

A Triad of Costimulatory Molecules Synergize to Amplify T-Cell Activation

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ABSTRACT

The activation of a T cell has been shown to require two signals via molecules present on professional antigen-presenting cells: signal 1, via a peptide/MHC complex; and signal 2, via a costimulatory molecule. Here, the role of three costimulatory molecules in the activation of T cells was examined. Poxvirus (vaccinia and avipox) vectors were used because of their ability to efficiently express multiple genes. Murine cells provided with signal 1 and infected with either recombinant vaccinia or avipox vectors containing a TRIad of COstimulatory Molecules (B7-1/ICAM-1/LFA-3, designated TRICOM) induced the activation of T cells to a far greater extent than cells infected with any one or two costimulatory molecules. Despite this T-cell “hyperstimulation” using TRICOM vectors, no evidence of apoptosis above that seen using the B7-1 vector was observed. Results using the TRICOM vectors were most dramatic under conditions of either low levels of first signal or low stimulator cell:T-cell ratios. Experiments using a four-gene construct also showed that TRICOM recombinants can enhance antigen-specific T-cell responses *in vivo*. These studies thus demonstrate for the first time the ability of vectors to introduce three costimulatory molecules into cells, thereby activating both CD4⁺ and CD8⁺ T-cell populations to levels greater than those achieved with the use of only one or two costimulatory molecules. This new threshold of T-cell activation has broad implications in vaccine design and development.

INTRODUCTION

The extent of the primary response of T cells, which involves their activation, expansion, and differentiation, is paramount to a successful immune response to an antigen. The initiation of an immune response requires at least two signals for the activation of naive T cells by APCs² (1–3). The first signal is antigen specific, delivered through the TCR via the peptide/MHC, and causes the T cell to enter the cell cycle. The second, or “costimulatory,” signal is required for cytokine production and proliferation. At least three distinct molecules normally found on the surface of professional APCs have been reported as capable of providing the second signal critical for T-cell activation: B7-1 (CD80), ICAM-1 (CD54), and LFA-3 (human CD58; murine CD48; Refs. 2–10). The T-cell ligands for these costimulatory molecules are distinct. B7-1 interacts with the CD28 and CTLA-4 molecules, ICAM-1 interacts with the CD11a/CD18 (LFA-1 β 2 integrin) complex, and LFA-3 interacts with the CD2 (LFA-2) molecules. These molecules have been individually shown to costimulate T-cell proliferation *in vitro* (4). However, because they may be expressed simultaneously on APCs, it has been difficult to examine relative potencies of individual costimulatory molecules during the induction of T-cell proliferation (2).

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² The abbreviations used are: APC, antigen-presenting cell; TCR, T-cell receptor; ICAM, intercellular adhesion molecule; LFA, leukocyte function-associated antigen; TRICOM, TRIad of COstimulatory Molecules; CEA, carcinoembryonic antigen; MOI, multiplicity of infection; pfu, plaque-forming unit(s); MAb, monoclonal antibody; IL, interleukin; TNF, tumor necrosis factor; GM-CSF, granulocyte/macrophage-colony stimulating factor; PCD, programmed cell death.

Because it has been proposed that both antigen and costimulatory molecules must be expressed in the same cell to properly engage the TCR and costimulatory receptor, respectively, an admixture of several recombinant vectors could be used to explore the potential cooperation of costimulatory molecules (6). The disadvantage of this approach, however, is that the admixture of three or more vectors or viruses has a statistically diminished probability of coinfecting the same cell. Thus, a multigene construct would be preferable for expression of multiple costimulatory molecule genes in the same cell. The use of retroviral vectors, however, requires multiple drug selection procedures. We report here, for the first time, the development of constructs containing and expressing a TRICOM (B7-1, ICAM-1, and LFA-3). The synergistic effect of these costimulatory molecules on the enhanced activation of T cells is demonstrated. More specifically, these three costimulatory molecule genes have been inserted into two vectors: vaccinia, which is replication competent; and avipox (fowlpox), which is replication defective. In each case, the degree of T-cell activation using vectors containing three costimulatory molecules was far greater than the sum of the constructs, each containing one costimulatory molecule.

MATERIALS AND METHODS

Recombinant Poxviruses. The individual recombinant vaccinia viruses containing the genes encoding either murine costimulatory molecule B7-1 (designated rV-B7-1), murine ICAM-1 (designated rV-ICAM-1), murine CD48 (designated rV-LFA-3), human CEA, or murine B7-1 have been described (11–14). Recombinant fowlpox viruses were constructed by the insertion of foreign sequences into the *Bam*HI J region of the genome of the POXVAC-TC (Schering Corporation) strain of fowlpox virus as described (15). In recombinant viruses containing a single foreign gene, the gene is under control of the vaccinia 40k promoter (16). rV-B7-1/ICAM-1 is a recombinant vaccinia virus that contains the murine *B7-1* gene under control of the synthetic early/late (sE/L) promoter (17) and the murine *ICAM-1* gene under control of the 40k promoter. rV-B7-1/ICAM-1/LFA-3 (designated rV-TRICOM) is a recombinant vaccinia virus that contains the murine *LFA-3* gene under control of the vaccinia 30k (M2L) promoter (18), the murine *ICAM-1* gene under control of the vaccinia I3 promoter (19), and the murine *B7-1* gene under control of the synthetic early/late (sE/L) promoter. rV-CEA/B7-1/ICAM-1/LFA-3 (designated rV-CEA/TRICOM) is the recombinant vaccinia-TRICOM construct containing the human *CEA* gene under the control of the 40k promoter, the murine *B7-1* gene under control of the sE/L promoter, the murine *LFA-3* gene under control of the I3 promoter, and the murine *ICAM-1* gene under control of the vaccinia 7.5k promoter (20). rF-CEA/B7-1/ICAM-1/LFA-3 (designated rF-CEA/TRICOM) is a recombinant fowlpox virus that was constructed similarly to rV-CEA/TRICOM. Nonrecombinant wild-type vaccinia virus (Wyeth strain) was designated V-WT, whereas nonrecombinant fowlpox virus was designated WT-FP.

Characterization of Recombinant Viruses: Fluorescent Analysis of Protein Surface Expression. Confluent MC38 cells, which are positive for class I (21), were infected with vaccinia constructs (V-WT, rV-B7-1, rV-ICAM-1, rV-LFA-3, and rV-TRICOM) or fowlpox constructs (WT-FP, rF-B7-1, rF-ICAM-1, and rF-CEA/TRICOM) at 5 MOI (pfu/cell) for 5 h. CEA was used in one rF construct as a marker gene only. After infection, cells were harvested and immunostained with FITC-conjugated MAb specific for murine B7-1, ICAM-1, or CD48 (PharMingen, San Diego, CA). Cell fluorescence was analyzed with a FACScan cytometer (Becton Dickinson, Mountain View, CA) with the CellQuest program.

In Vitro Costimulation Analysis. Female C57BL/6 mice (6–8 weeks of age) were obtained from Taconic Farms (Germantown, NY). Naive T cells were isolated as described previously (22). For certain experiments, T cells were further fractionated into CD4⁺ and CD8⁺ populations by negative selection using anti-CD4 or anti-CD8 paramagnetic beads (MiniMACS; Miltenyi Biotec, Auburn, CA). T cells were added at 10⁵/well in 96-well, flat-bottomed plates (Costar, Cambridge, MA). Stimulator cells consisted of uninfected MC38 cells or cells infected for 5 h with 5 MOI of vaccinia constructs (V-WT, rV-B7-1, rV-ICAM-1, rV-LFA-3, and rV-TRICOM) or fowlpox constructs (WT-FP, rF-B7-1, rF-ICAM-1, and rF-CEA/TRICOM) fixed with 2% paraformaldehyde and added at 10⁴/well. Cells in all wells were cultured in a total volume of 200 μ l of complete media (22) in the presence of several dilutions (5 to 0.625 μ g/ml) of Con A (Sigma) for 2 days. Control wells received T cells, stimulator cells, and media only. For indicated experiments, plate-bound anti-CD3 (1.5 to 0.012 μ g/well) was substituted for Con A. Cells were labeled for the final 12–18 h of the incubation with 1 μ Ci/well [³H]thymidine (New England Nuclear, Wilmington, DE) and harvested with a Tomtec cell harvester (Wallac Inc., Gaithersburg, MD). The incorporated radioactivity was measured by liquid scintillation counting (Wallac 1205 Betaplate; Wallac, Inc.) The results from triplicate wells were averaged and are reported as mean cpm \pm SE. For indicated experiments, the *in vitro* costimulation analysis was performed in the presence of either a MAb specific for the expressed costimulatory molecule or the matching isotype control antibody (Armenian hamster IgG, polyclonal). Antibodies used to block T-cell proliferation were hamster anti-B7-1, hamster anti-ICAM, or hamster anti-CD48, all from PharMingen. All antibodies were used at 25 μ g/ml final concentration.

C57BL/6 splenocytes were harvested and depleted of T cells by CD90 magnetic beads (Miltenyi Biotec). Spleen stimulator cell populations were prepared by infection with 25 MOI V-WT, rV-B7-1, or rV-TRICOM for 18 h, followed by irradiation (20 Gy). Allogeneic (BALB/c) or syngeneic (C57BL/6) responder T cells (10⁵/well) were prepared as described above and cocultured with graded numbers of spleen stimulator cells for 3 days and labeled for the final 12–18 h of the incubation with 1 μ Ci/well [³H]thymidine.

In other experiments, OVA (Ovalbumin_{257–264}, SIINFEKL)-specific responder T cells (10⁵/well) were cocultured with MC38 stimulator cell populations (MC38 or MC38 infected with V-WT, rV-B7-1, or rV-TRICOM), prepared as described above, and irradiated (300 Gy). OVA-specific T cells (10⁵/well) were cocultured with MC38 stimulator cells (10⁴/well) in the presence of either OVA peptide or control peptide VSVN (vesicular stomatitis virus N_{52–59}, RGYVYQGL) for 2 days and labeled for the final 12–18 h of the incubation with 1 μ Ci/well [³H]thymidine. The incorporated radioactivity was measured by liquid scintillation counting.

Cytokine Analysis. CD4⁺ and CD8⁺ T-cell populations were prepared as described above and added at 2.5 \times 10⁶/well in a six-well plate (Costar). Stimulator cell populations were prepared as above and added at 2.5 \times 10⁵/well. Cells were cultured in a total volume of 5 ml of complete media in the presence of 2.5 μ g/ml Con A for 24 h. Supernatant fluids were collected and analyzed for murine IL-2, IFN- γ , TNF- α , GM-CSF, and IL-4 by capture ELISA as described previously (23). Sensitivity of detection was 30, 100, 20, 20, and 20 pg/ml, respectively.

RNA populations from stimulated cells were also analyzed by multiprobe RNase protection assay. Defined riboprobes for murine cytokines were purchased from PharMingen. Assays were performed as described previously (24). Radioactivity contained in bands on dried polyacrylamide gels was quantified using a Storm system PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The net cpm for a given band was calculated by the following formula [cpm of cytokine gene – cpm of background] and was expressed as a percentage of the housekeeping gene transcript L32.

Apoptosis Assay. CD8⁺ cells were preincubated for 48 h in the presence of various stimulator cells as described in the *in vitro* costimulation analysis section and replated to 96-well plates for 24 h. Apoptosis was assessed using the terminal deoxynucleotidyl transferase-mediated nick end labeling assay, as described previously (25).

In Vivo Studies. Six- to eight-week-old female C57BL/6 mice (Taconic Farms) or C57BL/6 mice transgenic for human CEA (26) were immunized by tail scarification with either HBSS or with 10⁷ pfu of either rV-CEA, rV-CEA/B7-1, or rV-CEA/TRICOM. Lymphoproliferation activity of splenocytes was analyzed as described previously (22). In other experiments, C57BL/6 mice

were immunized as above, challenged 100 days later with 10⁶ MC38 cells expressing CEA (22), and monitored for survival.

RESULTS

Expression of Recombinant Costimulatory Molecules. To confirm that each of the recombinant vectors could express the appropriate costimulatory molecule transgene(s), the murine adenocarcinoma cell line MC38 was infected with the various recombinant vaccinia or fowlpox constructs, and cell surface expression of the transgene(s) was demonstrated by flow cytometry (Fig. 1). Uninfected cells (data not shown) and cells infected with wild-type vaccinia virus (V-WT) failed to express any of the three costimulatory molecules (Fig. 1). This observation was confirmed by PCR (data not shown). In contrast, cells infected with rV-B7-1, rV-ICAM-1, or rV-LFA-3 became positive for their respective transgenes (Fig. 1). Similar analysis of a construct containing two costimulatory molecules (rV-B7-1/ICAM-1) showed expression of B7-1 (78% positive with a mean fluorescent intensity of 1012) and ICAM-1 (70% positive with a mean fluorescence intensity of 690). Moreover, cells infected with the vaccinia multiple-gene construct rV-TRICOM coexpressed all three costimulatory molecules (Fig. 1). Coexpression of all three costimulatory molecules on >79% of cells was confirmed by three-color cytometric analysis. To determine whether the recombinant fowlpox viruses expressed their recombinant proteins, MC38 cells were infected with the fowlpox constructs in a similar manner (Fig. 1). Again, cells

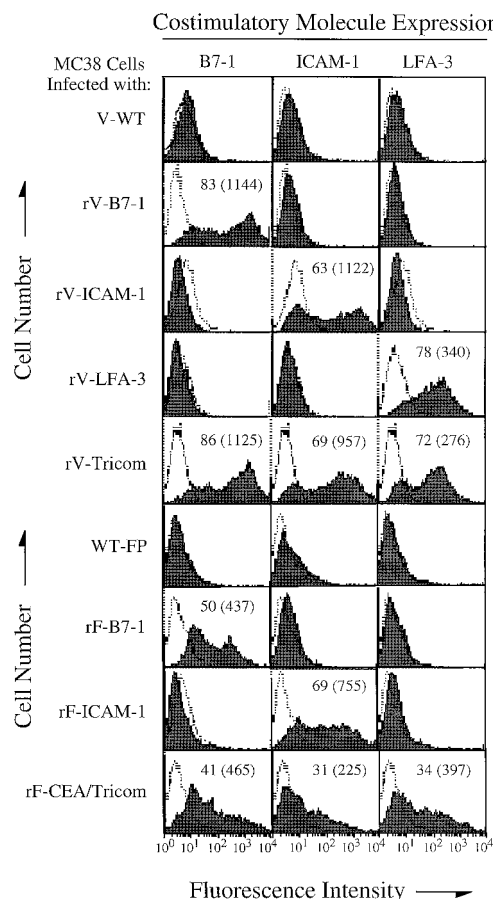


Fig. 1. Costimulatory molecule surface expression after infection with recombinant vectors. MC38 tumor cells were infected for 5 h at 5 MOI with the indicated virus. After infection, cells were immunostained with costimulatory molecule-specific FITC-labeled MAbs. Shaded areas, fluorescence intensity of the specific MAb; unshaded areas, fluorescence intensity of the appropriate isotype control antibody. Numbers in each panel, percentage of positive cells and mean fluorescent intensity (in parentheses).

infected with WT-FP failed to express any costimulatory molecule. Cells infected with rF-B7-1 became positive for B7-1 protein, and cells infected with rF-ICAM-1 became positive for ICAM-1 protein. A rF-LFA-3 vector was not constructed. However, cells infected with the fowlpox multiple-gene construct rF-CEA/TRICOM coexpressed all three costimulatory molecules (Fig. 1).

B7-1, ICAM-1, and LFA-3 Synergize to Enhance T-Cell Proliferation. The B7-1, ICAM-1, and LFA-3 molecules have been shown individually to costimulate T-cell proliferation. However, because they may be expressed simultaneously on APCs, it has been difficult to examine relative roles of individual costimulatory molecules during the induction of T-cell proliferation (2). To analyze the contribution of B7-1, ICAM-1, and/or LFA-3 molecules to the induction of naive T-cell proliferation, an *in vitro* model (7) was used where the first signal for T-cell activation was delivered via a pharmacological reagent (Con A). A panel of stimulator cells that differed only in costimulatory molecules was created using the MC38 cell line infected with various recombinant vaccinia (Fig. 2A) or fowlpox (Fig. 2B) viruses engineered to express costimulatory molecules. The second, or costimulatory, signal was delivered to the T cell via one or more costimulatory molecules expressed on the surface of these stimulator MC38 cells. As shown in Fig. 2A, both uninfected MC38 cells and MC38/V-WT induced marginal proliferation of T cells at all levels of Con A examined. MC38/LFA-3 induced a small (2.1-fold) but significant ($P < 0.05$) increase in T-cell proliferation. Delivery of signal-2 via MC38/ICAM-1 induced a 3.5-fold increase in T-cell proliferation at 2.5 $\mu\text{g/ml}$ Con A. MC38/B7-1 induced a 7.8- and 16-fold increase in proliferation at 2.5 and 1.25 $\mu\text{g/ml}$ Con A, respectively. However, MC38/TRICOM (MC38 cells coexpressing all three costimulatory molecules) induced a 17.5-fold increase in T-cell proliferation at 2.5 $\mu\text{g/ml}$ Con A and a 34-fold increase at 1.25 $\mu\text{g/ml}$ Con A. Moreover, at low Con A levels (0.625 $\mu\text{g/ml}$), expression of ICAM-1 and LFA-3 did not induce T-cell proliferation. Although B7-1 induced measurable proliferation (20,000 cpm) at 0.625 $\mu\text{g/ml}$ Con A, the coexpression of all three costimulatory molecules induced an even greater level of proliferation (100,000 cpm; Fig. 2A). These experiments were repeated four times with similar results.

MC38 stimulator cells were also prepared by infection with recombinant fowlpox vectors (Fig. 2B). Again, uninfected MC38, MC38/WT-FP, or MC38/rF-CEA induced marginal proliferation of T cells at all levels of Con A examined. MC38/rF-ICAM-1 supported a 2-fold increase, MC38/rF-B7-1 supported a 3.2-fold increase, and MC38/

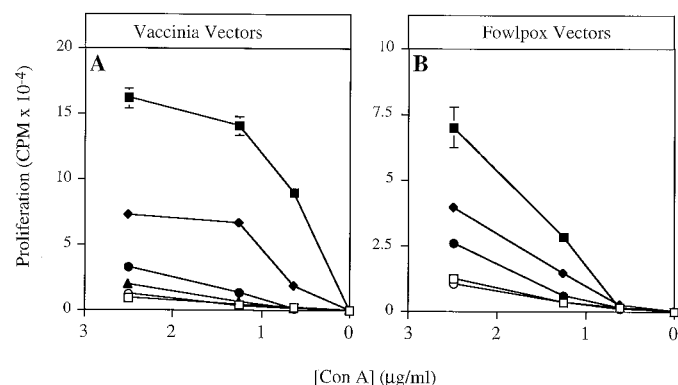


Fig. 2. Effect of multiple costimulatory molecules on T-cell proliferation. Naive murine T cells, in the presence of varying concentrations of Con A to provide the first signal, were cocultured with MC38 stimulator cells infected with either recombinant vaccinia (A) or recombinant fowlpox (B) vectors. Recombinant vectors were wild-type (*i.e.*, V-WT or WT-FP; \square), rV-LFA-3 (\blacktriangle), rV-ICAM-1 or rF-ICAM-1 (\bullet), rV-B7-1 or rF-B7-1 (\blacklozenge), and rV-TRICOM or rF-CEA/TRICOM (\blacksquare). \circ , uninfected MC38 cells. Bars, SD.

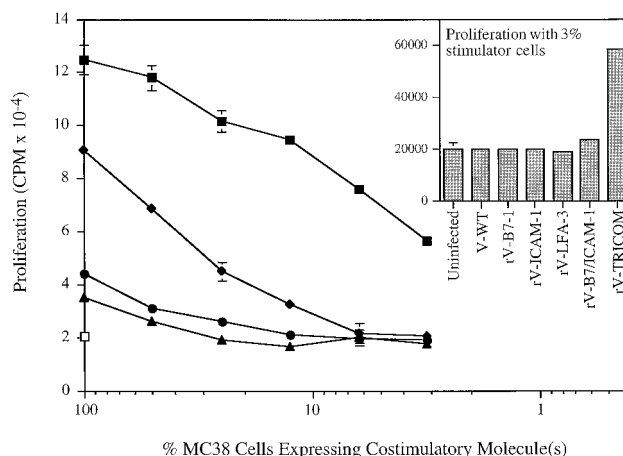


Fig. 3. Relative capacity of B7-1, ICAM-1, LFA-3, and the coexpression of all three costimulatory molecules (TRICOM) to deliver the second signal for T-cell proliferation. In the presence of Con A (2.5 $\mu\text{g/ml}$), 100,000 T cells were cocultured with 10,000 MC38 cells. The stimulator MC38 cells expressing one or all of the costimulatory molecules were added to the wells in various ratios in combination with V-WT-infected stimulator cells to a total of 10^4 MC38 cells/well. MC38 cells were infected with V-WT (\square), rV-LFA-3 (\blacktriangle), rV-ICAM-1 (\bullet), rV-B7-1 (\blacklozenge), or rV-TRICOM (\blacksquare). Inset, proliferation values obtained from a culture in which 3% of the MC38 stimulator cells were infected with the vectors shown. Thus, in this experiment, the final ratio of stimulator cells:T cells was 0.003. Bars, SD.

rF-CEA/TRICOM supported a 6-fold increase in T-cell proliferation at 2.5 $\mu\text{g/ml}$ Con A. Similar results were obtained when this experiment was repeated two additional times and when the first signal was delivered via immobilized anti-CD3 (data not shown).

To further confirm the specificity of the proliferative contribution of B7-1, ICAM-1, or LFA-3, MC38 stimulator cells were prepared by infection with V-WT, rV-B7-1, rV-ICAM-1, or rV-LFA-3 and cocultured with naive murine T cells and Con A in the presence or absence of MAb specific for the given costimulatory molecule. MC38/B7-1 enhanced T-cell proliferation 4.5-fold more than that of MC38/V-WT. This increased proliferation was inhibited 83% by the addition of a blocking MAb for murine B7-1. Similarly, MC38/ICAM-1 increased proliferation 2.25-fold, which was then reduced by 88% in the presence of anti-murine ICAM-1 MAb. Finally, MC38/LFA-3 increased proliferation 2.1-fold, which was then reduced by 98% in the presence of antimurine CD48 MAb. For each group, incubation with the appropriate isotype control antibody failed to block the noted proliferation. This experiment was repeated two additional times with similar results.

Determination of Costimulatory Molecule Capacity. Modification of the *in vitro* costimulation assay allowed a quantitative estimation of the relative capacity of B7-1, ICAM-1, and/or LFA-3 to deliver the second signal for T-cell proliferation. To that end, stimulator cells (MC38 cells infected with the various recombinant vaccinia viruses) were titrated by dilution with varying amounts of MC38 cells infected with V-WT and cocultured with a constant number of T cells in the presence of 2.5 $\mu\text{g/ml}$ Con A. The total MC38:T-cell ratio in these experiments remained constant at 1:10. As seen in Fig. 3, MC38/LFA-3 enhanced proliferation of T cells over that of MC38/V-WT to a dilution of 40% (*i.e.*, of the stimulator cells in the well, 40% were infected with rV-LFA-3 and the remaining 60% were infected with V-WT). MC38/ICAM-1 or MC38/B7-1 supported increased T-cell proliferation to dilutions of 13 and 6%, respectively. In contrast, MC38/TRICOM enhanced proliferation when $<3\%$ of stimulator cells contained the TRICOM vector (extrapolated to $<1\%$ via linear least squares analysis). Given the titration curves of these individual costimulatory molecules, it appeared that the extent of T-cell proliferation mediated by ICAM-1 and B7-1 is 3- and 6-fold, respectively,

more potent than that mediated by LFA-3 alone. Clearly, the strongest proliferation, however, is mediated by TRICOM. It should be noted (Fig. 3) that at relatively low stimulator cell concentrations (*i.e.*, when 3–6% of the MC38 cells are acting as stimulator cells), expression of LFA-3, ICAM-1, and even B7-1 alone does not enhance T-cell activation, whereas the TRICOM-expressing stimulator cells substantially enhance T-cell activation. The data in Fig. 3 (*inset*) show proliferation results obtained when 3% of the MC38 stimulator cells were infected with the vectors denoted. Because each well contained 10^4 total MC38 cells and 10^5 naive T cells, the actual stimulator:T-cell ratio in these cultures was 0.003. Note that the MC38 cells infected with the two-gene construct (rV-B7-1/ICAM-1) induced little, if any, proliferation of T cells under these conditions, whereas MC38/TRICOM increased proliferation substantially ($P < 0.0001$).

Costimulation of CD4⁺ and CD8⁺ T Cells. To further characterize the T-cell response to costimulatory molecules expressed singly or in combination, the ability of B7-1, ICAM-1, and LFA-3 to costimulate purified CD4⁺ and CD8⁺ T cells was tested. Fig. 4, A and B, respectively, show the proliferation of purified CD4⁺ and CD8⁺ cells activated with suboptimal concentrations of Con A. The stratification of stimulator cell effects on proliferation was similar for both CD4⁺ and CD8⁺ cells; MC38/LFA-3 stimulated the weakest proliferation, followed by MC38/ICAM-1 and MC38/B7-1. MC38/TRICOM were the most potent stimulator cells for both CD4⁺ and CD8⁺ T cells. These experiments were repeated three additional times with similar results. It should be noted that at very low concentrations of Con A (0.625 μ g/ml; Fig. 4, C and D), there was no significant enhancement in activation of CD4⁺ or CD8⁺ T cells when either ICAM-1, LFA-3, B7-1, or the B7-1/ICAM-1 dual recombinant was used to provide the second signal. However, substantial activation of both T-cell subsets was observed when the TRICOM vector was used. Similar results were noted when the first signal was delivered via immobilized anti-CD3 (data not shown).

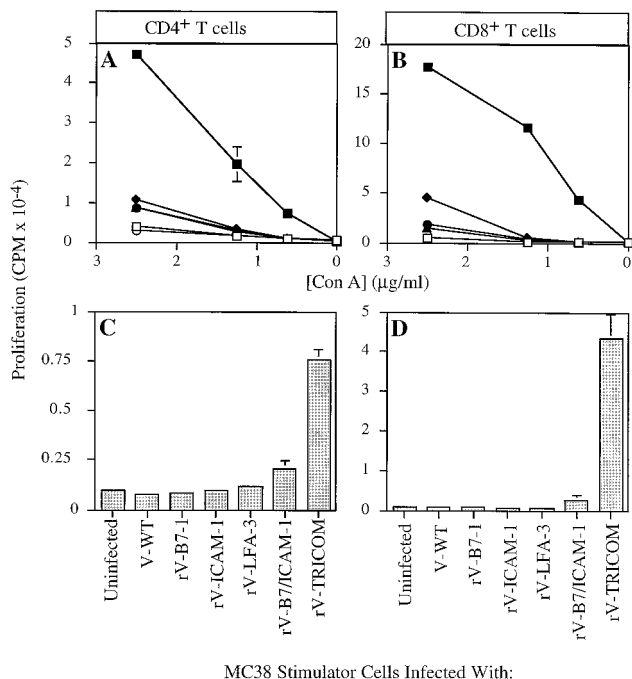


Fig. 4. Effect of costimulation on specific T-cell populations. Murine CD4⁺ (A) or CD8⁺ (B) T cells were cocultured with uninfected MC38 cells (○), or cells infected with V-WT (□), rV-LFA-3 (▲), rV-ICAM-1 (●), rV-B7-1 (◆), or rV-TRICOM (■) at a 10:1 ratio for 48 h in the presence of various concentrations of Con A. Bars, SD. C and D, proliferative responses of purified CD4⁺ and CD8⁺ cells, respectively, when cocultured in the presence of vector-infected MC38 stimulator cells at a low Con A concentration (0.625 μ g/ml). Bars, SD.

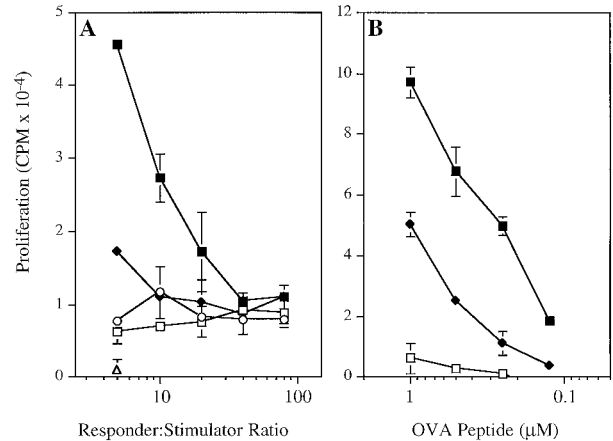


Fig. 5. Effect of costimulation on allospecific (A) or peptide-specific (B) T-cell proliferation. A. BALB/c (H-2^d) splenic T cells were cocultured with graded numbers of uninfected C57BL/6 (H-2^b) naive splenocytes (○) or C57BL/6 splenocytes infected with 25 MOI V-WT (□), rV-B7-1 (◆), or rV-TRICOM (■) for 72 h. These C57BL/6 stimulator cell populations were also cocultured with syngeneic C57BL/6 T cells (△). B. OVA_{257–264}-specific CD8⁺ T cells were cocultured with uninfected MC38 cells (not shown) or cells infected with V-WT (□), rV-B7-1 (◆), or rV-TRICOM (■) at a 10:1 ratio for 48 h in the presence of various concentrations of OVA_{257–264} peptide. Background proliferation (uninfected MC38 cells) was subtracted from all groups. Bars, SD.

Two additional models (peptide-specific and allospecific) were also used to demonstrate the efficacy of rV-TRICOM in enhancing T-cell proliferation. Naive splenocytes from C57BL/6 mice, depleted of T cells, were used as APCs either uninfected or infected with V-WT, rV-B7-1, or rV-TRICOM, followed by irradiation. Allogeneic (BALB/c) or syngeneic splenic T cells were used as responder cells in mixed lymphocyte reactions as described in “Materials and Methods.” As seen in Fig. 5A, C57BL/6 splenocytes infected with rV-TRICOM enhanced allospecific T-cell proliferation to far greater levels than uninfected splenocytes or splenocytes infected with V-WT or rV-B7-1. All stimulator cell populations, even those infected with TRICOM, that were incubated with syngeneic T cells resulted in no proliferation (<1000 cpm; Fig. 5A).

Peptide-specific proliferation of established effector T cells revealed similar results. When MC38 stimulator cells were pulsed with OVA peptide and cocultured with an OVA-specific T-cell line, MC38/B7-1 induced a 9-fold increase in peptide-specific T-cell proliferation over that of MC38/V-WT, whereas MC38/TRICOM induced a 20-fold increase in peptide-specific proliferation (Fig. 5B). Stimulator cells pulsed with control peptide VSVN did not support proliferation of the OVA-specific T-cell line (<1000 cpm).

Cytokine Studies. It has been reported that B7-1 costimulation prolongs IL-2 mRNA half-life and up-regulation of IL-2 transcription, resulting in production of considerable amounts of secreted IL-2 (3, 8). Additionally, T-cell costimulation with LFA-3 has been reported to have an effect on a variety of cytokines, notably IL-2 and IFN- γ (4). To determine qualitative and quantitative effects of costimulation by single or multiple costimulatory molecules on cytokine production, purified CD4⁺ and CD8⁺ T cells were cocultured with various stimulator cells expressing either B7-1, ICAM-1 or LFA-3, or expressing all three molecules (TRICOM) in the presence of 2.5 μ g/ml Con A. Supernatant fluids were analyzed for IL-2, IFN- γ , TNF- α , GM-CSF, and IL-4 after 24 h (Fig. 6). Uninfected MC38 (data not shown) and MC38/V-WT induced a marginal quantity of IL-2 from CD4⁺ cells, whereas MC38/B7-1 induced 3979 pg/ml (Fig. 6A). However, T-cell stimulation with MC38/TRICOM induced a 10-fold greater amount of IL-2 (Fig. 6A). Similarly, MC38/B7-1 induced a marginal quantity of IL-2 from CD8⁺ cells, whereas MC38/TRICOM induced a 20-fold greater amount (6182 pg/ml; Fig. 6B). IFN- γ

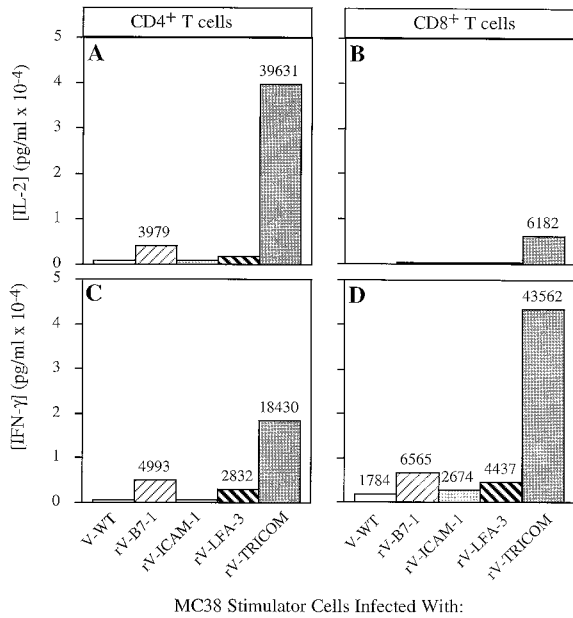


Fig. 6. Effect of costimulation on cytokine production. Murine CD4⁺ (A and C) or CD8⁺ (B and D) T cells were purified and cocultured with the indicated MC38 vector-infected stimulator cells for 24 h in the presence of 2.5 μg/ml Con A. Supernatant fluids were analyzed for production of IL-2 (A and B) and IFN-γ (C and D) by capture ELISA. Numbers above the columns, amount (pg/ml) of cytokine released.

production by stimulated T cells was also examined (Fig. 6, C and D). MC38/B7-1 and MC38/LFA-3 induced only moderate amounts of IFN-γ from CD4⁺ cells. In contrast, stimulation of CD4⁺ cells with MC38/TRICOM induced 4-fold more IFN-γ than stimulation with any other construct (Fig. 6C). Stimulation of CD8⁺ cells with MC38/TRICOM induced the greatest amount of IFN-γ, greater than 6-fold more than CD8⁺ cells stimulated with any of the other constructs

(Fig. 6D). Stimulation of either cell type with any construct failed to mediate significant changes ($P > 0.05$) in the levels of secreted TNF-α, GM-CSF, or IL-4 (data not shown). It appears that the predominant culmination of stimulation via the TRICOM construct was IL-2 secretion from CD4⁺ cells and IFN-γ secretion from CD8⁺ T cells. These experiments were repeated three additional times with similar results. Studies were also carried out comparing stimulator cells infected with the two-gene construct (rV-B7-1/ICAM-1) with the triad construct (rV-TRICOM) for their ability to enhance cytokine production by T cells. Only small differences were observed between the two in IFN-γ production by either CD4⁺ or CD8⁺ cells or in IL-2 production by CD8⁺ cells. However, a substantial difference was seen in the stimulation of IL-2 production by CD4⁺ cells (5000 pg/ml using MC38/B7-1/ICAM-1 versus 39600 pg/ml using MC38/TRICOM).

Cytokine expression in CD4⁺ and CD8⁺ T cells stimulated with single or multiple costimulatory molecules was also analyzed at the RNA level using the multiprobe RNase protection assay. A radiographic profile and quantitative analysis are depicted (Fig. 7). These experiments were repeated twice with similar results. Levels of IL-4, IL-5, IL-10, IL-15, and IL-6 were similar in CD4⁺ T cells stimulated with MC38/V-WT, MC38/B7-1, MC38/ICAM-1, MC38/LFA-3, or MC38/TRICOM (Fig. 7, A-B). IL-2 and IFN-γ expression levels were highest in CD4⁺ T cells stimulated with MC38/TRICOM when compared with CD4⁺ cells stimulated with MC38 cells expressing any single costimulatory molecule (Fig. 7, A and B). Slightly higher levels of IL-13, IL-9, and IL-6 were also noted in CD4⁺ cells stimulated with MC38/TRICOM. Expression of cytokine genes was also analyzed in stimulated CD8⁺ T cells. Of the cytokine RNAs analyzed, IL-2 and, in particular, IFN-γ levels were significantly higher when these cells were stimulated with MC38/TRICOM, compared with T cells stimulated with MC38 cells expressing any single costimulatory molecule (Fig. 7, A and C). Thus, the predominant synergistic effect

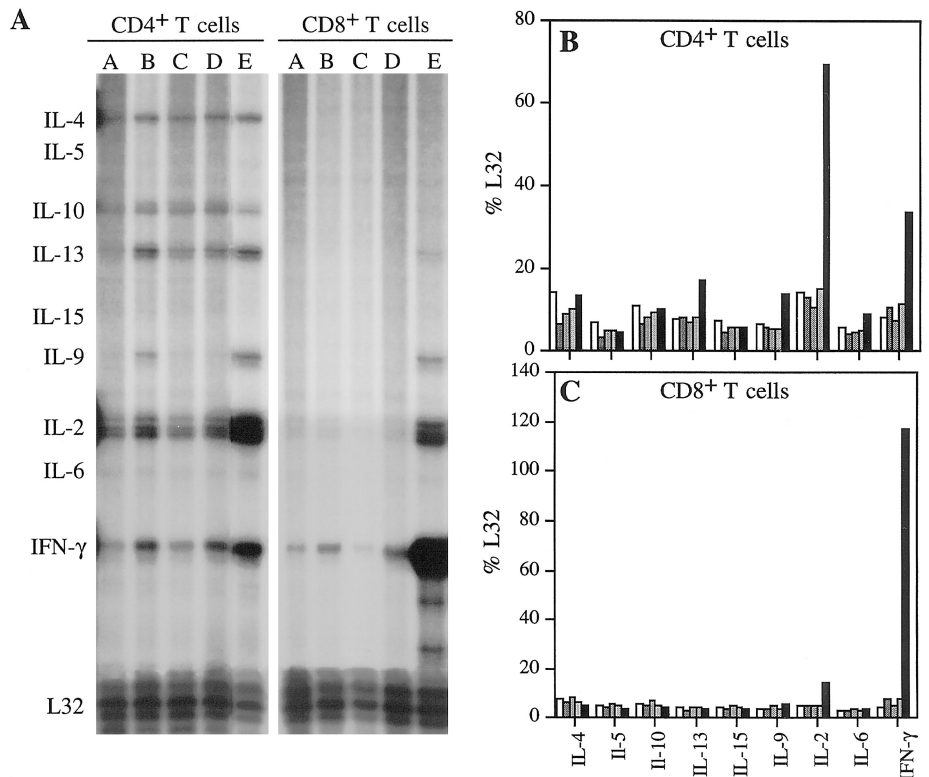


Fig. 7. Effect of costimulation on cytokine RNA expression. A, murine CD4⁺ or CD8⁺ T cells were cocultured with MC38 stimulator cells infected with V-WT (Lane A), rV-B7-1 (Lane B), rV-ICAM-1 (Lane C), rV-LFA-3 (Lane D), or rV-TRICOM (Lane E) at a T-cell:stimulator-cell ratio of 10:1 for 24 h in the presence of 2.5 μg/ml Con A. After culture, T-cell RNA was analyzed by multiprobe RNase protection assay. The quantitative representation of results from the autoradiograph is normalized for expression of the housekeeping gene L32 in B (CD4⁺ cells) and C (CD8⁺ cells). Order of histogram columns (from left to right): MC38/V-WT, MC38/B7-1, MC38/ICAM-1, MC38/LFA-3, and MC38/TRICOM (■).

of the triad of costimulatory molecules in cytokine production was IL-2 in CD4⁺ cells and IFN- γ in CD8⁺ T cells.

Apoptosis Studies. To determine whether stimulation of T cells with signal 1 and rV-TRICOM would lead to cell survival or PCD, T cells were activated with Con A for signal 1, cultured with either V-WT, rV-B7-1, or rV-TRICOM-infected MC38 cells, and replated for 24 h in medium to measure apoptosis. T cells activated by the combination of MC38 and Con A or MC38/V-WT and Con A in the absence of costimulatory signals exhibited high levels of spontaneous apoptosis (82.9 ± 3.8 and 78.9 ± 1 , respectively; Fig. 8, A and B). T cells activated by Con A and MC38/B7-1 or Con A and MC38/TRICOM exhibited substantially less spontaneous apoptosis (31.3 ± 3.8 and 30.7 ± 1 , respectively; Fig. 8, C and D).

In Vivo Studies. Studies were also conducted to determine whether an antigen-specific immune response could be enhanced using a TRICOM vector. A four-gene vaccinia recombinant was constructed that contained the human CEA gene and the B7-1, ICAM-1, and LFA-3 genes, designated rV-CEA/TRICOM (see "Materials and Methods"). Mice were vaccinated one time with 10^7 pfu rV-CEA (22), rV-CEA/B7-1 (11), or rV-CEA/TRICOM, and spleens were harvested 22 days later. As seen in Fig. 9 (inset), splenic T cells of mice vaccinated with rV-TRICOM showed higher levels of CEA-specific stimulation compared with T cells obtained from mice vaccinated with rV-CEA; ovalbumin and Con A were used as controls. An experiment was then conducted to determine whether rV-CEA/TRICOM could induce long-term immunity. Mice (five/group) were vaccinated one time with either V-WT, rV-CEA, or rV-CEA/TRICOM. One hundred days later, mice were challenged with a high dose (1×10^6) of MC38 colon carcinoma cells expressing CEA (22). All mice receiving V-WT and rV-CEA succumbed to tumors, whereas all mice vaccinated with rV-TRICOM were alive 50 days after challenge (Fig. 9).

CEA-transgenic mice (26, 27) in which the human CEA gene is expressed in normal adult gastrointestinal tissue, and who have CEA-positive sera were used to determine whether the rV-CEA/TRICOM vector could enhance T-cell responses to a self-antigen. CEA transgenic mice were separated into five mice/group (because of limited availability). Two mice were vaccinated once with 10^7 pfu rV-CEA, rV-CEA/B7-1, rV-CEA/TRICOM, or buffer and were euthanized on day 30 to analyze CEA-specific T-cell responses. T-cell responses obtained after vaccination with rV-CEA/TRICOM were substantially greater than those obtained with rV-CEA (Table 1). Responses to

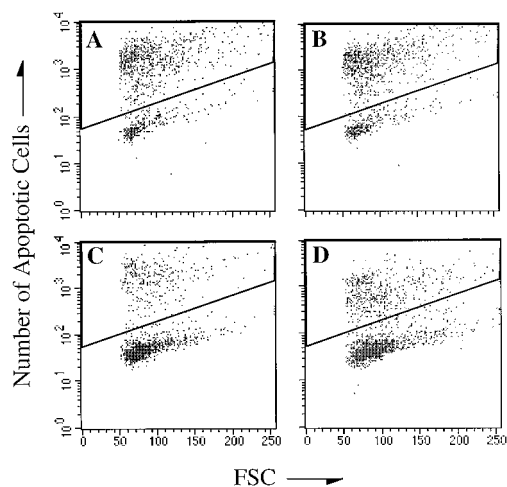


Fig. 8. Apoptosis of CD8⁺ cells activated by Con A and either MC38 (A), MC38/V-WT (B), MC38/B7-1 (C), or MC38/TRICOM (D). Each panel depicts the percentage of apoptotic cells (above line) in each group as measured by the terminal deoxynucleotidyl transferase-mediated nick end labeling assay. FSC, forward scatter.

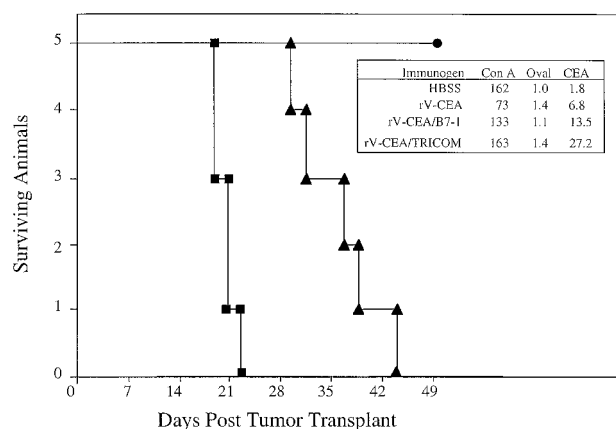


Fig. 9. C57BL/6 mice (five/group) were administered HBSS (■) or vaccinated with 10^7 pfu rV-CEA (▲) or rV-CEA/TRICOM (●). One hundred days later, mice were inoculated with 1×10^6 MC38 carcinoma cells expressing CEA (30), and survival was monitored. All mice other than the rV-CEA/TRICOM group developed tumors and were sacrificed when tumors exceeded 20 mm in length or width, or when the mice were moribund. Inset, in a second experiment, C57BL/6 mice (five/group) were vaccinated with 10^7 pfu rV-CEA, rV-CEA/B7-1, rV-CEA/TRICOM, or HBSS buffer. Lymphoproliferative responses from pooled splenic T cells were analyzed 22 days after vaccination. Values represent the stimulation index of the mean cpm of triplicate samples versus media. SD never exceeded 10%. Antigens used were Con A ($5 \mu\text{g/ml}$), CEA ($100 \mu\text{g/ml}$), and ovalbumin ($100 \mu\text{g/ml}$).

ovalbumin and Con A were used as controls. The remaining three CEA-transgenic mice in each group were used to determine whether antitumor responses to a CEA-expressing tumor could be enhanced using a TRICOM vector. These mice were first inoculated s.c. with 4×10^5 MC38 carcinoma cells expressing the CEA gene (22). Four days later, mice were vaccinated one time at a distal site with 10^7 pfu viral recombinant or buffer. No tumors grew in mice vaccinated with rV-CEA/TRICOM, whereas tumors continued to grow in mice vaccinated with buffer, rV-CEA and rV-CEA/B7-1 (Table 1). Although preliminary, these results support the *in vivo* activity of TRICOM vectors.

DISCUSSION

When a naive T cell encounters antigen, several distinct outcomes are possible including cytokine proliferation, cytokine secretion, differentiation into effector cells, inactivation, death, or unresponsiveness (anergy). The predominant outcome under physiological conditions may be determined by whether appropriate costimulatory signals are delivered to the responding T cell (9). At least three distinct molecules normally found on the surface of professional APC (B7-1, ICAM-1 and LFA-3) have been shown to be capable of providing the signals critical for T-cell activation (2–10). Here, the role of costimulatory molecules in naive T-cell activation was examined by using vectors engineered to express either B7-1, ICAM-1, LFA-3, or a combination of all three molecules (designated TRICOM).

Several groups have investigated the cooperation of two of these molecules in T-cell costimulation. Dubey *et al.* (9) have reported that costimulation by both B7-1 and ICAM-1 is a prerequisite for naive T-cell activation, whereas Cavallo *et al.* (10) determined that B7-1 and ICAM-1 must be coexpressed by tumor cells to establish an antitumor memory response. In addition, costimulation by B7-1 and LFA-3 has been shown to act additively both upon T-cell proliferation and cytokine production (4, 7). These previous studies were carried out using two costimulatory molecules and retroviral vectors. One gene was transduced into the target cell line and drug selected. These cells were then transduced again with a second recombinant retroviral construct, followed by selection with a different agent. This process

Table 1 Enhanced immune response and antitumor response of rV-CEA/TRICOM in CEA transgenic mice

C57BL/6 CEA-transgenic mice (five per group) were vaccinated via skin scarification with buffer or vaccinia recombinant (10^7 pfu) one time on day 0. On day 30, two mice were killed, and splenic T cells were analyzed for T-cell proliferative responses. Each value represents the stimulation index of the mean cpm of triplicate samples versus media. SD never exceeded 10%. On day -4, three mice per group were given 4×10^5 MC38 colon carcinoma cells expressing CEA. Tumor volume is given at days 14 and 35 after vaccination.

Immunogen	Stimulation index				Tumor volume	
	Con A	Oval	CEA	CEA	Day 14	Day 35
	(5 μ g/ml)	(100 μ g/ml)	(100 μ g/ml)	(25 μ g/ml)		
HBSS	109	1.0	1.3	2.0	698 \pm 928	3674 \pm 3107
rV-CEA	123	0.9	4.9	4.0	259 \pm 0	1112 \pm 1685
rV-CEA/B7-1	93	1.3	7.1	4.3	150 \pm 236	2696 \pm 1936
rV-CEA/TRICOM	111	1.1	19.2	15.9	0 \pm 0	0 \pm 0

often requires weeks or months. Using recombinant poxvirus vectors, one is able to achieve the coexpression of three costimulatory molecules 5 h after infection (Fig. 1). *In vitro* MC38 cells infected with either recombinant vaccinia or avipox TRICOM vectors were shown to enhance proliferation of T cells to a much greater extent than MC38 cells infected with vectors containing the gene for any single costimulatory molecule (Fig. 2). In addition, the relative strength of the second signal delivered to the T cell by the combination of costimulatory molecules appeared to be far greater than that delivered by MC38 cells expressing any single costimulatory molecule (Fig. 3). Dubey *et al.* (9) have demonstrated that at low stimulator:T-cell ratios, synergy was noted with B7-1 and ICAM-1. Our studies confirm these findings. However, at extremely low stimulator:T-cell ratios (Fig. 3) or weak signal 1 (0.625 μ g/ml Con A; Fig. 4, C and D), the two-gene construct (rV-B7-1/ICAM-1) had little effect, if any, on proliferation. In contrast, stimulation via the TRICOM construct had a substantial and statistically significant effect on proliferation. The predominant effect of stimulation via the TRICOM construct was IL-2 production from CD4⁺ cells and IFN- γ production from CD8⁺ T cells (Fig. 6), whereas few type 2 cytokines, if any, were produced. Cytokine expression analysis by RNase protection provided a profile compatible with the *in vitro* cytokine assay, manifested by substantially higher expression of IL-2 and IFN- γ in both CD4⁺ and CD8⁺ T cells stimulated with all three costimulatory molecules, compared with stimulation by any single costimulatory molecule (Fig. 7). These data are in accordance with previous studies demonstrating that in the context of low CD28 costimulation, T cells produced low levels of IL-1, whereas strong CD28 costimulation supported production of IL-2, IFN- γ , and IL-13 (28). Taken together, the studies reported here indicate that optimal naive T-cell responses require a higher level of costimulation than was previously thought, and that this could be provided by the combined action of three costimulatory molecules.

One question that is immediately raised is the potential of overstimulated T cells to undergo PCD. The results shown in Fig. 8, A and B, clearly demonstrate apoptosis in T cells stimulated with MC38 cells in the presence of Con A with or without V-WT infection (*i.e.*, in the absence of signal 2). Whereas Con A with MC38/TRICOM clearly stimulated CD8⁺ cells to far greater levels than Con A with MC38/B7-1 (Fig. 2) and resulted in the production of higher levels of IFN- γ and IL-2 (Figs. 6 and 7), this did not result in any greater degree of apoptosis (Fig. 8D). Our results are 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 undergo PCD in culture (29). This could be attributed to augmentation of cytokine production by these cells and potential up-regulation of survival genes. Further studies are presently under way to analyze the detailed mechanism of survival in these cells.

Perhaps the most studied T-cell costimulatory molecule is B7-1. This molecule's ability to enhance T-cell activation using retroviral

vectors, anti-CTLA-4 antibodies, and poxvirus vectors is well established. The studies reported here rank the order of T-cell stimulation by a single costimulatory molecule as B7-1 > ICAM-1 > LFA-3. However, the use of three costimulatory molecules was far superior to B7-1 alone in both proliferation and cytokine production for both CD4⁺ and CD8⁺ T cells. The studies reported here demonstrate the power of the multicostimulatory molecule effect to enhance T-cell proliferation in three very different systems and to use both naive and effector T-cell populations as responder cells. These three model systems are: (a) the activation of naive T cells using Con A or anti-CD3 as signal 1 (Fig. 2); (b) the use of OVA peptide as signal 1 and the activation of OVA-specific "effector" T cells from an established cell line (Fig. 5B); and (c) enhanced allospecific reactivity in a mixed lymphocyte reaction (Fig. 5A).

Initial *in vivo* experiments reported here indicate that rV-CEA/TRICOM is more efficient than rV-CEA/B7-1 in the induction of CEA-specific T-cell responses in both intact C57BL/6 mice and in CEA-transgenic C57BL/6 mice. Induction of long-term immunity to tumor challenge is indicated for rV-CEA/TRICOM in C57BL/6 mice (Fig. 9), and antitumor activity for rV-CEA/TRICOM versus rV-CEA/B7-1 or rV-CEA in CEA transgenic mice is indicated in Table 1. However, only limited amounts of the CEA transgenic mice were available and used in these studies. Additional comprehensive studies with more CEA transgenic mice and different doses and dose schedules of rV-TRICOM, rV-CEA/B7-1, and rV-CEA, along with different tumor burdens, are clearly indicated. These mice will also have to be carefully evaluated for evidence of autoimmunity. Because of the scarcity of CEA-transgenic mice, these will be long-term experiments.

There are several possible mechanisms for the synergy observed here between B7-1, ICAM-1, and LFA-3. The ICAM-1/LFA-1 interaction reportedly costimulates the TCR-mediated activation of T cells by sustaining the increase in the same intracellular second messengers as generated by TCR engagement. The ICAM-1/LFA-1 interaction is necessary to up-regulate expression of the IL-2R- α chain and CD28 on T cells, which is required to render them competent to respond to IL-2 and B7-1 costimulation. The B7-1/CD28 interaction delivers a TCR-independent costimulatory signal that increases both transcriptionally and posttranscriptionally the expression of IL-2 and other immunoregulatory lymphokines. The LFA-3/CD2 interaction induces tyrosine phosphorylation of several intracellular second messengers, Ca²⁺ mobilization, and cAMP production, resulting in elaboration of a variety of cytokines, notably IL-2 and IFN- γ (4). Thus, it appears that the three costimulatory molecules could be cooperating by enhancing the antigen-dependent activation of T cells, as well as their production of and response to autocrine and paracrine growth factors. Future studies should involve an analysis of the signal transduction pathways induced by T-cell costimulation via stimulator cell populations expressing B7-1, ICAM-1, and LFA-3, alone or in combination.

In conclusion, these studies demonstrate for the first time the ability of vectors to introduce three costimulatory molecules into a cell and

to rapidly and efficiently activate both CD4⁺ and CD8⁺ T-cell populations to levels far greater than those achieved when any one or two of these costimulatory molecules are used. This new threshold of T-cell activation, with the caveat of autoimmunity mentioned above, has broad implications in vaccine design and development for a range of diseases.

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