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A multimeric carcinoembryonic antigen signal inhibits the activation of human T cells by a SHP-independent mechanism: A potential mechanism for tumor-mediated suppression of T-cell immunity

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Carcinoembryonic antigen (CEA) is a well-known tumor antigen that is found in the serum of patients with various cancers and is correlated with an increased risk of cancer recurrence and metastasis. To understand the tumor environment and to develop antitumor therapies, CEA has been studied as an antigen to activate/tolerate specific T cells. In this study, we show that CEA can function as a coinhibitory molecule and can inhibit the activation of human peripheral blood mononucleated cell-derived T cells. The addition of CEA-overexpressing tumor cells or immobilized CEA dampened both cell proliferation and the expression of IL-2 and CD69 expression in T cells after TCR stimulation. The phosphorylation of ERK and translocation of NFAT were hampered in these cells, whereas the phosphorylation of proximal TCR signaling molecules such as ZAP70 and phospholipase Cγ was not affected by immobilized CEA. To determine the relevance of carcinoembryonic antigen-related cell adhesion molecule-1 and Src homology region 2 domain-containing phosphatase (SHP) molecules to CEA-mediated suppression, we tested the effect of the SHP inhibitor, NSC-87877, on CEA-mediated suppression of T cells; however, it did not reverse the effect of CEA. Collectively, these results indicate that CEA can function as a modulator of T-cell responses suggesting a novel mechanism of tumor evasion.

Key words: CEA, adhesion molecule, coinhibitory signal, tumor-associated antigen, TCR signaling

Abbreviations: CEA: carcinoembryonic antigen; CEACAM: carcinoembryonic antigen-related cell adhesion molecule; DC-SIGN: Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Nonintegrin; hnRNP: heterogeneous nuclear ribonucleoprotein; LAT: linker for activated T cells; PLCγ: phospholipase Cγ; SHP: Src homology region 2 domain-containing phosphatase

Additional Supporting Information may be found in the online version of this article.

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Carcinoembryonic antigen (CEA) is the founding member of the CEA-related cell adhesion molecule (CEACAM) family, which belongs to the immunoglobulin superfamily of cell adhesion molecules.[1] CEA is a 180-kDa glycoprotein, containing ~50% carbohydrate, and it consists of an Ig V-like N-terminal domain followed by six IgC2-like domains and a GPI anchor at the C-terminal end.[2] It mediates intercellular adhesion by homotypic or heterotypic binding to other CEACAM family members.[3] CEA is expressed during the stages of fetal development, and it is only found in the gastrointestinal epithelium of healthy adults; however, it can be highly expressed in various cancer cells, including in colorectal and gastric cancer.[4–6] This tumor-associated antigen is clinically important for the diagnosis of tumors and for determining cancer prognosis, as CEA can be shed from tumors then detected in the serum of patients.[7] Moreover, CEA has been proposed to functionally contribute to the metastatic process of colorectal cancer by disrupting tissue architecture and inhibiting anoikis, thus suggesting the potential of CEA as a therapeutic target.[8,9]

Previously, CEA was reported to regulate the responses of various types of immune cells. For instance, CEA can induce the production of cytokines such as IL-10, IL-6 and TNF-α by Kupffer cells, which are terminally differentiated macrophages that reside in the liver, thereby promoting tumor metastasis.[10] The heterophilic interaction of CEA with

What's new?

Carcinoembryonic antigen (CEA) is found in the serum of patients with various cancers and is correlated with an increased risk of cancer recurrence and metastasis. To shed light on the influence of CEA on T cells, here the authors investigate the co-inhibitory role of CEA during human T cell stimulation by TCR engagement. They show that CEA-overexpressing tumor cells or multimeric CEA significantly inhibit T cell activation, proliferation, and cytokine production. Collectively, the data suggest that multimeric CEA, such as cell-associated CEA, can hinder T cell stimulation upon TCR engagement, which may contribute to an immune escape mechanism for tumors.

CEACAM1, a member of the CEA family, in NK cells was observed to reduce NK-mediated cytotoxicity.[11] CEACAM1 acts as a coinhibitory molecule through its intracellular ITIM motif, and it can inhibit the production of IL-2 and the proliferation of expanded T cells or Jurkat cells *via* recruitment of Src homology region 2 domain-containing phosphatase (SHP)−1, when engaged with certain antibodies or bacterial ligands.[12–14] Recent studies have demonstrated that CEACAM1 can associate with β-catenin in Jurkat T cells and inhibit Fas-induced apoptosis, suggesting a SHP-1-independent mechanism for CEACAM1-mediated T-cell suppression.[15]

Despite advancement in the understandings of CEA, the studies demonstrating the influence of CEA on T cells are limited and controversial. Pickford *et al.*[16] have reported that human peripheral blood mononucleated cell (PBMC) has a T-cell population that responds to CEA as an antigen, and in some donors, this response is held in check by regulatory T-cell-secreted IL-10 *via* CEA-specific recognition by TCR. However, soluble circulating CEA is able to contribute to the tolerance of antigen-specific CD8 T cells by being taken up and presented by liver sinusoidal endothelial cells in mice.[17] In our study, we investigate the coinhibitory role of CEA during human T-cell stimulation by TCR engagement. We show that the activation and proliferation of T cells on stimulation with anti-CD3 antibody are considerably inhibited when T cells are cocultured with CEA-overexpressing tumor cells. Notably, recombinant CEA protein inhibits T-cell responses during TCR stimulation more efficiently under conditions where cells are cultured with immobilized multimeric CEA than when cultured with a soluble form of CEA. The CEA-induced regulation of the T-cell response is accompanied by the reduced activation of ERK and NFAT, whereas TCR-proximal signaling events are not affected. Collectively, these data suggest that multimeric CEA, such as cell-associated CEA, can hinder T-cell stimulation on TCR engagement, which may contribute to an immune escape mechanism for tumors.

Material and Methods

Cell culture

PBMC was isolated from healthy donors by density-gradient centrifugation using Histopaque®-1077 Hybri-Max™ (Sigma-Aldrich, St. Louis, MO, USA). This study was approved by the human ethics committee at the Seoul National University. CD4+ T cells and CD8+ T cells were negatively purified using magnetic purification kits (Miltenyi Biotec, Bergisch Gladbach, Germany). Purified T cells (4×10^4) were stimulated with plate-bound anti-human CD3 antibody (2 μg/ml) and soluble anti-human CD28 antibody (5 μg/ml; Biolegend, San Diego, CA, USA) in all experiments except when assessing signal transduction. Recombinant human CEA (5 μg/ml; R&D Systems, Minneapolis, MN, USA) was immobilized on a plate for multimeric signal along with anti-CD3 antibody or added in the culture for a soluble form at 10 μg/ml (low) or 30 μg/ml (high). In chemical inhibitor assays, CD4+ T cells were preincubated for 15 min with 10 or 50 μM of NSC-87877 (Tocris Bioscience, Bristol, UK) and were then stimulated with anti-CD3/CD28 antibodies as described above in the presence of the inhibitors at the same concentration. For CEACAM1 crosslinking, D14HD11 (Abcam, Cambridge, UK) was used. After 20 hr of culture, supernatants were collected, and released IL-2 was measured by ELISA. At the same time, cells were also collected and analyzed for cell surface CD69 expression by flow cytometry using a BD FACS Calibur™ flow cytometer (BD Bioscience, San Jose, CA, USA). Proliferation of human T cells was detected using the Vybrant® CFDA SE Cell Tracer Kit (Molecular Probes™, Invitrogen, Eugene, OR, USA) followed by FACS analysis. The MC38 and MC38-cea2 (MC38-CEA) murine colorectal tumor cell lines were kindly provided by Dr. J. Schlom (Division of Tumor Immunology and Biology, NIH, USA). The CEA-expressing CHO (CHO-CEA) Chinese hamster ovary cell lines and their parental CHO-K1 cells were also kindly provided by Dr. Kwon (Division of Radiation Oncology, Korea Institute of Radiological and Medical Sciences, Seoul, South Korea). CD4+ T cells (4×10^4) were plated in anti-CD3-coated wells along with the tumor cells at a 1:1 ratio in the presence of anti-CD28 antibodies, and the cells were then stimulated for 20 hr. IL-2 in the supernatants and CD69 expression on the cell surface were then evaluated. For long-term culture, tumor cells were fixed with 4% formaldehyde for 15 min in room temperature. After several washes, the fixed tumor cells and CFSE-labeled CD4+ T cells were plated at a 1:1 or 1:2 ratio in the presence of anti-CD3/CD28 antibodies, and proliferation was measured by flow cytometry on Day 4. For allogenic experiments, we transfected HeLa cells (Korea Cell Line Bank, Seoul, Korea) with pcDNA3-CEA (provided by Dr. T. G. Kim) or pcDNA3-Mock using Lipofectamine LTX and PLUS reagents (Life

Technologies). Transfection was performed 24 hr after plating HeLa cells in six-well plates according to the manufacturer's protocol. Cells were incubated with the 2 μg of DNA and the transfection reagents in a cell culture incubator at 37°C for a further 24 hr. T cells (4×10^4) were cocultured with the tumor cells at a 2:1 ratio for 20 hr, and IL-2 in the supernatants was then evaluated.

Cytokine ELISA and FACS analysis

The levels of IL-2, IFN-γ and IL-4 in the supernatants of cultures were evaluated using a BD OptEIA™ Set (BD Bioscience) according to the manufacturer's instructions. For the analysis of cell surface proteins, cells were stained with anti-human CD69-APC, anti-CD66a/c/e-PE (Biolegend), anti-human CEACAM5-PE and anti-human CEACAM1-PE (R&D Systems) according to the manufacturers' instructions.

Western blot analysis

Purified 3×10^6 CD4+ T cells were stimulated with anti-CD3/CD28 antibodies as described above and were lysed with 1% Triton lysis buffer containing phosphatase and protease inhibitors at 15 or 30 min after stimulation. SDS-PAGE was performed on 12% polyacrylamide gels, and the proteins were transferred to a PVDF membrane. Transferred membranes were blocked with 5% BSA, as recommended by primary antibody suppliers (Abcam). Blocked membranes were incubated with each antibody, washed and incubated with HRP-conjugated goat anti-rabbit IgG or anti-mouse IgG antibodies. The membranes were developed using WEST-one reagent (iNtRON Biotechnology, Gyeonggi-Do, Korea) and were detected with X-ray film. Following detection, membranes were stripped and washed to perform additional blots with the same membrane to ensure the consistency of the data.

Real-time polymerase chain reaction

Total RNA, isolated from naïve or cultured CD4+ T cells using the TRIzol reagent (Life Technologies), was reverse transcribed into cDNA by oligo(dT) priming using M-MLV RT (Life Technologies) according to the manufacturer's protocol. The primers for *CEACAM1* were 5′-GGG CAA GCG ACC AGC GTG AT-3′ (forward) and 5′-TGG TTG TGT GGG TTG CTG GGC-3′ (reverse). *Hprt* were 5′-TGG GAC GTC TGG TCC AAG GAT TCA-3′ (forward) and 5′-CCG AAC CCG GGA AAC TGG CCG CC-3′ (reverse). Quantitative real-time polymerase chain reaction was performed on a LightCycler optical system (Roche, Basel, Switzerland) using SYBR Premix Ex Taq (Takara, Otsu, Japan). The expression levels of the target *CEACAM1* gene were normalized relative to *Hprt* expression.

Immunofluorescence staining

CD4+ T cells were cultured and adhered to a poly-D-lysine (Sigma-Aldrich)-coated slide chamber for 5 hr with or without immobilized CEA in the presence of anti-CD3/CD28 antibodies as described above. The cells were fixed and permeabilized with methanol and blocked with 2% BSA in PBST for 1 hr. The cells were then stained with anti-NFAT2 (Abcam) overnight at 4°C followed by staining with DAPI (Life Technologies). After a final wash, a coverslip was mounted on the slides with ProLong® Antifade (Life Technologies). The fluorescence images were acquired with a LSM710 microscope (Carl Zeiss, Oberkochen, Germany) at the National Center for Inter-university Research Facilities (NCIRF).

siRNA transfection

We used a commercially available siRNA targeting the *CEACAM5* sequence (Bioneer, Daejeon, Korea) or control siRNA with a scrambled sequence that should not lead to the specific degradation of any known cellular mRNA. For siRNA treatments, LoVo colon adenocarcinoma cell lines (Korea Cell Line Bank, Seoul, Korea) were seeded at 400,000 cells per well in six-well plates 24 hr prior to the treatment. To knock down the target gene, LoVo cells were transfected with 50 pmol *CEACAM5* or control siRNA using Lipofectamine RNAiMAX reagents (Life Technologies) as instructed by the manufacturer. Transfection efficiency and experiments were performed after a further 48 hr.

Statistics

All results are expressed as the mean values ± SEM. To compare the differences between two groups, a *t*-test or paired *t*-test was used.

Results

Activation of CD4+ T cells through TCR stimulation is dampened in the presence of CEA-overexpressing tumor cells

Because CEA is expressed on the apical luminal surface of normal colon epithelial cells, T cells may not encounter CEA under normal conditions. However, once colon carcinoma cells which express CEA over their entire surface invade and enter the blood circulation, there is an increased change that cells expressing this antigen will encounter the immune system. To examine whether the surface expression of CEA on tumor cells can affect human CD4+ T-cell responses, we added CEA-expressing tumor cells to CD4+ T-cell cultures during their stimulation with anti-CD3 and anti-CD28 antibodies. To avoid the possible influences of human growth factors from tumor cells on T cells, we used a murine adenocarcinoma cell line, MC38, and a CEA-transduced MC38 cell line (Fig. 1*a*). Compared to CD4+ T cells cocultured with MC38, CD4+ T cells cultured with MC38-CEA showed decreased levels of CD69 expression and IL-2 production in the early activation stage (Figs. 1*b* and 1*c*). When we used tumor cells that were fixed before culture with CD4+ T cells to rule out effects other than the interaction of surface molecules, the prefixed MC38-CEA cells inhibited early activation of CD4+ T cells, consistent with the above result (Fig. 1*d*). We further tested whether CEA-expressing tumor cells were able to affect the proliferation of T cells after TCR

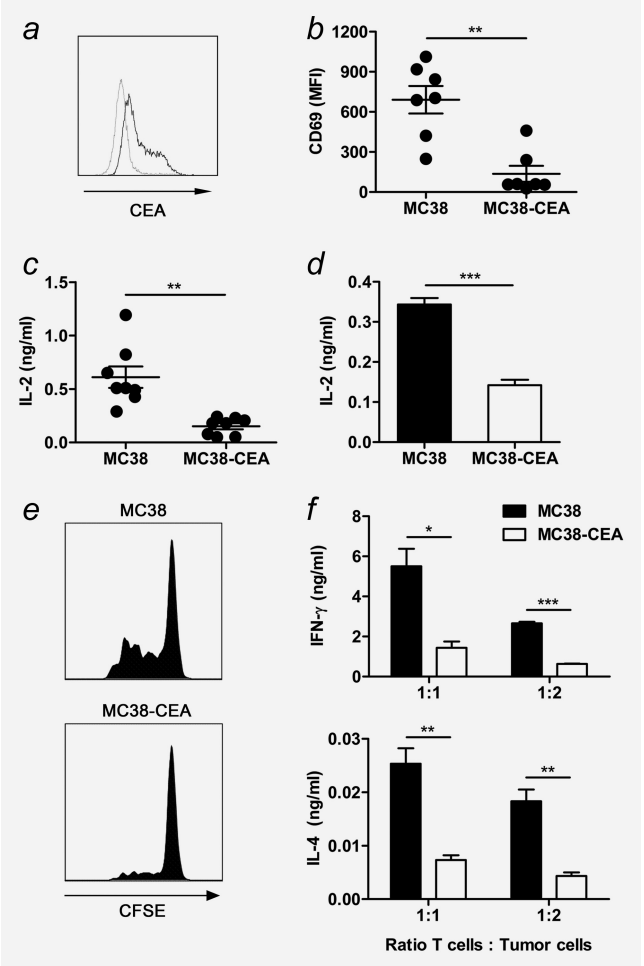

Figure 1. The inhibitory effect of CEA-overexpressing tumor cells on CD4+ T cells during TCR priming. (*a*) Expression of CEA on the surface of MC38 (dotted line) and MC38-CEA (solid line) cells was detected with an anti-CEA antibody by flow cytometry. (*b* and *c*) Purified human CD4+ T cells were incubated with plate-bound anti-CD3 antibody (2 μg/ml) and soluble CD28 antibody (5 μg/ml) in the presence of MC38 or MC38-CEA tumor cells at a 1:1 ratio for 20 hr, followed by flow cytometry analysis to measure CD69 expression on the cell surface (*b*) and ELISA for IL-2 secretion in the supernatant (*c*). (*d*) MC38 and MC38-CEA cells were fixed with 4% formaldehyde for 15 min at room temperature before incubation with T cells. Fixed tumor cells were then washed with PBS and added to CD4+ T cells in the presence of anti-CD3 and anti-CD28 antibodies. After 20 hr of culture, the cells were collected and analyzed as described in (*b*). (*e*) CFSE-labeled CD4+ T cells were stimulated with anti-CD3 and anti-CD28 antibodies in the presence of prefixed MC38 or MC38-CEA cells at 1:1 or 1:2 ratios for 4 days. The cells were then collected, and CFSE dilution was detected by flow cytometry. (*f*) Cell supernatants were analyzed by IFN-γ or IL-4 ELISA. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. (*d–f*) Data are representative of at least two independent experiments.

stimulation. Because the high rate of tumor cell division hinders T-cell survival, only prefixed tumor cells were used for longer T-cell stimulation. Four days after TCR stimulation, the presence of prefixed MC38-CEA cells decreased the proliferation of CD4+ T cells compared to the control tumor (Fig. 1*e*). The levels of effector cytokines produced by differentiated CD4+ T cells, including IFN-γ and IL-4, were also lower in the supernatants of cultures containing MC38-CEA cells (Fig. 1*f*). Similarly, CEA-transfected CHO cells induced the suppression of IL-2 production from T cells (Supporting Information Figs. S1A–S1C). We also performed a similar experiment with LoVo tumor cells, a human colorectal adenocarcinoma cell line that naturally express CEA at high level, to assess the influence of lower CEA expression on T cells. CEA-specific siRNA was transfected into LoVo cells, and the CEA downregulation was confirmed by surface FACS staining (Supporting Information Fig. S1D). When freshly isolated CD4+ T cells were cocultured with the LoVo cells and with anti-CD3 and anti-CD28 antibodies, T cells cultured with CEA-knockdown LoVo cells produced more IL-2 than cells cultured with control LoVo cells (Supporting Information Fig. S1E). Collectively, these results demonstrated that CEA expression on the surface of tumors decreases CD4+ T-cell activation, proliferation and cytokine production. Additionally, we tested the effect of CEA when it is present on the surface of the same cells that can activate T cells. CEA transduction resulted in the reduced allogenic response of T cells responding to HeLa cells, indicating that the inhibitory effect of CEA is also efficient in case that tumor cells express both CEA and T-cell antigen (Supporting Information Fig. S1G).

Immobilized CEA inhibits IL-2 production and CD69 upregulation of human T cells more efficiently than soluble CEA

We further determined the effect of the CEA molecule itself on T-cell activation by using recombinant protein. To this end, recombinant CEA was used in two forms to treat T cells during TCR stimulation with anti-CD3/CD28 antibodies: immobilized on the surface of culture plates or added in a soluble form to the culture media to mimic cell-associated CEA or released CEA, respectively. In the early stage of TCR stimulation, immobilized CEA was more effective than soluble CEA in suppressing IL-2 production and CD69 expression levels (Figs. 2*a* and 2*b*). The coinhibitory effect of CEA on T cells occurred independently of costimulatory molecules, as CEA inhibited IL-2 production from T cells stimulated only with anti-CD3 antibody, regardless of the presence of anti-CD28 antibody (Fig. 2*c*). It has been reported that CEA elicits secretion of IL-10, which suppresses the proliferation of memory T cells.[16] However, we observed that the production of IL-10 was also reduced by immobilized CEA, implying that IL-10 is not involved in CEA-mediated T-cell suppression (Fig. 2*d*). This phenomenon may be due to the possibility of direct TCR interactions between plate-bound antibodies and T cells or the optimal coating of anti-CD3 antibody onto the culture plate itself being interfered by immobilizing CEA onto the culture plate. To address this issue, commonly used proteins such as BSA were immobilized onto the culture plate, and IL-2 production was

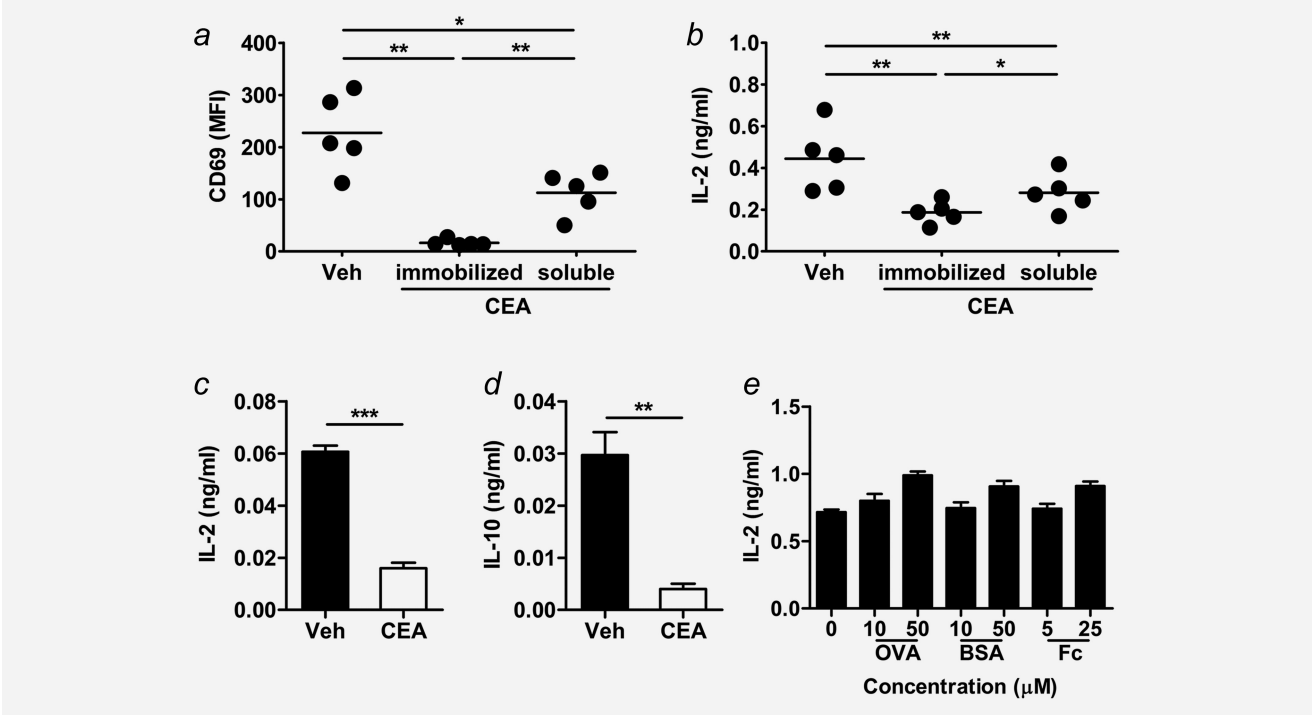

Figure 2. Immobilized CEA inhibits the activation of human CD4+ T cells more efficiently than soluble CEA. (*a* and *b*) Recombinant CEA was used in a form that was bound to plates (immobilized CEA) or added to the culture media (soluble CEA) during the activation of human CD4+ T cells. CD4+ T cells were incubated with immobilized or soluble form CEA along with plate-bound anti-CD3 and soluble anti-CD28 antibodies for 20 hr, followed by flow cytometry analysis for CD69 expression on the cell surface (*a*) and ELISA for IL-2 production in the supernatant (*b*). (*c*) CD4+ T cells were incubated with immobilized CEA and anti-CD3 without anti-CD28 antibodies for 20 hr, followed by ELISA to measure IL-2 production in the supernatant. (*d*) CD4+ T cells were incubated with immobilized CEA along with anti-CD3 and anti-CD28 antibodies for 20 hr, followed by ELISA to measure IL-10 production in the supernatant. (*e*) CD4+ T cells were incubated with immobilized OVA, BSA or Fc molecule in the presence of anti-CD3 and anti-CD28 antibodies for 20 hr, followed by ELISA to measure IL-2 production in the supernatant. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. Data are representative of at least two independent experiments.

measured after TCR stimulation. None of these nonspecific proteins inhibited IL-2 production from CD4+ T cells (Fig. 2*e*). We also tested lamininα4 as a highly glycosylated protein control, and we confirmed that it had no effect on T-cell activation (Supporting Information Fig. S2). Therefore, it can be concluded that the CEA molecule can inhibit T-cell stimulation and that immobilized CEA is more efficient than soluble CEA in suppressing CD4+ T cells.

Immobilized CEA, but not soluble CEA, inhibits T-cell proliferation and effector cytokine production of T cells

To further investigate the ability of CEA to inhibit T-cell stimulation, immobilized or soluble CEA was used to treat CD4+ T cells, and cell proliferation was assessed after 4 days of stimulation with anti-CD3/CD28 antibodies. In accordance with the above experiments using CEA-expressing tumor cells, immobilized CEA decreased the percentage of proliferated T cells on TCR stimulation (Fig. 3*a*). Surprisingly, the addition of soluble CEA did not have any influence on the proliferation. Similarly, immobilized CEA substantially reduced the release of cytokines from CD4+ T cells during TCR stimulation, whereas soluble CEA did not (Fig. 3*b*). We observed that soluble CEA slightly reduced IL-4 production at a high dose but it was insufficient to efficiently suppress proliferation and cytokine production; however, it was capable of modulating IL-2 during the early activation of T cells. These findings imply that immobilized CEA, such as a cell-associated form of CEA, should be considered to have a more coinhibitory effect rather than soluble forms of CEA. Moreover, the addition of immobilized CEA also led to decreased IL-2 production and reduced proliferation of CD8+ T cells after TCR stimulation, indicating that its effect is not limited to CD4+ T cells (Fig. 4).

Treatment with CEA reduces the activation of ERK and nuclear translocation of NFAT

To explore the mechanistic basis of the coinhibitory regulation mediated by multimeric CEA, we first examined whether treatment with CEA promoted the apoptosis of T cells after TCR stimulation (Fig. 5*a*). Although we observed that the intensity of Annexin V increased over the stimulation time, there was no difference between groups (Veh 8.9 ± 0.02 *vs.* CEA 8.8 ± 0.18 at 3 hr; Veh 24.3 ± 8.02 *vs.* CEA 26.3 ± 9.38 at 17 hr), indicating that the regulation mediated by CEA was not related to increased apoptosis of T cells. We next investigated whether multimeric CEA regulates signaling molecules downstream of TCR signaling. As activation of ERK is critical for TCR signaling-induced transcription of IL-2,[18]

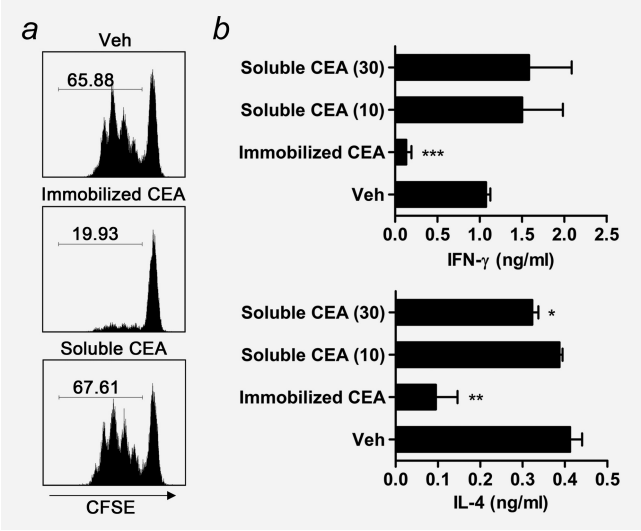

Figure 3. Immobilized CEA, but not soluble CEA, inhibits T cell proliferation and effector cytokine production of T cells. (*a*) CFSE-labeled CD4+ T cells were stimulated with anti-CD3 and anti-CD28 antibodies in the presence of immobilized or soluble CEA for 4 days. The cells were then collected, and CFSE dilution was analyzed by flow cytometry. (*b*) The supernatant was analyzed by ELISA for IFN-γ or IL-4. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ compared to Veh.

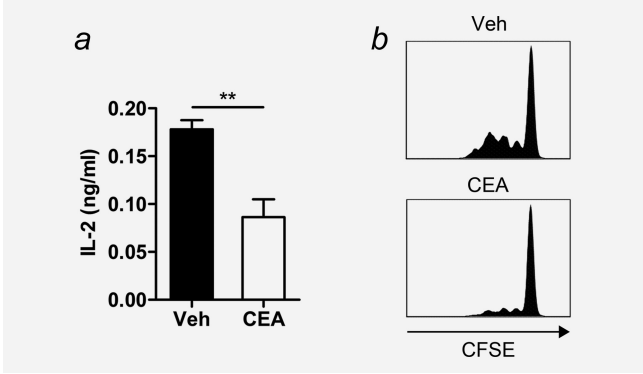

Figure 4. Immobilized CEA inhibits the activation of CD8+ T cells. (*a*) CD8+ T cells were incubated with immobilized CEA along with anti-CD3 and anti-CD28 antibodies for 20 hr, followed by ELISA for IL-2 production in the supernatant. (*b*) CFSE-labeled CD8+ T cells were stimulated with anti-CD3 and anti-CD28 antibodies in the presence of CEA for 3 days. The cells were then collected and analyzed for CFSE dilution using flow cytometry. **$p < 0.01$

the phosphorylation of ERK was evaluated after TCR engagement in the presence of multimeric CEA. Phosphorylated ERK was increased on TCR stimulation, but it was reduced in the presence of CEA (Fig. 5*b* and Supporting Information Fig. S3C). As a distal event of TCR stimulation, dephosphorylated NFAT translocates into nucleus from the cytosol which is regulated by calcineurin. Once in the nucleus, NFAT can regulate gene expression in coordination with various transcription factors, including AP-1.[19] We analyzed the subcellular localization of NFAT in TCR-stimulated CD4+ T cells in the presence of CEA by microscopy and found that the nuclear translocation of NFAT was also inhibited by CEA (Fig. 5*c*). Collectively, treatment with CEA leads to suboptimal activation of ERK and NFAT, thereby resulting in the suppression of T-cell activation.

TCR-proximal signaling molecules are not affected by treatment with CEA, and the inhibition of CD4+ T-cell activation occurs through SHP-independent mechanisms

It has previously been demonstrated that CEA modulates the cytolytic activity of NK cells through a direct interaction with CEACAM1.[11] CEACAM1 is known to control T-cell responses by recruiting SHP-1 and hindering the phosphorylation of tyrosine kinase signaling molecules involved in the TCR signal, particularly ZAP70.[12] Therefore, we investigated whether the regulatory effect of CEA was related to CEACAM1–SHP-dependent inhibition of TCR signaling. The detected RNA levels of *CEACAM1* (Fig. 6*a*) and its surface expression (Fig. 6*b*) were both very low level in unstimulated primary CD4+ T cells; however, they were induced after several days of stimulation, as shown in previous studies.[12,20] Many studies regarding the inhibitory function of CEACAM1 have used prestimulated T cells to maximize the functional activity of CEACAM1. To determine whether CEACAM1 and SHP still participate in CEA-induced suppression of T cells despite the limited presence of CEACAM1 on the surface of freshly isolated CD4+ T cells, cells were treated with a SHP inhibitor. Treatment of CD4+ T cells with NSC-87877 did not reverse the decrease in IL-2 production induced by CEA, although a slight tendency toward an overall increase in both CEA-treated and control conditions was observed (Fig. 6*c*). We confirmed that NSC-87877 was active under these conditions by showing that it was able to reverse CEACAM1 crosslinking-induced suppression of T-cell activation when it was used to treat 5-day-cultured T cells (Supporting Information Fig. S3A). Furthermore, to define the involvement of CEACAM1, soluble CEACAM1 protein was tested for its ability to counteract the effects of CEA, as several studies have used soluble CEACAM1 molecules to neutralize the contact with its ligands.[21,22] Soluble CEACAM1 protein failed to reverse CEA-induced suppression of IL-2 production (Supporting Information Fig. S3B). Additionally, the phosphorylation of TCR-proximal molecules such as ZAP70, linker for activated T cells (LAT), phospholipase Cγ (PLCγ) and VAV was not regulated on anti-CD3/CD28 stimulation in the presence of immobilized CEA (Fig. 6*d* and Supporting Information Fig. S3C). The absence of CEA-dependent effects on TCR-proximal signaling molecules also supports the idea that CEA suppresses the activation of CD4+ T cells through a SHP-independent mechanism, as SHP molecules are recruited and function near the cell membrane.

Discussion

Our study provides evidence for a coinhibitory function of CEA in the responses of human primary T cells. The role of CEA has long been understood in relation to its effects on

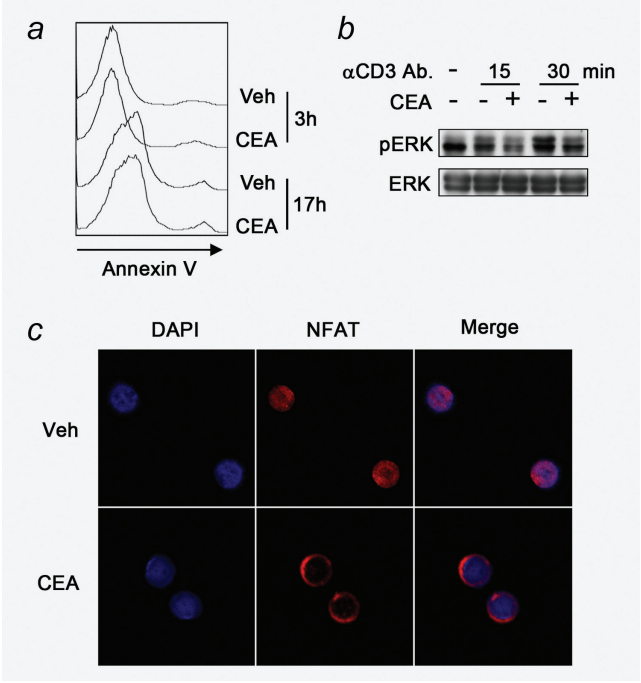

Figure 5. Reduced activation of ERK and nuclear translocation of NFAT by CEA treatment. (*a*) Purified CD4+ T cells were plated on immobilized CEA along with plate-bound anti-CD3 and soluble anti-CD28 antibodies for 3 or 17 hr. These cells were stained with Annexin V followed by flow cytometry analysis. (*b*) CD4+ T cells were collected at the indicated time points after anti-CD3/CD28 stimulation, treated with immobilized CEA and lysed with Triton-X-based buffer. The total and phosphorylated forms of ERK were detected by Western blot analysis. (*c*) CD4+ T cells were stimulated with anti-CD3 and anti-CD28 antibodies in the presence of immobilized CEA for 4 hr in a poly-D-lysine-coated slide chamber. Cells were fixed and permeabilized with methanol and stained with anti-NFAT2 antibody. Images were acquired by confocal microscopy. Data are representative of at least two independent experiments.

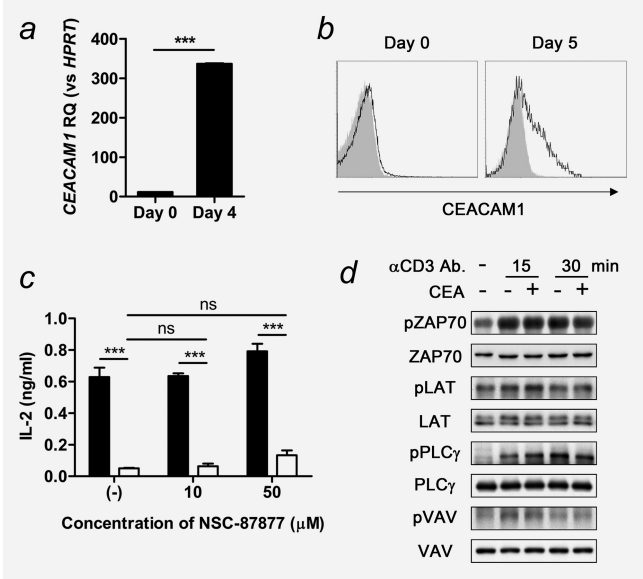

Figure 6. Phosphorylation of TCR-proximal signaling molecules is not affected by the presence of CEA. (*a*) *CEACAM1* and *Hprt* mRNA expression levels were detected in total cDNA from freshly purified CD4+ T cells or prestimulated CD4+ T cells by real-time polymerase chain reaction. (*b*) CD4+ T cells were stained with anti-CEACAM1 antibody (ASL-32, solid line) or its isotype control (filled) followed by flow cytometry analysis. (*c*) CD4+ T cells were treated with the indicated dose of NSC-87877 for 20 hr during stimulation with anti-CD3 and anti-CD28 antibodies in the presence of CEA (blank) or Veh (filled). After incubation, the IL-2 levels in the supernatant were analyzed by ELISA. (*d*) CD4+ T cells were collected at the indicated time points after anti-CD3/CD28 stimulation in the presence of immobilized CEA and lysed. The amounts of total and phosphorylated forms of each molecule were detected by Western blot analysis. ***$p < 0.001$, ns: not significant. (*b* and *d*) Data are representative of at least two independent experiments.

the tumor itself (*e.g.*, in recurrence and metastasis) including its antigenicity.[23–25] In this study, we focused on the possibility that a tumor-associated antigen could play a role in modulating T-cell responses. Our data demonstrate that CEA can regulate adaptive immune responses by inhibiting TCR stimulation in T cells. We show that CEA-overexpressing tumor cells or multimeric CEA significantly inhibit T-cell activation, proliferation and cytokine production. Based on our observations, it is conceivable that multimeric CEA dampens the activation of T cells as a coinhibitor when there are tumor burdens or circulating tumor cells near the site of T-cell activation. Therefore, CEA-overexpressing tumor cells could take advantage of easier migration and settlement by avoiding the optimal T-cell surveillance. Indeed, a recent report has demonstrated the importance of tumor cell CEA surface expression for metastasis showing CEA-mediated promotion of tumor implantation in mice.[26] Furthermore, clinical research in patients has discovered significantly increased levels of surface CEA in biopsies of metastasized colorectal cancer from the liver.[27] These observations, together with our study, suggest the possibility that suppressed T-cell functions caused by CEA may be responsible for tumor metastasis and stabilization. Intriguingly, we also show that the inhibitory effect is limited when CEA proteins are administered in a soluble form. Clinically, a 20 ng/ml or higher concentration of CEA in the serum of patient is considered to be a reference value for predicting tumor recurrence or metastasis.[28] Because the concentration we tested far exceeded such a dose, we may assume that the soluble form of CEA in serum has little coinhibitory effect on T cells. However, given the ability of soluble circulating CEA to give rise to tolerance of antigen-specific CD8 T cells *in vivo*, its functional importance should be clarified by further studies.[17]

CEA is known to mediate homophilic interactions with itself or heterophilic interactions with several other CEACAM family members, including CEACAM1.[29] Notably, Stern *et al.*[11] have demonstrated that the direct interaction of CEA with CEACAM1 on NK cells inhibits NK-cell activity, showing a functional interaction between the two molecules. Because expression of CEA is restricted to epithelial cells,[29]

we originally hypothesized that CEACAM1 has a role in the CEA-induced suppression of T cells through heterophilic interactions rather than homophilic interactions. Several possible underlysing mechanisms can be hypothesized for CEA-induced suppression of TCR signaling in the case that CEACAM1 acts as a receptor for CEA. Previous studies have noted that SHP is recruited to the ITIM motif in the cytoplasmic domain of CEACAM1 on ligation to the antibody or bacterial ligand, thus subsequently mediating the regulation of various types of receptor kinase signaling pathways *via* the phosphatase activity of SHP. In our study, SHP inhibitors were incapable of reversing the CEA-induced suppression of T-cell activation. Furthermore, irrespective of the presence of CEA, TCR-proximal signaling molecules were activated normally, indicating that other SHP-independent mechanisms may underlie the effect of CEA. The interaction between CEACAM1 and β-catenin is functionally important for the regulation of Fas-mediated signal transduction *via* the redistribution of the actin cytoskeleton.[15] Additionally, it has been found that cytoplasmic accumulation of β-catenin regulates T-cell stimulation.[30] However, it is unlikely that β-catenin is involved in CEA-mediated TCR signal transduction interference, as CEA does not decrease the activation of LAT and PLCγ, which are mainly affected by cytoplasmic β-catenin. CEACAM1 has also been shown to associated with Shc, a SH2-containing cytoplasmic adaptor protein, thereby resulting in the regulation of the Ras mitogenesis pathway through the sequestration of Grb2 recruited to the insulin receptor.[31] Shc participates in Lck-dependent TCR signaling, regardless of LAT, and it is essential for IL-2 expression after TCR stimulation *via* the Ras/ERK activation pathway.[32] Based on these studies, it can be assumed that CEACAM1 binding to multimeric CEA may affect the Ras-dependent TCR signal transduction mechanism by delocalizing Shc and TCR, as LAT-dependent signaling remains normal. However, it is important to note that expression of CEACAM1 is very low in freshly isolated T cells (Figs. 6*a* and 6*b*) but is upregulated after stimulation.[33] Indeed, many studies of CEACAM1 function have used prestimulated T cells or cell lines.[12,20] Because our study is focused on early activation events in freshly isolated primary T cells within 1 day, before CEACAM1 is induced extensively, the expression of CEACAM1 might be too low to contact with CEA and exert an effect. We confirmed this idea by showing that soluble CEACAM1 protein affected neither T cell activation nor CEA-mediated inhibition (Supporting Information Fig. S3B). Collectively, in resting T cells or very early activated T cells, CEA is unlikely to interact with CEACAM1 or to use SHP indicating that other receptors might be involved in its inhibitory effect. Several molecules have been discovered to interact with CEA. Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Nonintegrin
(DC-SIGN), a C-type lectin found on dendritic cells, binds to Lewis glycans of CEA in colorectal tumors.[34,35] CEA receptor, an ortholog of the heterogeneous nuclear RNA-binding protein M4 (hnRNP M4) on Kupffer cells, acts as a receptor for CEA by recognizing the peptide sequence PELPK at the hinge region between the N and A1 domains.[36,37] In the context of T-cell activation, they can be excluded as interaction partners because it is known that they are not expressed on the surface of T cells. Further studies will provide a better insight into possible binding partners and the mechanism by which multimeric CEA affects T-cell activation.

Ongoing studies have revealed many important roles of T cells in tumor immunity. Although CD8+ T cells are the most important cells that eradicate tumor cells, direct killing mechanism of tumors by CD4+ T cells has been shown in certain conditions.[38] Many indirect mechanisms by which CD4+ T cells aid in cancer immunity are well known; for instance, they help CD8+ function and recruitment of NK cells.[39–42] Thus, both CD4+ T cells and CD8+ T cells critically contribute to tumor surveillance. Herein, we suggest that CEA is not only a diagnostic factor for colon cancer metastasis but that it is also possible to function as a modulator of antitumor T-cell responses. It is meaningful to show the effect of CEA on freshly isolated T cells from human blood, as such experiments are more likely to accurately predict the events *in vivo* than experiments using prestimulated T cells or T-cell clones. We used stimulatory antibodies in the presence of CEA for activation because of the diversity of antigen receptors on total primary T cells thereby mimicking the environment in which CEA-producing tumor cells are near the site of T-cell activation. However, because tumor cells can concomitantly express CEA and its own antigens to antigen-specific T cells and activate them, it is intriguing to find an effect of CEA protein and specific antigens displayed on the same tumor cells. Indeed, we observed that CEA-expressing HeLa cells showed a reduced allogenic response when cocultured with primary T cells (Supporting Information Fig. S1G). Furthermore, revealing the role of CEA in an *in vivo* model would strengthen our knowledge about the tumor evasion mechanisms of CEA-expressing tumors and would therefore help to develop strategies to control them. Additional *in vivo* studies regarding T-cell behaviors under the environment of CEA-expressing tumor cells should be conducted, as they will deepen our understandings on the mechanisms underlying the effect of CEA on T cells.

Author Contribution

K.-A.L., E.-A.B., and Y.-C.S. designed the research and wrote the manuscript. K.-A.L., E.-A.B., Y.-C.S., E.-K.K., and Y.-S.L. performed the experiments. T.-G.K. and C.-Y.K. edited and commented on the manuscript.

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