Vaccines with Enhanced Costimulation Maintain High Avidity Memory CTL

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

J Immunol 2005; 175:3715-3723; doi: 10.4049/jimmunol.175.6.3715

References
This article cites 52 articles, 31 of which you can access for free at:
http://www.jimmunol.org/content/175/6/3715.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Vaccines with Enhanced Costimulation Maintain High Avidity Memory CTL

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

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 (Thyl.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 virus (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.


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
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/6j (C57BL/6) mice were obtained from the National Cancer Institute, Frederick Cancer Research Facility. Female Thy.1.1 mice (formerly called C57BL/6/P-L-Thy1+/Cy) were purchased from the Jackson Laboratory. This C57BL/6j congenic strain carries the T lymphocyte-specific Thy1+ (Thy.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, 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.

Marine 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 tumour cell line EL4 (H-2a, thymoma, ATCC TIB-39) was purchased from American Type Culture Collection and was maintained in RPMI 1640 complete medium.

**Recombinant viruses**

The recombinant vaccinia viruses 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 vaccine 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 (DAPIYTNV) (18), and OVA epitope (SINFEKL) 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 (Millex-LG, hydrophilic PTFE membrane; Millipore) 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, Thy.1.1, Thy.1.2, 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 Thy.1.1 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 (Thy.1.2) 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 C8D8+ T cell precursor frequency and avidity were determined immediately by intracellular IFN-γ staining as described below. All viruses were administered at 1 × 10^8 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, Thy.1.1 mice were vaccinated with rV-LacZ/TRICOM (1 × 10^6 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 depleting CD62L+CD2-Labeled cells (Miltenyi Biotec) as previously described (19). Purified Thy.1.1 memory T cells were washed twice, resuspended in PBS, and stained with surface markers as indicated in Fig. 2 before adoptive transfer to Thy.1.2 (C57BL/6) mice. All C57BL/6 (Thy.1.2) mice received the same number of Thy.1.1 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 Thy.1.1 memory T cells, mice were vaccinated with PBS, rF-TRICOM, rF-LacZ, or rF-LacZ/TRICOM (each at 1 × 10^6 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, Thy.1.1 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). Thy.1.1 T cells were purified from the pan-T cells by depleting Thy.1.2 T cells, using Thy.1.2 beads and AutoMACS (Miltenyi Biotec). Purified Thy.1.1 T cells were used for CTL induction and avidity titration using the cytolytic method (20, 21).

**FIGURE 1.** Immunization with TRICOM-based vaccines in naive mice induced high avidity Ag-specific CTL. A, C57BL/6 mice were vaccinated once with buffer (HBSS; ▼), rV-LacZ (▲), rV-LacZ/B7-1 (●), or rV-LacZ/TRICOM (▲). After 30 days, splenocytes were harvested. The β-gal-specific C8D8+ T cell precursor frequency and avidity were determined by intracellular IFN-γ staining. B, β-gal-specific C8D8+ T cell avidity, as determined by lytic assay. 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. CTL avidity was determined using lytic assay as described in Materials and Methods. □, rV-LacZ; ▲, rV-LacZ/TRICOM.
intracellular IFN-γ staining after coculture with B cells pulsed with graded concentrations of peptide. Avidity, expressed as MC₅₀ 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 cautery 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 × 10⁸ 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 × 10³ PFU of rF-CEA or rF-CEA/TRICOM, admixed with 1 × 10⁸ PFU IF-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 mounted 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 (CD62Llow/CD44high; Fig. 2). Ag-specific T cells (CD8+Tet+), as determined by β-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 μg/ml β-gal-peptide for 24 h, and IFN-γ 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-γ after peptide stimulation. Booster vaccinations with rF-LacZ increased IFN-γ production by Thy1.1 T cells by 2.77-fold compared with the PBS group (Fig. 3A, left panel). IFN-γ 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-γ 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.

FIGURE 3. Functional analysis of Ag-specific memory T cells after booster with rF-LacZ or rF-LacZ/TRICOM vaccines. Memory T cells were prepared and adoptively transferred as described in Materials and Methods and in Fig. 2. Four weeks after one booster vaccination with the indicated vaccine, T cells were isolated for functional assay. For IFN-γ production, purified memory Thy1.1 T cells (A, left panel) and endogenous Thy1.2 T cells (A, right panel) were stimulated with irradiated B cells pulsed with β-gal peptide (1 μg/ml) for 24 h. IFN-γ production was detected using a cytometric bead array as described in Materials and Methods. Note the difference in scale between the panels of A. B. For intracellular IFN-γ staining, purified pan-T cells were stimulated with irradiated B cells pulsed with control OVA peptide or β-gal peptide (1 μg/ml) for 6 h. At the end of the incubation, cells were stained with anti-CD8, Thy1.1, Thy1.2, and anti-IFN-γ Abs. IFN-γ-producing CD8+ T cells in gated Thy1.1+ and Thy1.2+ T cell populations were analyzed using CellQuest software. These data are representative of two similar experiments.
A

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

FIGURE 4. Effects of multiple booster vaccinations with rF-LacZ vs rF-LacZ/TRICOM on the expansion of Ag-specific memory T cells. Memory T cells were purified from rF-LacZ/TRICOM-vaccinated Thy1.1 mice as described in Materials and Methods and Fig. 2 and were adoptively transferred to C57BL/6 mice (Thy1.2). One week after adoptive transfer, mice were vaccinated three times with the indicated vaccines at 2-wk intervals. Five days after second and third vaccinations, three mice from each group were killed, and splenocytes were prepared for monitoring the expansion of CD8+β-gal tetramer+ T cells in the gated Thy1.1 T cell population.

FIGURE 5. The effect of repeated booster vaccinations on the expansion of Ag-specific memory CD8+ T cells and avidity maturation. Memory T cells (Thy1.1) were prepared and adoptively transferred to Thy1.2 mice as described in Materials and Methods and Fig. 2. Four weeks after three booster vaccinations at 2-wk intervals with either rF-LacZ or rF-LacZ/TRICOM, splenocytes were purified and stained with the indicated Abs for Ag-specific memory T cell expansion. Thy1.1 T cells were purified and stimulated in vitro with β-gal peptide for 5 days, and CTL avidity was titrated using a cytolytic assay. A, Thy1.1 and β-gal tetramer staining of splenocytes from mice boosted with rF-LacZ or rF-LacZ/TRICOM. B, Peptide titration of CTL avidity. C, Normalization of data in B for avidity calculation. These data are representative of two similar experiments.

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 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 × 10−9 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
FIGURE 6. CTL avidity titration in freshly isolated memory T cells after booster vaccinations with rF-LacZ or rF-LacZ/TRICOM using intracellular IFN-γ staining. Thy1.1 mice were vaccinated with rV-LacZ/TRICOM. Thy1.1+ memory T cells were prepared and adoptively transferred to Thy1.2 mice as described in Materials and Methods and Fig. 2. Four weeks after three booster vaccinations with either rF-LacZ or rF-LacZ/TRICOM at 2-wk intervals, Thy1.1+ T cells were purified as described in Materials and Methods and stimulated with irradiated B cells pulsed with graded concentrations of β-gal peptide for 6 h. Cells were then stained with anti-Thy1.1, CD8, and anti-IFN-γ Abs. A, Phenotypic analysis of intracellular IFN-γ staining using graded concentrations of β-gal peptide. B, Dose-response curve of data collected from A. C, Avidity titration curves via normalization of data (20, 21, 25) from B. These experiments were repeated twice with similar results.

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

FIGURE 7. Induction of antitumor responses in CEA transgenic (CEA-Tg) mice by recombinant poxviral vectors. CEA-Tg mice bearing 14-day established peripancreatic metastases were divided into three treatment groups. Tumors were transplanted by intrasplenic injection of MC-38 colon carcinoma cells that were transduced with CEA (day 0). Group 1 (n = 10; □) received an rV-CEA/TRICOM prime vaccination (T), followed by three weekly boosts with rF-CEA/TRICOM (T). Group 2 (n = 10; ○) received an rV-CEA/TRICOM prime vaccination, followed by three weekly boosts with rF-CEA/TRICOM (T). Group 3 (n = 10; □) received an HBSS prime vaccination, followed by three weekly boosts with HBSS. In groups 1 and 2, prime vaccinations were administered with rGM-CSF and low dose IL-2, and all booster vaccinations were admixed with rF-GM-CSF and low dose IL-2. Mice in each group were monitored weekly for survival.
tion of Th1-type cytokines by effector/memory CD4+ T cells. In the lung, proliferation and sensitization of memory CD4+ T cells in blood from chronic beryllium disease required CD28 costimulation for proliferative and cytokine responses to beryllium. It is thus clear that costimulation (especially signaling through CD28) promotes the proliferation and expansion of naive T cells. However, the effects of increased costimulatory signals on the expansion and survival of memory T cells have been controversial (6–8). Sabzevari et al. (8) reported that effector/memory CD4+ T cells from PCC TCR transgenic mice were more susceptible to apoptosis induced by APC expressing CD80 in the presence of high affinity cognate peptide compared with naive 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, specific 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+ 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 cells 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 rV-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 nonrafts is required.
for efficient T cell activation (46). It was reported that CD28 costimulation 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 rF-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-based 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.

Acknowledgments

We thank Debra Weingarten for her editorial assistance in the preparation of this manuscript.

Disclosures

S. Yang, J. W. Hodge, D. W. Grosenbach, and J. Schlom are all employees of the Laboratory of Tumor Immunology and Biology (LTIB) at the National Cancer Institute (NCI), National Institutes of Health. The LTIB, NCI has a Collaborative Research and Development Agreement (CRADA) with Theron Biologics, which provided the (noncommercial) vectors used.

References
