Multiple Costimulatory Modalities Enhance CTL Avidity

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Multiple Costimulatory Modalities Enhance CTL Avidity

James W. Hodge, Mala Chakraborty, Chie Kudo-Saito, Charlie T. Garnett, and Jeffrey Schlom

Recent studies in both animal models and clinical trials have demonstrated that the avidity of T cells is a major determinant of antitumor and antiviral immunity. In this study, we evaluated several different vaccine strategies for their ability to enhance both the quantity and avidity of CTL responses. CD8+ T cell quantity was measured by tetramer binding precursor frequency, and avidity was measured by both tetramer dissociation and quantitative cytolytic function. We have evaluated a peptide, a viral vector expressing the Ag transgene alone, with one costimulatory molecule (B7-1), and with three costimulatory molecules (B7-1, ICAM-1, and LFA-3), with anti-CTLA-4 mAb, with GM-CSF, and combinations of the above. We have evaluated these strategies in both a foreign Ag model using β-galactosidase as immunogen, and in a “self” Ag model, using carcinoembryonic Ag as immunogen in carcinoembryonic Ag transgenic mice. The combined use of several of these strategies was shown to enhance not only the quantity, but, to a greater magnitude, the avidity of T cells generated; a combination strategy is also shown to enhance antitumor effects. The results reported in this study thus demonstrate multiple strategies that can be used in both antitumor and antiviral vaccine settings to generate higher avidity host T cell responses. The Journal of Immunology, 2005, 174: 5994–6004.

The use of vaccines as therapeutic interventions for infectious diseases and cancer is in an active state of investigation. Numerous vaccine modalities are being evaluated in both preclinical models and in clinical trials. A principal evaluation of the efficiency of a therapeutic vaccine is its ability to generate Ag-specific T cell responses, with emphasis currently being placed on the level of generation of CTL. This has been evaluated for the most part by tetramer binding, ELISPOT, or other assays to monitor cytokine secretion by CTL, and by the ability of CTL to lyse target cells. Although the vast majority of studies have quantitatively evaluated these responses, few studies using different vaccines and vaccine strategies have evaluated or defined the actual avidity of the T cells generated.

As previously defined, (1–4) CTL that can recognize peptide/MHC only at high Ag density are termed low avidity CTL, while those that can recognize peptide/MHC at low densities are termed high avidity CTL. Studies have demonstrated that high avidity CTL are more effective in the elimination of tumor cells and viral clearance (1, 5–9). It has also been shown that when high avidity CTL are selected by tetramer-positive sorting of high TCR density, these higher avidity CTL can lyse targets more efficiently than their lower avidity counterparts (7, 10). Higher avidity CTL have also been selected from immunized mice by culturing heterogeneous splenic lymphocyte populations with low peptide densities (1). Although the methods above have been used to select for higher avidity CTL in heterogeneous T cell populations, only one report exists on a methodology of enhancing costimulation to enhance T cell avidity in vivo. In that study, Oh et al. (11) used peptide-pulsed splenocytes that overexpressed costimulatory molecules to vaccinate mice. The use of anti-CTLA-4 Ab to block the inhibitory role of CTLA-4 has also been hypothesized to select for higher affinity T cell clones (12, 13). However, to our knowledge, no experimental data thus far have been reported to support this hypothesis.

In this study, we have evaluated several different vaccines and vaccine strategies for their ability to enhance the quantity and avidity of CTL responses. CD8+ T cell quantity was measured by tetramer binding precursor frequency, and avidity was measured by both tetramer dissociation and cytolytic function. These methods were used to monitor T cell precursor frequency and avidity directly from fresh mouse splenocytes (tetramer binding and dissociation assay), as well as after a short in vitro restimulation (CTL assay). We have evaluated a peptide, a vector expressing the Ag transgene alone, with one costimulatory molecule (B7-1), and with three costimulatory molecules (B7-1, ICAM-1, and LFA-3, designated TRICOM), the use of anti-CTLA-4 mAb, the use of GM-CSF, and combinations of the above. We have evaluated these vaccines in both a nonself model using β-galactosidase (β-gal) 2 as the immunogen, and in a “self” model, using carcinoembryonic Ag (CEA) as the immunogen in CEA-transgenic (Tg) mice. Finally, using this model, we provide evidence that several of these strategies are complimentary not only in enhancing the quantity and avidity of T cells generated, but also in mediating antitumor effects in the absence of autoimmunity.

Materials and Methods

Animals

Female C57BL/6 mice were obtained from the National Cancer Institute, Frederick Cancer Research Facility. Female C57BL/6 mice Tg for human CEA have been previously described (14). Mice were housed and maintained under pathogen-free conditions in microisolator cages until used for experiments at 6–8 wk of age.

Tumor cells

For quantitative cytotoxicity assays, the target tumor cell line EL-4 (H-2b, thymoma, ATCC TIB-39) pulsed with different concentrations of peptide was used. For in vivo studies, murine colon adenocarcinoma MC38 cells (H-2b) expressing human CEA (designated MC38-CEA+) were used (15).

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2 Abbreviations used in this paper: β-gal, β-galactosidase; CEA, carcinoembryonic Ag; Tg, transgenic.

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Recombinant poxviruses

The recombinant vaccinia viruses designated rV-CEA (16) and rV-CEA/B7-1 (17) have been described. rV-CEA/TRICOM contains the murine B7-1, ICAM-1, and LFA-3 genes in combination with the human gene CEA as described elsewhere (18). The recombinant vaccinia viruses designated rV-LacZ, rV-LacZ/B7-1, and rV-LacZ/TRICOM were constructed in a similar manner, and contain the LacZ gene encoding β-gal. 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 elsewhere (18). The recombinant fowlpox containing the murine GM-CSF gene (designated rF-GM-CSF) has been described (19).

Peptides

The H-2Kb-restricted peptides CEA (CAP-M8, CEA526–533, EAQNTTYL (21)) and vesticular stomatitis virus nucleoprotein (NP45–59, RGYVYQGL (21)) were purchased from Synpep.

Novel Ag system.

C57BL/6 mice were vaccinated with buffer (HBSS), rV-CEA, rV-CEA/B7-1, or rV-CEA/TRICOM. In another experiment, mice were vaccinated with rV-CEA and administered anti-CTLA-4 mAb and/or rF-GM-CSF as described above.

Determination of CD8⁺ T cell precursor frequency

To evaluate the frequency of β-gal-specific CTL in C57BL/6 mice (n = 3 per group) after vaccination, splenocytes were stained with FITC-conjugated anti-CD3e mAb (BD Pharmingen), CyChrome-conjugated anti-CD8 mAb (BD Pharmingen), and human MHC class I tetramer (National Institutes of Health Tetrimer Facility, National Institutes of Allergy and Infectious Diseases). To evaluate the frequency of CEA-specific CTL in CEA-Tg mice after vaccination, splenocytes were stained with FITC-conjugated anti-CD3e mAb, CyChrome-conjugated anti-CD8 mAb, and PE-conjugated CEA/H-2Kb-tetramer (iTAG, Beckman Coulter). Immunofluorescence staining was performed after FcR blocking with anti-CD16/CD32 mAb (BD Pharmingen). The immunofluorescence was compared with the appropriate isotype-matched controls and analyzed with CellQuest software using a FACS Calibur cytometer (BD Biosciences). Results were depicted as percent CD8⁺/tetramer⁺ T cells of CD3⁺ T cells. Precursor frequency was also expressed as percent tetramer⁺ T cells of CD8⁺ T cells (Table I).

Determination of CD8⁺ T cell avidity: tetramer dissociation

Tetramer decay/dissociation was performed largely as described by Holmberg et al. (23) Briefly, splenocytes were stained with FITC-conjugated anti-CD3e mAb, CyChrome-conjugated anti-CD8 mAb and PE-conjugated-ed tetramer as described above. Cells were washed twice and kept on ice until they were mixed with appropriate excess anti-Kb or Db and then incubated at 37°C to allow tetramer dissociation. Dissociation was followed for 0–180 min, at which time the cells were fixed with Cytofix (BD Pharmingen). Results were expressed as the percentage of tetramer-positive cells over time. To normalize groups within each experiment, data were also expressed as the percentage of maximum tetramer binding over time. Finally, the natural logarithm of the normalized data was plotted against time. The dissociation half-life of each tetramer was derived from the slope of the natural logarithm, and was expressed in minutes.

Table I. Effect of multiple co stimulatory modalities to enhance CTL avidity

<table>
<thead>
<tr>
<th>Vaccine Modality</th>
<th>Precursor Frequency/10⁶ CD8 T Cells</th>
<th>Tetramer Dissociation Time (minutes)</th>
<th>Peptide Concentration for CTL (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer (HBSS)</td>
<td>800</td>
<td>ND</td>
<td>NA</td>
</tr>
<tr>
<td>β-Gal peptide/IFA</td>
<td>1,500</td>
<td>50</td>
<td>720</td>
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<tr>
<td>rV-LacZ</td>
<td>2,650</td>
<td>85</td>
<td>160 × 1</td>
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<tr>
<td>rV-LacZ/B7-1</td>
<td>3,800</td>
<td>127</td>
<td>27 × 6</td>
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<tr>
<td>rV-LacZ/TRICOM</td>
<td>5,900</td>
<td>300</td>
<td>3 × 53.3</td>
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<tr>
<td>rV-LacZ + isotype control mAb</td>
<td>2,424</td>
<td>70</td>
<td>ND</td>
</tr>
<tr>
<td>rV-LacZ + CTLA-4 mAb</td>
<td>4,191</td>
<td>90</td>
<td>ND</td>
</tr>
<tr>
<td>rV-LacZ/B7-1 + isotype control mAb</td>
<td>3,393</td>
<td>118</td>
<td>ND</td>
</tr>
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<td>5,709</td>
<td>235</td>
<td>ND</td>
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<td>rV-LacZ/TRICOM + CTLA-4 mAb</td>
<td>8,536</td>
<td>310</td>
<td>ND</td>
</tr>
<tr>
<td>rV-LacZ/TRICOM + GM-CSF</td>
<td>8,200</td>
<td>360</td>
<td>0.05 × 3,200</td>
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<tr>
<td>rV-LacZ/TRICOM + GM-CSF + CTLA-4 mAb</td>
<td>13,500</td>
<td>475</td>
<td>0.003 × 53,333</td>
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<tr>
<td>Buffer (HBSS)</td>
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<td>ND</td>
<td>NA</td>
</tr>
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<td>rV-CEA</td>
<td>321</td>
<td>88</td>
<td>510 × 1</td>
</tr>
<tr>
<td>rV-CEA/B7-1</td>
<td>584</td>
<td>135</td>
<td>110 × 4.6</td>
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<tr>
<td>rV-CEA/TRICOM</td>
<td>769</td>
<td>233</td>
<td>5 × 102</td>
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<tr>
<td>rV-CEA</td>
<td>455</td>
<td>70</td>
<td>950 × 1</td>
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<td>rV-CEA + CTLA-4 mAb</td>
<td>784</td>
<td>105</td>
<td>237 × 4</td>
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<tr>
<td>rV-CEA/B7-1</td>
<td>674</td>
<td>120</td>
<td>135 × 7</td>
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<tr>
<td>rV-CEA/TRICOM + CTLA-4 mAb</td>
<td>1,303</td>
<td>370</td>
<td>0.4 × 1,275</td>
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<tr>
<td>rV-CEA/TRICOM + GM-CSF</td>
<td>1,289</td>
<td>315</td>
<td>0.6 × 850</td>
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<tr>
<td>rV-CEA/TRICOM + GM-CSF + CTLA-4 mAb</td>
<td>1,690</td>
<td>480</td>
<td>0.02 × 25,500</td>
</tr>
</tbody>
</table>
Determination of CD8$^+$ T cell avidity: cytotoxicity assay

To evaluate the avidity of β-gal-specific CTL in C57BL/6 mice after vaccination, splenocytes were pooled and dispersed into single-cell suspensions, and then stimulated with β-gal peptide (10 μg/ml). To evaluate the frequency of CEA-specific CTL in CEA-Tg mice after vaccination, splenocytes were pooled and dispersed into single-cell suspensions, and then stimulated with CEA peptide (10 μg/ml). Six days later, bulk lymphocytes were separated by centrifugation through a Ficoll-Hypaque gradient. Using these recovered lymphocytes, tumor-killing activity was tested as described previously (16). Briefly, the recovered lymphocytes (2.4 × 10$^6$ cells/well) and 111In-labeled target tumor cells (EL-4, 3 × 10$^5$ cells/well) were cocultured at a constant E:T ratio (80:1) in the presence of diminishing concentrations of peptide (10–0 μM) for 5 h (96-well U-bottom plates), and radioactivity in supernatants was measured using a gamma-counter (Corbaplate; Packard Instruments). In a separate plate, lymphocytes and target cells (E:T 80:1) were coincubated with control peptides: OVA for β-gal and the vesicular stomatitis virus nucleoprotein for the CEA peptide. The percentage of tumor lysis was calculated as follows: percent tumor lysis = [(experimental cpm − spontaneous cpm)/(maximum cpm − spontaneous cpm)] × 100. Nonspecific 111In release in response to each control peptide was subtracted from that induced by the appropriate Ag peptide. The data were averaged and graphed as the difference in percent specific lysis. To normalize groups within each experiment, data were also expressed as percentage of maximum lysis vs peptide concentration. Finally, the natural logarithm of the normalized data was plotted against peptide concentration. The avidity of each T cell population was defined as the negative log of the peptide concentration that resulted in 50% maximal target lysis (5) and was expressed in nanomoles.

Statistical analysis

Significant differences were statistically evaluated using ANOVA with repeated measures using Statview 4.1 (Abacus Concepts). Correlation coefficients were determined using Statview. Evaluation of survival patterns in mice bearing MC38-CEA$^+$ tumors was performed by the Kaplan-Meier method.

Results

Peptide vs vector

Studies were conducted in the β-gal model to compare the ability of β-gal peptide vs rV-LacZ to generate T cell responses of different or similar avidities. As seen in Fig. 1A, rV-LacZ enhanced tetramer-positive CD8$^+$ T cells in vaccinated mice by ~2-fold compared with peptide-vaccinated mice. This was accompanied by a slight increase in tetramer dissociation time in the rV-LacZ

![Image](http://www.jimmunol.org/Downloaded from http://www.jimmunol.org/)
group. Furthermore, lysis was enhanced 4-fold in rV-LacZ-vaccinated mice (Fig. 1, B and C, □ vs ■, and Table I).

**Vector-driven costimulation**

We next compared CTL responses in mice vaccinated with rV-LacZ, rV-LacZ/B7-1, and rV-LacZ/TRICOM (rV-LacZ/B7-1/ICAM-1/LFA-3). We have previously shown, using other models and T cell proliferation assays, that vectors containing three costimulatory molecules are more efficient than vectors devoid of, or containing one or two costimulatory molecules in generating greater quantities of T cells (18). This is extended in this study with the analysis of tetramer binding T cells; there is a modest increase in the number of tetramer binding T cells with more costimulation (Fig. 1A). However, profound differences are seen when one compares tetramer dissociation (Fig. 1B) and quantitative lytic activity (Fig. 1C) of rV-LacZ and rV-LacZ/TRICOM. Indeed, while there is ~2-fold increase in precursor frequency among the three vectors, there is a 6-fold increase in avidity in rV-LacZ vs rV-LacZ/B7-1, and a 53-fold increase in avidity comparing rV-LacZ with rV-LacZ/TRICOM (Fig. 1, A–C, and Table I). One could speculate that the in vitro stimulation of T cells with peptide-pulsed APC, required to obtain enough cells for cytolytic titrations, could skew the population toward higher or lower avidity T cells. To control for this, we analyzed tetramer dissociation from T cells directly obtained from spleens and after the 1 wk in vitro stimulation. Although there was, as expected, an expansion of tetramer-positive T cells after in vitro stimulation (Fig. 2, A and B), there was no difference observed in tetramer dissociation before or after in vitro stimulation (Fig. 2, C and D).

Although β-gal is a well-established experimental foreign Ag, we then sought to determine whether similar findings would be obtained with the use of a weaker Ag in a “self” system. To this end, studies were performed with recombinant vector constructs containing the CEA transgene in CEA-Tg mice, in which CEA is expressed as a self Ag in fetal and normal gastrointestinal tissues (14, 24). Tetramer-positive T cells from splenocytes of immunized mice increased ~2-fold with the use of rV-CEA/B7-1 or rV-CEA/TRICOM vs rV-CEA (Fig. 1D). However, tetramer dissociation increased substantially, from 88 min with the use of rV-CEA, to 135 min for rV-CEA/B7-1, to 233 min with the use of rV-CEA/TRICOM as immunogen (Fig. 1E). Moreover, this was accompanied by a 100-fold increase in functional avidity (510 nM for rV-CEA, to 110 nM for rV-CEA/B7-1, to 5 nM for rV-CEA/TRICOM) (Fig. 1F and Table I).

**Effect of positive vs negative costimulatory regulation on T cell avidity**

Anti-CTLA-4 Ab has been shown to have antitumor effects in several models using moderately antigenic and highly immunogenic tumors (13, 25). However, the growth of poorly immunogenic tumors is not profoundly influenced by the sole use of anti-CTLA-4 mAb (26). Studies were first conducted here to define how to use anti-CTLA-4 Ab with a live recombinant viral vector. These studies revealed that administering anti-CTLA-4 mAb at the same time as rV-LacZ/TRICOM (day 0) was superior to administering it on day 3, −3, or −6 in relation to vaccine, in terms of inducing enhanced β-gal-specific IFN-γ production (rV-LacZ/TRICOM, 1900 pg/ml/24 h vs rV-LacZ/TRICOM plus anti-CTLA-4 mAb, 3400 pg/ml/24 h) and lysis by CD8+ T cells.

Using the optimal dose scheduling, we then compared positive costimulation (rV-LacZ/B7-1) vs negative costimulatory signal regulation (rV-LacZ plus anti-CTLA-4), using rV-LacZ as a control. Both vaccination strategies elicited slightly higher tetramer-positive T cells in vaccinated mice than the use of rV-LacZ alone (Fig. 3A). Vaccination with rV-LacZ/B7-1 resulted in T cells with a slightly higher tetramer dissociation constant than vaccination with rV-LacZ plus anti-CTLA-4 mAb (127 vs 90 min) (Fig. 3C and Table I).

We next evaluated the manipulation of positive vs negative costimulatory signals in the CEA self-Ag system. Vaccination of mice with rV-CEA/B7-1, or rV-CEA plus anti-CTLA-4 mAb, gave T cells with similar tetramer-positive precursor frequencies, and those frequencies were only slightly higher than those of mice vaccinated with rV-CEA (Fig. 3B). Tetramer dissociation was also slightly higher for T cells from mice vaccinated with rV-CEA/B7-1 than T cells from mice receiving rV-CEA plus anti-CTLA-4 mAb, and both dissociations were greater than T cells from rV-CEA-vaccinated mice (Fig. 1D). However, this was accompanied by clear differences in lytic ability between the groups (135 nM for rV-CEA/B7-1, 237 nM for rV-CEA plus anti-CTLA-4 mAb, and 950 nM for rV-CEA; Table I).

**Combined use of positive and negative costimulatory strategies for optimal T cell avidity**

We then asked whether the combined use of positive and negative costimulatory strategies would enhance T cell avidity. Toward this goal, mice were vaccinated with rV-LacZ/TRICOM ± anti-CTLA-4 mAb. As seen in Fig. 4A, there was an increase in tetramer-positive T cells with the addition of anti-CTLA-4 mAb to rV-LacZ/TRICOM. There was no effect of isotype control mAb on any parameters measured (Table I). Moreover, there was a clear advantage in the use of the combination of rV-LacZ/TRICOM plus anti-CTLA-4 in terms of both tetramer dissociation (Fig. 4B) and quantitative lytic activity (Fig. 4C). Similar findings were seen in the self-Ag system. Although there was less than a 2-fold increase.
in CEA-specific tetramer-positive T cells from mice vaccinated with rV-CEA/TRICOM vs rV-CEA/TRICOM plus anti-CTLA-4 (Fig. 4D), there was a profound difference in tetramer dissociation (Fig. 4E), and a 10-fold difference in functional avidity in T cells receiving both rV-CEA/TRICOM and anti-CTLA-4 vs rV-CEA/TRICOM alone (Fig. 4F and Table I).

GM-CSF
GM-CSF has been used in numerous preclinical and clinical studies to enhance vaccine efficacy by recruiting DC to regional nodes and thus increasing the potency of regional APC. Although numerous studies have determined that greater quantities of Ag-specific T cells can be generated by using GM-CSF (recombinant protein or vector-driven rF-GM-CSF) with vaccine, (27, 28) none has evaluated whether there is any effect of GM-CSF on the avidity of those T cells. Ahlers et al. (28) have reported that when mice received peptide vaccines in combination with multiple cytokines, including GM-CSF, IL-12, and TNF-α, optimal induction of CTL was achieved, although the avidity of the CTL elicited by the peptide vaccination was not directly affected by the exogenous cytokine administration. To examine the effect of GM-CSF with poxvirus vaccines, mice were thus vaccinated with rV-LacZ/TRICOM ± rF-GM-CSF. As seen in Fig. 5A, a slight increase in tetramer binding precursor frequency is observed. However, there is a substantial increase in tetramer dissociation (360 vs 300 min; Fig. 5B) and a 60-fold increase in lytic ability (0.05 vs 3 nM; Fig. 5C and Table I) in the use of rV-LacZ/TRICOM plus GM-CSF as compared with rV-LacZ/TRICOM alone. Similar results were seen in the self Ag system. Only a slight increase in tetramer-positive T cells was seen with the addition of GM-CSF to rV-CEA/TRICOM (Fig. 5D). However, tetramer dissociation increased from 233 to 315 min with the addition of GM-CSF (Fig. 5E), and lysis increased 10-fold with the addition of GM-CSF to rV-CEA/TRICOM in vaccination (Fig. 5F and Table I).

Combined costimulatory modalities
Finally we asked whether the combined use of rV-LacZ/TRICOM, anti-CTLA-4 mAb, and rF-GM-CSF would result in a still higher avidity T cell response. Compared with the use of rV-LacZ/TRICOM alone, a 2-fold increase in tetramer-positive precursor frequency is seen with the addition of anti-CTLA-4 Ab and rF-GM-CSF (Fig. 6A). However, there is a substantial increase in tetramer dissociation (300 min for rV-LacZ/TRICOM vs 475 min for the triple combination) (Fig. 6B). This is associated with a 1000-fold increase in functional avidity of resulting CTL when comparing vaccination with rV-LacZ/TRICOM (3.0 nM) vs vaccination with rV-LacZ/TRICOM plus rF-GM-CSF plus anti-CTLA-4 (0.003 nM) (Fig. 6C and Table I).

If one evaluates the various modalities used to enhance T cell costimulation, these studies demonstrate a 5.1-fold increase in tetramer-positive T cells in the use of rV-LacZ as a vaccine vs vaccination with rV-LacZ/TRICOM plus rF-GM-CSF plus anti-CTLA-4 (Table I). However, if one evaluates T cell avidity, these differences are greatly magnified. Dissociation of tetramer-positive
T cells increases from 85 min for mice vaccinated with rV-LacZ to 475 min for mice vaccinated with rV-LacZ/TRICOM (○) or rV-LacZ/TRICOM in combination with anti-CTLA-4 mAb (○). After 30 days, splenocytes were harvested. A, β-gal-specific CD8⁺ T cell precursor frequency as determined by tetramer staining. Inset number indicates percent β-gal tetramer⁺/CD8⁺ T cells of CD3⁺ T cells. B, β-gal-specific CD8⁺ T cell avidity as determined by tetramer dissociation. Inset depicts results that are normalized as the percentage of maximum tetramer binding. C, β-gal-specific CD8⁺ T cell avidity as determined by CTL assay. Inset depicts results that are normalized as the percentage of maximum lysis. D–F, CEA-Tg mice were vaccinated with rV-CEA/TRICOM (○) or rV-CEA/TRICOM in combination with anti-CTLA-4 mAb (○). After 30 days, splenocytes were harvested. D, CEA-specific CD8⁺ T cell precursor frequency as determined by tetramer staining. Inset number indicates percent CEA tetramer⁺/CD8⁺ T cells of CD3⁺ T cells. E, CEA-specific CD8⁺ T cell avidity as determined by tetramer dissociation. Inset depicts results that are normalized as the percentage of maximum tetramer binding. F, CEA-specific CD8⁺ T cell avidity as determined by CTL assay. Inset depicts results that are normalized as the percentage of maximum lysis.

FIGURE 4. Contribution of anti-CTLA-4 mAb to TRICOM costimulation on Ag-specific T cell precursor frequency and T cell avidity. A–C, C57BL/6 mice were vaccinated with rV-LacZ/TRICOM (●) or rV-LacZ/TRICOM in combination with anti-CTLA-4 mAb (○). After 30 days, splenocytes were harvested. A, β-gal specific CD8⁺ T cell precursor frequency as determined by tetramer staining. Inset number indicates percent β-gal tetramer⁺/CD8⁺ T cells of CD3⁺ T cells. B, β-gal-specific CD8⁺ T cell avidity as determined by tetramer dissociation. Inset depicts results that are normalized as the percentage of maximum tetramer binding. C, β-gal-specific CD8⁺ T cell avidity as determined by CTL assay. Inset depicts results that are normalized as the percentage of maximum lysis. D–F, CEA-Tg mice were vaccinated with rV-CEA/TRICOM (○) or rV-CEA/TRICOM in combination with anti-CTLA-4 mAb (○). After 30 days, splenocytes were harvested. D, CEA-specific CD8⁺ T cell precursor frequency as determined by tetramer staining. Inset number indicates percent CEA tetramer⁺/CD8⁺ T cells of CD3⁺ T cells. E, CEA-specific CD8⁺ T cell avidity as determined by tetramer dissociation. Inset depicts results that are normalized as the percentage of maximum tetramer binding. F, CEA-specific CD8⁺ T cell avidity as determined by CTL assay. Inset depicts results that are normalized as the percentage of maximum lysis.
would result in any advantage in antitumor activity. A stringent CEA-Tg mouse model was used for these studies, one in which we have previously shown that CEA/TRICOM vaccine alone has no significant antitumor effect (29). As can be seen in Fig. 7, 40% (4 of 10) of the mice receiving the CEA/TRICOM vaccine regimen with rF-GM-CSF and anti-CTLA-4 mAb (Fig. 7, F) remained alive and apparently healthy through the 22-wk observation period. However, only 10% (1 of 10) of the mice that received the vaccine regimen with rF-GM-CSF (Fig. 7, E) survived past 6 wk (P < 0.04). None of the mice that received no therapy (Fig. 7, D) or anti-CTLA-4 mAb alone (Fig. 7, C) survived past 6 wk. When comparing the tumor volumes of mice 28 days after tumor transplant (inset), 7 of 10 mice receiving the vaccine regimen with rF-GM-CSF and CTLA-4 mAb had tumor volumes <400 mm³, while only 2 of 10 mice that received the vaccine regimen with rF-GM-CSF, and 1 of 10 that received anti-CTLA-4 mAb alone, had tumor volumes <400 mm³.

Because CEA is expressed in normal gastrointestinal epithelium in CEA-Tg mice at levels similar to those in humans, and CEA protein is found in the serum of CEA-Tg mice at levels seen in human colon carcinoma patients, studies were undertaken to determine whether there was any evidence of autoimmunity observed in mice cured of their tumor following the triple modality strategy (CEA/TRICOM plus GM-CSF plus anti-CTLA-4 mAb) shown in Fig. 7. Mice cured of tumor were followed for 5 mo; nonvaccinated age-matched controls were also used. There were no differences evident in any symptoms in either group, including body weight (vaccine group average weight at day 150 was 25 ± 2 g vs 24.3 ± 2 g for the age-matched control group). Vaccinated mice developed anti-CEA, anti-vaccinia virus, and anti-fowlpox Ig responses. However, vaccinated and cured mice did not develop any detectable Abs to nuclear ribonucleic protein, histone, dsDNA or ssDNA, SCL-70

FIGURE 5. Contribution of GM-CSF to TRICOM-mediated costimulation on Ag-specific T cell precursor frequency and T cell avidity. A–C, C57BL/6 mice were vaccinated with rV-LacZ/TRICOM (●) or rV-LacZ/TRICOM in combination with rF-GM-CSF (○). After 30 days, splenocytes were harvested. A, β-gal-specific CD8⁺ T cell precursor frequency as determined by tetramer staining. Inset number indicates percent β-gal tetramer+/CD8⁺ T cells of CD3⁺ T cells. B, β-gal-specific CD8⁺ T cell avidity as determined by tetramer dissociation. Inset depicts results that are normalized as the percentage of maximum tetramer binding. C, β-gal-specific CD8⁺ T cell avidity as determined by CTL assay. Inset depicts results that are normalized as the percentage of maximum lysis. D–F, CEA-Tg mice were vaccinated with rV-CEA/TRICOM (●) or rV-CEA/TRICOM in combination with rF-GM-CSF (○). After 30 days, splenocytes were harvested. D, CEA-specific CD8⁺ T cell precursor frequency as determined by tetramer staining. Inset number indicates percent CEA tetramer+/CD8⁺ T cells of CD3⁺ T cells. E, CEA-specific CD8⁺ T cell avidity as determined by tetramer dissociation. Inset depicts results that are normalized as the percentage of maximum tetramer binding. F, CEA-specific CD8⁺ T cell avidity as determined by CTL assay. Inset depicts results that are normalized as the percentage of maximum lysis.
(DNA topoisomerase 1), and no circulating immune complexes were observed. Thus, no evidence of autoimmunity was observed in mice that received the CEA/TRICOM vaccine regimen with rF-GM-CSF and anti-CTLA-4 mAb.

**Discussion**

Recent studies in both animal models and clinical trials have demonstrated that functional T cell avidity is a major determinant for antiviral and antitumor immunity (30–33). Therefore, vaccine approaches to tumor or viral immunotherapy should focus on modalities that can selectively induce and expand 1) greater levels of tumor Ag-specific T cell precursor frequencies and, more importantly, 2) higher avidity T cells. Two major methods have been proposed to analyze avidity of T cells; tetramer dissociation and lysis of target cells pulsed with different peptide concentrations. However, there is contradiction in the literature as to whether these two methods always coincide (23, 34–38). Recent studies (39) have also suggested that CD107A may be a marker for high avidity human T cells, but no corresponding marker has been identified for murine cells. Although several studies have shown how to select for high avidity T cells from vaccinated mice or patients, few studies have demonstrated a method of inducing higher avidity T cells in vivo. In those studies, this has mainly been accomplished by altering the amino acid sequence of the test Ag (1, 5–9, 40–42). In only one previous study has costimulation been used to enhance avidity; in that study (11), B cells pulsed with peptide and overexpressing costimulatory molecules were shown to induce higher avidity T cells than uninfected peptide-pulsed B cells. In the studies reported in this paper, we have used seven different vaccine strategies...
and two independent methods to measure avidity. Moreover, we have compared results in a foreign and a self-Ag system.

Numerous preclinical studies have demonstrated antitumor effects in some rodent models with the use of anti-CTLA-4 Ab (26, 43–48). Several theories have been put forth (12, 13) as to the role of action of GM-CSF with vaccine in an antitumor setting. The studies reported in this paper are the first to show that the administration of GM-CSF with vaccine will actually increase the avidity of Ag-specific T cells (Figs. 5 and 6 and Table I).

We show in this study that the avidity of T cells for a specific β-gal epitope was enhanced by the use of a rV-LacZ vs peptide in adjuvant (Fig. 1 and Table I). This result could be attributable to at least three factors: 1) helper epitopes in the LacZ gene delivered by rV-LacZ, 2) nonspecific help by the vaccinia virus proteins, and 3) better cytosolic expression and presentation of the epitope by the recombinant vaccinia virus. However, using the same recombinant poxvirus vector, additional studies showed the avidity of T cells produced by vaccination with rV-LacZ/TRICOM was greater than that of rV-LacZ/B7-1, which was greater than rV-LacZ (Fig. 1 and Table I).

It is interesting to note that the differences observed in enhancing precursor frequency or avidity using the different vaccine strategies were quite similar for the nonself (LacZ) and self (CEA) systems. However, there were both greater numbers of T cells and more avid T cells seen for each vaccine using the foreign Ag LacZ model compared with the CEA self model. For example, rV-LacZ vaccination resulted in a precursor frequency of 2,650 tetramer-positive cells/10^5 CD8 cells, while rV-CEA vaccination resulted in 321 tetramer-positive cells/10^5 CD8 cells (Table I). Likewise, the combined vaccination regimen of rV-LacZ/TRICOM plus GM-CSF plus anti-CTLA-4 precursor frequency was 13,500 tetramer-positive cells/10^5 CD8 cells vs 1,690 frequency for the CEA-based combination therapy. Quantitative cytolytic avidities were always stronger for the nonself Ag: 160 nM for rV-LacZ vs 510 nM for rV-CEA. Furthermore, using the TRICOM/GM-CSF/anti-CTLA-4 strategy, cytolytic avidity was ~10-fold greater in the nonself than the self system (0.003 for the LacZ vaccinations vs 0.02 for the CEA vaccinations, Table I).

The vaccine strategies used in this study are all currently being evaluated clinically as single agents or in combination with a second agent. CEA/TRICOM vaccines and PSA/TRICOM vaccines (±GM-CSF) are currently in clinical trials in patients with advanced carcinoma (Refs. 58–61 from www.clinicaltrials.gov) These phase I studies are providing preliminary evidence of objective clinical response, drops in tumor markers, and increased survival (62, 63). Anti-CTLA-4 has been in clinical trials with a peptide vaccine in melanoma with indications of antitumor activity accompanied by severe autoimmunity (64). Perhaps the use of lower doses of anti-CTLA-4 with more potent TRICOM vectors will achieve antitumor activity with less severe autoimmunity.

The studies reported in this paper also provide evidence that multiple strategies can be used in combination to enhance T cell avidity; to our knowledge, these are the first studies to demonstrate such a phenomenon. These studies also demonstrate that the difference between success and failure in an antitumor situation can hinge on just such additive effects. It should be pointed out that similar strategies can be used in the development of vaccines directed against many viral-mediated diseases, either in the prevention or therapeutic setting.

Unfortunately, many therapeutic anticancer and antiviral vaccine regimens to date have used one or at most two vaccine strategies. The strategies reported in this study, alone or in combination, do not require the use of costly and labor-intensive ex vivo manipulation and/or expansion of dendritic cells or T cells, and can readily be used as prevailed reagents. Hopefully, the studies reported in this paper and by others will provide the proof of concept to institute more sophisticated and rigorous vaccine strategies using such multiple modalities.
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