Amplification of the lytic potential of effector/memory CD8⁺ cells by vector-based enhancement of ICAM-1 (CD54) in target cells: implications for intratumoral vaccine therapy

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We demonstrated that enhanced expression of the costimulatory molecules CD80, CD54 and CD48 (designated rF-TRICOM) on target cells, as delivered via a recombinant fowlpox vector, results in an increased state of stimulation of CD8⁺ T cells, and consequent increased lysis of target cells. CTL studies in conjunction with antibody-blocking studies demonstrated that the enhanced effector activity of these CD8⁺ T cells is mediated mainly through CD54. Intracellular staining of CD8⁺ cells that interact with target cells infected with rF-TRICOM showed that they contain higher amounts of perforin and have a higher level of perforin message. Enhanced expression of costimulatory molecules (specifically CD54) on target cells using rF-TRICOM vectors also leads to the formation of stable conjugates/synapses between targets and T cells. The interaction of T cells with target cells that overexpress costimulatory molecules upon infection with rF-TRICOM leads to enhanced signaling through Lck, ZAP70, and STAT-1 in CD8⁺ T cells and heightened lytic activity of CD8⁺ cells through the formation of a greater number of immunological synapses. This, in turn, leads to enhanced signaling in T cells. Finally, studies were conducted in mice in which CEA is a self-antigen in an attempt to understand the potential clinical relevancy of intratumoral vaccine therapy. Mice were transplanted subcutaneously with CEA expressing tumors. Intratumoral (i.t.) vaccination was administered 8 days post tumor transplant. Mice vaccinated i.t. with rF-TRICOM demonstrated significantly reduced tumor growth and 40% of the mice had complete tumor regression. The antitumor effects were further improved by the addition of tumor antigen (CEA) in the vaccination by utilizing rF-CEA/TRICOM, with 80% of the mice experiencing complete tumor regression. These studies thus support the concept of intratumoral vaccination employing vectors expressing costimulatory molecules. *Cancer Gene Therapy* (2004) **11**, 665–680. doi:10.1038/sj.cgt.7700741

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T-cell activation is a complex process involving various ligand–receptor interactions. At least two distinct signals are needed for these interactions and for T-cell activation. Signal 1 is mediated through the T-cell receptor (TCR) binding to the MHC-peptide complex. Signal 2 is generated upon the interaction of T cells and costimulatory molecules. One of the best-described costimulatory pathways is mediated by interactions of CD80 with its ligand CD28. While there have been

numerous studies that address the role of CD80 in T-cell activation, the importance of other molecules such as CD54 and CD48 in providing costimulation and signal transduction is not as well understood.^{1–3} The difficulty in analyzing the specific contribution of each of these accessory molecules has been compounded by various issues, such as their ability to act as adhesion molecules, the redundancy of these molecules, and the differences in the specific requirements of accessory molecules for different subpopulations of T cells.

Although there have been a number of studies on the role of accessory molecules in T-cell activation,^{4–10} only a few studies have begun to address the role of the costimulatory molecules on enhancing the stimulation of effector/memory cells.^{11–15} To this end, we have investigated the consequence of infected tumor target cells with fowlpox (FP) viruses containing various costimulatory molecules on enhancing the activity of the effector/memory CD8⁺ cells in the lysis of those target cells.

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The use of replication incompetent recombinant fowlpox (rF) vectors expressing costimulatory/adhesion molecules has been well documented in preclinical studies.^{16–18} It has been demonstrated that using an rF vector, which contains three costimulatory/adhesion molecules (CD80, CD54 and CD48, designated rF-TRICOM (triad of costimulatory molecules)), is more advantageous than using a vector containing a single costimulatory molecule in the enhancement of naive CD4⁺ and CD8⁺ T-cell receptor mediated proliferation.^{16–18} These findings have led to clinical studies using rF-TRICOM in various settings, including current clinical trials using rF-TRICOM vector via direct injection of tumors. The rationale of these studies is to enhance the ability of the solid tumors that lack costimulatory molecules to become more efficient APCs.

The question we raised here is whether infection of target cells with rF-TRICOM vectors would also increase the target-cell/T-cell interactions and subsequently enhance the affinity of antigen-specific T cells. To answer this question, we employed two different *in vitro* models. The first is the TCR transgenic (Tg) CD8⁺ T-cell line specific for Flu peptide (NP68), and as targets the L-929D^b fibroblast line or MC38 tumor cell line; the second is a CD8⁺ T-cell line specific for an epitope of carcinoembryonic antigen (CEA) and CEA peptide-pulsed targets (L-929D^b fibroblast line). In studies reported here we demonstrated that infection of target cells with rF-TRICOM vectors enhanced the lytic activity of interactive T cells, and that this enhanced activity is principally mediated through CD54. Moreover, infection of target cells with rF-TRICOM led to more conjugate/synapse formation between target cells and T cells and the enhancement of signals through the TCR. When CEA-transgenic mice were transplanted subcutaneously (s.c) with CEA expressing tumors, intratumoral (i.t.) vaccination with rF-TRICOM on day 8 significantly reduced tumor growth with 40% of the mice demonstrating complete tumor regression. The antitumor effects were further improved by the addition of tumor antigen (CEA) in the vaccination by utilizing rF-CEA/TRICOM, with 80% of the mice experiencing complete tumor regression.

Materials and methods

Mice and tumor cells

Flu TCR-Tg mice (F5 TCR-Tg) mice, which are TCR transgenic for NP68 peptide, were obtained from Taconic Farms (Germantown, NY) and bred in a National Cancer Institute facility under pathogen-free conditions. C57BL/6 mice transgenic for human CEA were obtained from a breeding pair provided by Dr John Thompson (Institute of Immunobiology, University of Freiburg, Germany). The generation and characterization of the CEA-transgenic mouse has been previously described.¹⁹

Parental murine colon adenocarcinoma MC38 cells, MC38 expressing human CEA (designated MC38-CEA⁺) were used.²⁰ Before implantation to mice, these adherent

cells were trypsinized and washed in Hank's balanced salt solution (HBSS).

MAbs and flow cytometry

To evaluate CD80, CD54 and CD48 and MHC class I H-2D^b expression on target cells, cell suspensions were stained with directly conjugated antibodies (Abs). Fluorochrome Abs to CD8, MHC class I H-2D^b, CD48, CD54 and CD80, blocking Abs to CD48, CD54, CD80 and CD11a and the isotype control Abs were purchased from BD Pharmingen (San Diego, CA), and samples were analyzed with FACS Caliber (Becton Dickinson, Mountain View, CA) using the cell quest software.

Peptide

The MHC class I H-2D^b NP68 (366–374, ASNENMDAM) and CAP-M8 (CEA526–533, EAQNTTYL) were synthesized and HPLC-purified by American Peptide (Sunnyvale, CA).

Development of F5 cytolytic T cells

Spleens were removed from F5 4 to 6-week-old Tg mice. Splenocytes were teased apart using a 0.4-mm mesh screen and washed once in complete RPMI. Splenocytes were resuspended in 3 ml of ACK/spleen and placed on ice for 5 minutes to lyse the red blood cells. The remaining cells were washed three times in cRPMI and after the final wash resuspended at a concentration of 2×10^6 splenocytes/ml in cRPMI. The splenocytes were plated in six-well plates (2.5 ml/well) and 2.5 ml of cRPMI with 0.0004 μ g/ml NP68 peptide was added to each well. After 5 days the cells were harvested and washed twice in cRPMI. Cells were then underlaid with Ficoll-Hypaque. After centrifugation, the layer of cells at the interface of RPMI and Ficoll-Hypaque was removed and these cells were washed three times in cRPMI. The cells were counted and placed in six-well plates in 2 ml of cRPMI at a concentration of 2.5×10^6 cells/ml and rested for 2 days. On day 7, 2 ml of cRPMI containing 20 U/ml IL2, 4×10^{-4} μ g/ml NP68 and $5\text{--}7.5 \times 10^6$ /ml irradiated splenocytes were added to the rested T cells. In order for these naive CD8⁺ cells to gain effector function, the cycle was repeated at days 5 and 7 of each of the 2 or 3 weeks the cells remained in culture.

Recombinant poxviruses and infection of target cells

The fowlpox viruses FP-WT (fowlpox-wild type), rF-CD80 (B7-1), rF-CD54 and rF-TRICOM have been described previously.¹⁸ The fowlpox virus encoding the human CEA gene in combination with B7-1, CD54, and CD48 (rF-CEA/TRICOM) has been described.¹⁸ The recombinant fowlpox virus containing the gene for murine GM-CSF (designated rF-GM-CSF) has been described.²¹ All viruses were kindly provided by Dr Dennis Panicali of Therion Biologics Corp. (Cambridge, MA). Target cells (L-929D^b fibroblasts or MC38 tumor cells) were washed and resuspended in OptiMem ($1\text{--}2 \times 10^6$ /ml) (Gibco/BRL, Gaithersburg, MD) and

mock infected or infected with FP-WT, rF-CD80 or rF-TRICOM at 60 multiplicity of infection (MOI) (PFU/cell) for 5 hours. Complete medium was added after infection and cells were incubated at 37°C overnight. After infection, the cells were harvested and used for various assays. Mock-infected targets were treated similarly without receiving the virus.

Chromium release assays

Standard chromium-51 (^{51}Cr) release assays were performed using the F5 cytotoxic T lymphocyte (CTL) cell line and as targets either L-929D^b fibroblasts or MC38 tumor cells infected with no fowlpox virus (mock), FP-WT, rF-CD80, rF-CD54 or rF-TRICOM. Target cells were labeled with 250–300 μCi ^{51}Cr /5 $\times 10^6$ cells for 1 hour. Labeled targets were washed three times and counted. Targets were plated in 96-well round bottom plates at 10^4 targets/100 μl /well. The concentration of NP68 was either a final high concentration of 0.01 $\mu\text{g}/\text{ml}$ or a final low concentration of 2.5×10^{-5} $\mu\text{g}/\text{ml}$, and 50 μl of peptide was added to each well. Effector CTLs (100 μl) were plated at the appropriate concentration to create the necessary E:T ratio. The plates were incubated at 37°C, 5% CO_2 for 5 hours and subsequently harvested (Skatron, Sterling, VA) and counted in a gamma counter. The percentage of specific release of ^{51}Cr was determined by the standard equation, % specific lysis = (experimental release – release)/(maximum release – spontaneous release) $\times 100$.

Intracellular staining

Intracellular staining of perforin on F5 CTLs was performed using an intracellular staining kit (BD Pharmingen). Briefly, F5 CTLs were rested overnight. The cells were then passed over a Ficoll-Hypaque gradient, washed three times in cRPMI and counted; 0.5 ml of T cells were plated in 48-well plates at a concentration of $4 \times 10^6/\text{ml}$. The appropriate concentration of NP68 was added to the antigen-presenting cells (APCs) (mock, FP-WT, rF-CD80 or rF-TRICOM-infected fibroblasts) and 0.5 ml NP68 and targets were added to the plates at an E:T ratio of 1:10. Golgi Stop was added to cultures. At indicated time points, the cells were harvested and washed twice in PBS and Fc blocked for 15 minutes. The appropriate extracellular stains were added and the cells were incubated at 4°C for 30 minutes. After this step, all subsequent steps were performed with ice-cold materials and incubations were conducted at 4°C. Cells were subsequently washed three times in PBS and cytofix/cytoperm was added for 30 minutes. The cells were washed twice in perm/wash and placed at 4°C overnight. After overnight incubation the cells were washed and resuspended in 100 μl of perm/wash and the anti-perforin Ab (P1-8 Kamiya, Seattle, WA) was added to the cells. The cells were incubated for 1 hour and subsequently washed twice in perm/wash. Secondary antibody was added at a dilution of 1:200 and incubated at 4°C for 30 minutes (Jackson ImmunoResearch Laboratories, West Grove, PA). After the final incubation, the cells were

washed twice in perm/wash followed by two washes with PBS and then fixed in 1% paraformaldehyde. Following fixation the cells were analyzed by FACS (Becton Dickinson).

Conjugate formation assay

Target cells were washed in serum-free media and were stained for 5 minutes at RT in 10 μM of PKH26 Red (Sigma, St Louis, MO). Reaction was stopped by the addition of serum and cells were washed twice with complete media. CD8⁺ cells were also stained with anti-CD8⁺ FITC Ab (BD Pharmingen) for 15 minutes and washed twice in FACS buffer. Stained target cells and CD8⁺ cells were incubated together in the presence of 0.1 $\mu\text{g}/\text{ml}$ of peptide for 30 minutes at 37°C. Samples were fixed using ice-cold 0.5% paraformaldehyde in PBS and analyzed by FACS.

Quantitative PCR

Assessment of increased perforin message was performed by utilizing the perforin primers from a relative RT PCR kit (Ambion, Austin, TX). F5 CD8⁺ cells were cocultured with target cells either mock infected or infected with one of the following: FP-WT or rF-TRICOM; target cells were incubated with NP68 (2.5×10^{-5} $\mu\text{g}/\text{ml}$) for 3.5 hours at an E:T of 10:1. RNA was isolated from each sample and cDNA was generated using an oligo-dT random primer. The number of perforin copies was quantified using Sybrgreen (ABI, Applied Biosystems incorporated, Palo Alto, CA) and 300 nM of perforin specific primers or control primers to 18S RNA. The samples were placed in an ABI 7600 Thermocycler. A three-step quantitative PCR was performed at 94°C for 30 seconds, 62°C for 30 seconds, and 72°C for 30 seconds for 35 cycles. The perforin copy number is expressed as per 10^6 copies of control 18S RNA. Unstimulated F5 CD8⁺ cells (no treatment) were used as a control and demonstrated minimal perforin message.

Immunofluorescence and microscopy

Fibroblasts infected with FP-WT or rF-TRICOM were incubated in the presence or absence of NP68 peptide (0.1 $\mu\text{g}/\text{ml}$) at 37°C for 1 hour. After incubation the APCs were washed and mixed with the T cells at a 1:1 ratio. The cell mixture was centrifuged at 500 r.p.m. for 5 minutes to allow conjugate formation and incubated for 30 minutes at 37°C for synapse formation. The conjugates were adjusted to 1×10^6 cells/ml and plated on coverslips pre-coated with poly-L-lysine (Sigma, St Louis, MO). After adherence, the cover slips were washed with PBS and fixed for 1 hour with 4% paraformaldehyde, followed by two quenching steps of 5 minutes each with 50 mM of NH_4Cl . Cells were permeabilized with 1% NP-40 in wash buffer (PBS + 3% FCS + 0.01% saponin) for 10 minutes. The cells were labeled with the following anti-mouse Abs: rat anti-CD11a (Southern Biotechnology Associates, Inc., Birmingham, AL), hamster anti-CD3 (BD Pharmingen) and mouse antiphosphotyrosine (Upstate Biotechnology,

Lake Placid, NY). Primary antibodies were incubated with the following secondary antibodies: donkey anti-rat couple with Alexa 488, rabbit anti-mouse coupled with Alexa 568 (Molecular Probes, Inc., Eugene, OR) and rabbit anti-hamster coupled with Cy5 (Jackson Immuno Research Laboratories). Samples were examined with a Zeiss Axioplan 2 microscope with $\times 63$ objective.

Western blots

Western blots were performed using the power blots (BD Pharmingen). Equal amounts of cell lysates were separated on 10% SDS-PAGE and transferred to nylon membrane for immunoblotting. India ink staining of the transferred proteins served as a control for loading. The membranes were then blocked with 5% milk in TBS and incubated with the indicated Abs.

Intratumoral vaccination studies

CEA transgenic mice were subcutaneously (s.c.) implanted with MC38-CEA⁺ tumor cells (3×10^5 cells/mouse) into the right flank. Mice were vaccinated intratumorally (i.t.) with rF-TRICOM or rF-CEA/TRICOM on days 8, 15 and 22 after tumor implantation. Each virus was injected at 10^8 PFU/mouse admixed with 10^7 PFU/mouse of rF-GM-CSF. Tumor size was measured using calipers 1–2 times a week. Tumor volumes were calculated as follows: Tumor volume (mm^3) = $0.5 \times \text{length} \times \text{width}^2$. In all experiments, mice were killed when either size (length or width) of tumors exceeded 20 mm.

Statistical analysis

Statistical significance for differences was evaluated using analysis of variance (ANOVA) with repeated measures using Statview 4.1. (Abacus Concepts Inc., Berkeley, CA).

Results

Enhanced expression of costimulatory molecules upon infection of target cells with various recombinant fowlpox vectors

Studies were first undertaken to determine whether infecting the target fibroblasts or tumor cells with various recombinant fowlpox vectors would result in enhanced expression of the respective transgene in these cells. The L-929D^b fibroblasts and MC38 tumor cells were either mock infected (no FP virus) overnight or infected overnight with 60 MOI of FP-WT (containing no costimulatory molecules), rF-CD80 (containing the B7-1 gene), or rF-TRICOM (which contains three costimulatory molecule genes, CD80, CD54 and CD48). Subsequently, the infected cells were analyzed by flow cytometry for surface expression of four different markers: MHC class I, CD80, CD54 and CD48. As shown in Figure 1a, infection with the various vectors resulted in a slight decrease in MHC class I on fibroblast L-929D^b cells. Moreover, fibroblasts that were mock

infected or infected with FP-WT had no increase or only a slight increase in the surface expression of the three costimulatory molecules as compared with the isotype control (Fig 1b–d). When the cells were infected with rF-CD80, the mean fluorescence intensity (MFI) of CD80 was substantially increased (Fig 1b), whereas the percentage of positive cells and the MFI of MHC class I, CD54 and CD48 remained unchanged (Fig 1a–d). When the fibroblasts were infected with rF-TRICOM, the MFI of CD80 increased and the number of CD54-positive cells increased from 0 to 90%. In total, 40% of the cells also became positive for CD48 expression (Fig 1b–d). Studies were then carried out to determine whether MC38 cells act similarly to fibroblasts upon infection with various fowlpox vectors. As shown in Figure 1, panels e–h, MC38 cells expressed MHC class I but no costimulatory molecules. Infection of MC38 cells with control FP-WT virus did not alter the expression of any of these markers. Infection of MC38 cells with rF-CD80 enhanced the expression of CD80 in terms of both the percentage of positive cells and MFI (Fig 1f). None of the other markers were appreciably changed (Fig 1g, h). Infection of MC38 with rF-TRICOM resulted in the enhanced surface expression of CD80, CD54 and CD48 in terms of both the percentage of positive cells and the MFI (Fig 1f–h).

Expression of costimulatory molecules on target cells and its effect on the cytolytic activity of CD8⁺ cells

To investigate how the expression of costimulatory molecules on a target cell affects CD8⁺ effector cell activity, two different CD8⁺ effector T-cell lines were evaluated in cytotoxicity assays. A flu peptide (NP68) TCR-Tg cell line (F5 CD8⁺ passed only two times in culture and rested 2 days in the absence of peptide and IL-2) and a CEA peptide-specific cell line were evaluated for their ability to lyse two target cell lines that were either mock infected or infected with one of the above-mentioned rF vectors. When the F5 effector CD8⁺ cells were used at a single E:T ratio of 10:1 and the amount of NP68 peptide was titrated on L-929D^b fibroblast targets, those targets infected with rF-TRICOM were lysed more efficiently than those that were either mock infected or infected with FP-WT or rF-CD80 (Fig 2a). In total, 60% of L-929D^b targets infected with rF-TRICOM were lysed at 8×10^{-5} $\mu\text{g/ml}$ of NP68 peptide. As the peptide dose decreased into the femtogram levels, the percent lysis of the rF-TRICOM-infected L-929D^b fibroblast cells decreased concomitantly. However, when the cells were infected with rF-CD80, FP-WT or mock infected, less than 30% of the cells were lysed with increased peptide concentration. In the absence of peptide, the amount of nonspecific lysis was similar for all L-929D^b target groups. When the T cells were titrated and a single concentration of NP68 (2.5×10^{-5} $\mu\text{g/ml}$) was loaded onto L-929D^b targets, a trend similar to that mentioned above was observed (Fig 2b). If L-929D^b targets were infected with rF-TRICOM, F5 CD8⁺ cells at an E:T ratio of 20:1 or 10:1 lysed target cells at greater than 90%. As the ratio of effector cells to target cells decreased to

Figure 1 Expression of costimulatory molecules upon infection of target cells with various recombinant fowlpox vectors. L-929D^b fibroblast cells (a–d), and MC38 tumor cells (e–h), were either mock infected (green line) or infected overnight with 60 PFU/cell of FP-WT (pink dashed line), rF-CD80 (FP-B7-1; blue line) or rF-TRICOM (FP-TRICOM; orange line). The cells were stained for H2-D^b MHC class I expression (a, e), CD80 (b, f), CD54 (c, g) or CD48 (d, h), and analyzed by three-color FACS analysis. This experiment is representative of numerous FACS analyses performed on the rF-infected target cells.

Figure 2 Expression of rF-TRICOM within target cells increased the efficiency of F5 effector CD8⁺ T cells from TCR-Tg mice to specifically lyse NP68 loaded targets. A 5-hour ⁵¹Cr release cytotoxicity assay was performed using target cells of either L-929D^b fibroblasts (**a** and **b**) or MC38 tumor cells (**c** and **d**). Panel **a**: L-929D^b cells were either mock infected (open circles), or infected with FP-WT (open squares), rF-CD80 (closed circles) or rF-TRICOM (closed squares) and were cultured with different concentrations of NP68 peptide and F5 TCR-Tg CD8⁺ T-cell line as effector at a constant E:T ratio of 10:1. Panel **b**: Infected L-929D^b cells were cultured with 0.025 ng/ml of NP68 and F5 TCR-Tg CD8⁺ T-cell line at varying E:T ratios. Panel **c**: MC38 tumor cells were either mock infected (open circles), or infected with FP-WT (open squares), rF-CD80 (closed circles) or rF-TRICOM (closed squares) and cultured with varying concentrations of NP68 peptide and F5 TCR-Tg CD8⁺ T-cell line as effector at a constant E:T ratio of 10:1. Panel **d**: Infected MC38 tumor cells were cultured with 0.025 ng/ml of NP68 and F5 TCR-Tg CD8⁺ T-cell line at varying E:T ratios. The efficiency of the F5 CTLs to lyse the various targets was tested in a 5-hour ⁵¹Cr release assay.

1.25:1, the percent lysis (50%) of rF-TRICOM-infected target cells was close to two-fold greater than that of either mock infected or FP-WT- or rF-CD80-infected target cells (Fig 2b).

Further studies were performed using MC38 tumor cells as targets to determine whether the same phenomenon would occur using a murine tumor target. As demonstrated in Figure 2c, when the F5 effector CD8⁺ cells were used at a single E:T ratio of 10:1 and the

amount of NP68 peptide was titrated on MC38 target cells, those targets infected with rF-TRICOM were lysed more efficiently than those that were either mock infected or infected with FP-WT or rF-CD80. Similarly, as shown in Figure 2d, when peptide concentration was maintained at a constant level (NP68 at 2.5×10^{-5} μ g/ml), F5 CD8⁺ cells lysed a greater percentage of rF-TRICOM-infected target cells at lower E:T ratios than mock, FP-WT or rF-CD80 targets.

In these studies, in the absence of peptide (0 ng/ml NP68; Figure 2a and c), we observed no MHC-nonrestricted killing by F5 Flu Tg CD8⁺ cells. Additionally, using irrelevant peptide such as ovalbumin (OVA) did not lead to any CTL killing of either of the targets in our studies (data not shown).

To examine enhanced T-cell affinity responses in a non-TCR-Tg T-cell line, the H2-D^b restricted CEA peptide-specific CD8⁺ T cell line CAPm8 was also evaluated for its ability to lyse L-929D^b peptide-pulsed targets that were mock infected or infected with the various rF vectors (Fig 3). A trend similar to that observed using Flu TCR-Tg CD8⁺ T cells was seen with this system. Targets infected with rF-TRICOM were more efficiently lysed as compared with other targets. Thus, in these separate cases (Figs 2 and 3), the effector CD8⁺ cells more efficiently lysed those targets expressing three accessory molecules, as compared with those targets expressing only CD80 and class I or no costimulatory molecules.

Enhanced conjugate formation between CD8⁺ cells and targets infected with rF-TRICOM

We further evaluated how the presence of TRICOM on target cells affected the interaction between T cells and TRICOM-infected target cells. L-929D^b fibroblasts infected with FP-WT or rF-TRICOM were incubated with

F5 CD8⁺ effector cells (1:1 ratio) in the absence or presence of NP68 peptide (0.1 µg/ml) for 30 minutes. T cells were double stained for CD11a (green, Figure 4a, d, g and j) and CD3 (red, Figure 4b, e, h and k). As demonstrated in Figure 4, upon incubation of CD8⁺ cells with FP-WT-infected L-929D^b cells in the absence or presence of peptide (Fig 4c, f) there was little conjugate formation. The interaction of CD8⁺ cells with rF-TRICOM-infected target cells in the absence of NP68 peptide was also minimal (Fig 4g-i). However, incubation of CD8⁺ cells with rF-TRICOM-infected target cells in the presence of peptide led to a significantly higher conjugate formation between T cells and target cells (Fig 4l). This elevation also led to increased numbers of T cells interacting with an individual target cell (Fig 4l). In order to quantitate the number of conjugates formed between T cells and target cells, L-929D^b fibroblast targets infected with FP-WT or rF-TRICOM (stained with Red PKH dye) were incubated with F5 CD8⁺ (stained with FITC CD8⁺) effector cells (1:1 ratio) in the absence or presence of NP68 peptide (0.1 µg/ml) for 30 minutes and were analyzed by FACS. As demonstrated in Figure 5a, upon incubation of FP-WT-infected L-929D^b with CD8⁺ cells, very few conjugates were formed (6% double stained for CD8⁺ FITC and PKH L-929D^b). However, incubation of rF-TRICOM-infected L-929D^b cells with CD8⁺ cells led to a significantly higher amount of conjugates formed (34% double stained cells, Figure 5b).

We then asked if this increased conjugate formation between T cells and target cells leads to enhanced T-cell signaling. L-929D^b fibroblasts infected with FP-WT or rF-TRICOM were incubated with F5 CD8⁺ effector cells (1:1 ratio) in the presence of NP68 peptide (0.1 µg/ml) for 30 minutes. As demonstrated in Figure 6, CD8⁺ cells were stained for CD11a (green, Figure 6a and e), CD3 (blue, Figure 6b and f) and phosphotyrosine (red, Figure 6c and g). In the presence of NP68 peptide and FP-WT target cells, the T-cell surface molecules (CD11a and CD3) were reorganized at the area of T-cell to target contact (synapse, Figure 6d). In concert with the increase of CD11a and CD3 in the contact area in the presence of peptide and FP-WT-infected target cells, phosphotyrosine staining was also observed within the T cells (Fig 6d). When CD8⁺ cells were incubated with FP-TRICOM-infected target cells in the presence of NP68 peptide, there was an increased number of T cells interacting with target cells, a redistribution/organization of CD11a and CD3 and phosphotyrosine at the site of conjugates (Fig 6h). This suggests that the presence of costimulatory molecules may optimize signaling through the supramolecular activation cluster/complex (SMAC) formation between T cells and target cells.

Enhanced efficiency of T cells upon interaction with target cells expressing costimulatory molecules is through perforin-related pathways

The increase in the lytic response of the CD8⁺ effectors to the enhanced expression of costimulatory/adhesion molecules on the target cells implied potential increases in

Figure 3 Enhanced expression of the triad of accessory molecules CD80, CD48 and CD54 led to increased cytotoxic activity of CAPm8 cell line. A 5-hour ⁵¹Cr release cytotoxicity assay was performed using target cells of L-929D^b fibroblasts. The target cells were mock infected (open circles), or infected overnight with 60 PFU/cell of one of the following fowlpox vectors: FP-WT (open squares), rF-CD80 (closed circles) or rF-TRICOM (closed squares). A CEA peptide-specific CD8⁺ T-cell line (CAPm8) was used as effector at varying E:T ratios and at a constant concentration of a CEA peptide (0.1 µg/ml).

T-cell lytic molecules. Because the ^{51}Cr assays were of short duration (5 hours), we examined the amount of perforin message as well as the accumulation of intracellular perforin protein within CD8^+ effector cells. To ascertain the level of intracellular perforin, flow cytometric analysis was performed on F5 CD8^+ cells exposed to target cells infected with FP-WT or rF-TRICOM in the

presence of NP68 peptide. After 1 hour of stimulation, all of the CD8^+ cells activated with various target cells had increased levels of intracellular perforin as compared with baseline levels (Fig 7a). However, as evidenced by the histogram shift in the MFI (Fig 7a), there was a higher level of detectable perforin protein at 1 hour in those F5 CD8^+ cells stimulated with rF-TRICOM-infected target cells compared with mock-infected or FP-WT-infected cells. The early time point was chosen to look at the differences before the cells released their perforin content to kill the target cells.

To determine whether there was also an increase in the expression of perforin message, we performed quantitative PCR on the F5 CD8^+ T-cell line (Fig 7b). Unstimulated F5 effectors were observed to have a low copy number of perforin message. Upon *in vitro* stimulation with mock-infected L-929D^b targets (loaded with $2.5 \times 10^{-5} \mu\text{g/ml}$ NP68), perforin message increased (5000 copies/ 10^6 18S RNA). When the targets were infected with FP-WT, the amount of perforin copies increased further. The amount of perforin mRNA copies in rF-TRICOM-stimulated effector CD8^+ cells, however, was significantly increased compared with the amount of message observed in FP-WT- and mock-stimulated effectors (Fig 7b).

We also evaluated the extent of perforin involvement in effector cell lysis of target cells expressing costimulatory molecules using concanamycin A (CMA), which is known to block perforin pathway. F5 CD8^+ cells were treated with CMA for 1 hour at 37°C and subsequently used in a 5 hour ^{51}Cr release assay. rF-TRICOM-infected L-929D^b fibroblasts loaded with $2.5 \times 10^{-5} \mu\text{g/ml}$ NP68 were used as targets. Treating the F5 CD8^+ cells with CMA reduced rF-TRICOM target cell lysis significantly at various E:T ratios (Fig 8). These results further demonstrated that the interaction of costimulatory molecules on the target cells with the T cells increased perforin production and perforin-mediated lysis.

Figure 5 Increased conjugate formation between target cells infected with rF-TRICOM and CD8^+ cells. L-929D^b cells were infected with FP-WT construct, stained with PKH and cocultured with FITC-labeled CD8^+ T cells at 1:1 ratio for 30 minutes in the presence of peptide ($0.1 \mu\text{g/ml}$) and were analyzed for conjugate formation by two-color FACS analysis (panel a). L-929D^b cells were infected with rF-TRICOM construct, stained with PKH and cocultured with FITC labeled CD8^+ T cells at 1:1 ratio for 30 minutes in the presence of NP68 ($0.1 \mu\text{g/ml}$). After conjugate formation, the samples were analyzed by two-color FACS analysis (panel b).

CD54 expression on target cells affects cytolytic activity of CD8^+ cells

To determine which one or combination of costimulatory/adhesion molecules expressed by the rF-TRICOM vector rendered target cells more susceptible to lysis, Ab-blocking experiments were performed. As seen in Table 1, when rF-TRICOM-infected targets were treated with anti-CD48 blocking Ab (anti-LFA-3), there was no decrease in lysis of these target cells by CD8^+ cells.

Figure 4 Expression of rF-TRICOM within target cells led to increased synapse formation between CD8^+ cells and target cells. Panels a–f: L-929D^b cells were infected with FP-WT construct and co-cultured with CD8^+ T cells at 1:1 ratio for 30 minutes in the absence (a–c) or presence (d–f) of NP68 ($0.1 \mu\text{g/ml}$). After conjugate formation, the samples were plated on cover slips and were double stained for CD11a (panels a and d, green) and CD3 (panels b and e, red). Superimposition of green CD11a and red CD3 led to the appearance of yellow, representing the interaction of the cells through TCR and ICAM and conjugate formation (panels c and f). Panels g–l: L-929D^b cells were infected with rF-TRICOM construct and cocultured with CD8^+ T cells at 1:1 ratio for 30 minutes in the absence (panels g–i) or presence of NP68 ($0.1 \mu\text{g/ml}$, panels j–l). After conjugate formation, the samples were plated on cover slips and cells were double stained for CD11a (green, panels g and j) and CD3 (red, panels h and k). Panels i and l: superimposition of green CD11a and red CD3 led to the appearance of yellow, representing the interaction of the cells through TCR and ICAM and conjugate formation.

Figure 6 Expression of rF-TRICOM within target cells led to enhanced phosphotyrosine expression. Panels **a–d**: L-929D^b cells were infected with FP-WT and cocultured with CD8⁺ T cells at 1:1 ratio for 30 minutes in the presence of NP68 (0.1 μ g/ml). After conjugate formation, the samples were plated on cover slips and cells were triple stained for CD11a (green, panel **a**), CD3 (blue, panel **b**) and phosphotyrosine (red, panel **c**) to visualize the molecules' redistribution. Superimposition of green CD11a, red phosphotyrosine and blue CD3 led to the appearance of the pink, which depicts the area of conjugate formation between T cells and target cells (panel **d**). Panels **e–h** represent the interaction of CD8⁺ cells with rF-TRICOM-infected L-929D^b cells in the presence of NP68 peptide. Similar to previous samples, after conjugate formation, the samples were plated on cover slips and cells were triple stained for CD11a (green, panel **e**), CD3 (blue, panel **f**) and phosphotyrosine (red, panel **g**) to visualize the molecules' redistribution. Superimposition of green CD11a, red phosphotyrosine and blue CD3 led to the appearance of the pink, which depicts the area of conjugate formation between T cells and target cells (panel **h**).

Similarly, treatment of target cells with anti-CD80 (anti-B7-1) blocking Ab did not reduce the lysis of target cells. By comparison, treatment of T cells with anti-CD11a Ab (to block the receptor for CD54) or anti-CD54 Ab led to an appreciable decrease in lysis of rF-TRICOM-infected target cells (53 and 30% inhibition). No decrease was observed when the matched control Ab for the CD11a or ICAM blocking Ab was used. These experiments thus indicate that the major effects observed on susceptibility of targets to lysis were attributable to vector-mediated expression of CD54. As a consequence of these studies, an rF-CD54 vector was constructed. Target cells were then infected with available individual vectors: FP-WT, rF-CD80, rF-CD54 and rF-TRICOM. As shown in Figure 9, if target cells were infected with either FP-WT or rF-CD80, F5 CD8⁺ T cells were able to lyse 40–44% of the infected target cells in CTL assays. When target cells were infected with rF-CD54 or rF-TRICOM, however, CD8⁺ cells were able to lyse 88–98% of cells. These results support the Ab-blocking experiments and further indicate that CD54 expression on target cells was mainly responsible for enhanced cytolytic activity of CD8⁺ cells.

Enhanced expression of costimulatory molecules on target cells and its effect on T-cell signaling

To address the mechanisms that are involved in the enhanced cytolytic activity of CD8⁺ cells in conjunction

with enhanced expression of costimulatory molecules on target cells, we evaluated several signaling molecules involved in effector T-cell activation. F5 CD8⁺ effector cells were stimulated for 30 minutes with peptide loaded (2.5×10^{-5} μ g/ml) L-929D^b fibroblasts infected with FP-WT or rF-TRICOM. As shown by Western blots (equal amount of protein loaded on each lane as described in Materials and methods) in Figure 10, three major molecules, ZAP-70, Lck and STAT-1, increased in band intensity at 30 minutes post-activation with rF-TRICOM-infected target cells as compared with FP-WT-infected target cells. Two of the signaling molecules, ZAP-70 and Lck, are part of the supramolecular complex and STAT-1 is involved in interferon regulation. Interestingly, all of these changes were observed at 30 minutes postactivation of CD8⁺ T cells at the time of conjugate formation; there was little to no change in any of these molecules at 10 minutes postactivation (data not shown). These results suggest that enhanced expression of costimulatory molecules on target cells facilitates a stronger interaction of T cells with target cells. This, in turn, leads to the enhancement of specific signal pathways in T cells.

Efficacy of intratumoral vaccination with TRICOM vectors

Finally, studies were conducted in mice in which CEA is a self-antigen in an attempt to understand the potential

Figure 7 Perforin levels increased in CD8⁺ cells in response to enhanced expression of accessory molecules. Panel **a**: Flow cytometric analyses of intracellular perforin were performed using F5 CD8⁺ cells. These cells were first cocultured for 1 hour with a low dose of NP68 peptide (2.5×10^{-5} μ g/ml) and target cells (L-929D^b; E:T, 10:1) that were mock infected (green line), or infected with Fp-WT (blue line) or rF-TRICOM (orange line). Panel **b**: Assessment of increased perforin message was performed using quantitative PCR. F5 CD8⁺ cells were cocultured with target cells either mock infected or infected with one of the following: Fp-WT or rF-TRICOM; target cells were incubated with NP68 (2.5×10^{-5} μ g/ml) for 3.5 hours at an E:T of 10:1. Isolated RNA from each sample was quantified using perforin specific primers and control primers to 18S RNA. The perforin copy number is expressed as per 10^6 copies of control 18S RNA. Unstimulated F5 CD8⁺ cells (no treatment) were used as a control and demonstrated minimal perforin message.

clinical relevancy of intratumoral vaccine therapy. These CEA-transgenic mice express CEA in normal gastrointestinal tissue and fetal tissue in a manner similar to that expressed in human beings.¹⁹ Additionally, CEA-transgenic mice have CEA protein in sera at levels similar to

Figure 8 Enhanced cytotoxic activity of CD8⁺ cells upon interaction with target cells expressing a triad of costimulatory molecules is blocked in the presence of concanamycin A (CMA). A 5-hour ⁵¹Cr release assay was conducted using F5 CD8⁺ T cells that were either treated with 200 nM of CMA (open squares) or left untreated (closed squares). L-929D^b cells infected with rF-TRICOM were loaded with 2.5×10^{-5} μ g/ml NP68 and used as targets. The error bars are the standard deviation of triplicate wells. The results presented are representative of three experiments.

Table 1 Blocking CD54 or its receptor CD11a decreases the cytotoxic efficiency of CD8⁺ cells^a

Treatment: Target cells+CD8 ⁺ cells	% L-929D ^b lysis $\pm$ SD	% MC38 lysis $\pm$ SD
FP-WT target cells+CD8 ⁺ cells	53 $\pm$ 5.6	58 $\pm$ 6.3
rF-TRICOM target cells+CD8 ⁺ cells	98 $\pm$ 6.7	73 $\pm$ 6.9
rF-TRICOM target cells treated with anti-CD54+CD8 ⁺ cells	72 $\pm$ 4	56 $\pm$ 6
rF-TRICOM target cells+CD8 ⁺ cells treated with anti-CD11a	46 $\pm$ 6	54 $\pm$ 5
rF-TRICOM target cells treated with anti-CD80+CD8 ⁺ cells	98 $\pm$ 7	66 $\pm$ 6
rF-TRICOM target cells treated with anti-CD48+CD8 ⁺ cells	90 $\pm$ 8.9	67 $\pm$ 4
rF-TRICOM target cells treated with control Ab for anti-CD54 Ab	93 $\pm$ 4.6	76 $\pm$ 7
rF-TRICOM target cells+CD8 ⁺ cells treated with control Ab for anti-CD11a	99 $\pm$ 6	84 $\pm$ 3
rF-TRICOM target cells treated with control Ab for anti-CD80 Ab	91 $\pm$ 4	73 $\pm$ 3
rF-TRICOM target cells treated with control Ab for anti-CD48	98 $\pm$ 6	71 $\pm$ 4

^aA 5-hour ⁵¹Cr release cytotoxicity assay was performed using L-929D^b or MC38 cells as target cells infected with rF-TRICOM. This result is representative of three separate experiments.

Figure 9 CD54 expression on target cells affects the cytolytic activity of CD8⁺ cells. A ⁵¹Cr release cytotoxicity assay was performed using target cells of L-929D^b fibroblasts. The target cells were infected overnight with 60 PFU/cell of one of the following fowlpox vectors: FP-WT (open circles), rF-CD80 (closed circles), rF-CD54 (open squares) or rF-TRICOM (closed squares). These target cells were cultured with varying concentrations of NP68 peptide and F5 TCR-Tg CD8⁺ T-cell line as effector at a constant E:T ratio of 10:1.

those found in patients with advanced colorectal carcinoma (5–100 ng/ml). Previous studies have shown that subcutaneous (s.c.) vaccination using CEA/TRICOM vectors could eliminate established tumors in CEA-transgenic mice when vaccination was begun 3 or 4 days post s.c. tumor transplant at a distal site from vaccination but not when therapy was initiated 8 days following transplant.²² Previous studies have shown no antitumor effect when either FP-WT or buffer (PBS) was administered intratumorally.²² In the studies presented here, vaccination was administered 8 days post s.c. tumor transplant. As a control, this was repeated here; mice were administered PBS i.t. on days 8, 15 and 22 after tumor implantation (Fig 11a). When mice were vaccinated i.t. with rF-TRICOM three times at weekly intervals, tumor growth was suppressed in some mice, and two of five tumors disappeared (Fig 11b); this antitumor effect was significant compared with that in the PBS-treated group ($P=0.0034$). When mice received i.t.-vaccination with rF-CEA/TRICOM at 3 weekly intervals, however, tumor growth was significantly suppressed in all mice ($P=0.00001$ as compared with PBS) and four of five mice were completely cured (Fig 11c). These data demonstrated the efficacy of i.t.-vaccination using vectors encoding either costimulatory molecules alone, or in combination with tumor antigen (CEA).

Discussion

Previous studies have demonstrated that adhesion molecules are important for the interaction between CTLs and

Figure 10 Changes in signaling molecules occur in response to high levels of costimulatory molecules on the surface of the target cells correlating to CD8⁺ cytotoxicity. An immunoblot screen was performed to detect changes in signaling molecules involved in effector cell activation and function. F5 CD8⁺ effector cells were cocultured for 30 minutes with NP68 (2.5×10^{-5} μ g/ml) loaded L-929D^b target cells that were infected with either FP-WT or rF-TRICOM. CD8⁺ cells were isolated from fibroblasts and were analyzed for expression of various proteins using Western blots. Equal amounts of protein were loaded in each lane.

target cells.^{14,23–26} However, the role of adhesion molecules and their interaction with TCR has remained elusive. Bohmig *et al*²⁷ have shown that interference with distinct adhesion molecules (LFA-1, LFA-3 and ICAM) during antigen contact leads to antigen-specific functional inactivation of allospecific T cells. Others have suggested that human CD8 virus-specific CTL can utilize either

Figure 11 Antitumor effects of intratumoral vaccination with CEA/TRICOM vectors in a self-antigen system. CEA transgenic (CEA-Tg) mice were transplanted subcutaneously with MC38-CEA⁺ tumors. Panel a: Control mice were administered PBS intratumorally (IT) 8 days following tumor transplant. Mice were then administered PBS intratumorally on days 15 and 22. Panel b: Mice were vaccinated (IT) with rF-TRICOM admixed with rF-GM-CSF 8 days following tumor transplant. Mice were boosted (IT) with rF-TRICOM admixed with rF-GM-CSF on days 15 and 22. Panel c: Mice were vaccinated (IT) with rF-CEA/TRICOM admixed with rF-GM-CSF 8 days following tumor transplant. Mice were boosted (IT) with rF-CEA/TRICOM admixed with rF-GM-CSF on days 15 and 22. Tumor volume was monitored.

LFA-3/CD2 or ICAM-1/LFA-1 as a major adhesion pathway in CTL reactions.^{27,28} Studies reported here, however, demonstrate for the first time that enhanced adhesion molecule expression on target cells (using fowlpox vectors such as rF-TRICOM) can further enhance the affinity of antigen-specific CD8 cells (already high affinity Flu TCR-Tg CD8 cells) without leading to anergy or inactivation of T cells during the antigen contact phase. We also show that this enhancement is directly related to ICAM-1 and not LFA-3 (unlike previous studies²⁸) or CD80 pathways and leads to enhanced expression of perforin in antigen-specific T cells. Furthermore, our data specifically demonstrate that interaction through TCR and ICAM-1/LFA-1 pathways leads to enhanced colocalization of phosphotyrosine proteins within the synapses followed by enhanced signaling through LCK, ZAP70 and STAT-1. In this study we demonstrated that TCR interaction with peptide/MHC in conjunction with accessory molecules (such as CD54 on target cells) plays an important role in the increase of functional response of effector CD8⁺ cells by enhancing some of the signaling pathways. Ab-blocking studies in conjunction with CTL study further demonstrated that CD80 or CD48 did not play a major role in increasing the effector function of activated/effector T cells, whereas CD54 played a significant role. This result is in contrast to those obtained with APCs infected with various T-cell costimulatory molecules. In those studies, CD80 was shown to play a major role in the proliferation of naive T cells as compared with CD54 or

CD58.^{16–18} The TCR Tg F5 Flu CTLs used in these studies have a high affinity for NP68 peptide and were specifically used to generate a high number of memory T cells. These cells were generated *in vitro* and passed only two times in culture. We have also compared F5 CTLs that were in culture for 12 passages vs two to three passages and found no differences in their killing activity (data not shown). Furthermore, we have conducted experiments with T cells that were isolated and activated for only 3 days; however, this was not a sufficient amount of time for CD8⁺ cells to gain effector function (data not shown). Therefore, these cells were not long-term cell lines; they were activated *in vitro* for a minimum time only to gain effector functions.

For the past few years, the role of CD54 in providing costimulatory signals on APCs has remained controversial. Original studies by Springer and Shaw demonstrated that CD54 (ICAM-1) played a significant part in antigen-independent conjugate formation. They also showed that by using allospecific T cells, CD54 Ab inhibited lysis of certain targets in an antigen-independent conjugate formation.^{14,23,24} Moreover, it was demonstrated that melanoma cells that did not express CD54 were relatively resistant to T-cell lysis using antibody crosslinking killing assays.²⁹ A study by Nishio *et al*³⁰ has demonstrated that overexpression of CD80 (B7) and CD54 on syngeneic cells triggered MHC-nonrestricted lysis by MHC-restricted CTLs without TCR/CD3 engagement. They further demonstrated that both CD80 and CD54 interactions were required for MHC-non-restricted cytotoxicity. Interestingly, our data confirm the role of CD54 in the increased lysis of targets. However, in contrast to the findings of Nishio *et al*,³⁰ increased affinity of T cells for target cells expressing CD54 in our experiments was MHC restricted. As shown in Figure 2, in the absence of peptide, we observed no MHC-nonrestricted killing by F5 Flu Tg CD8⁺ cells. Additionally, using irrelevant peptide such as ovalbumin (OVA) did not lead to any CTL killing of either of the targets in our studies (data not shown). Furthermore, our Ab-blocking studies demonstrated that blocking of CD80 or LFA-3 (CD48) did not affect the enhanced activity of T cells, whereas blocking CD54 significantly reduced the enhanced lytic ability of T cells. This suggests that, in contrast to the results from Nishio *et al*,³⁰ CD80 appears not to be required in the enhanced activity of T cells. We have further demonstrated that enhanced affinity of CD8⁺ cells is attributable to increased perforin pathway and an increased conjugate formation between T cells and target cells. This perhaps leads to enhanced TCR signaling through ZAP70, LCK and STAT pathway. While the Ab-blocking experiments in the studies reported here demonstrate the principal role of CD54 to enhance the susceptibility of target cells, it should be pointed out that the use of the TRICOM vectors does not rule out a potential role for CD80 or LFA-3. A number of studies have reported that CD54 is not capable of fully delivering a costimulatory signal leading to the full activation of T cells.^{31–33} Other studies have proposed that CD54 plays a role in the activation of naive T cells but not effector T cells.³⁴ In a recent study, it

has been suggested that the interaction of CD54 on APCs with CD11a on T cells enhanced the cytolytic activity of these CD4⁺ cells, resulting in a heightened functional response to their cognate peptide-MHC.³⁵ It was demonstrated in that study that *in vitro* priming of OVA-specific TCR-Tg CD4⁺ T cells with APCs expressing CD54 led to the generation of effector CD4⁺ cells. Upon adoptive transfer of these T cells into OVA Tg mice, these T cells were capable of infiltrating and lysing islet cells that expressed OVA, leading to the onset of diabetes. Priming of TCR-Tg CD4⁺ cells with APCs expressing CD80 alone did not lead to the generation of CD4⁺ cells responsible for the destruction of the islet cells. Thus, it appears that APC surface CD54 binding to T-cell surface CD11a may promote avidity maturation in these CD4⁺ cells such that they have a heightened functional response to their cognate peptide-MHC.³⁵ Whereas the above-described studies addressed the role of CD54 on APC and T-cell activation, the studies reported here address the role of CD54 on target cell lysis. Our studies further demonstrate that the expression of CD54 on target cells can lead to enhanced antigen presentation and increased functional activity of effector/memory CD8⁺ cells. Interestingly, our results show that CD54 may indeed be a major player in the activation of effector/memory cells, whereas CD80 and CD48 do not seem to be required for enhanced activation of effector/memory CD8⁺ cells. Therefore, our studies seem to suggest that the costimulatory requirement for enhanced functional activity of effector/memory CD8⁺ cells is different than that of naive cells.

One of the controversial issues is whether the enhanced activation of T cells upon interaction with target cells expressing CD54 is mediated through intracellular adhesion, allowing more efficient TCR signaling. The interaction of peptide MHC and costimulatory molecules such as CD80 (B7-1), CD54 (ICAM-1) and CD48 (LFA-3) on the APC with TCR, CD28, CD11a and CD2 on T cells forms SMACs through which proliferation, activation and response signals may flow into the T-cell.^{36,37}

Recently, the formation of SMACs on CD8⁺ T cells was shown to occur in the presence of physiological amounts of peptide.³⁸ Using microscopy, we demonstrated here that these molecules were present in the synapses formed between CD8⁺ effector cells and target cells expressing enhanced CD54; phosphotyrosine proteins appeared to colocalize within the synapses and increased upon enhanced expression of CD54 in target cells. Therefore, it can be suggested that the redistribution of these molecules into distinct synapses may facilitate the integration of signals from several receptors. Moreover, it may provide a mechanism to account for the ability of T cells to be activated under low TCR occupancy. Indeed, the results reported here from CTL assays demonstrate that upon interaction of T cells with target cells expressing enhanced costimulatory molecules such as CD54, CD8⁺ cells required a significantly lower concentration of peptide to lyse the target cells.

It has been suggested that enhancement of the activation of CD8⁺ T cells through CD54 may not

require an organized molecular synapse.³⁹ However, our data suggest that the presence of CD54 in immunological synapses does lead to the enhancement of the functional activity of CD8⁺ effectors. This could be attributed to the fact that we used APCs for activation and presentation to T cells as opposed to an APC-independent system.³⁹

The colocalization of Lck with the CD8⁺ coreceptor is important in the development of lipid rafts and in T-cell signaling and activation.⁴⁰ The Lck molecule facilitates phosphorylation of the TCR immunoreceptor tyrosine-based activation motifs and the accumulation and phosphorylation of ZAP-70. Furthermore, blocking of Lck activity can directly block perforin release.⁴¹ In our investigations, both ZAP-70 and Lck expression were increased in CD8⁺ effectors that were in contact with target cells expressing enhanced costimulatory/accessory molecules, specifically CD54. Therefore, based on these results, one can hypothesize that increased conjugate/synapse formation between APCs and T cells may lead to enhanced signaling through Lck and ZAP-70, which, in turn, may lead to an increased perforin release and enhanced functional activity of the effector/memory T cells. The data presented here, along with previous reports that demonstrated the role of CD54 in the activation of NF-AT and NF- κ B signaling,^{42,43} further suggest that engagement of LFA-1 on T cells by CD54 on target cells can provide sufficient costimulatory signals to enhance T-cell activation.

The studies reported here indicate that an increase in functional activity of CD8⁺ T cells upon contact with APCs infected with rF-TRICOM appears to be highly dependent on CD11a/CD54 interactions during T-cell priming. The enhanced activity of CD8⁺ cells coincides with the heightened expression of a number of signaling molecules such as Lck, ZAP-70 and STAT-1, and the formation of immunological synapses/conjugates. These signals can play a role in the upregulation of perforin mRNA and protein, which in turn leads to the enhanced functional activity of CD8⁺ T cells. To examine the potential enhanced functional activity, we compared the antitumor effects induced by an i.t.-vaccination regimen in a CEA-transgenic tumor model where the Tumor associated antigen is a self-antigen, thus simulating cancer patients with CEA-positive tumors (Fig 11). Two of five mice vaccinated i.t. with rF-TRICOM and four of five mice vaccinated i.t. with rF-CEA/TRICOM were cured. Kaufman *et al*⁴⁴ have administered a viral vector encoding costimulatory molecules directly into melanoma lesions in clinical trials, suggesting that potential TAAs can be recognized without initial identification of melanoma antigens, and that the immune response may be directed to multiple melanoma antigens. Our data support these observations; intratumoral administration of rF-TRICOM significantly reduced the growth rate of tumors (Fig 11b). However, we suggest that this antitumor activity can be further enhanced by the addition of a tumor antigen in the viral vectors. This was demonstrated when the tumor growth was further reduced in mice vaccinated with rF-CEA/TRICOM (Fig 11c); $P=0.0774$ vs rF-TRICOM). These data are

in agreement with that of Kudo-Saito et al,²² who showed that the addition of the antigen (CEA) in the intratumoral vaccination regimen significantly improved antitumor responses.²² In addition, these data confirm and extend the observations of Uzendoski et al,⁴⁵ who examined the role and importance of ICAM-1 in tumor immunity, with emphasis on its ability to induce specific and systemic immunity and immunologic memory against the growth of the ICAM-1-negative wild-type tumor population. In those studies, ICAM-1 was transiently introduced into the ICAM-1-negative tumor cell line by infection with a recombinant vaccinia virus (rV), rV-ICAM-1. Syngeneic C57BL/6 mice that rejected a primary tumor challenge of rV-ICAM-1-infected MC38 also rejected a rechallenge of the ICAM-1 negative wild-type MC38 tumor cells at a distal site, up to 5 weeks after the initial administration of the immunogen.

Even though vaccine approaches to cancer therapy are at relatively early stages, the intratumoral delivery of vectors such as rF-TRICOM or rF-CEA/TRICOM may lead to the enhancement of host immune responses to various tumors. Therefore, the results of our studies are important in revealing the mechanisms that play a role in the enhancement of immune responses upon intratumoral administration of vaccines containing T-cell costimulatory molecules.

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