Costimulatory Molecules as Adjuvants for Immunotherapy

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1. ABSTRACT

Tumor-associated antigens (TAAs) are by definition either weakly immunogenic or functionally nonimmunogenic. Therefore, efforts have concentrated on the development of vaccine strategies in which the presentation of TAAs to the immune system results in far greater activation of T cells than that occurring naturally in the host. Several strategies are being explored in our laboratory and others to enhance the immunogenicity of TAAs. These are: (a) placing the gene coding for the tumor antigen, as a transgene, into poxvirus vectors. (b) The use of diversified prime and boost vaccine strategies employing two different types of poxvirus vectors. (c) The use of T-cell costimulation; accomplished by placing transgenes for different T-cell costimulation molecules into viral vectors along with the transgenes for the TAA. (d) Altering the amino acid sequence of the TAA to enhance the host immune response. (e) The use of cytokines, and in particular GM-CSF, as a biologic adjuvant. This review will focus on the current state of the use of costimulatory molecules as adjuvants for immunotherapy, and in particular, as immunomodulators for cancer vaccines.

2. COSTIMULATORY MOLECULES: SIGNAL 2

When a naive T cell encounters antigen, several distinct outcomes are possible, including proliferation, cytokine secretion, differentiation into effector cells, inactivation, death, and unresponsiveness (anergy). The predominant outcome under physiologic conditions may be determined based on which (if any) appropriate costimulatory signals are delivered to the responding T cell. The initiation of an immune response requires multiple signals for the activation of naive T cells by antigen presenting cells (APC) (Figure 1). The first signal is antigen specific, delivered through the T-cell receptor (TCR) via the peptide/MHC, and causes the T cell to enter the cell cycle. The second, or "costimulatory," signal(s) is required for T cell cytokine production and proliferation. Several distinct molecules normally found on the surface of professional APC have been reported as capable of providing the second signals critical for T-cell activation (Figure 2). Among these are genes within the B7 family (B7-1, B7-2, B7-H1, B7-H3, B7-DC), and genes within the TNF-family (CD70) (1). Molecules typically associated with adhesion, such as ICAM-1 and LFA-3, have also
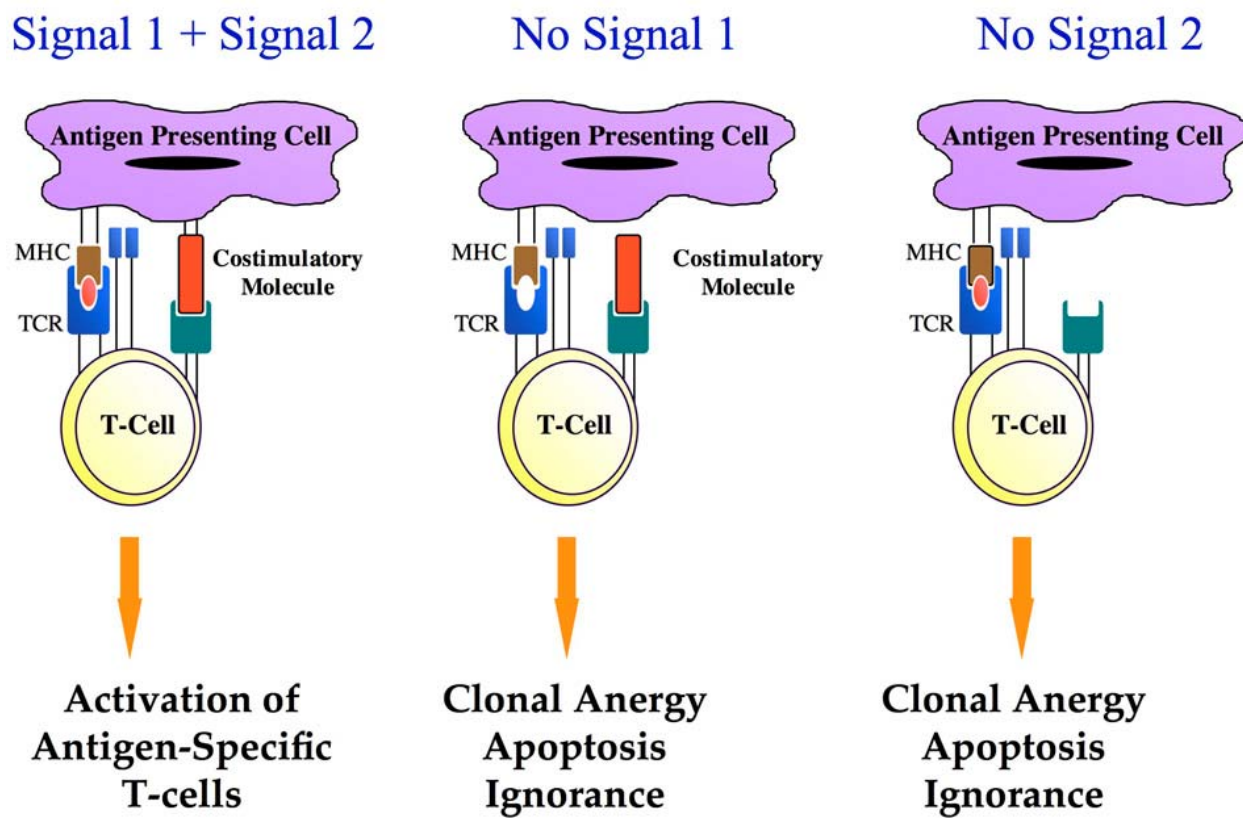
Figure 1. T-cell dependence on costimulation: Two-signal model. Reproduced with permission from (54).

shown their ability to act as classical costimulatory molecules through activation of signal transduction pathways (2). More recently, classes of molecules have been identified on the surface of APC that modulate apoptosis of the activated T cell in addition to contributing to costimulation. These 'signal 3' molecules include OX40L, 41BBL, and CD40L (1).

T-cell costimulation in the context of vaccines has now been shown to be delivered via several modalities and delivery systems (i.e., recombinant retroviral vectors, recombinant poxviral vectors, and anti-CTLA-4 antibodies) to enhance anti-tumor immunity in experimental models and clinical studies. In this article, we will deal principally with the use of recombinant poxvirus vectors to deliver an array of costimulatory molecules via direct vaccination and by intratumoral administration.

3. RECOMBINANT VACCINE VECTORS

Our laboratory has concentrated on three human carcinoma TAAs as vaccine targets: (a) carcinoembryonic antigen (CEA), (b) MUC-1, a pan-carcinoma mucin, and (c) prostate-specific antigen (PSA). While CEA and MUC-1 are characterized as being overexpressed in tumors versus adult normal tissues, PSA is characterized as a tissue-specific antigen expressed in normal prostatic epithelium as

well as in prostatic carcinoma. This article, however, will describe preclinical and clinical studies on the use of CEA as a target antigen. Similar studies are ongoing employing vaccines encoding genes for PSA and MUC-1 as vaccine targets.

CEA is an 180,000d glycoprotein. It is a homoadhesion molecule, which has been implicated in the metastatic process. CEA is overexpressed on the vast majority (> 95%) of human colorectal, gastric and pancreatic carcinomas. It is also overexpressed on approximately 50% of mammary carcinomas, 70% of non-small cell lung carcinomas, the majority of head and neck squamous cell carcinomas, as well as other carcinomas. It is extensively expressed in fetal gut and in much lower levels, as compared with tumor tissue, in normal colonic mucosa. This differential expression may constitute a "therapeutic window" for the use of vaccines directed against CEA. As will be detailed below, moreover, there has been no evidence of autoimmunity thus far in clinical studies employing CEA-based vaccines.

Preclinical and clinical studies have employed two types of recombinant poxvirus-based vectors: vaccinia and avipox. Vaccinia has been used in the worldwide eradication of smallpox and has been administered to more than 1 billion people. Figure 3 describes the schema in
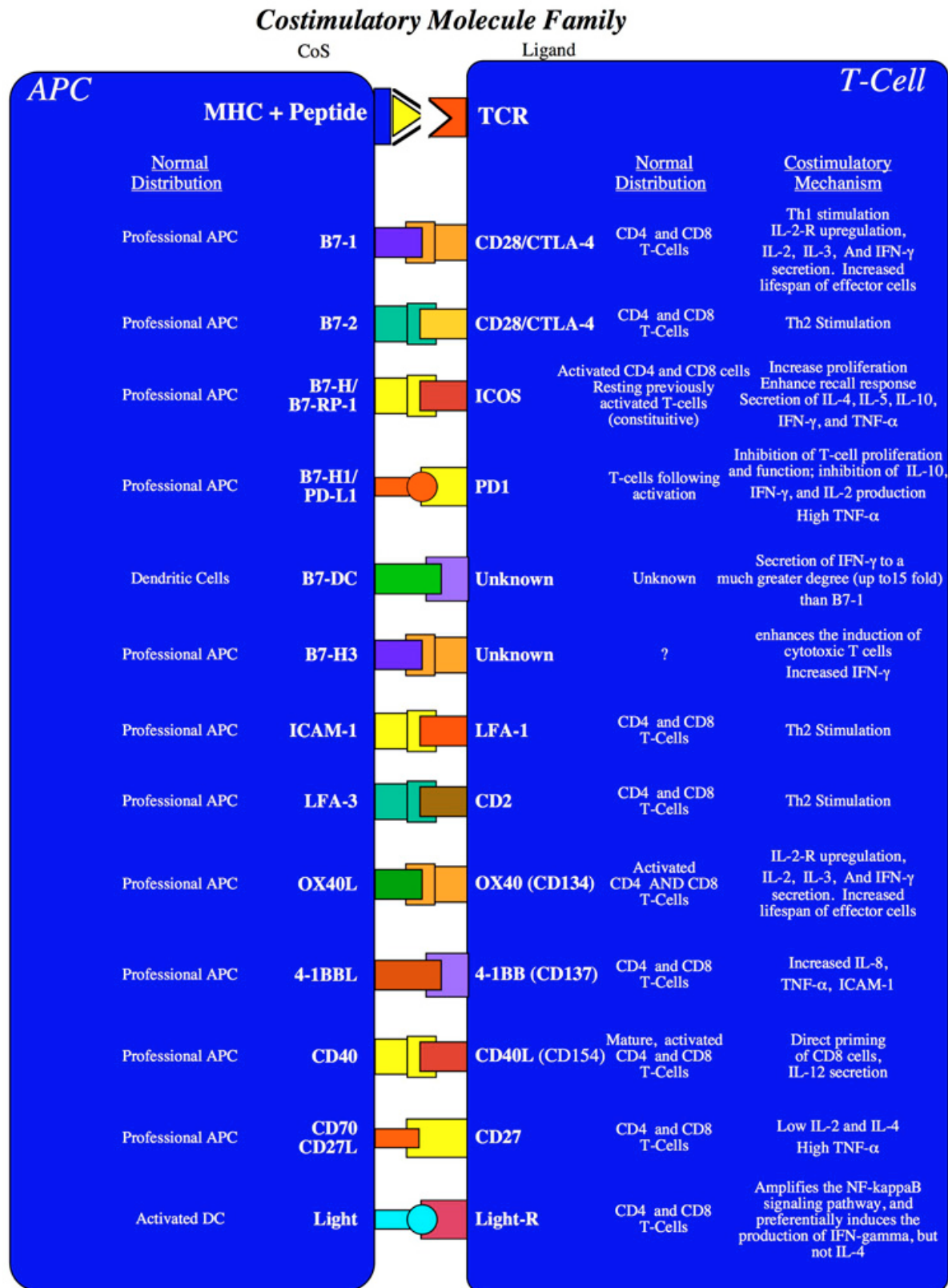
Costimulatory Molecule Family

Figure 2. Costimulatory molecule family. Depicted are costimulatory molecules reported to have a positive influence on T-cell activity.
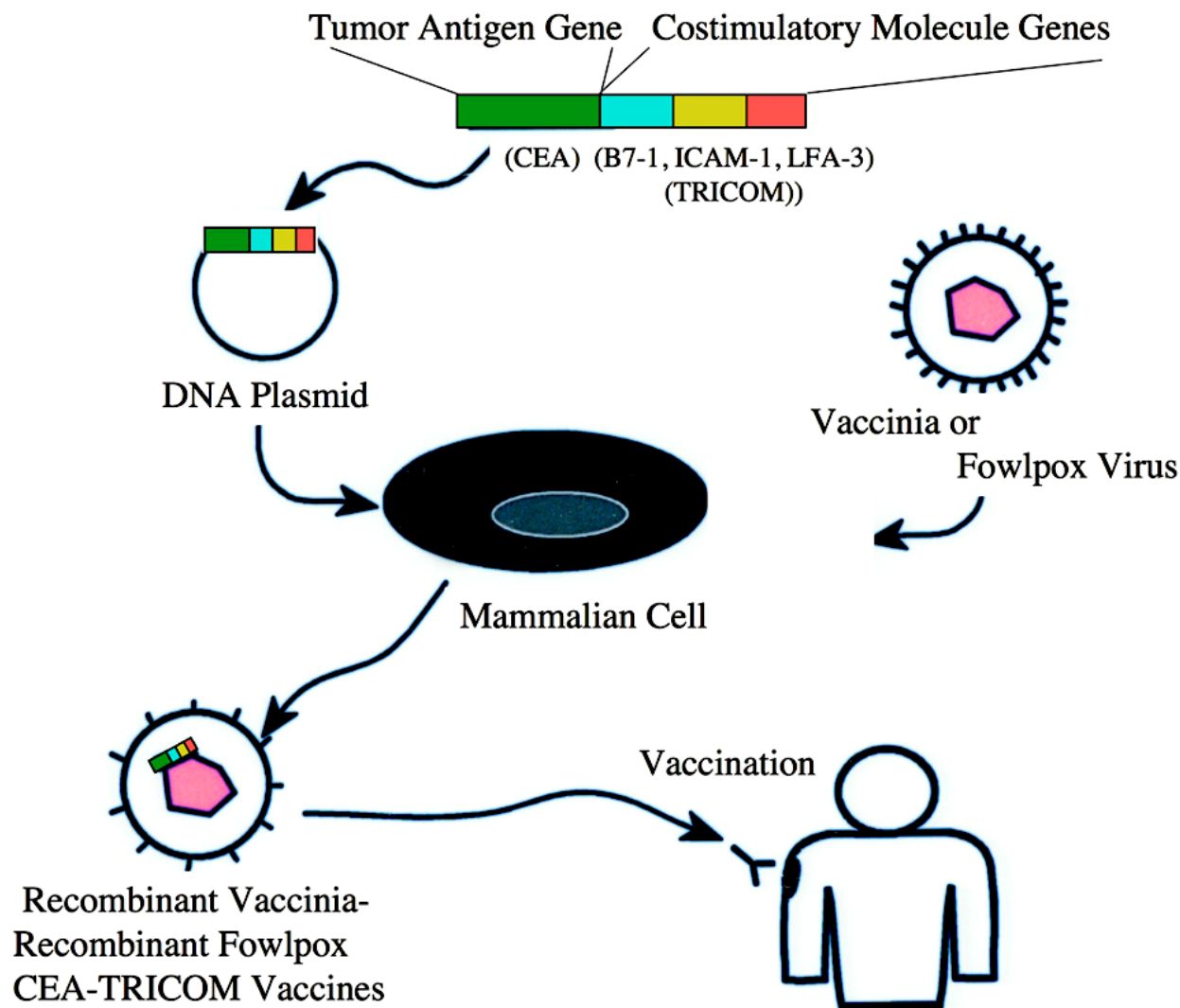
Figure 3. Generation of a recombinant poxviral (vaccinia or fowlpox) vector containing genes for tumor antigen and costimulatory molecules.

which recombinant poxvirus vectors are engineered for clinical use. Preclinical studies have demonstrated that when a TAA transgene is placed into the vaccinia genome, its expression leads to a more vigorous host T-cell immune response to the TAA transgene than would be achieved otherwise. This is most likely due to the acute inflammatory response directed against the highly immunogenic vaccinia proteins, which in turn creates a pro-inflammatory environment of cytokines, chemokines, effector cells, etc., at the vaccination site. An example of this was demonstrated by the administration of CEA protein multiple times to mice transgenic (Tg) for the human CEA gene (these CEA transgenic mice express CEA as a self antigen in fetal and normal GI tissues at levels similar to that seen in humans); no T-cell response is elicited to the CEA protein. However, when the CEA transgene was expressed via a recombinant vaccinia construct (i.e., rV-CEA), a vigorous T-cell response was observed in the CEA-Tg mouse (3). While these and many

other studies demonstrate the advantage of using recombinant vaccinia as a vaccine vector, its repeated use is limited by the same property that defines its efficacy as a priming agent. The vigorous host immune response to the vaccinia proteins limits the ability of vaccinia to replicate when given multiple times and thus limits the expression of the transgene. Both preclinical and clinical studies have now demonstrated that recombinant vaccinia virus can elicit a strong immune response in hosts who have previously received a smallpox vaccine. This has been shown to be attributable to the fact that the recombinant vaccinia vaccines are administered in approximately 2 logs higher concentration than that of the smallpox vaccine (4).

Preclinical (5) and now clinical (6) studies have demonstrated that recombinant avipox vectors can be used efficiently as boosts to vaccinations following a priming vaccination with a recombinant vaccinia vaccine. There are two major types of avipox vectors: fowlpox (rF-) and
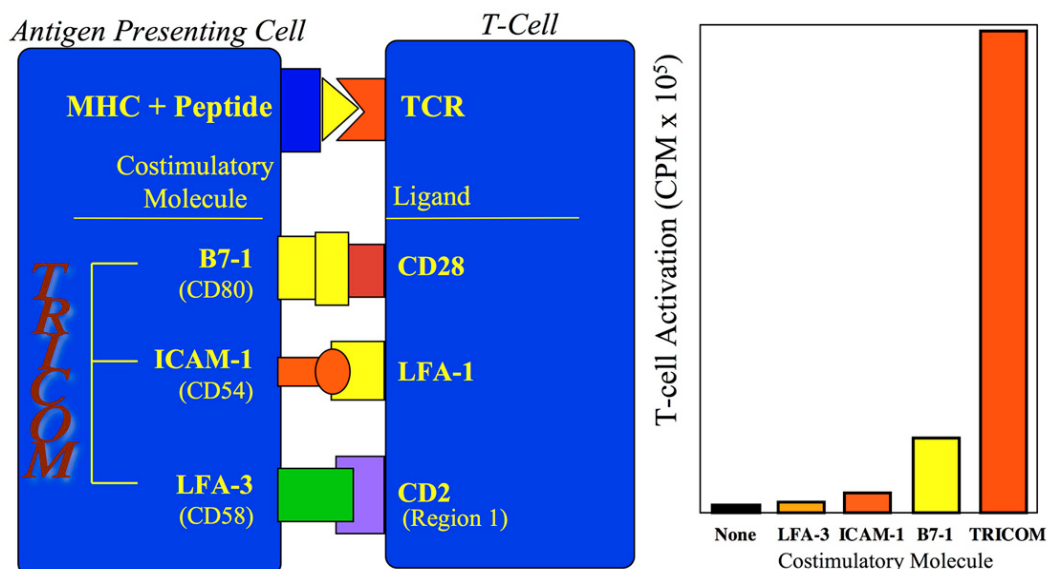
Figure 4. T-cell activation and multiple costimulatory molecules. Left panel, three different T-cell costimulatory molecules (composing TRICOM) and their respective ligands on T cells. Right panel, Activation of naive murine T cells using Con A as signal 1, and no, one or three (TRICOM) different costimulatory molecules as signal 2. APC were infected with pox vectors containing individual costimulatory molecules or TRICOM. Reproduced with permission from (11).

canarypox (ALVAC). Both are derived from avian species and cannot replicate in mammalian cells. Because most of the avipox proteins are not expressed in mammalian cells, these vectors can be used repeatedly without host-neutralizing immune responses. Recombinant fowlpox vectors, moreover, have been engineered to express transgenes on early promoters. Thus, following infection of mammalian cells, these vectors express the transgenes for 14–21 days after which infected cells die.

A major advantage of all poxviruses is their large size; thus, one can insert multiple transgenes. We have now shown, for example, efficient expression of five different transgenes in both vaccinia and avipox recombinant vectors (7). Poxviruses have also been shown to efficiently infect APC including murine and human B cells (8) and dendritic cells (9). Finally, poxvirus vectors replicate in the cytoplasm and do not integrate into host DNA.

Preclinical studies have demonstrated that priming with rV-CEA (V) and administering multiple booster vaccinations with avipox-CEA (A) is superior to the continued use of one vaccine (5, 10). A small randomized clinical trial was then undertaken to define whether this strategy was also efficacious in patients with advanced CEA-expressing carcinoma. The two randomized cohorts (n= 9 patients per cohort) were VAAA versus AAAV (6). All vaccinations were given at monthly intervals. The endpoint was the generation of CEA-specific immune responses directed against a 9-mer CEA peptide (designated CAP-1) using an overnight ELISPOT assay. The results obtained from these two randomized cohorts were as follows: (a) Patients in the VAAA cohort had a statistically significant (p<0.01) increase in CEA-specific T cells (post vs. pre-vaccination) as compared with patients in

the AAAV cohort; (b) treatment with VAAA resulted in longer survival than treatment with AAAV (p=0.05); (c) survival duration was unrelated to pre-vaccination CEA-specific T-cell levels (p=0.77), and (d) the generation of CEA-specific T-cell responses was associated with increased survival (p=0.03) after accounting for disease status. Although randomized, it should be emphasized that treatment groups were small and thus results can only be considered trends. These studies confirmed, however, that the continued use of the avipox-CEA vector resulted in continued increases in CEA-specific CD8^{+} responses.

4. T-CELL COSTIMULATION WITH MULTIPLE COSTIMULATORY MOLECULES (TRICOM VECTORS)

As noted above (Section 1), the generation of a vigorous immune response to a given antigen, especially a weak antigen such as a TAA, has been shown to require at least two different signals (Figure 1). Signal 1 consists of the peptide MHC complex of the APC interacting with the T-cell receptor on the surface of the T cell. Signal 2 is the interaction of a costimulatory molecule with its ligand on the T cell. It is important to point out that both signal 1 and signal 2 must be present on the same cell for efficient T-cell activation. T-cell costimulatory molecules are expressed on professional APC such as dendritic cells and B cells, and are not expressed on the vast majority of solid tumors. Thus, TAAs such as CEA, expressed on the tumor in the absence of costimulation, would not activate T cells and most likely would anergize T cells. We have now developed recombinant avipox vectors containing one, two and three different T-cell costimulatory molecules. Their respective ligands on T cells are shown in Figure 4. *In vitro* and *in vivo* studies have demonstrated that the use of the T-
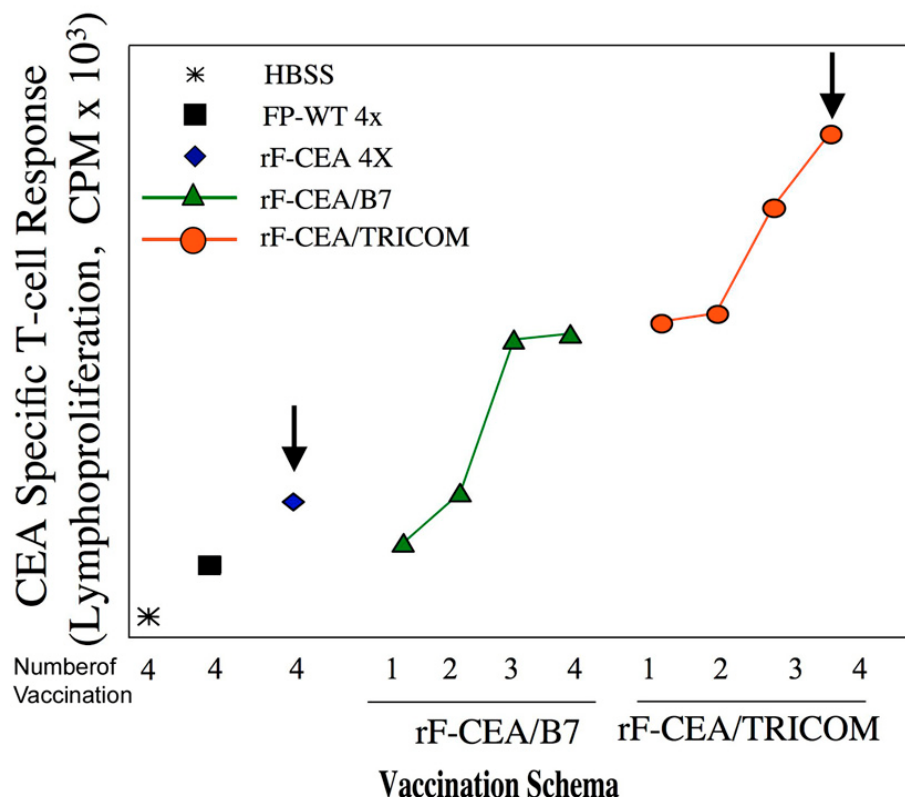
Figure 5. Comparison of potency of vaccines encoding no, one or 3 costimulatory molecules. CEA-specific T-cell responses following multiple vaccinations with recombinant fowlpox vectors rF-CEA, rF-CEA/B7-1, or rF-CEA/TRICOM. C57BL/6 mice were vaccinated s.c. one, two, three, or four times at 2-week intervals with avipox wild-type (FP-WT) (closed square), avipox-CEA (diamond), avipox-CEA/B7-1 (triangles), avipox-CEA/TRICOM (circles), or HBSS buffer (asterisk). Two weeks following the last vaccination, purified splenic T cells were tested for reactivity to CEA protein, or negative control protein ovalbumin (all values negative) in a lymphoproliferation assay. Reproduced with permission from (12).

cell costimulatory molecule B7-1 along with the expression of signal 1 enhances T-cell activation (11). We then showed (10, 11) that the use of a <u>TRI</u>ad of <u>CO</u>stimulatory <u>M</u>olecules (designated TRICOM, consisting of costimulatory molecules B7-1, ICAM-1, and LFA-3) enhances T-cell activation to far greater levels than can be achieved with any one or two such molecules. This triad of molecules was selected because each costimulatory molecule has an individual ligand on T cells and each has been shown previously to prime a unique signaling pathway in T cells (Figure 2). The level of activation of T cells seen with TRICOM vectors has been shown to be synergistic, i.e., more than additive when compared with the use of individual T-cell costimulatory molecules (Figure 4).

Figure 5 demonstrates the relative potencies in the induction of T-cell responses *in vivo* using vectors containing a TAA and either none, one or three costimulatory molecules (12). Four vaccinations of avipox-CEA (designated (fowlpox) rF-CEA) induce a modest CD4+ response against CEA protein. Four vaccinations of rF-CEA/B7 induce a still greater level of CEA-specific T-cell responses. One vaccination of rF-CEA/TRICOM, however, is shown to induce the same level of CEA-

specific T cells as four vaccinations of rF-CEA/B7. Continued vaccination with rF-CEA/TRICOM further enhances T-cell responses. These studies (Figure 5) also offer further evidence that continued vaccination with avipox vectors leads to continued enhancement of immune responses directed against the transgene.

The CEA transgenic (Tg) mouse model (CEA-Tg) offers an excellent reflection of the expression of human CEA in patients. In CEA-Tg mice, the entire human CEA transgene is integrated into the germ line and is under the control of the endogenous CEA promoter. Thus, CEA-Tg mice express human CEA as a "self" entity. CEA is expressed in fetal tissue in a manner very similar to humans, and is expressed in normal tissues, especially gastrointestinal epithelium, in a manner extremely similar to humans. CEA protein is also found in serum of normal CEA-Tg mice at levels similar to those found in the sera of advanced cancer patients; this thus reflects an excellent example of peripheral tolerance. Studies have utilized an experimental metastases model in CEA-Tg mice in which MC38 colon carcinoma cells, transduced with a retroviral vector to express CEA, are injected intrasplenically, and spleens are immediately removed (13). This results in the development of peripancreatic tumors expressing CEA. As
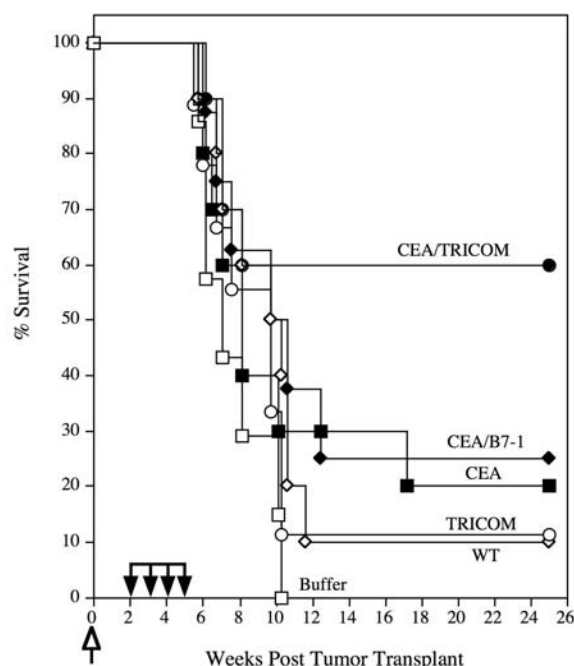
Figure 6. Induction of antitumor responses in CEA transgenic (CEA-Tg) mice by recombinant poxviral vectors encoding costimulatory molecules. CEA-Tg mice bearing 14-day established peripancreatic metastases were divided into five treatment groups. Tumors were transplanted by intrasplenic injection of MC-38 colon carcinoma cells that were transduced with CEA (arrow, day 0). Vaccine schedule is indicated by the open arrows (weeks 2–5). Group 1 received an rV-CEA/TRICOM prime vaccination followed by three weekly boosts with rF-CEA/TRICOM. Group 2 received an rV-CEA/B7-1 prime vaccination followed by three weekly boosts with rF-CEA/B7-1. Group 3 received an rV-CEA prime vaccination followed by three weekly boosts with rF-CEA. Group 4 received an rV-TRICOM prime vaccination followed by three weekly boosts with rF-TRICOM. For groups 1-4, all prime vaccinations were administered with recombinant GM-CSF and low-dose IL-2, and all boost vaccinations were admixed with rF-GM-CSF and low-dose IL-2. Group 5 received only HBSS buffer injections. Reproduced with permission from (13).

can be seen in Figure 6, 50% of mice are dead at approximately 6 weeks following tumor implantation, with all mice dead at approximately 10 weeks post-tumor transplant (13). In these studies (Figure 6), vaccines were given using the previously described diversified prime and boost strategies; i.e., rV-CEA/TRICOM prime, rF-CEA/TRICOM boosts. As can be seen, only moderate anti-tumor effects are seen using the CEA-based vaccine without the addition of costimulation. The use of CEA/B7 vaccines resulted in only a slight increase in anti-tumor effects. The use of CEA/TRICOM vaccines, however, resulted in a statistically significant increase in anti-tumor effect when compared with the CEA or CEA/B7 vaccines (13). We have now shown that both GM-CSF and low-dose IL-2 are required for optimal anti-tumor effects (10), with

GM-CSF being the most substantial contributor because of its ability to increase dendritic cells to regional nodes of the vaccination site. Figure 7 demonstrates a concordance in the ability of these vectors to mount CEA-specific CD4$^+$ and CD8$^+$ T-cell responses, with anti-tumor response. We have now demonstrated that both a CEA-specific CD8$^+$ response and CD4$^+$ response are primarily responsible for the anti-tumor effects seen in Figure 6 (13).

Because CEA is expressed in normal GI epithelium of CEA-Tg mice, it was extremely important to define whether there was any evidence of autoimmunity or other toxicity as a result of vaccination. To this end, mice that have been cured of their CEA-expressing tumors (see Figure 6) were extensively evaluated for evidence of autoimmunity. These CEA-Tg mice were of particular interest as they were shown to mount an immune response vigorous enough to reject an established tumor. Mice were examined approximately 1 year post-tumor transplant and vaccination, and compared with age-matched controlled CEA-Tg mice. No differences were observed between the treated group (n=5) and the control group (n=5) in the analysis of 50 different tissues by histopathology. Moreover, there were no differences in body weights, urinalysis (11 different parameters), serum chemistry (9 different parameters) and blood chemistry (7 different parameters) between experimental and control groups. No differences were observed in auto-antibodies specific for double-stranded DNA, histone, Scl-70, Sm/nRNP, and CIC in control versus experimental groups. There was a slight elevation of anti-single stranded DNA in the vaccinated group; however, antibodies to single-stranded DNA have not been implicated with any disease processes in either mice or humans. There was also no evidence of neo-antibodies specific for B7-1, ICAM-1, LFA-3 or GM-CSF in the vaccinated group (13).

5. COSTIMULATORY MOLECULES WITH ADDITIONAL FUNCTIONS: SIGNAL 3 AND BEYOND

Recent findings suggest that certain costimulatory molecules, in contrast to B7, act predominantly on activated T cells and have little or no effect on their initial activation (Figure 2). In particular, OX40L, 41BBL, and CD70, all members of the tumor necrosis factor (TNF) superfamily (Figure 2), appear to have somewhat distinct roles as costimulators of activated CD4$^+$ and CD8$^+$ subsets of T cells, respectively (14). One of the suggested effects of these 'signal-3' molecules is to extend the lifespan of the stimulated effector cell by suppressing genes associated with apoptosis. Studies on the role of OX40L, when used in combination with other costimulatory molecules, in the activation of T cells have been conducted (7). Specifically, experiments were performed to examine the effect of OX40L costimulation on the initial activation of both CD4$^+$ and CD8$^+$ T cells and on the restimulation of memory/effector T cells. To facilitate these studies, fowlpox virus vectors were constructed that express OX40L alone (rF-OX40L) or in combination with other costimulatory molecules (B7-1, ICAM-1 and LFA-3), referred to as rF-TRICOM/OX40L.
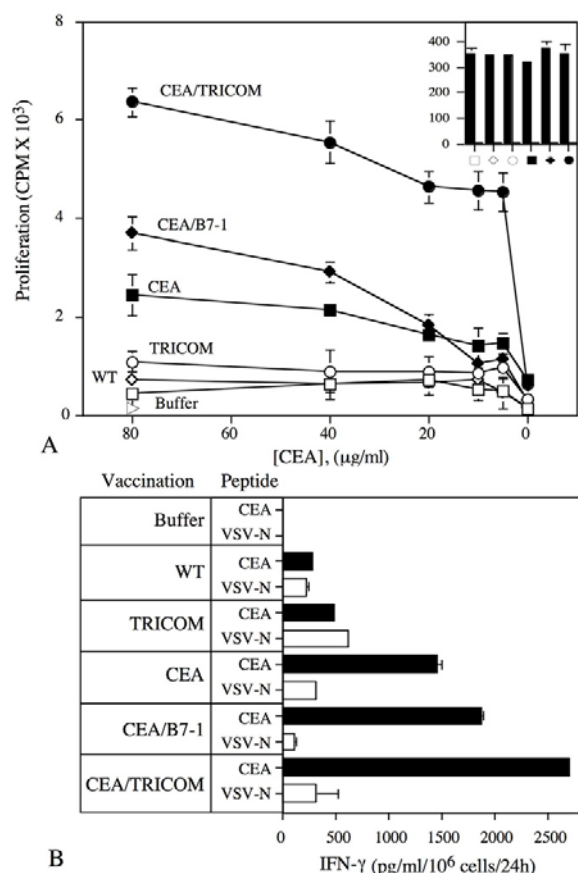
Figure 7. CEA-specific T-cell responses from CEA-Tg mice vaccinated with TRICOM vectors. CEA-Tg mice were divided into six vaccination groups: Group 1 (closed circles) received an rV-CEA/TRICOM prime vaccination followed by three weekly boosts with rF-CEA/TRICOM. Group 2 (closed diamonds) received an rV-CEA/B7-1 prime vaccination followed by three weekly boosts with rF-CEA/B7-1. Group 3 (closed squares) received an rV-CEA prime vaccination followed by three weekly boosts with rF-CEA. Group 4 (open circles) received an rV-TRICOM prime vaccination followed by three weekly boosts with rF-TRICOM. Group 5 (open diamonds) received a V-WT prime vaccination followed by three weekly boosts with FP-WT. For groups 1-5, all prime vaccinations were administered with recombinant GM-CSF and low-dose IL-2, and all boost vaccinations were admixed with rF-GM-CSF and low-dose IL-2. Group 6 (open squares) received only HBSS buffer injections. *In vitro* assays were performed 3 weeks following the last vaccination. Each group contains splenic T cells pooled from three mice. *Panel A,* Lymphoproliferation of splenic T cells in response to CEA protein. Proliferation in response to the T-cell mitogen Con A (2.5 µg/ml) is shown in the inset panel. *Panel B*, IFN-gamma production by T cells in response to the 8-mer CEA$_{526-533}$ peptide or control peptide VSV-N (open bars). Reproduced with permission from (13).

These viruses were utilized in numerous studies for CD4$^+$ and CD8$^+$ T-cell activation to include proliferation, cytokine release, and protection from apoptosis and gene

expression. These studies (7) demonstrated that (a) OX40L plays a role in sustaining the long-term proliferation of CD8$^+$ T cells in addition to the known effect on CD4$^+$ T cells following activation, and (b) the anti-apoptotic effect of OX40L on T cells is likely the result of sustained expression of anti-apoptotic genes while genes involved in apoptosis are inhibited. In addition, the combined use of a vector driving the expression of OX40L with three other costimulatory molecules (B7-1, ICAM-1, and LFA-3) both enhances initial activation and then further potentiates sustained activation of naive and effector T cells.

These data, taken together, might suggest that the addition of even more costimulatory molecules to TRICOM vectors would result in further enhancement of T-cell activation. This, however, is not necessarily the case. CD70 has been reported to support proliferation of naive T-lymphocytes, and enhances CTL activity. Indeed, when CD70 was examined as a single costimulatory molecule, T-cell proliferation was increased (15). However, when CD70 was used in combination with TRICOM to enhance T-cell proliferation (Figure 8), the combination actually decreased T-cell activity by greater than 40% of that observed with TRICOM alone. These studies indicate that the overexpression of more costimulatory molecules to APC does not necessarily result in increased T-cell function.

6. INDUCTION OF HIGH AVIDITY CTL WITH THE USE OF TRICOM VECTORS

High avidity CTL have been shown to be most effective in clearing viruses in cancer cells (16). The studies outlined above clearly demonstrate that the use of TRICOM vectors can enhance the quantity of antigen-specific T cells generated, but do not address the quality of such T cells. Oh *et al.* set out to better understand the mechanisms involved in the induction of high avidity CTLs (17). In that study, signals from MHC Class I (signal 1) and costimulatory signals (signal 2) were adjusted by varying antigen dose and by the use of TRICOM poxvirus vectors, respectively. Increasing signal 1 resulted in an increased frequency of CD8$^+$ CTL but not an increase in CTL avidity. The infection of APC with TRICOM vectors to produce a strong signal 2, however, was shown to be necessary for the induction of high avidity CD8$^+$ CTL that killed target cells more efficiently. Moreover, signal 2, i.e., the use of TRICOM, was shown to play a more crucial role in the absence of a strong signal 1. Only CTL induced with TRICOM killed tumor cells endogenously expressing low levels of antigen. The use of TRICOM vectors was also shown to contribute to the induction of high avidity CTL in both primary and secondary responses. These studies (17) thus demonstrate the importance of the quality, as well as the quantity, of T cells in the generation of an immune response, especially directed against a weak antigen such as a TAA.

7. MODIFICATION OF TUMOR CELLS TO EXPRESS MULTIPLE COSTIMULATORY MOLECULES

Utilizing whole tumor cells as a vaccine, where the entire tumor antigen repertoire is represented, presents
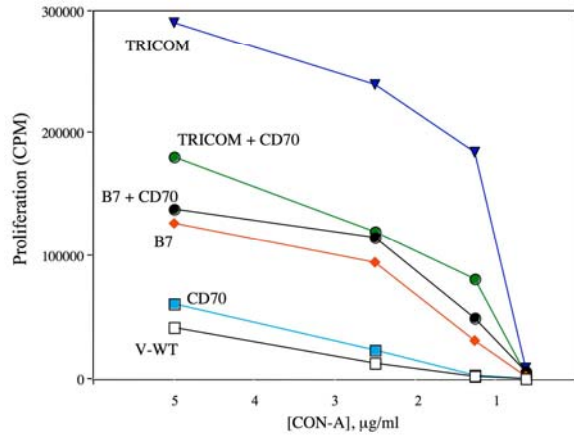
Figure 8. More costimulation is not always better. Activation of naive murine T cells using Con A as signal 1, and B7-1 or TRICOM as signal(s), with and without the addition of the costimulatory molecule CD70 as signal 3. APC were infected with pox vectors containing individual costimulatory molecules or TRICOM.

an alternative to antigen-defined vaccination strategies, and bypasses the necessity of tumor antigen identification. Direct over-expression of tumor antigen and costimulatory molecules on tumor cells could potentiate strong anti-tumor T-cell activity. This was examined using tumor cells *in-situ,* employing a vaccine regimen comprised of priming mice subcutaneously (s.c.) with rV-CEA/TRICOM and boosting intratumorally (i.t.) with rF-CEA/TRICOM. Kudo-Saito *et al.* compared the antitumor effects induced by a systemic vaccination regimen (s.c.) and intratumoral (i.t.) vaccination, and a sequential s.c/i.t. vaccination regimen utilizing rV-CEA/TRICOM as the s.c. prime vaccination and rF-CEA/TRICOM as the i.t. boost (18). Vaccination was started on day 8 after s.c. implantation of CEA-Tg mice with CEA-positive tumors (Figure 9). When i.t.-vaccination with rF-CEA/TRICOM was started on day 15, tumor growth was not significantly inhibited as compared with that in the PBS-treated group (*P*=0.9884 to Figure 9A), and no mice were cured (Figure 9B). However, when rV-CEA/TRICOM was utilized as a prime on day 8 before i.t.-vaccination with rF-CEA/TRICOM on day 15, tumor development was significantly inhibited as compared with that seen when mice were vaccinated i.t. on day 15 without priming (*P*=0.0001 to Figure 9B), and 14 of 20 mice were cured (Figure 9C). In contrast, when mice were boosted s.c. with rF-CEA/TRICOM following priming with rV-CEA/TRICOM, tumor growth was not strongly inhibited, and the anti-tumor activity was significantly lower than that seen in i.t.-boosted mice after priming (Figure 9D; *P*=0.0001 to Figure 9C). These studies demonstrate that i.t.-vaccination with a fowlpox vector containing a tumor antigen and multiple costimulatory molecule combined with systemic priming is an effective modality in the therapy of tumors. This vaccine regimen holds promise not only for the therapy of subcutaneous tumors but also for other tumors accessible by surgery.

8. USE OF TRICOM VECTORS IN A CANCER PREVENTION SETTING

As noted above (Section 5), preclinical murine models expressing the complete human *CEA* gene as a transgene have been generated and CEA is expressed predominately along the GI tract, as in humans. Greiner *et al.* utilized CEA-Tg mice that were bred with *Apc*MIN (MIN) mice that are heterozygous for a mutant allele of the mouse homologue of the human *APC* gene (19). MIN mice carry a germ-line mutation of the murine *Apc* gene, which results in the formation of multiple intestinal adenomas (19, 20). In humans, a homologous germ-line mutation in the tumor suppressor gene, adenomatous polyposis coli (*APC*), predisposes individuals to an inherited form of colon cancer, familial adenomatous polyposis (FAP), characterized by the early development of multiple colorectal adenomas, some of which can subsequently form carcinomas (21). Somatic mutations of the *APC* gene are found in the early stages of 85–90% of sporadic colorectal cancers (21). Mice (CEA-Tg/MIN) carrying both the MIN and human *CEA* genes develop multiple intestinal neoplasms, which overexpress CEA to levels that are reminiscent of those reported for tubulovillous intestinal adenomas from patients. In that study, an immunotherapeutic protocol consisted of a primary vaccination with rV-CEA/TRICOM followed by booster vaccinations with rF-CEA/TRICOM. GM-CSF was administered as a biological adjuvant with all vaccinations. That vaccine regimen generated strong CEA-specific host immune responses in CEA-Tg/MIN mice, which resulted in (a) a delayed onset of adult anemia and weight loss, (b) improved overall survival, and (c) a significant reduction in the number of intestinal tumors. When analyzed at necropsy, those CEA-Tg/MIN mice that received the CEA-based vaccine had a significant reduction in the number of intestinal tumors when compared with CEA-Tg/MIN mice that received the non-CEA-based vaccine or vehicle control. Of the 13 CEA-Tg/MIN mice that received the CEA-based vaccine, 5 had dramatic responses (0–3 tumors), 4 had partial responses (4–25 tumors), and the remaining 4 had no response. The administration of the vaccine devoid of the CEA transgene did not suppress tumor formation. No evidence of autoimmunity directed against normal tissues expressing CEA was observed in mice in which the CEA-based vaccine significantly reduced intestinal tumor load. The CEA-Tg/MIN mice thus present a model in which different CEA/costimulatory molecule-based vaccine strategies can be tested on the spontaneous onset of intestinal tumorigenesis.

Both chemotherapeutic and immunological-based approaches have been independently explored as potential strategies for the intervention of colorectal cancer. One chemotherapeutic/prevention approach that has proven successful in both experimental models and the treatment of familial adenomatous polyposis in humans is the selective targeting of the cyclooxygenase (COX) pathway. A regular intake of nonsteroidal anti-inflammatory drugs (NSAIDs), such as aspirin, ibuprofen, and sulindac, inhibits both COX enzymes with an associated reduction of cancer risk (22). Celecoxib, one of the most studied COX-2
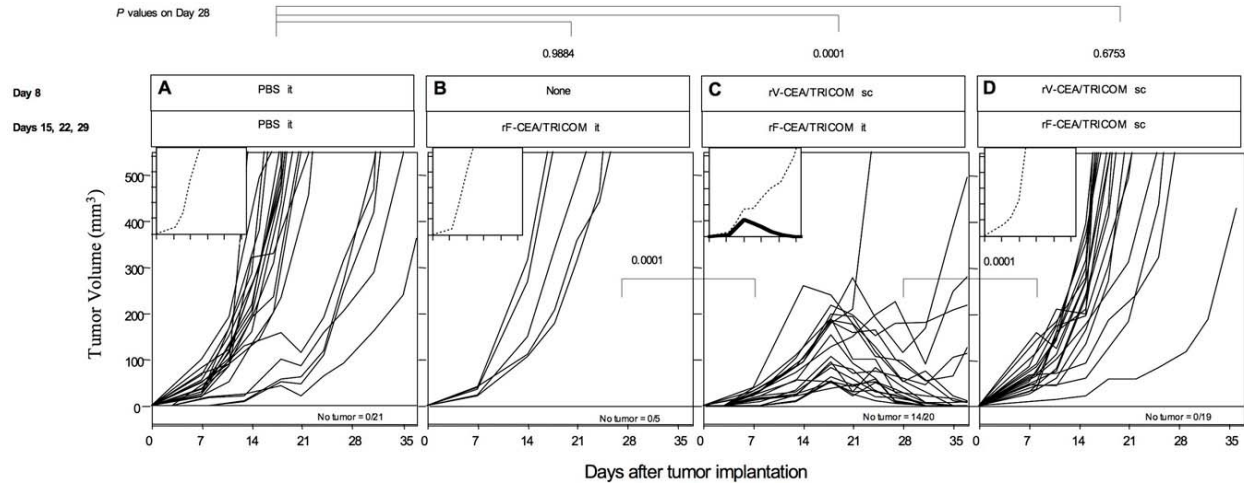
Figure 9. Efficacy of intratumoral (i.t.) vaccination following subcutaneous (s.c.) priming with rV-CEA/TRICOM on advanced tumors in a self-antigen system. CEA-transgenic mice were implanted s.c. with MC38-CEA$^+$ tumors on day 0. *Panel A,* Control mice were administered i.t. PBS on days 8, 15, 22 and 29. *Panel B,* Mice were vaccinated i.t. with rF-CEA/TRICOM on days 15, 22 and 29. *Panel C,* Mice were vaccinated s.c. with rV-CEA/TRICOM on day 8. Then, mice were boosted i.t. with rF-CEA/TRICOM on days 15, 22 and 29. *Panel D,* Mice were vaccinated s.c. with rV-CEA/TRICOM on day 8. Then, mice were boosted s.c. with rF-CEA/TRICOM on days 15, 22 and 29. Each virus was admixed with rF-GM-CSF. Tumor volume was monitored 1-2 times a week. These data are the compilation of 4 separate experiments. Inset panels: mean tumor volumes of mice responding to (heavy line) or failing (dotted line) vaccine therapy. Reproduced with permission from (18).

inhibitors, has been shown to reduce the multiplicity and size of intestinal tumors (primarily adenomas) in the multiple intestinal neoplasia (MIN) mouse model.

Zeytin *et al.* (23) have demonstrated that COX-2 and CEA can be simultaneously targeted in a combined chemoimmuno-based approach to cancer prevention and/or therapy. Here, the COX-2 inhibitor celecoxib was administered to mice via dietary supplement. Treatment of CEA-Tg/MIN mice with celecoxib combined with a CEA-based vaccine significantly reduced tumor multiplicity and prolonged survival. Beginning at 30 days of age, the administration of a diversified prime/boost recombinant CEA/TRICOM poxvirus-based vaccine regimen (Figure 10B) or celecoxib-supplemented diet (Figure 10C) as single agents reduced the number of intestinal tumors in CEA-Tg/MIN mice by 54% and 65%, respectively. Combining the CEA/TRICOM–based vaccine regimen with celecoxib led to an additional reduction in intestinal tumor multiplicity, 95% reduction when compared with untreated CEA-Tg/MIN mice (Figure 10D). In fact, 6 of 16 CEA-Tg/MIN mice administered monthly injections of the CEA/TRICOM and celecoxib were tumor-free at 150–160 days of age. Another cohort of CEA-Tg/MIN mice that was administered the combined treatment regimen had a significant improvement in survival. At 18 months of age, 100% (12 of 12) of CEA-Tg/MIN mice that continued to receive monthly booster vaccinations of rF-CEA-TRICOM combined with rF-GM-CSF, as well as being maintained on the 1000-ppm celecoxib-supplemented diet, remained alive (Figure 10E). As before, both tumor reduction and improved overall survival were achieved without any evidence of autoimmunity directed at CEA-expressing or other normal tissues.

Taken together, these data provide a strong argument for future experimental as well as clinical efforts to combine cancer vaccines utilizing multiple costimulatory molecules with NSAID targeting of COX expression. The most immediate population that may benefit from a combined CEA-based vaccine with celecoxib is of patients diagnosed with FAP. Celecoxib (Celebrex) is being prescribed for FAP patients based on a 30% reduction in polyp burden (24).

9. COSTIMULATORY MOLECULES IN CLINICAL TRIALS

Costimulatory molecules are currently being examined as immunostimulants in over 20 clinical trials (Table 1). In these trials, the costimulatory molecules are being delivered by (a) adenovirus vectors, (b) recombinant soluble proteins, (c) encoded in autologous or allogeneic whole tumor cell vaccines, (d) recombinant poxvirus vectors, vaccinia, and/or fowlpox (avipox; ALVAC), or (e) antibodies directed against negative costimulatory signals such as anti-CTLA-4.

As noted in Section 7, whole tumor cells modified to express costimulatory molecules present an attractive modality for cancer therapy. To that end, Antonia *et al.* (25) have described an autologous renal cell carcinoma (RCC) tumor-cell vaccine that was genetically modified to overexpress B7-1 to provide costimulation to tumor-reactive T cells. There, primary tumors or metastases were resected from patients and infected with a recombinant adenoviral vector containing human B7-1. In that trial, 26% (4 of 15) of patients had either a partial

Costimulatory Molecules as Adjuvants for Immunotherapy

Table 1. Costimulatory Molecules In Clinical Trials

Costimulatory Molecule	Modality	Tumor	Phase	Reference
CD-40 Ligand	Ad-CD40L	Leukemia	I	30, 38
CD40L	rhuCD40L	Solid Tumor	I	29
B7-1 (CD80)	adenovirus-B7-1/ tumor cell vaccine	Renal Cell Carcinoma	II	39
B7-1	ALVAC-CEA-B7-1	Adenocarcinoma	I	40, 41, 42
B7-1	autologous tumor cell vaccine	Renal Cell Carcinoma	I	25
B7-1	allogeneic tumor cell vaccine	Breast Carcinoma	I	26
B7-1	rV-B7-1 (Intratumoral)	Melanoma	I	28
B7-1	rV-PSA/ rF-PSA/ rF-B7-1	Prostate Carcinoma	II	43
B7-1, B7-2	rV-MART-1/gp100/tyrosinase	Melanoma	I/II	31
B7-1	Anti-CTLA-4 antibody	Melanoma, Ovarian Carcinoma	I	33, 34
B7-1/ TRICOM (B7-1, ICAM-1, LFA-3)	rF-B7-1/ rF-TRICOM (Intratumoral)	Solid Tumor	I	44
TRICOM	DC infected with rF-CEA/TRICOM	Metastatic Cancer	I	45
TRICOM	rF-TRICOM (Intravesical)	Bladder Cancer, Melanoma	I/II	46, 47
TRICOM	rV-CEA/TRICOM, rF-CEA/TRICOM	Solid Tumor, Lung Carcinoma	I	48, 49
TRICOM	rV-MUC-1/ rV-TRICOM	Breast Carcinoma	I	50
TRICOM	rV-PSA/TRICOM, rF-PSA/TRICOM	Prostate Carcinoma	II	51, 52
TRICOM	rV-CEA/MUC-1/TRICOM, rF-CEA/MUC-1/TRICOM	Pancreatic Adenocarcinoma	II/III	36, 53

response or stable disease, while 9 patients continued to have progressive disease. Dols et al. (26) have described a similar strategy utilizing vaccination of women with metastatic breast cancer, using a B7-1-modified, HLA-A2-matched, allogeneic, breast cancer cell line. This vaccine was used to vaccinate 30 women with previously treated stage IV breast cancer. This immunization strategy proved to be safe and feasible, and induced tumor-specific immune responses in a minority of patients; however, no objective tumor regressions were observed.

Intratumoral delivery of vectors encoding costimulatory molecules as a means of modifying tumor cells in-situ has also been translated to the clinical setting in the form of local delivery of poxvirus vaccines for melanoma (27). Specifically, a Phase I trial of intralesional rV-B7-1 vaccine in the treatment of malignant melanoma is being examined (28).

Another costimulatory molecule being examined in a series of Phase I studies is recombinant human CD40 ligand (CD40L). Vonderheide et al. (29) conducted a study in which patients with advanced solid tumors or intermediate- or high-grade non-Hodgkin's lymphoma (NHL) received recombinant human CD40L subcutaneously daily for 5 days. Subsequent courses were given until disease progression. Thirty-two patients received CD40L at three dose levels, and a total of 65 courses were administered. Two patients (6%) had a partial response on study (one patient with laryngeal carcinoma and one with non-Hodgkin's lymphoma). For the patient with laryngeal cancer, a partial response was sustained for 12 months before the patient was taken off therapy. Three months later, the patient was found to have a complete response and remains biopsy-proven free of disease at 24 months. Twelve patients (38%) had stable disease after one course, which was sustained in four patients through four courses.

In another study by Wierda et al. (30), chronic lymphocytic leukemia (CLL) cells were made to express

CD40-ligand by transduction with a replication-defective adenovirus vector (Ad-CD154). Treatment consisted of a one-time bolus infusion of autologous Ad-CD154-transduced leukemia cells. On average, treated patients experienced a greater than 240% increase in absolute blood T-cell counts within 1 to 4 weeks of treatment. Moreover, treatment increased the numbers of leukemia-specific T cells, demonstrated by autologous ELISPOT assay and mixed lymphocyte reactions. These biologic effects were associated with reductions in leukemia cell counts and lymph node size.

Zajac et al. (31) has conducted a Phase I/II clinical trial of a vaccinia virus expressing multiple HLA-A0201-restricted tumor-associated epitopes and costimulatory molecules in metastatic melanoma patients. In that study, the vaccine consisted of a recombinant vaccinia virus encoding the endoplasmic reticulum-targeted HLA-A0201-restricted Melan-A/MART-1, gp100, and tyrosinase epitopes, together with B7-1 and B7-2 costimulatory molecules (Table 1). Seventeen of 18 patients completed the 3-month trial. Three patients displayed regression of individual metastases, seven had stable disease, and progressive disease was observed in seven patients.

Costimulation by B7-1, in addition to activating T cells, upregulates a negative costimulatory receptor: cytotoxic T lymphocyte-associated antigen 4 (CTLA-4). The attenuation of T-cell activation by CTLA-4 could limit the potency of tumor immunity. An alternative method of modulating costimulation would be to block the negative signals associated with CTLA-4. In murine systems, the administration of antibodies that block CTLA-4 function inhibited the growth of moderately immunogenic tumors and, in combination with cancer vaccines, increased the rejection of poorly immunogenic tumors (32). CTLA-4 blocking antibody has been examined clinically (Table 1). Hodi et al. (33) reported that infusion of a CTLA-4 blocking antibody into nine previously immunized advanced cancer patients stimulated extensive tumor
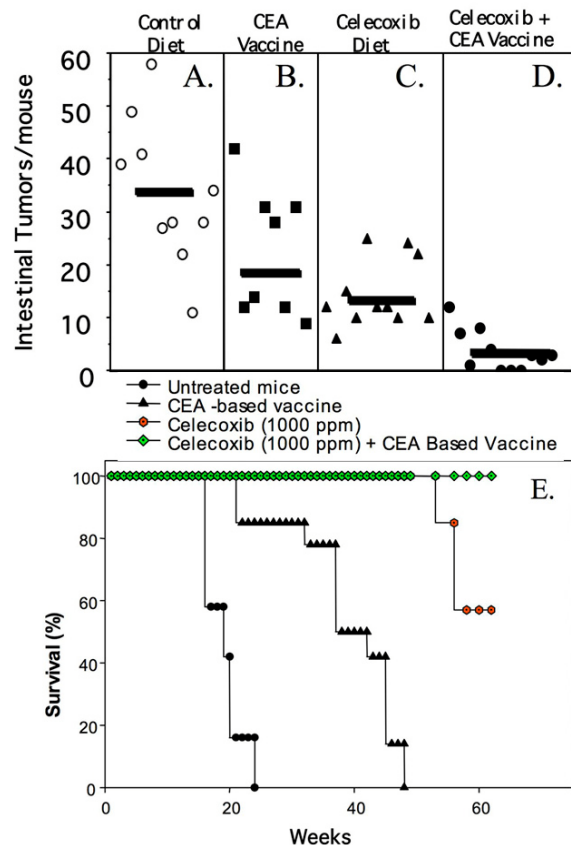
Figure 10. Combination therapy in a cancer prevention setting: Vaccines encoding multiple costimulatory molecules and chemotherapy. Total number of intestinal tumors in CEA-Tg/MIN mice that were fed either a control diet (*Panels A–B*) or a celecoxib-supplemented diet (*Panels C–D*). Mice also received the CEA/TRICOM vaccine regimen (*Panels B* and *D*). Closed symbols represent individual mice and horizontal lines (—) the mean number of tumors for each treatment group. Reproduced with permission fromreference (23). *Panel E,* Long-term survival of CEA-Tg/MIN mice fed either the control or celecoxib-supplemented diets ± the CEA/TRICOM vaccine regimen. CEA-Tg/MIN mice were untreated (closed circles), fed the celecoxib-supplemented diet alone (red circles), or injected with the CEA/TRICOM vaccine regimen (*n* = 12); (closed triangles). To assess the combination of these modalities, CEA-Tg/MIN mice were fed the celecoxib-supplemented diet and also injected with the CEA/TRICOM vaccine regimen (*n* = 12); (green symbols). Reproduced with permission from (23).

necrosis in three of three metastatic melanoma patients and the reduction or stabilization of CA-125 levels in two of two metastatic ovarian carcinoma patients. Phan *et al.* (34) has examined anti-CTLA-4 blocking antibody in 14 patients with metastatic melanoma in conjunction with vaccination with two peptides from the gp100 melanoma-associated antigen. There, the administration of anti-CTLA-4 antibody induced grade III/IV autoimmune manifestations in six patients (43%), including dermatitis, enterocolitis, hepatitis, and hypophysitis, and mediated

objective cancer regression in three patients (21%; two complete and one partial responses). This study further established CTLA-4 as an important molecule regulating tolerance to "self" antigens in humans and suggested a role for CTLA-4 blockade in breaking tolerance to human cancer antigens for cancer immunotherapy.

TRICOM vaccines are currently being investigated in patients with advanced forms of cancer in a series of Phase I and II clinical trials (Table 1). The initial Phase I trial of a TRICOM vaccine was performed using the TRICOM-CEA construct that had been modified to contain an improved HLA-A2 motif within the CEA protein, referred to as CEA(6D) (35). No maximum tolerated dose was reached during the course of this trial. The first tier of the trial was to enroll patients to receive rF-CEA(6D)/TRICOM alone in a dose escalation fashion. Once the safety was established for the fowlpox component, subsequent patients received a single rV-CEA/TRICOM injection followed by a series of monthly rF-CEA/TRICOM injections. Both vaccines were well tolerated. The final cohorts had the cytokine GM-CSF added at the site of the injection on days 1 through 4 during every 28-day treatment cycle.

Of the original 58 patients with refractory advanced cancer who were treated, 11 had stabilization of their previously progressive disease. Of the 46 patients who progressed initially, 25 elected to remain on vaccine therapy through 4 additional cycles of vaccine, and 12 of these additional patients then stabilized (Figure 11). This suggested that the impact of the vaccination regimen is not fully felt in the patient population until after a series of 4 vaccinations were administered. As part of this trial, after the initial 6 vaccinations, the 12 patients who were still benefiting from the therapy were shifted to an every-3-month boosted schedule. Interestingly, all of these patients progressed on this administration schedule and when 11 of the 12 patients were reverted to the every-28-day treatment schedule, six restabilized, suggesting that the vaccine needs to be administered on a more frequent basis. The immune responses observed in this clinical trial were higher than those observed in previous clinical trials using vaccinia and avipox-CEA vaccines with no costimulation. Likewise, there was a suggestion of a survival benefit in those patients who had achieved a better immune response (35). There was one patient with an advanced refractory small-cell lung carcinoma who had a 15-month complete response (35, 36). While preliminary (Phase I setting), there was a trend toward better survival in those patients receiving the rV-CEA/TRICOM prime, rF-CEA/TRICOM boosts, and GM-CSF vaccinations (Figure 11). Interestingly, these are the same regimens that also demonstrated the best immune responses and antitumor activity in preclinical models.

A second generation of TRICOM vaccines has been developed (37). These vaccines not only take advantage of the vaccinia prime and fowlpox boost vaccine regimen as well as TRICOM costimulation, but now also encode two tumor antigens: CEA and MUC-1. This vaccine has been tested in a series of two Phase I clinical trials carried out in pancreatic patients. These trials demonstrated high levels of
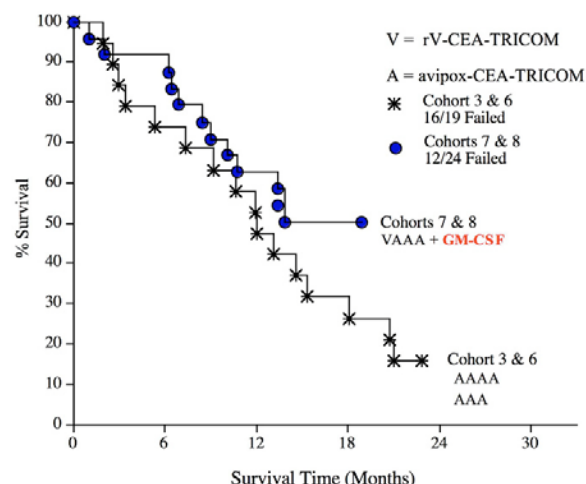
Figure 11. Vaccines with multiple costimulatory molecules in clinical trials. Overall survival by cohorts with or without GM-CSF. Cohorts 3 and 6 (16/19 failed) received no GM-CSF with vaccines; cohorts 7 and 8 (12/24 failed) received vaccines plus GM-CSF. Reproduced with permission from (35).

safety and preliminary evidence of increased survival (37). Based on these results, a Phase III randomized trial in second-line metastatic pancreatic cancer patients has been initiated.

Another tumor antigen, prostate specific antigen (PSA), has been incorporated with TRICOM, i.e., rV-PSA/TRICOM prime followed by rF-PSA/TRICOM boosting, and is being evaluated for the treatment of androgen-independent prostate cancer. This vaccine modality is currently being examined in Phase II trials. Other clinical trials are examining the combined use of TRICOM vaccines in conjunction with chemotherapeutic agents or in combination with external-beam radiation.

Thus, the overall goal is the use of vaccine regimens in combination with front-line cancer therapies, thereby reducing the interval between disease diagnosis and the initiation of vaccine, thus bringing the use of cancer vaccines closer to its original intention, for use in patients in the minimal disease setting.

10. CONCLUSIONS

The studies reviewed here demonstrate that several different strategies employing costimulation can be used to enhance the immunogenicity of a weak immunogen, which is by definition the case for virtually all TAAs. These findings can, in turn, be employed toward the development of new vaccines and vaccine strategies for the therapy and/or prevention of a range of human cancers. These studies also demonstrate the efficacy of the combinatorial approach of using several strategies in tandem to achieve maximum immune responses, leading to anti-tumor activities. Many of the parameters investigated in hypothesis-driven preclinical studies, employing appropriate preclinical models, were shown to be translated

to science-driven clinical trials. The uses of multiple costimulatory molecules appear to be able to potentiate T-cell responses. The utilization of these newly developed more potent vaccines and a novel vaccine strategy has formed the scientific base for further evaluation, perhaps in combination with conventional therapies, for a range of human cancers.

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