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Antigen-presenting cells containing multiple costimulatory molecules promote activation and expansion of antigen-specific memory CD8⁺ T cells

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Abstract

We have previously demonstrated that multiple immunizations with vector-based vaccines containing transgenes for tumor Ags and a triad of costimulatory molecules (TRICOM) enhance the expansion and functional avidity of Ag-specific memory CD8⁺ T cells in a mouse model. However, the effect of enhanced costimulation on human memory CD8⁺ T cells is still unclear. The study reported here was an in vitro investigation of the proliferation and function of CEA-specific human memory CD8⁺ T cells following enhanced costimulation. Our results demonstrated that TRICOM costimulation enhanced production of multiple cytokines and expansion of CEA-specific memory CD8⁺ T cells. The lytic capacity of memory CTLs toward CEA⁺ tumors was also significantly enhanced. IL-2R α (CD25) was upregulated dramatically following APC-TRICOM stimulation, suggesting that the enhanced expansion of memory CD8⁺ T cells may be mediated by increased expression of IL-2R on memory T cells. The enhanced cytokine production and proliferation following TRICOM signaling was completely blocked by the combination of neutralizing Abs against B7-1, ICAM-1, and LFA-3, the costimulatory molecules comprising TRICOM. No difference in T-cell apoptosis was observed between APC-TRICOM and APC-wild-type groups, as determined by annexin V, Bcl-2, and active caspase-3 staining. Results indicated that enhanced costimulation greatly expanded human CEA-specific CD8⁺ T cells and enhanced T-cell function, without inducing increased apoptosis of CEA-specific memory CD8⁺ T cells.

Keywords

Costimulation; Memory; T Cells; Cytotoxic; Human; Cell Proliferation

Introduction

Induction of efficient long-term immune memory is the aim of all vaccine protocols. Costimulatory molecules play a critical role in initiating and maintaining optimal immune responses (1,2). It has previously been shown that naïve T cells are more dependent on costimulation for proliferation and activation than are effector/memory T cells. In a study employing APCs with decreased costimulatory capacity (3), higher peptide concentrations were required to induce proliferation of naïve T cells than were required for effector/memory

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Disclosures

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T cells. In effector/memory T cells, higher peptide concentrations usually result in apoptosis. Studies of the effect of costimulatory molecules on effector/memory T cells have yielded conflicting results. Iezzi et al. (3) demonstrated that signaling through CD28 partially protected effector/memory CD4⁺ T cells from flu hemagglutinin peptide TCR-transgenic (Tg)³ mice from apoptosis induced by high peptide concentration and prolonged peptide stimulation. In contrast, Sabzevari et al. (4) reported that effector/memory CD4⁺ T cells from pigeon cytochrome c TCR-Tg mice were more susceptible to apoptosis induced by APCs expressing B7-1 in the presence of high-affinity cognate peptide compared to naïve CD4⁺ T cells. It should be noted, however, that both studies (3,4) were performed in vitro using relatively strong Ags, and that both analyzed effector/memory CD4⁺ T cells from TCR-Tg mice.

Previous in vivo studies have shown that recombinant poxvirus vectors can be efficiently employed in diversified prime-and-boost strategies to enhance Ag-specific murine T-cell responses. These studies used replication-competent recombinant vaccinia (rV) as prime and replication-defective recombinant fowlpox (rF) as boost. In subsequent studies, insertion of the transgenes for a triad of costimulatory molecules (designated TRICOM), consisting of B7-1 (CD80), ICAM-1 (CD54) and LFA-3 (CD58), further enhanced CD8⁺ T-cell responses (5,6). More recently, using adoptive transfer of Thy1.1⁺ memory T cells to Thy1.2⁺ mice, we demonstrated that repeated immunization with TRICOM-based vaccines not only expanded Ag-specific memory T cells, but also significantly enhanced the functional avidity of memory T cells (7).

It is generally accepted that proliferation and activation of Ag-primed memory T cells are more dependent on costimulation in humans than in mice (8–12). Damle et al. (13) demonstrated that ICAM-1 and VCAM-1 are required to initiate anti-TCR-driven human CD4⁺ T-cell proliferation, while B7-1 and LFA-3 facilitate sustained proliferation of Ag-primed human memory T cells. Semnani et al. (14) showed that only memory CD4⁺ T cells were costimulated by LFA-3, whereas both naïve and memory T cells were costimulated by ICAM-1. All these studies were performed using anti-CD3- or anti-TCR-driven CD4⁺ proliferation in combination with immobilized costimulatory molecules on plates (13,14). To our knowledge, less is known about the role of costimulation in the proliferation, function, and survival of Ag-specific human effector/memory CD8⁺ T cells (hereafter designated memory T cells). In the present study, we employed cognate peptide-pulsed, TRICOM-infected human fibroblast cells as APCs to investigate the role of costimulation in the activation of human memory T cells. The study employed purified memory pan-T cells. We first generated CEA-specific T cells using peptide-pulsed autologous dendritic cells (DCs) from normal HLA-A2⁺ donors. We then studied the proliferation, lytic activity, and cytokine production profile of purified Ag-specific memory T cells following stimulation with peptide in the presence of costimulation, as well as the role of individual costimulatory molecules in the activation of memory CD8⁺ T cells. The results of these studies have implications for the design of clinical trials in which vaccines containing vector- or DC-based costimulatory molecules are used as boosts to maintain Ag-specific memory CD8⁺ T cells.

Materials and Methods

Cell lines

The human melanoma cell line SKMEL24 (HLA-A2⁺, CEA[−]) and colon cancer cell lines SW1463 and SW480 (both HLA-A2⁺, CEA⁺) were used as targets in a ⁵¹Cr release assay. The human fibroblast cell line MRC5 (HLA-A2⁺, CEA[−], CD80[−], CD54^{low}, and CD58^{low}) was used to present exogenous peptide to Ag-specific memory CD8⁺ T cells. All cell lines were purchased from ATCC (Rockville, MD). All cells were cultured in RPMI supplemented with 10% FCS (Invitrogen, Carlsbad, CA), antibiotics (penicillin 100 U/ml, streptomycin 100 µg/

ml, amphotericin B 0.25 µg/ml, gentamicin 50 µg/ml, L-glutamine 450 µg/ml, and sodium bicarbonate 2.5 mg/ml in 75 cm² T flasks (Costar, Cambridge, MA).

HLA typing

HLA-A*02 subtyping was performed by the Blood Bank of the National Institutes of Health by PCR using previously described sequence-specific primers (15,16). All donors were HLA-A*0201⁺.

Recombinant viruses

The study employed rF-TRICOM containing transgenes for the human costimulatory molecules B7-1 (CD80), ICAM-1 (CD54) and LFA-3 (CD58), as previously described (17). The wild-type (WT) fowlpox viral construct was used as a control.

Peptides

The HLA-A*0201-binding CEA agonist peptide CAP1-6D (YLSGADLNL, designated CEA peptide) has been previously described (18,19). The flu peptide (GILGFVFTL) was derived from influenza matrix protein (20). Both peptides, used to pulse DCs or target cells as indicated, were synthesized by SynPep (Dublin, CA) and were > 95% pure.

Ab, tetramer staining, and flow cytometry assay

Fluorochrome-labeled antihuman CD2, CD8, CD11a, CD28, CD54, CD58, CD80, CD45RA, CCR7, Bcl-2, active caspase-3, HLA-A2(BB7.2), and IFN-γ were used for phenotypic analysis. All of the Abs were purchased from BD Pharmingen (San Diego, CA). PE-labeled antihuman IL-2Rα and APC-labeled antihuman IL-2Rβ were purchased from R&D Systems (Minneapolis, MN). PE-labeled HLA-A2 CAP1-6D tetramer (designated CEA tetramer) was provided by the National Institutes of Health Tetramer Core Facility (Atlanta, GA). APC-labeled HLA-A2 CAP1 pentamer was purchased from ProImmune (Springfield, VA), and HLA-A2 flu tetramer was purchased from Beckman Coulter (San Diego, CA). For flow cytometric cell-surface analysis, 2–5 × 10⁵ cells were incubated on ice with the appropriate Abs for 45 to 60 min, washed twice, and analyzed by FACSCalibur or LSR-II (BD Biosciences, Mountain View, CA). Background staining was assessed using isotype control Abs. Data were analyzed using CellQuest or FlowJo software.

Generation of DCs from PBMCs

DCs were generated from PBMCs as previously described (21), with some modifications (22), including the addition of 100 ng/ml GM-CSF (PeproTech, Rocky Hill, NJ) and 20 ng/ml IL-4 (PeproTech). Day 6 DCs generated with GM-CSF/IL-4 were matured by incubating for 24 h with 1 µg/ml CD40L plus 1 µg/ml cross-linking Ab enhancer (Alexis Biochemicals, San Diego, CA).

CTL generation

CTLs were generated using autologous DCs, as previously described (23). In brief, pan-T cells isolated using a Pan T Cell Isolation Kit (Miltenyi Biotec, Auburn, CA) were stimulated with autologous DCs pulsed with CEA peptide (20 µg/ml) at a T:DC ratio of 20–30:1 for three to four cycles of in vitro stimulation (IVS) at 7- to 10-day intervals. IL-2 (20 U/ml) was added 3 days after each IVS except the first. CTL activity was screened using T2 cells pulsed with native CEA peptide CAP1 (YLSGANLNL) in a ⁵¹Cr release assay 7 days after the final IVS.

Purification of memory T cells

Bulk CTL cultures were incubated with CD45RA-labeled beads for 15 min at 4°C. Memory CTLs were isolated by depleting CD45RA⁺ cells with an autoMACS Separator (Miltenyi Biotec), according to the manufacturer's instructions.

Cytotoxicity assays

Cultured CTLs were tested for cytotoxicity in a standard 4-h ⁵¹Cr release assay (23). Tumor cells (1–2 × 10⁶/ml) were labeled with 100 µCi sodium ⁵¹Cr for 1 h at 37°C. Peptide-pulsed targets (1 × 10⁶/ml in the presence of peptide) were labeled with 100 µCi sodium ⁵¹Cr for 2 h at 37°C. Target cells (5000 targets/well) were added to wells containing effector CTLs. Percent specific ⁵¹Cr release was calculated as previously described (23).

Cytokine/chemokine detection

MRC5 cells were either untreated or infected with 40 PFUs of F-WT or rF-TRICOM. Cells were cultured overnight at 37°C, then washed three times with medium (see *Cell lines* above). Purified memory T cells were cocultured for 24 h with either F-WT- or rF-TRICOM-infected MRC5 cells, pulsed with or without various concentrations of CEA peptide, at a T:MRC5 ratio of 10:1. Supernatants from T-cell cultures were collected 24 h after stimulation with APC-pulsed peptide. A panel of 22 cytokines/chemokines was detected using a Human Cytokine/Chemokine Multiplex Immunoassay Kit (LINCO Research, St. Charles, MO) on a Luminex 100 IS System (Luminex Corp., Austin, TX), according to the manufacturer's instructions. Data were analyzed using MasterPlex QT2.0 software.

Intracellular IFN-γ staining

Purified T cells were cocultured for 6 h with either F-WT- or rF-TRICOM-infected MRC5 cells pulsed with or without graded concentrations of cognate peptide. GolgiStop (BD Pharmingen) was added to the cultures during the last 5 h of incubation. Following incubation, cells were collected and stained with tetramer and CD8⁺ cell-surface markers. Cells were then permeabilized with Cytofix/Cytoperm (BD Pharmingen), followed by anti-IFN-γ staining. Data were analyzed using CellQuest software.

CFSE cell proliferation assay

Isolated memory T cells were labeled with CFSE (Molecular Probes, Eugene, OR) according to the manufacturer's instructions. CFSE-labeled cells were cocultured for 6 days with either F-WT- or rF-TRICOM-infected APCs pulsed with CEA peptide in the presence or absence of IL-2 (20 U/ml). In some experiments, neutralizing Abs against CD54 (clone 15.2, Serotec, Oxford, UK), CD58 (Cat. No. AF1689, R&D Systems), and/or CD80 (clone 37711, R&D Systems) were included in the cultures at a concentration of 10 µg/ml each. T cells were then harvested and stained with CD8⁺ and tetramer. Tetramer/CD8 double-positive (tet⁺/CD8⁺) T cells were gated for cell proliferation analysis.

Apoptosis assay: annexin V, intracellular Bcl-2 and active caspase-3 staining

Isolated memory T cells were cultured for 24 h with either F-WT- or rF-TRICOM-infected APCs pulsed with CEA peptide. For the apoptosis assay, cells were stained with CD8⁺ and CEA tetramer followed by annexin V staining. For Bcl-2 and active caspase-3 staining, cells were stained first with PE-Cy7-CD8⁺ and APC-labeled CEA pentamer, followed by intracellular staining with FITC-active caspase-3 and PE-Bcl-2. CEA-specific CD8⁺ T cells were gated for analysis of annexin V⁺ (apoptotic) cells and expression of active caspase-3 (proapoptotic marker) and Bcl-2 (antiapoptotic factor).

Results

Phenotypic analysis of purified memory T cells

CEA-specific CTLs were generated from peripheral blood T cells of healthy HLA-A2⁺ donors, using CEA peptide-pulsed autologous DCs as APCs. Seven days after the last stimulation, T cells were rested in medium containing IL-2 (20 U/ml), without peptide, for approximately 2 weeks. Memory T cells were then purified as described in Materials and Methods. Typically, > 95% of purified cells were CD45RA⁺/CD45RO⁺ (Fig. 1A). Among the gated CEA-specific T cells (tet⁺/CD8⁺) (Fig. 1B), > 90% were memory T cells with the phenotype of CD45RA⁺/CCR7⁺ (Fig. 1C). CEA-specific memory CD8⁺ T cells expressed high levels of CD2 and CD11a and a low level of CD28, the ligands for CD58, CD54, and CD80, respectively (Fig. 1D–F). These T cells were used for all experiments in this study.

Phenotypic analysis of APCs

More than 10 human cell lines were screened for use as parental APCs in this study; none completely satisfied the ideal criteria of being HLA-A2⁺, CD54⁺, CD58⁺, and CD80⁺. The closest match was an HLA-A2⁺ human fibroblast cell line, MRC5. Parental MRC5 was CD54^{low}, CD58^{low}, and CD80⁺ (Fig. 2). Infection of MRC5 with the control vector F-WT did not affect the expression of HLA-A2, CD54, CD58, and CD80, while cells infected with rF-TRICOM expressed high levels of CD54, CD58, and CD80 (Fig. 2). A pilot study showed that parental MRC5 and F-WT-infected MRC5 had a similar capacity to induce T-cell proliferation and cytokine production. Therefore, MRC5 infected with F-WT- and rF-TRICOM (hereafter designated APC-WT and APC-TRICOM) were used as APCs in all the experiments in this study.

Enhanced costimulation increases production of multiple cytokines and chemokines from Ag-specific memory T cells

We first investigated whether enhanced costimulation would facilitate cytokine production from CEA-specific memory T cells. Eight of the 22 cytokines and chemokines detected by Luminex showed significant dose-dependent increases following stimulation with CEA peptide-pulsed APC-TRICOM, compared to APC-WT (Fig. 3). For example, at a peptide concentration of 0.1 μM, GM-CSF, IFN-γ, IL-13, and IL-5 increased 15- to 30-fold, and IL-4, IP-10 and MIP-1α increased 5- to 7-fold (Fig. 3). MCP-1, IL-3, IL-6, IL-8, and TNF-α also increased, to a lesser degree, following APC-TRICOM costimulation, compared to the corresponding APC-WT groups (Fig. 3). Memory T cells stimulated with either APC-WT or APC-TRICOM pulsed with various concentrations of CEA peptide showed no observable CEA-specific release of 10 other cytokines and chemokines among the 22 cytokine-chemokine panel, including eotaxin, IL-1α, IL-1β, IL-2, IL-7, IL-10, IL-12p40, IL-12p70, IL-15, and RANTES (data not shown).

Increasing the cognate peptide density on APC-WT resulted in a dose dependent increase in the production of some cytokines/chemokines such as IFN-γ and IP-10. However, the magnitude of cytokine/chemokine product induced by APC-WT at the highest peptide concentration (0.1 μM) used in this study was much less than that induced by APC-TRICOM at the lowest peptide concentration (0.001 μM) (Fig. 3). Further increase of peptide concentration to 1 μM led to apoptosis of most of the T cells for both the APC-WT and APC-TRICOM groups. Interestingly, increasing the peptide concentration in the APC-WT group did not lead to any production above background for most cytokines/chemokines such as IL-3, IL-4, IL-5, IL-6, IL-8, IL-13 and TNF-α (Fig 3).

Enhanced costimulation significantly reduces amount of peptide required for optimal IFN- γ production

Engagement of costimulatory molecules with their ligands generally amplifies the signal initiated by TCR engagement with a cognate peptide-MHC complex. We examined the number of IFN- γ -producing cells in purified memory CTLs costimulated with APC-TRICOM to determine whether costimulation reduced the required amount of peptide. Memory T cells were stimulated in vitro with APC-TRICOM pulsed with 1–100 nM CEA peptide. After 6 h, 52.3% of CEA-specific memory CD8⁺ T cells produced IFN- γ in the APC-TRICOM group at a peptide concentration of 1 nM (Fig. 4). In contrast, only 1.7% of CEA-specific memory T cells stimulated with APC-WT produced IFN- γ at the same peptide dose (Fig. 4). Increasing the dose of peptide in the APC-WT groups increased the number of IFN- γ -producing cells. The number of IFN- γ -producing cells in the APC-WT group under the highest peptide concentration (0.1 μ M) was still less than that in the APC-TRICOM group under the lowest concentration (0.001 μ M) (Fig. 4). However, higher peptide concentrations did not induce further increases in IFN- γ -producing cells in the APC-TRICOM groups. (See Discussion for possible reasons for this discrepancy.) In short, the peptide dose required to achieve the level of CEA-specific memory CD8⁺ T-cell activation observed in the absence of costimulation was reduced at least 100-fold in the APC-TRICOM groups.

Short-term stimulation with APC-TRICOM enhances the lytic capacity of Ag-specific memory CTLs toward tumor cells

We have previously demonstrated that TRICOM costimulation facilitates maturation and functional avidity of Ag-specific CTLs (7,17). Here we tested the lytic capacity of CEA-specific memory CTLs following short-term TRICOM costimulation. As shown in Fig. 5, coculturing CEA-specific memory CTLs with APC-TRICOM/CEA enhanced lysis of CEA⁺/HLA-A2⁺ human colon carcinoma cells SW1463 and SW480, compared to coculturing with APC-WT/CEA. However, the memory CTLs could not recognize the CEA⁺/HLA-A2⁺ human melanoma cell line SKMEL24 (Fig. 5). The results have clearly demonstrated that antigen-specific memory T cells generated or boosted with TRICOM containing vaccines have enhanced capacity to recognize cognate antigen bearing tumor cells which do not express TRICOM.

Enhanced costimulation promotes expansion of CEA-specific memory CTLs and expression of IL-2R α

To determine whether TRICOM costimulation facilitates the expansion of CEA-specific memory CTLs, we cocultured CFSE-labeled purified memory T cells with CEA peptide-pulsed APC-TRICOM for 6 days in the presence of 20 U/ml IL-2 (Fig. 6A). This process induced dose-dependent proliferation of CTLs, as demonstrated by the dilution of CFSE (Fig. 6C). At peptide concentrations of 1 to 100 nM, enhanced costimulation with APC-TRICOM promoted greater CTL proliferation than with APC-WT loaded with the same dose of peptide. Enhanced costimulation of memory CTL proliferation was consistently supported by the expansion of CEA-specific CTLs (tet⁺/CD8⁺) at the end of culture (Fig. 6A). In the absence of IL-2, APC-TRICOM costimulation did not significantly promote memory CTL proliferation (data not shown).

We also examined IL-2R expression following APC-TRICOM costimulation. As seen in Table I, CEA-specific memory CD8⁺ T cells from three different donors showed greater increases in IL-2R α expression when stimulated with APC-TRICOM than when stimulated with APC-WT, in terms of both percentage of IL-2R α ⁺ cells and receptor density, expressed as mean fluorescence intensity (MFI), in a dose-dependent manner. Interestingly, APC-TRICOM costimulation did not alter expression of IL-2R β at the peptide doses tested (Table I).

IL-15 is a growth and survival factor of memory CD8⁺ T cells. We thus investigated whether APC-TRICOM would facilitate IL-15-induced memory CTL expansion. In contrast to IL-2, which did not affect the number of tet⁺/CD8⁺ T cells in the absence of cognate peptide, IL-15 maintained low-level proliferation of memory CD8⁺ T cells. In the presence of 50 ng/ml IL-15, CEA-specific CTLs increased from ~ 2.5% to ~ 3.5% without peptide stimulation. In both the APC-WT and APC-TRICOM groups (Fig. 6B and 6D), > 90% of CEA-specific CTLs in the presence of IL-15 divided > 4 times at a peptide concentration of 100 nM. Although there was no discernible difference in T-cell proliferation between the APC-WT and APC-TRICOM groups at higher concentrations of CEA peptide (Fig. 6B and 6D), the costimulatory effect of APC-TRICOM on T-cell proliferation was clearly evident at CEA concentrations of 1 and 10 nM.

Enhanced costimulation does not impair Ag-specific memory CTLs

Previous murine studies employing strong Ags and T cells from TCR-Tg mice (4) have demonstrated that too much costimulation can lead to apoptosis of memory T cells. To investigate whether TRICOM costimulation would induce apoptosis of T cells directed against this human TAA, purified T cells were cocultured with APC-TRICOM pulsed with various concentrations of CEA peptide for 24–72 h and stained with annexin V (Fig. 7). Annexin V⁺ cells increased gradually as CEA peptide increased. However, at the same concentration of CEA peptide, enhanced costimulation did not result in more CEA-specific CTLs undergoing apoptosis after 24 h of incubation. Similar results were also observed after 72 h of incubation (data not shown). No differences were observed between APC-WT-stimulated and APC-TRICOM-stimulated memory T cells in expression of the apoptosis inhibitor Bcl-2 and the proapoptosis factor active caspase-3, as determined by intracellular staining.

The costimulatory effect of TRICOM can be blocked by Abs against CD54, CD58, and CD80

We have previously shown that vaccines containing TRICOM synergistically facilitate Ag-driven primary immune responses (5). In this study, we investigated the role of each of the three costimulatory molecules comprising TRICOM in the expansion of CEA-specific memory CTLs and cytokine production following peptide stimulation, by including saturated concentrations of blocking Ab individually or in combination.

Abs against CD54, CD58, and CD80 inhibited expansion of CEA-specific memory CTLs at different degrees when the Abs were used individually, whereas the combination of all three Abs almost completely abolished TRICOM-induced proliferation (Table II). All three Abs inhibited T-cell activation, as evidenced by the cumulative number and number of divisions of CEA-specific CTLs at the end of incubation (data not shown). Approximately 56% of CEA-specific CTLs divided ≥ 4 times in the presence of TRICOM costimulation (TRICOM/CEA group) or with isotype Abs (TRICOM/CEA + isotype), and the percentage of tet⁺/CD8⁺ T cells was ~ 11.5%. In the presence of the blocking Ab against CD58, only 34% of memory T cells divided ≥ 4 times and the percentage of tet⁺/CD8⁺ T cells was reduced to 5.9%. In the presence of the blocking Ab against either CD54 or CD80, ~ 40% of memory T cells divided ≥ 4 times and the percentage of tet⁺/CD8⁺ T cells was reduced slightly to ~ 10%. When all three Abs were used together in the presence of TRICOM, the CFSE curve and the percentage of tet⁺/CD8⁺ T cells at the end of culture were comparable to those of the APC-WT/CEA group (Table II). We also evaluated the role of a single costimulatory molecule in TRICOM in the activation of T cells using specific antibody blocking. The activation of memory CTL was determined by cytokine/chemokine production following stimulation with cognate peptide presented on APC-TRICOM. As seen in Fig. 8, the Ab against CD54, CD58 and CD80 demonstrated a different inhibitory effect on cytokine/chemokine production if an individual Ab was added to the cultures separately. Generally speaking, costimulatory molecule CD58 plays a major role in inducing the production of the majority of the cytokines/chemokines, as compared with

CD54 and CD80. Combined use of all three Abs completely inhibited all the enhanced cytokine/chemokine production induced by TRICOM (Fig. 8). Interestingly, adding the three Abs separately to the cultures had no inhibitory effects on IL-6 production. However, the combined use of the three Abs completely blocked the enhanced IL-6 production (Fig. 8). The results clearly showed that there are synergistic effects among the three costimulatory molecules in TRICOM in activating memory CTL responses.

Discussion

This study investigated the role of TRICOM in the activation, proliferation, and survival of Ag-specific human memory CD8⁺ T cells. The function of accessory molecules in T-cell activation and proliferation has been studied using a variety of approaches in both mouse and human settings (1,2,8,13,14,24). However, little is known about the effect of enhanced costimulation on the expansion and survival of Ag-specific human memory CD8⁺ T cells. The present study has several unique aspects. First, because accessory molecules may have different effects on naïve and memory T cells, we used purified memory T cells (> 95% CD45RA⁻/CD45RO⁺/CCR7⁻). This excluded the possibility that changes in proliferation or function were a result of Ag-specific cells generated de novo from naïve T cells. Second, to study the costimulatory effect of the accessory molecules, we used cells pulsed with cognate peptide as APCs, rather than mAbs or ligands immobilized on a plastic surface, in combination with either anti-TCR or CD3 (3,8,9,13,14,25). Third, we used blocking Abs individually or in combination to investigate the contribution of each costimulatory molecule to observed changes in function. Finally, we used multifaceted parameters to examine Ag-specific memory CD8⁺ T-cell proliferation and activation. For example, we analyzed a) proliferation according to division times and accumulated cell numbers, b) cytokine and chemokine production by Luminex assay and intracellular IFN- γ staining, and c) lytic capacity toward tumor cells and apoptosis by annexin V, Bcl-2, and active caspase-3 staining. Our results demonstrated that enhancing costimulation by infecting APCs with rF-TRICOM facilitates antigenic peptide-induced proliferation of memory CD8⁺ T cells, induces production of multiple cytokines and chemokines by memory T cells, and enhances lytic capacity without inducing further apoptosis of memory T cells. Our results suggest that while all three molecules in TRICOM contribute to the expansion and enhanced function of CEA-specific memory CD8⁺ T cells, CD58 is a major costimulator of Ag-specific memory CD8⁺ T cells. It should be noted, however, that these findings may be a result of the relative avidity and/or titers of the commercial Abs used.

Using the Luminex technique, we demonstrated that enhanced costimulation dramatically increases the production of multiple cytokines and chemokines from CEA-specific memory CD8⁺ T cells in a peptide dose-dependent manner (Fig. 3). Without enhanced costimulation, stimulation with 1 to 100 nM of CEA peptide induced minimal cytokine and chemokine release from memory T cells. Our results demonstrate that accessory molecules on APCs must engage with their ligands on T cells for optimal activation of Ag-specific memory CTLs. This finding contradicts the generally held belief that activation of memory T cells is less dependent on costimulation than is activation of naïve T cells (8–12). This belief is based on studies that used a first signal from either anti-CD3 or strong foreign Ags and a second signal from CD28/B7-1 engagement in CD4⁺ responses in mouse models (1,8,9,11), and thus might not apply to human CD8⁺ responses. There are several possible explanations for any discrepancies between the two observations. First, CD4⁺ and CD8⁺ T cells have different requirements for costimulation (2). In antiviral studies, it has been shown that CD4⁺ T cells, but not CD8⁺ T cells, are adversely affected by the absence of CD40, OX40, or CD28 costimulation, and that CD8⁺ T-cell responses are more dependent on 41BBL signaling (2). In studies of anti-TCR-induced T-cell responses, Deeths et al. (24) showed that B7-1 costimulates both CD4⁺ and CD8⁺ T cells, while ICAM-1 is much more effective in costimulating both naïve and memory CD8⁺ T cells. Second, the role of CD28 in CD8⁺ T-cell responses to viruses varies with the viral model, while the

effects of CD28 on CD4⁺ T-cell responses are unequivocal (1). Third, human and murine T cells may differ in their dependence on costimulation. Damle et al. (13) demonstrated that anti-TCR-driven proliferation and IL-2 production of human memory CD4⁺ T cells (CD45RO⁺) are more dependent on costimulation mediated by B7-1, ICAM-1, LFA-3, or VCAM-1 than are naïve CD4⁺ T cells (CD45RA⁺).

The present study illuminates an important function of costimulation. Both the Luminex assay of cytokine and chemokine production and intracellular IFN- γ staining indicate that TRICOM costimulation significantly reduces the level of antigenic peptide needed for optimal T-cell activation (Fig. 3 and Fig. 4). Groups receiving no costimulation required at least 100-fold more peptide to achieve the same level of memory T-cell activation as groups receiving enhanced costimulation. This is especially true for intracellular IFN- γ staining (Fig. 4). TRICOM costimulation also enhanced the lytic capacity of CEA-specific memory CTLs toward human carcinoma cells that endogenously process and present CEA (Fig. 5). These results suggest that enhanced costimulation is required for functional avidity and maturation of human memory CD8⁺ T cells.

We also showed that three signals are required to promote effective proliferation of Ag-specific human memory T cells. In this study, in addition to enhancing the functional activity of Ag-specific memory CD8⁺ T cells, TRICOM costimulation also promoted CEA peptide-driven memory T-cell proliferation in a peptide dose-dependent manner in the presence of T-cell growth factors IL-2 or IL-15 (Fig. 6). Although TRICOM costimulation alone dramatically enhanced CEA-driven production of multiple cytokines from memory CD8⁺ T cells, it could not induce significant proliferation and expansion of CEA-specific memory CD8⁺ T cells. This may be due to the fact that memory T cells have enhanced effector function capacity (lysis, cytokine production) and decreased proliferation potential compared to naïve T cells (26). Without an Ag signal, TRICOM costimulation plus IL-2 or IL-15 could not significantly drive Ag-specific memory T-cell proliferation. Because CEA as a self-Ag is a weaker first signal, it is not clear whether the third signal (growth factor) is required to drive memory T-cell proliferation against strong Ags, such as viral Ags. Interestingly, TRICOM costimulation did not enhance the production of T-cell growth factors such as IL-2, IL-7, IL-12, and IL-15, although production of many other cytokines and chemokines was significantly increased.

Ab blocking experiments showed that of the three molecules in TRICOM, LFA-3 is the major costimulator of CEA-driven memory T-cell expansion and cytokine production (Table II and Fig. 8). This observation differs from human studies using either anti-TCR or CD3 as signal 1 on memory CD4⁺ T cells (13,14). Damle et al. (13) reported that B7-1 played a critical role in costimulating proliferation of memory CD4⁺ T cells, and Semnani et al. (14) demonstrated that ICAM-1 was an important costimulator for memory CD4⁺ T-cell proliferation. This may be attributable to different costimulation requirements for CD4⁺ and CD8⁺ T cells, as discussed above. It is also possible that different subsets of memory T cells (i.e., effector/memory, central memory) have different requirements for costimulation.

The question could be raised as to whether stimulation of T cells with TRICOM APCs leads to permanent changes in CTL function, i.e., if such CTL were re-stimulated with APC-WT versus APC-TRICOM, what would be their relative functions? Also, what are the implications of this? We have previously reported studies (7) employing two preclinical models concerning this question. Briefly, in those studies we have shown that primary vaccination in Thy1.1 (C57BL background) mice with a recombinant vaccinia virus (rV-) expressing a model Ag (LacZ) and TRICOM enhanced the level and avidity of T cells from naïve vaccinated C57BL/6 (Thy1.2) mice. Adoptive transfer of Thy1.1 memory CD8⁺ T cells into naïve Thy1.2 C57BL/6 mice was followed by booster vaccinations with either a recombinant fowlpox (rF-)-expressing LacZ (rF-LacZ) or booster vaccinations with rF-LacZ/TRICOM. Analysis of levels

of beta-galactosidase tetramer-positive T cells and functional assays (IFN- γ expression and lytic activity) determined that booster vaccinations with rF-LacZ/TRICOM were superior to booster vaccinations with rF-LacZ in terms of both maintenance and enhanced avidity of memory CD8⁺ T cells. In another set of experiments, using a self-Ag (CEA vaccines in CEA Tg mice bearing CEA-expressing tumors), we have demonstrated that the use of booster vaccinations with TRICOM vaccines bearing enhanced costimulatory capacity had superior antitumor effects than booster vaccinations devoid of TRICOM. These studies thus have implications in the design of more effective vaccine strategies, i.e., one should not only prime using TRICOM vaccines but also continue boosting with TRICOM vaccines (7).

Using multiple parameters, the present study shows that enhanced costimulation facilitates optimal proliferation, expansion, and activation of memory CTLs, and demonstrates for the first time that enhanced costimulation can increase the expansion and function of human Ag-specific memory CTLs. Repeated immunizations with vaccines containing enhanced vector- or DC-based costimulation may thus be able to activate not only naïve T cells but also memory T cells.

Abbreviations

Tg, transgenic; rV, recombinant vaccinia; rF, recombinant fowlpox; TRICOM, triad of costimulatory molecules; DCs, dendritic cells; WT, wild-type; IVS, in vitro stimulation; MOI, multiplicity of infection; MFI, mean fluorescence intensity.

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FIGURE 1.

Phenotypic analysis of CEA-specific memory $CD8^+$ T cells. CEA-specific T cells were generated from PBLs of healthy donors using peptide-pulsed autologous DCs. Memory T cells were purified by depleting $CD45RA^+$ cells. Purified T cells were stained with $CD8^+$ and CEA tetramer combined with either $CD45RA$, $CD45RO$, and $CCR7$, or $CD2$, $CD11a$, and $CD28$ for phenotypic analysis. All live cells were gated for $CD45RA$ and $CD45RO$ analysis (A). CEA-specific $CD8^+$ T cells were also gated (B) for $CD45RA$ vs. $CCR7$ (C), $CD2$ (D), $CD11a$ (E), and $CD28$ (F) analysis. D–F, open curve: isotype control; solid curve: specific Ab staining.

FIGURE 2.

Phenotypic analysis of APCs. MRC5, a human fibroblast cell line, was either untreated or cultured overnight with F-WT or rF-TRICOM. Cells were stained with fluorochrome-labeled HLA-A2, CD58, CD54 and CD80, then analyzed with CellQuest software. Solid curve: isotype control; open curve: specific Ab staining.

FIGURE 3.

Cytokine/chemokines production by CEA-specific memory T cells following TRICOM costimulation. Purified memory T cells were stimulated for 24 h with CEA peptide-pulsed APCs infected with either F-WT or rF-TRICOM. Supernatants were collected at the end of culture and 22 cytokines/chemokines were detected by Luminex assay. Only cytokines or chemokines with Ag-specific production are shown.

FIGURE 4.

TRICOM costimulation significantly reduces peptide requirement for optimal IFN- γ production. Purified memory T cells were stimulated for 6 h with CEA peptide-pulsed APCs infected with either F-WT or rF-TRICOM. Cells were stained with fluorochrome-labeled CD8⁺ and CEA tetramer, followed by intracellular anti-IFN- γ staining. IFN- γ -producing cells were analyzed using FlowJo software. *A*, FACS analysis of intracellular IFN- γ staining. *B*, IFN- γ -producing cells in tet⁺/CD8⁺ T-cell populations (data calculated from staining shown in *A*).

FIGURE 5.

Lytic capacity of CEA-specific memory T cells following stimulation with CEA peptide-pulsed APCs. Purified memory T cells were stimulated for 5 days with CEA peptide-pulsed APCs infected with either F-WT or rF-TRICOM in the presence of IL-2 (20 U/ml). At the end of cocultures, the memory T cells were collected, washed with media and rested in IL-2 (20 U/ml) containing media for 3–5 days before ^{51}Cr release assay for lytic capacity. Lytic capacity was tested against melanoma cell line SKMEL24 ($\text{A2}^+/\text{CEA}^-$) and colon carcinoma cell lines SW1463 and SW480 (both $\text{A2}^+/\text{CEA}^+$) using a 4-h standard ^{51}Cr release assay. Results represent three independent experiments.

FIGURE 6.

CEA-specific memory T-cell proliferation and expansion following TRICOM costimulation. CFSE-labeled purified memory T cells were stimulated for 6 days with CEA peptide-pulsed APCs infected with either F-WT or rF-TRICOM in the presence of IL-2 (20 U/ml, *A* and *C*) or IL-15 (50 ng/ml, *B* and *D*). Cells were stained with CD3, CD8, and CEA tetramer for analysis of CEA-specific CD8⁺ T-cell expansion (*A* and *B*). Tet⁺/CD8⁺ cells were then gated for CFSE proliferation assay (*C* and *D*). Results in *A* and *C* represent five independent experiments; results in *B* and *D* represent two independent experiments. Data shown in *A* and *B* were calculated from experiments *C* and *D*, respectively. *C* and *D*, black curve: APCs without peptide; green curve: APC/CEA 1 nM peptide; red curve: APC/CEA 10 nM peptide; blue curve: APC/CEA 100 nM peptide.

FIGURE 7.

Effect of TRICOM costimulation on apoptosis of CEA-specific memory T cells. Purified memory T cells were stimulated for 24 h with CEA peptide-pulsed APCs infected with either F-WT or rF-TRICOM. Cells were stained with fluorochrome-labeled CD8⁺, CEA tetramer, and annexin V. Tet⁺/CD8⁺ cells were gated for analysis of apoptosis. Gray curves: memory T cells cultured with APCs infected with either F-WT or rF-TRICOM without CEA peptide. Black curves: memory T cells cultured with CEA peptide-pulsed APCs infected with either F-WT or rF-TRICOM.

FIGURE 8.

Enhanced cytokine and chemokine production by TRICOM costimulation was completely blocked by anti-CD54, anti-CD58 and anti-CD80. Purified memory T cells were stimulated with CEA peptide-pulsed APCs infected with either F-WT or rF-TRICOM for 24 h in the absence or presence of an individual antibody against CD54, CD58 or CD80, or a combination of all three Abs. Supernatants were collected at the end of culture and cytokines/chemokines were detected using Luminex assay.

Table I
TRICOM costimulation enhancement of IL-2R α on memory T cells

CEA peptide	0 nM	1 nM	10 nM	100 nM
Donor 1				
IL-2Rα				
WT	35% (3521)	50% (5356)	75% (7108)	92% (10262)
TRICOM	42% (4144)	70% (7098)	95% (11534)	99% (16298)
IL-2Rβ				
WT	72% (299)	68% (298)	68% (314)	70% (337)
TRICOM	78% (326)	79% (336)	84% (367)	81% (360)
Donor 2				
IL-2Rα				
WT	6% (2496)	17% (1991)	62% (2860)	
TRICOM	9% (2659)	55% (3530)	67% (13531)	
IL-2Rβ				
WT	56% (452)	85% (558)	74% (527)	
TRICOM	74% (511)	82% (538)	80% (493)	
Donor 3				
IL-2Rα				
WT	26% (2300)	29% (2571)	65% (2666)	
TRICOM	22% (2488)	54% (2535)	87% (4989)	
IL-2Rβ				
WT	64% (485)	81% (664)	65% (517)	
TRICOM	67% (483)	83% (544)	65% (517)	

IL-2R α and IL-2R β expression on CEA-specific memory T cells following TRICOM stimulation. Purified memory CTLs were stimulated for 24 h with CEA peptide-pulsed APCs infected with either F-WT or rF-TRICOM. Cells were then stained with fluorochrome-labeled CEA tetramer, CD8⁺, anti-IL-2R α and anti-IL-2R β . CEA tetramer⁺ T cells were gated for IL-2R α and IL-2R β expression. Numbers in parentheses represent mean fluorescence intensity (MFI).

Table II

Enhanced expansion and proliferation of CEA-specific memory CTLs induced by CEA peptide in the presence of TRICOM can be blocked by specific Abs

Treatment	% Tet ⁺ /CD8 ⁺
No peptide	2.39
WT/CEA	3.35
TRICOM/CEA	11.53
TRICOM/CEA + isotype control Ab	11.45
TRICOM/CEA + anti-CD54	9.32
TRICOM/CEA + anti-CD58	5.88
TRICOM/CEA + anti-CD80	9.82
TRICOM/CEA + all 3 Ab	4.62

CFSE-labeled CEA-specific CTL lines were stimulated for 6 days with CEA peptide-pulsed APCs infected with either F-WT or rF-TRICOM in the presence or absence of indicated blocking Abs (10 µg/ml). Cells were then stained with CEA tetramer and CD8⁺. CEA tetramer⁺ T cells were gated for proliferation analysis. Experiments were conducted with CTLs from four donors, with the same results.