CD8+ T cells.

Tumor therapy studies

The studies described above deal with a well-established experimental model Ag. β-Gal, however, is a foreign Ag and does not reflect the type of situation one encounters dealing with the vast...
majority of self tumor-associated Ags. We thus designed experiments to determine whether the phenomenon observed of boosting with vectors containing enhanced T cell costimulation would result in enhanced antitumor effects using vaccines to a self-Ag. The CEA-Tg mouse containing a CEA-expressing carcinoma has been previously used as such a model. CEA-Tg mice express CEA in both fetal tissue and adult gastrointestinal epithelium in a manner similar to that seen in humans. Fourteen days after transplant with CEA-expressing MC38 colon carcinoma cells, CEA transgenic mice received a primary vaccination with rV-CEA/TRICOM. Groups of mice (n = 10/group) then received three booster vaccinations with either rF-CEA or rF-CEA/TRICOM. As shown in Fig. 7, there was a clear and statistical difference in the survival of mice receiving rF-CEA/TRICOM booster vaccinations (designated TTTT, Fig. 7) compared with mice receiving booster vaccinations with rF-CEA (designated TCCC, Fig. 7; p = 0.025). Taken together, these studies support the observations described above using the β-gal Ag system that boosting with vaccines containing enhanced costimulation enhances the level of memory T cells, enhances the avidity of memory T cells, and can manifest itself in enhanced antitumor activity.

Discussion

Previous studies have demonstrated that high avidity CTL are essential for the effective clearance of viral infections as well as for the elimination of tumor cells (21, 25–29). However, high avidity CTL are also susceptible to activation-induced cell death (25, 30, 31). Therefore, the maintenance of high avidity CTL in vivo has been a challenge when designing effective vaccines for both viral infection and cancer. In the present study we have provided evidence that multiple boosts with vaccines containing a triad of costimulatory molecules to enhance signal 2 not only expanded Ag-specific memory CD8+ T cells, but also promoted the avidity maturation of the memory CTL in vivo.

CTL functional avidity, which has also been called recognition efficiency (28), is defined functionally, based on the peptide requirement of a CTL population. Although the term functional avidity may not be completely accurate to describe both the effectiveness and overall binding capacity between an Ag-specific CTL and its specific target, it is now widely used in the literature (see review article (32)). Thus, in this study we have used the term functional avidity to describe the capacity and efficiency of a CTL to recognize and lyse target cells in an Ag-specific fashion.

Through the use of direct injection of recombinant vectors expressing TRICOM and a model Ag β-gal, the present study confirmed and extended the phenomenon observed by Oh et al. (20) and Hodge et al. (10) that primary vaccinations with vaccines containing costimulation result in increases in both the magnitude...
creatin of Th1-type cytokines by effector/memory CD4+ T cells. However, Iezzi et al. (6) demonstrated that signaling through CD28 partially protected the effector/memory CD4+ T cells from TCR transgenic mice, specifically for influenza hemagglutinin peptide, from apoptosis induced by high concentrations of the cognate peptide and/or prolonged peptide stimulation. A more recent report by Fontenot et al. (33) demonstrated that memory CD4+ T cells in blood from chronic beryllium disease required CD28 costimulation for proliferative and cytokine responses to beryllium. In the lung, proliferation and secretion of Th1-type cytokines by effector/memory CD4+ T cells were functionally independent of CD28 costimulation, and a proportion of the CD4+ T cells were CD28-. In addition, in some patients, CD28 signaling resulted in decreased proliferation and cytokine production by lung memory CD4+ T cells (33). Fontenot et al. (33) suggested that the effect of costimulation on memory CD4+ T cells might be dependent on the activation stages of memory T cells.

The study reported by Yu et al. (34) may provide an explanation for some of the differences observed in these different studies (6, 8) as well as those observed in this study. There, it was shown that the outcome of CD28 signaling on T cell activation and expansion depended on Ag affinity. CD28 signaling enhanced T cell activation and expansion when TCR interacted with low and intermediate affinity alloantigen in vivo, whereas the same signal enhanced T cell activation, but inhibited T cell expansion and increased T cell apoptosis when TCR interacted with the high affinity alloantigen in vivo. Although β-gal is a foreign Ag to regular C57BL/6 mice, it is a relatively weak Ag compared with PCC. Kwok et al. (35) demonstrated that a β-gal-based vaccine was very immunogenic in BALB/c mice, whereas the same vaccine constructs barely induced specific immune responses in C57BL/6 mice, as used in our study. One can hypothesize that signaling through costimulation protects T cells from death by up-regulating survival factors (36–38) and maintains the T cell response over a long term. In contrast, the costimulatory signal facilitates Ag-activated T cell apoptosis when the peptide-TCR signal exceeds a certain threshold. The results shown in Fig. 3 clearly demonstrate that rF-LacZ/TRICOM boosted memory CD8+ T cells to greater levels than rF-LacZ. This observation is consistent with reports that stimulation with TRICOM-containing vectors markedly reduces the level of apoptosis in CD8+ T cell populations (39) and is in agreement with those of previous studies, which found that costimulation through the CD28 receptor appears to play an important role in enhancing the resistance of activated T cells to undergoing apoptosis in culture (38, 40).

Thus the seemingly divergent results of Sabzevari (8) and those reported in this study may be reconciled by the fact that the use of a strong (avid) signal 1, such as PCC, along with costimulation can result in a great degree of apoptosis of memory T cells, whereas the use of costimulation with a relatively weak signal 1 (β-gal), as reported in this study, can actually maintain or even enhance the level and avidity of memory T cells. In the present studies we focused on the maintenance/survival of adoptively transferred memory T cells in Thy1.2 recipient mice. Compared with the non-TRICOM vaccine, multiple boosts with the TRICOM-based vaccine slightly increased the number of Ag-specific Thy1.1+ memory T cell and dramatically enhanced the functional avidity of memory CTL. Based on the modestly enhanced effect on memory T cell maintenance/survival seen in this study (Figs. 3 and 4), one can argue that enhanced costimulation is not necessarily inducing apoptosis of memory CTL for all Ags, especially relatively weak ones. In addition, these two studies underscore that caution is needed when generalizing the phenomenon of memory T cell maintenance/avidity using only one model system.

It is unclear at this point why the percentage of CD8+/β-gal tetramer+ T cells did not increase between the second and third booster vaccinations with rF-LacZ/TRICOM (Fig. 4). Perhaps the combination of apoptosis of some memory T cells along with the expansion of other memory T cells resulted in the maintenance of the number of memory T cells. However, it is clear that in mice boosted two or three times, the actual amount of memory T cells and their avidity was greater for those mice receiving rF-LacZ/TRICOM booster vaccinations than for those receiving rF-LacZ devoid of costimulation (p < 0.01). Future studies using cytokines such as IL-15 or IL-7 (41–44) may demonstrate a greater potentiation of memory T cells using these vaccines. Alternatively, vectors containing costimulatory molecules other than those used in this study may be used in booster vaccinations to further potentiate memory T cells.

In the studies we report, avidity measured by cytolytic assay was made after 5–6 days of T cell stimulation with peptide-pulsed APC. In vitro stimulation might potentially alter avidity. To address this issue, in our previous studies (10) avidity was measured by a tetramer dissociation assay to compare the avidities of T cells obtained immediately from mice vs T cells cocultured with peptide-pulsed APC for 5–6 days. Those studies showed no difference in avidity, as measured by tetramer dissociation assay, between the freshly isolated T cells and 5- to 6-day cultured T cells. The data presented in Fig. 1 show a 4-fold increase in CTL avidity, as measured by intracellular IFN-γ staining, after vaccination with rV-LacZ/TRICOM vs rF-LacZ and a 16-fold increase, as determined by lytic assay, using T cells cultured in vitro for 5–6 days. There are at least two possible explanations for this. First is the possibility of an alteration of CTL avidity after in vitro peptide stimulation. However, the data noted above by Hodge et al. (10) might argue against this. The second explanation is that IFN-γ production and lytic activity reflect different features of CTL. Previous studies (45) showed that different clones of flu M1-specific T cells demonstrated different peptide sensitivity in cytolytic assay, whereas the different sensitivities were not seen in IFN-γ release.

The effect of TRICOM on the avidity of T cells after primary vaccination has been evaluated using both a stronger epitope (P18-I10, an HIV immunodominant epitope) and a weaker epitope (CEA peptide in CEA-Tg mice) (10, 20). The differences observed using vectors containing TRICOM vs those devoid of TRICOM on avidity are 2.5-fold for the HIV peptide and 100-fold for the CEA peptide. Thus, the 16-fold difference seen in the studies we report for β-gal peptide appears to support the concept that enhancing costimulatory molecules have a greater effect on increasing avidity of weaker Ags (such as self tumor-associated Ags). The actual increase in the number of Ag-specific T cells produced using the LacZ/TRICOM vector (Fig. 4) is moderate. However, the major point to consider is that the functional avidity of these cells is quite distinct from that of T cells immunized with non-TRICOM-containing vector, as shown in Figs. 5 and 6.

The mechanisms underlying the enhanced avidity of CTL by increased costimulation are not clear at the present time. Membrane compartmentalization between rafts and nonrafs is required.
for efficient T cell activation (46). It was reported that CD28 co-stimulation induced recruitment of Lck and lipid rafts as well as their accumulation at the immunological synapse (47, 48). Cawthon et al. (49) found that high avidity CTL colocalized substantially more TCR with CD8 compared with low avidity CTL. The ability of high avidity CTL to respond functionally to fewer TCR engagement events than low avidity CTL is directly related to integrating lipid rafts on their surfaces. Taken together, the results suggest that clustering of membrane and intracellular kinase-rich lipid rafts at the site of TCR engagements induced by costimulation may be attributed to the enhanced avidity of CTL observed in the present study.

Although the β-gal system used in this study is a well-established model Ag, we also conducted studies to support the observations using a self-Ag system. The β-gal system was used to define numbers and avidity of Ag-specific T cells because of the availability of β-gal-specific tetramer to identify specific T cells. CEA-Tg mice were used to define antitumor effects in a well-defined tumor system using a self-Ag. Using the CEA transgenic mouse bearing a CEA-expressing tumor, primary vaccination with rV-CEA/TRICOM and booster vaccinations with rF-CEA/TRICOM were clearly shown to be more efficacious than the use of a primary vaccination with rV-CEA/TRICOM and booster vaccinations with rF-CEA. Taken together, these studies support the observations described above using the β-gal Ag system that boosting with vaccines containing enhanced costimulation enhances the level of memory T cells, enhances the avidity of memory T cells, and can manifest itself in enhanced antitumor activity.

The studies reported also provide experimental data and support the current clinical protocols using CEA/TRICOM- and PSA/TRICOM-based vaccines in patients with advanced carcinomas. In a recently completed clinical trial described by Marshall et al. (50), patients with progressive metastatic disease were first vaccinated with rV-CEA/TRICOM and then given multiple booster vaccinations with rF-CEA/TRICOM. Although the primary end point of that trial was safety, 40% of patients were stable after 4 mo, and survival-up to 2 years for some patients appeared to correlate with CEA-specific T cell responses. There, 9 of 10 HLA-A2-positive patients demonstrated increased CEA-specific T cell levels after the three booster vaccinations, as determined by ELISPOT assay. Although it is not known whether the increased levels of CD8 T cells in these patients after vaccination expanded from a memory pool or arose from naïve T cells, the data presented in our study (Figs. 1, 3, 5, and 6) support the hypothesis that these T cells originated from both sources. Furthermore, the stabilization of disease appeared to be correlated with continuous boosts with TRICOM-containing CEA vaccines (50). Although the data presented in our study demonstrate that in the mouse model, memory T cells are maintained in both quantity and avidity by continuous boosting with TRICOM-containing vectors, additional vaccine strategies may be necessary to achieve this in humans with advanced stage malignancies.

An ongoing phase I/II trial in patients with metastatic prostate cancer involves primary vaccination with rV-PSA/TRICOM, followed by multiple booster vaccinations with rF-PSA/TRICOM, and has reported both clinical and biochemical (drops in serum prostate-specific Ag) responses (51, 52). Considering that high avidity CTL are essential for the elimination of tumor cells, these clinical trial data are consistent with the observations reported in this study that multiple boosts with vectors or cell-based vaccines containing relatively weak tumor Ags, but with enhanced costimulatory properties, may maintain and promote the avidity maturation of Ag-specific memory T cells.

In conclusion, our results clearly demonstrate that repeated vaccinations with vectors inducing enhanced costimulation not only induced naïve T cells into the effector CTL pool, but also expanded Ag-specific memory CD8 T cells. More importantly, memory CD8 T cells from mice boosted with these vaccines demonstrated greater functional avidity. The present study thus provides insight into the design of more efficient tumor vaccines.

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