Carcinoembryonic Antigen-Related Cellular Adhesion Molecule 1 Isoforms Alternatively Inhibit and Costimulate Human T Cell Function

Daohong Chen, Hideki Iijima, Takashi Nagaishi, Atsushi Nakajima, Sara Russell, Raktima Raychowdhury, Victor Morales, Christopher E. Rudd, Nalan Utku and Richard S. Blumberg

J Immunol 2004; 172:3535-3543; doi: 10.4049/jimmunol.172.6.3535
http://www.jimmunol.org/content/172/6/3535

References
This article cites 45 articles, 25 of which you can access for free at:
http://www.jimmunol.org/content/172/6/3535.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Carcinoembryonic Antigen-Related Cellular Adhesion Molecule 1 Isoforms Alternatively Inhibit and Costimulate Human T Cell Function

Daohong Chen, Hideki Iijima, Takashi Nagaishi, Atsushi Nakajima, Sara Russell, Raktima Raychowdhury, Victor Morales, Christopher E. Rudd, Nalan Utku, and Richard S. Blumberg

Carcinoembryonic Ag-related cellular adhesion molecule 1 (CEACAM1) represents a group of transmembrane protein isoforms that consist of variable numbers of extracellular Ig-like domains together with either a long cytoplasmic (cyt) tail containing two immunoreceptor tyrosine-based inhibitory motifs or a unique short cyt tail. Although CEACAM1 has been reported to be expressed on the surface of T lymphocytes upon activation, its roles in T cell regulation are controversial due to the lack of functional characterization of each individual CEACAM1 isoform. We thus cotransfected Jurkat T cells with CEACAM1 isoform-encoding constructs and an IL-2 promoter-bearing plasmid or a small interference RNA targeting src homology domain 2 containing phosphatase 1. In a luciferase reporter assay and through measurements of cytokine secretion (IL-2, IL-4, and IFN-γ), CEACAM1 containing either a long or a short cyt tail inhibited or costimulated, respectively, TCR/CD3 complex plus CD28 mediated activation with the inhibitory functions of the long cyt tail dominating. The inhibitory function of CEACAM1, was dependent upon src homology domain 2 containing phosphatase 1 activity, required both tyrosine residues within the immunoreceptor tyrosine-based inhibitory motif domains of the cyt tail and was mediated through the mitogen-activated protein kinase pathway. CEACAM1-mediated inhibition could be functionally reconstituted by incubation of PBMC with either a CEACAM1-specific mAb or CEACAM1-Fc fusion protein in the presence of an allogeneic or mitogenic stimulus, respectively. These studies indicate that the long and short cyt tails of CEACAM1 serve as inhibitory and costimulatory receptors, respectively, in T cell regulation. The Journal of Immunology, 2004, 172: 3535–3543.
cells (22–27). The function of CEACAM1 on T lymphocytes is unclear. Although it is tempting to consider CEACAM1 as an inhibitory receptor given the presence of two ITIMs within the long cytail, ligation of CEACAM1 with either CEACAM1-specific Abs or bacterial products known to bind CEACAM1 heterophilically have resulted in either T cell activation (23, 26) or inhibition (24, 25, 27). One possible explanation for these contradictory results is that they reflect the consequences of ligating different CEACAM1 isoforms. Virtually all studies performed to date have examined primary mouse or human T cells, which are known to express various combinations of both the long and short cytail domains. More specifically, there have been no carefully performed functional assessments of the downstream pathways associated with the CEACAM1 long and short cytail domain-containing isoforms including their interactions in T cells and the specific role of the ITIM domains. Therefore, in this study, we sought to determine the functional behavior of CEACAM1 expressing three extracellular Ig-domains that differ only in expression of either a long (CEACAM1–3L) or short (CEACAM1–3S) cytail in the Jurkat cell line, which has been previously shown to be devoid of CEACAM1 expression even after activation (26). We found that the human CEACAM1–3L isoform has the properties of an ITIM-bearing inhibitory receptor in that inhibition of TCR/Cd3 complex signaling is affected by this isoform and is dependent upon the presence of two functional tyrosine residues in the cytail complex. These two ITIMs are functionally linked to the inhibition of TH1 and TH2 cytokine secretion and, via SHP-1, to the specific inhibition of mitogen-activated protein kinases (MAPK) comprising the c-Jun N-terminal kinase (JNK) and extracellular response kinase (ERK). In contrast, the short cytail of human CEACAM1 behaves as an activating receptor whose function is dominated by the inhibitory function of the long cytail domain-containing isoform. These studies help to clarify the function of CEACAM1 in T lymphocytes and support consideration of the CEACAM1 long cytail-containing isoforms as inhibitory receptors and the short cytail-containing isoforms as costimulatory receptors.

Materials and Methods

Cells and cell culture

Human PBMC were purified from whole blood of unrelated donors by centrifugation on a Ficoll/Hypaque gradient (Amersham Pharmacia, Piscataway, NJ) by standard methods. Jurkat cells stably transfected with SV40-large T Ag (Jurkat-T) have been previously described (28). COS-7 cells were obtained from the American Tissue Type Culture Collection (Pronasas, VA) and cultured in complete medium containing DMEM.

Reagents and Abs

The mouse anti-human CEACAM1 mAbs, 5F4 and 26H7, are of the IgG1 isotype and generated as previously described (24). Mouse anti-human CEACAM1–3L and -3S expression constructs were generated in pEF6/V5-HisB vector (Invitrogen, Carlsbad, CA) by PCR amplification of the template pPAN3.1 DNA (23) with the following primers. The 5’ and 3’ ends of the primers for site-specific cloning. The CEACAM1–3L/Y459A mutant containing an alanine substitution of Y459 was generated by PCR-directed mutagenesis of the CEACAM1–3L template DNA using the following primers: 5’-GAGTGAATGAACTGTCCTCTACGCTCAAACT-3’ (forward primer), 5’-GTCAAGATGAGGACTCTTGCCTCTAC-3’ (reverse primer) with the QuickChange Site-Directed Mutagenesis kit (Invitrogen). cDNAs encoding CEACAM1–3L/Y486A containing an alanine substitution of Y486 and CEACAM1–3L/Y459AY486A containing alanine substitutions of Y459 and Y486 were generated by PCR amplification of the CEACAM1–3L and CEACAM1–3LY459A templates, respectively, with the following primers: 5’-GATGGAATGAACTGTCCTCTAC-3’ (forward primer), and 5’-CCTCTAGATCTGCTATTCTTTTTCAGTGTCCTC-3’ (reverse primer).

Generation of CEACAM1-Fc-fusion protein

The ScFvExpress-sec vector, which can induce the synthesis and secretion of proteins in eukaryotic cells using an EF-BOS promoter in front of the human IgG1 secretory leader peptide sequence (30), was digested with Sall and NorI, dephosphorylated with shrimp alkaline phosphatase (Amersham Pharmacia), and ligated to a Sall-NorI fragment (693 bp) containing the cDNA sequence of the human Fc (kindly provided by Dr. W. Lencer, Children’s Hospital Medical Center, Boston, MA). The extracellular domain of the human BGgb (CEACAM1–3L) coding sequence was amplified from pTPAN1, a pTZ19 vector with an XbaI restriction site, resulting in a fragment (1.3 kb) containing the cDNA sequence for human BGP (24). The primers used for this amplification were PANSAL (5’-GATGGAATGAACTGTCCTCTAC-3’), GAATTCATGCCAGCTCATCTCATCT (Forward Primer), and ADAPSAABA (5’-AGATTGGTGCAGTGGGCCGGGACCCAACCGCAACCGGATCCATCTACATTATCAAGGACGC-3’). The amplified fragment was digested with Sall, gel purified using NAC45 paper (Schleicher & Schuell, Kenne, NJ), ligated to the dephosphorylated Sall-linearized scHuF3 vector and transformed into competent E. coli cells. One of these plasmids (pBGPFc1) was used for transfection of CHO cells using Effectene (Qiagen, Valencia, CA) for production of CEACAM1-Fc fusion protein.

MLR and PHA stimulation

Allogeneic MLR and PHA stimulation of PBMCs was performed by standard methods as previously described (31). Responder PBMCs were stimulated with equal numbers of irradiated (3000 rad, 13 min) stimulator PBMCs at concentrations of 106 cells/ml for 24 h. PBMCs were exposed to PHA (2 μg/ml) for 24 h.

Transfection studies

COS-7 cells were transfected with 2 μg of each plasmid DNA per well in a six-well tissue culture plate using Lipofectamine Plus reagent (Life Technologies) according to the manufacturer’s instructions and Western blotting performed by standard methods with an anti-CEACAM1 mAb, 5F4, at a concentration of 10 μg/ml using an enhanced chemiluminescence reagent (New England Nuclear, Boston, MA) for detection. Jurkat-T cells (106) were transfected with the 1 μg of plasmids encoding mutated or nonmutated forms of CEACAM1 cloned into pE6FV5-His B or empty vector alone as control in combination with 0.5 μg of NFAT/ AP1/LUC reporter plasmid using GenePorter transfection reagent (Gene Therapy System, San Diego, CA). Twenty-four hours after transfection, cells (5 × 104/condition) were stimulated with anti-human CD3 mAb (0.4–10 μg/ml) and anti-human CD28 mAb (0.08–10 μg/ml) or isotype-matched control Abs followed by goat anti-mouse Fc Ab (3 μg/ml) for 6 h at 37°C. For the control mAbs for anti-CD3 and anti-CD28, mouse IgG1 and mouse IgG2a were used, respectively. Six hours after stimulation, cells were solubilized with 100 μl of cell culture lysis reagent (Promega, Madison, WI) and cell extracts were separated by SDS-PAGE. The difference in luciferase activity determined using a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA). The protein concentration of the lysates was determined by bicinchoninic acid protein assay reagent (Pierce) and the luciferase results normalized for the protein content. Alternatively, the cells were collected for Western blot analysis at various times following transfection or the supernatants of the transfectants removed for ELISA (BD PharMingen) based cytokine production assays at either days 1, 2, or 3 following transfection.

Western blotting

The samples were washed twice in PBS and lysed in 200 μl of 1% Triton X-100. The protein content was determined using a bicinchoninic acid protein assay kit (Pierce). Proteins (20 μg) were separated by SDS-PAGE on 10% polyacrylamide gels and then transferred to nitrocellulose membrane. Following blocking of nonspecific binding by 5% nonfat milk, the
membranes were treated with pERK, pJNK, or p-p38 Abs for 1 h at room temperature. The membranes were then washed and incubated with HRP-conjugated, goat anti-mouse IgG (1:2000; DAKO, Carpinteria, CA) and developed using Western blot chemiluminescence reagent (NEN Life Science Products, Boston, MA). Following demonstration of pJNK, p-p38, or pERK, the nitrocellulose membranes were stripped by treatment with 250 mM glycine-HCl, pH 2.0, 1% SDS for 30 min and total MAPK levels were detected in the same membranes using rabbit anti-ERK, JNK, or p38 Abs, respectively. Densitometry was performed for standardization of the phosphorylated MAPK species relative to the total MAPK fractions.

**SHP-1 gene silencing by means of small interfering (si) RNA**

To selectively silence human SHP-1 gene expression in Jurkat-T cells, a specific siRNA approach was developed. The cDNA sequence was targeted at human SHP-1 (5'-UGCCGGCUAGACUGAGAAGdTdT-3') and an anti-sense strand (5'-dTdTACCUGACUAGACUCUGG-3') (Xeragon, Germantown, MD). siRNA (1 μg) was used to transfect 5 × 10⁶ Jurkat-T cells using the TransMessenger Transfect kit (Qiagen) following the manufacturer's instructions. To confirm the posttranscriptional gene silencing effect of SHP-1 siRNA, transfected Jurkat-T cells were harvested 48 h after transfection. Total cellular RNA was isolated using TRIzol (Invitrogen) following the manufacturer's instructions. RNA (100 ng) was subjected to reverse-transcription using the Advantage RT-for-PCR Kit (Clontech Laboratories, Palo Alto, CA). The sense and antisense primers for human SHP-1 were 5'-GGGAGAATGTTCTTGTAGCG-3' and 5'-GGAGAAGTTTGC(GA)CTCTGA-3' and 5'-GGGAGAATGTTCTTGTAGCG-3' (634 bp). The conditions for the PCR were as follows: after initial denaturation at 95°C for 5 min, the thermocycler (MJ Research, Watertown, MA) was programmed for 35 cycles: 1 min at 95°C, 1 min at 55°C and 2 min at 72°C. The reaction was concluded with a final extension step at 72°C for 7 min. PCR products were separated and visualized with 1.5% agarose gel containing 0.01% ethidium bromide. OD of the cDNA bands were determined by the computerized image-analysis system and normalized to RT-PCR products of β-actin.

**Results**

Transfection of Jurkat cells with human CEACAM1-3L and/or CEACAM1-3S isoforms inhibit and activate, respectively, NFAT-AP1 reporter activity induced by anti-CD3 and anti-CD28

Previous studies have shown that the Jurkat cell line cannot be induced to express CEACAM1 making it a useful tool to examine the function of the human (h)CEACAM1–3L and hCEACAM1–3S isoforms in human T cells (26). As shown in Fig. 1A, this was confirmed by the fact that Jurkat-T cells did not express CEACAM1 either before or after (3–24 h) activation with a variety of stimulatory conditions but did so after transfection with either the CEACAM1–3S or CEACAM1–3L isoforms, COS-7 cells, as a control (Fig. 1B), or the Jurkat-T cell line (Fig. 1C) were thus transfected with either an empty vector, as control, or plasmids containing DNA fragments encoding the CEACAM1–3L and/or CEACAM1–3S isoforms. As can be seen in Fig. 1C, confirming previous studies with Jurkat cells (26), Jurkat-T cells failed to express CEACAM1 unless transfected with the CEACAM1-encoding plasmids. Moreover, an evaluation of the time course of CEACAM1 protein expression after transient transfection in the Jurkat-T cells showed detectable evidence of CEACAM1 after 24 h with peak expression at 48–72 h for both the CEACAM1–3L and CEACAM1–3S isoforms (Fig. 1D). These studies (Fig. 1, B–D) show that the CEACAM1–3L- and CEACAM1–3S-encoding plasmids were equally efficient at driving CEACAM1 protein synthesis.

To define the effect of CEACAM1 expression in the Jurkat-T cell line, the CEACAM1-transfected Jurkat-T cells were cotransfected with a plasmid containing luciferase controlled by the

**FIGURE 1.** Expression of CEACAM1 in transfected COS and Jurkat-T cells. A, CEACAM1 expression on Jurkat-T cells was assessed by flow cytometry with the 5F4 mAb and a FITC-labeled secondary Ab either before or after activation with IL-2 (12 h), anti-CD3 plus anti-CD28 (12 h), or PHA plus PMA (24 h) stimulation. Transfection of Jurkat-T cells with CEACAM1-S and CEACAM1–3L-encoding plasmids is shown as a positive control. B, COS cells were transfected with either the CEACAM1–3L or CEACAM1–3S cDNAs and Western blotting performed with the 5F4 mAb 48 h after transfection. Control untransfected COS cells are shown. C, Jurkat-T cells were transfected with either the CEACAM1–3L (3L) or CEACAM1–3S (3S) isoforms and Western blotting performed at day 1 (B) or at days 1, 2, 3, and 4 (D) after transfection with the 5F4 mAb. The blot in D was reprobed with a β-actin-specific Ab as a loading control. C represents untransfected Jurkat-T cells.
Inhibitory and stimulation of Jurkat-T cells by CEACAM1

FIGURE 2. Inhibition and stimulation of Jurkat-T cells by CEACAM1–3L and CEACAM1–3S isoforms, respectively. A. Jurkat-T cells were transfected with the NFAT-AP1 luciferase reporter vector plus either an empty vector or vectors containing the CEACAM1–3L cDNA alone, CEACAM1–3S cDNA alone or CEACAM1–3L and CEACAM1–3S cDNA. After 24 h, the transfected cells were stimulated with either control IgG Abs (left panel) or anti-CD3 (2 μg/ml) plus anti-CD28 (2 μg/ml) mAbs in the presence of a goat anti-mouse Fc Ab (GmM) as crosslinker. After 6 h, luciferase activity was analyzed and expressed as a ratio of total protein content. * p < 0.01. B. Jurkat-T cells were transfected with NFAT-AP1 luciferase reporter and either an empty vector or vector containing the CEACAM1–3S cDNA. After 24 h, the transfected cells were stimulated with anti-CD3 (2 μg/ml) and either control IgGlx or anti-CD28 at doses of 0, 0.08, 0.4, 2, or 10 μg/ml. Luciferase activity was assessed as described in A. * p < 0.01.

The observation that transfection of the CEACAM1–3S isoform resulted in spontaneous activation of the NFAT-AP1 reporter in the absence of anti-CD3 and CD28 stimulation (Fig. 2A, left panel) suggested a stimulatory function for this isoform independently of CEACAM1–3L. In addition, under the experimental conditions examined in Fig. 2A (right panel), there was a trend toward a costimulatory effect of the CEACAM1–3S isoform in the presence of anti-CD3 plus anti-CD28 cross-linking in at least three replicative experiments. We therefore next considered the possibility that CEACAM1–3S might costimulate TCR/CD3 complex signaling. Therefore, we directly examined this possibility by transfecting Jurkat-T cells with the NFAT-AP1 luciferase reporter and either the empty vector, as control, or vector containing the CEACAM1–3S cDNA in the presence or absence of a stable concentration of anti-CD3 (2 μg/ml) and increasing concentrations of anti-CD28 (0.0–10.0 μg/ml) or an isotype control Ab. As can be seen in Fig. 2B, in the absence of anti-CD28 Ab, CEACAM1 costimulated TCR/CD3 complex signaling as revealed by increased luciferase reporter activity in the presence of the CEACAM1–3S isoform relative to the vector control. In the presence of anti-CD28 stimulation, the CEACAM1–3S isoform was able to further costimulate anti-CD3-induced activation of the NFAT-AP1 luciferase reporter at suboptimal concentrations of anti-CD28-induced costimulation. For example, the relative luciferase activity noted for anti-CD3 stimulation of the Jurkat cells at 2 μg/ml of the anti-CD28 Ab in the presence of the CEACAM1–3S isoform was greater than that observed with 10 μg/ml of the anti-CD28 Ab when the CEACAM1–3S isoform was absent. Taken together, these studies indicate that whereas the CEACAM1–3S isoform is costimulatory to T cell activation, the CEACAM1–3L isoform behaves as an inhibitory receptor whose function is dominant over the CEACAM1–3S isoform at least when present at equimolar ratios.

Inhibitory function of the human CEACAM1–3L isoform in T cells is dependent upon both functional ITIM domains

The human CEACAM1–3L cyt tail contains two tyrosine residues as part of ITIMs (5, 7). Previous studies have directly linked these ITIMs to inhibitory functions in mouse epithelial cells (32) and human B lymphocytes (19) and indirectly to T cells due to the ability of the CEACAM1 cyt tail to associate with SHP-1 and SHP-2 in human and mouse T cells (25, 27). However, the role of the two ITIMs in T cell function has not been directly examined. To determine whether the two ITIMs are important in the inhibitory function of the CEACAM1–3L isoform, alanine substitutions were made at the Y459 and/or Y486 residues in the cyt tail of the CEACAM1–3L isoform. These three CEACAM1–3L mutants (3L-Y459A, 3L-Y486A, and 3L-Y459A/Y486A) were examined for their ability to inhibit TCR/CD3 complex-mediated stimulation of T cell. As can be seen in Fig. 3A, deletion of both the Y459 and Y486 residues but not either alone resulted in abrogation of the ability of the CEACAM1–3L isoform to inhibit spontaneous (left panel) and anti-CD3 plus anti-CD28 stimulated (right panel) activation of NFAT-AP1 luciferase reporter activity. The biologic significance of this inhibition was shown by the ability of the CEACAM1–3L isoform to inhibit IL-2 secretion induced by anti-CD3 plus anti-CD28 stimulation of the Jurkat-T cell line, the loss of this inhibition in the context of deletion of both the Y459 and Y486 residues in the cyt tail (YY) and the opposing effects of the −L and −S isoforms on IL-2 secretion when transfected together (LS). (Fig. 3B). These studies indicate that the two tyrosine residues of the cyt tail of human CEACAM1–3L are functional in T cells and likely to be part of ITIMs given that their elimination leads to a loss of inhibitory function.

Ligation of CEACAM1 on a primary T cell is functionally inhibitory

The fact that the stimulatory and inhibitory functions of the CEACAM1–3S and CEACAM1–3L isoforms, respectively, were elicited in the absence of addition of any exogenous factors suggested that the newly expressed CEACAM1–3S or CEACAM1–3L isoforms on the transfected Jurkat-T cell line were being ligated homophilically...
in a homotypic interaction between the transfected Jurkat-T cells. Moreover, given the fact that human T cells have been previously shown to simultaneously express CEACAM1 with both a long and short cyt tail (26), given the results described above, it would be predicted that the functional consequence of CEACAM1 ligation is dependent on tyrosine residues in the cyt tail. A, Jurkat-T cells were transfected an NFAT-AP1 reporter activity assessed as described in Fig. 2 after transfection with either the wild-type CEACAM1–3L cDNA or mutated CEACAM1–3L cDNAs containing alanine substitutions at Y459 and/or Y486 residues. The transfected cells were stimulated with either control (mlgG1, mlgG2a) or anti-CD3 plus anti-CD28 mAbs (2 μg/ml). *, p < 0.05; **, p < 0.01. B, Jurkat-T cells were transfected with the NFAT-AP1 luciferase reporter and either a vector control (V) or vectors containing the CEACAM1–3S (S), CEACAM1–3L (L), and/or the CEACAM1–3L containing alanine substitutions of both tyrosine residues in the cyt tail (YY) in the presence or absence of control siRNA duplexes (LC) or siRNA duplexes specific for SHP1 (Li) vs nonspecific (control) siRNA duplexes (Lc) and assessed as described in A for the secretion of IL-2 at 24 h after T cell stimulation. The results are expressed as the concentration of IL-2 (picograms per milliliter) normalized to 10^6 cells. LS indicates simultaneous transfection with both CEACAM1-L and CEACAM1–3S isoforms. *, p < 0.05.

FIGURE 3. Inhibitory function of CEACAM1–3L isoform is dependent on tyrosine residues in cyt tail. A, Jurkat-T cells were transfected an NFAT-AP1 reporter activity assessed as described in Fig. 2 after transfection with either the wild-type CEACAM1–3L cDNA or mutated CEACAM1–3L cDNAs containing alanine substitutions at Y459 and/or Y486 residues. The transfected cells were stimulated with either control (mlgG1, mlgG2a) or anti-CD3 plus anti-CD28 mAbs (2 μg/ml). *, p < 0.05; **, p < 0.01. B, Jurkat-T cells were transfected with the NFAT-AP1 luciferase reporter and either a vector control (V) or vectors containing the CEACAM1–3S (S), CEACAM1–3L (L), and/or the CEACAM1–3L containing alanine substitutions of both tyrosine residues in the cyt tail (YY) in the presence or absence of control siRNA duplexes (LC) or siRNA duplexes specific for SHP1 (Li) vs nonspecific (control) siRNA duplexes (Lc) and assessed as described in A for the secretion of IL-2 at 24 h after T cell stimulation. The results are expressed as the concentration of IL-2 (picograms per milliliter) normalized to 10^6 cells. LS indicates simultaneous transfection with both CEACAM1-L and CEACAM1–3S isoforms. *, p < 0.05.

FIGURE 4. Ligation of CEACAM1 with either specific mAb or CEACAM1-Fc fusion protein is associated with T cell inhibition. A, An allogeneic MLR was performed in the presence of varying concentrations (micrograms per milliliter) of either the 5F4 mAb or isotype matched control mAb and proliferation assessed by [H]thymidine incorporation after 72 h. MLR, control MLR without added Abs; PHA, positive control; Stim, stimulator cells alone; Resp, responder cells alone. *, p < 0.05 vs MLR. B, Human PBMC were cultured 48 h with PHA. Proliferation was assessed in the presence or absence of either CEACAM1-Ig or CTLA4-Ig on day 3 by pulsing with 20 μg/well of BrdU for the last 16 h. Positive (Pos) control is PHA stimulation alone. Negative (Neg) is unstimulated cells. *, p < 0.05 vs pos control.
isoform on the levels of pJNK and pERK consistent with the opposing functional effects of the CEACAM1–3L and CEACAM1–3S isoforms as described above.

Since pJNK and pERK have been shown to affect Th1 and Th2 differentiation and cytokine production, respectively (33, 34), we next determined whether these effects on MAPK pathways by CEACAM1 also correlated with effects on Th1 and Th2 cytokine production. In the absence of anti-CD3/CD28 Ab stimulation, IFN-γ and IL-4 levels were all below 50 pg/ml/10⁶ cells in the various groups analyzed (Fig. 6, A and B, respectively). In contrast, in the presence of anti-CD3/CD28 Ab stimulation, the Jurkat-T cells exhibited a ~3-fold increase in the secretion of IFN-γ (Fig. 6A) and IL-4 (Fig. 6B). As noted above, transfection with the CEACAM1–3S isoform caused a further slight up-regulation in secretion of these two cytokines above the vector control. In contrast, transfection with the CEACAM1–3L isoform resulted in significant inhibition of both IFN-γ and IL-4 secretion with a decrease in the inhibition when the two ITIM domains were deleted by mutation of the Y459 and Y486 residues (YY) and opposing effects of the –L and –S isoforms (LS) when transfected together. These studies suggest that CEACAM1–3L, by virtue of the two ITIM domains in the cyt tail, inhibits pJNK and pERK MAPK activity and the downstream activation of both Th1 and Th2 cytokine secretion when examined in a transfected cellular system as shown here.

**ITIM-associated regulation of MAPK activation by CEACAM1 is dependent on SHP-1**

Since CEACAM1 has been shown to associate with SHP-1 in epithelial cells through direct interactions with the ITIM domains of the long cyt tail and SHP-1 is known to negatively regulate MAPK pathways (35), we reasoned that the inhibition of TCR-mediated activation of MAPK pathways by CEACAM1–3L may occur through SHP-1. This possibility was assessed by analyzing the ability of the CEACAM1–3L isoform to inhibit the activation of the two ITIM regions as caused by alanine substitutions of the Y459 and Y486 residues (Fig. 7B). These studies prove that CEACAM1–3L inhibition of MAPK activation after ligation of the TCR/CD3 complex in the presence of CD28 costimulation is due to ITIM-dependent regulation of SHP-1.

**Discussion**

Mouse and human T cells have been recently appreciated to express CEACAM1 as a consequence of activation by a variety of means (22–27). However, both stimulatory and inhibitory functions have been ascribed to CEACAM1 expression on T cells. By a careful examination of CEACAM1 isoforms containing the same extracellular domain but either the long or short cyt tail characteristic of this receptor family, we were able to ascribe inhibitory functions to the long cyt tail and stimulatory functions to the short
cyt tail. Given that the CEACAM1–3L variant could inhibit the stimulatory activity of the CEACAM1–3S isoform, our studies suggest that CEACAM1 expression in T cells may be a tunable system whose functional outcome is determined by the relative proportion of the long and/or short cyt domain-containing isoforms. This characteristic of CEACAM1 draws clear parallels between this group of molecules and the relationship between killer inhibitory receptors (KIR) and killer activating receptors (KAR) (35). Although the functional features of the KIRs and KARs may be similar to that proposed for CEACAM1–3L and CEACAM1–3S, the structural bases for these functional properties are quite different. Like KARs, the inhibitory function of CEACAM1–3L is likely mediated by ITIMs within the cyt tail as shown here. In contrast to the KARs which are derived from separate gene products that are related to KIRs and contain a positively charged residue in the transmembrane domain, CEACAM1–3S is generated by alternate splicing and does not contain a positively charged transmembrane residue. This further suggests that the activating properties of CEACAM1–3S in T cells is not likely due to association with co-associating activating molecules such as DAP10 or DAP12, which contain a negatively charged transmembrane residue (36). The mechanistic basis for the apparent stimulatory activity of the CEACAM1–3S isoform on T cells thus remains to be established but may be related to the activity of serine/threonine kinases (8).

Previous studies that have characterized CEACAM1 isoform expression in human and mouse T cells have used RT-PCR amplification, cloning, and sequencing since there are no mAbs available that are able to discriminate CEACAM1 isoforms. These studies have shown that human and mouse T cells express the BGP a, b, c, and d (or CEACAM1–4L, 3L, 4S and 3S, respectively) splice variants (26, 37). In the limited data available, the long cyt domain-containing isoforms appear to be expressed in excess over the short cyt domain-containing isoforms. Given our results in the Jurkat system that the inhibitory functions of the long cyt tail are dominant over the stimulatory functions of the short cyt tail, it might be predicted that the outcome of CEACAM1 expression on a T cell is a dampening effect on TCR/CD3 complex function. Moreover, this inhibitory effect is likely to be stimulated homophilically based upon results with the Jurkat transfection system and the human CEACAM1-Fc fusion protein as shown here, which draws comparisons to CD22, a similarly functioning molecule (38). To further confirm that the mechanism of inhibition is homophilic ligation of CEACAM1 and stimulation of an inhibitory pathway as opposed to inhibition of an activating pathway, future studies should be aimed at examining the effects of monovalent reagents such as Fab that would bind directly to the homophilic binding site without cross-linking. Whether there are circumstances in which the costimulatory functions of the short cyt tail domain-containing isoforms dominate remains to be determined but would be predicted to be the case under certain conditions and may suffice to explain previous stimulatory functions ascribed to this molecule.

Like the inhibitory functions of CEACAM1 observed in mouse intestinal epithelial cells (7, 32) and human B cells (19), the tyrosine residues within the ITIMs appear to be important for CEACAM1–3L function in T cells. However, there are interesting differences which suggest that the mechanism(s) by which CEACAM1–3L isoform functions in human T cells may be distinct. First, in contrast to the long cyt tail in mouse intestinal epithelial cells, which requires the short cyt tail-containing isoforms to dominate, the inhibitory function of the long cyt tail in T cells is not likely due to association with co-associating activating molecules such as DAP10 or DAP12, which contain a negatively charged transmembrane residue (36). The mechanistic basis for the apparent stimulatory activity of the CEACAM1–3S isoform on T cells thus remains to be established but may be related to the activity of serine/threonine kinases (8).

Previous studies that have characterized CEACAM1 isoform expression in human and mouse T cells have used RT-PCR amplification, cloning, and sequencing since there are no mAbs available that are able to discriminate CEACAM1 isoforms. These studies have shown that human and mouse T cells express the BGP a, b, c, and d (or CEACAM1–4L, 3L, 4S and 3S, respectively) splice variants (26, 37). In the limited data available, the long cyt domain-containing isoforms appear to be expressed in excess over the short cyt domain-containing isoforms. Given our results in the Jurkat system that the inhibitory functions of the long cyt tail are dominant over the stimulatory functions of the short cyt tail, it might be predicted that the outcome of CEACAM1 expression on a T cell is a dampening effect on TCR/CD3 complex function. Moreover, this inhibitory effect is likely to be stimulated homophilically based upon results with the Jurkat transfection system and the human CEACAM1-Fc fusion protein as shown here, which draws comparisons to CD22, a similarly functioning molecule (38). To further confirm that the mechanism of inhibition is homophilic ligation of CEACAM1 and stimulation of an inhibitory pathway as opposed to inhibition of an activating pathway, future studies should be aimed at examining the effects of monovalent reagents such as Fab that would bind directly to the homophilic binding site without cross-linking. Whether there are circumstances in which the costimulatory functions of the short cyt tail domain-containing isoforms dominate remains to be determined but would be predicted to be the case under certain conditions and may suffice to explain previous stimulatory functions ascribed to this molecule.

Like the inhibitory functions of CEACAM1 observed in mouse intestinal epithelial cells (7, 32) and human B cells (19), the tyrosine residues within the ITIMs appear to be important for CEACAM1–3L function in T cells. However, there are interesting differences which suggest that the mechanism(s) by which CEACAM1–3L isoform functions in human T cells may be distinct. First, in contrast to the long cyt tail in mouse intestinal epithelial cells, which requires the short cyt tail-containing isoforms to dominate, the inhibitory function of the long cyt tail in T cells on TCR/CD3 complex function can be detected independently of the short cyt tail. The function of the long cyt tail has also been examined in human B cell lines by expressing a chimera of the extracellular domain of FcyRIlb and the long cyt tail of CEACAM1 (19). In these studies, the long cyt tail of CEACAM1 inhibited calcium mobilization in B cells initiated by stimulation of the B cell receptor complex. This inhibition was dependent upon either SHP-1 or SHP-2 and the Y459 residue with the Y486 residue being dispensable. In contrast, our studies in T cells showed that both the Y459 and Y486 contribute significantly toward the inhibitory function of CEACAM1. The role of the two ITIMs in controlling the inhibitory function of CEACAM1 is further supported by our observation that the mouse CEACAM1–2L splice variant controls the effector function of T cells in the CD4+CD45RB<sup>high</sup> transfer model but only in the presence of the ITIMs and SHP-1 (T. Nagaishi and R. S. Blumberg, unpublished observations).

Our studies also revealed that the ITIM-dependent inhibitory functions of the CEACAM1–3L isoform are linked to MAPK

**FIGURE 7.** Inhibitory function of CEACAM1–3L isoform depends upon ITIMs in cyt tail and SHP-1. A, Jurkat-T cells were either untransfected or transfected with control oligomers or oligomers capable of silencing SHP-1 mRNA levels. At 24 h after transfection, the Jurkat-T cells were assessed relative to the total levels of JNK and ERK. *p < 0.01. B, Jurkat-T cells were transfected with either the vector control (V), the CEACAM1–3S isoform (S), the CEACAM1–3L isoform in the presence of a control oligomer, the CEACAM1–3S isoform in the presence of an oligomer capable of silencing SHP-1 mRNA levels as shown in A or the CEACAM1–3L isoform containing mutations of Y459 and Y486 residues in the cyt tail. Twenty-four hours after transfection, the cells were stimulated with anti-CD3 and anti-CD28 and the levels of pJNK (top panels) and pERK (bottom panels) assessed relative to the total levels of JNK and ERK. *p < 0.01.
pathways and specifically ERK and JNK but not p38. ERK was first identified as a downstream mediator of the Ras oncogene (33). Furthermore, this pathway is required for Th2 differentiation as shown by Yamashita and colleagues using ERK activation-compromised H-Ras transgenic mice (39). In contrast, JNK significantly regulates Th1 differentiation, as revealed by the fact that T cells from JNK−/− mice produce significantly less IFN-γ (40) and, as a corollary, JNK1-deficient mice exhibit an exaggerated Th2 response (34). Our findings that the CEACAM1–3L isoform significantly suppresses both JNK and ERK activation and regulates both Th1 and Th2 cytokine secretion after TCR/CD3 complex mediated activation is consistent with these observations. These results also extend recent observations obtained from rat pheochromocytoma PC12 cells which showed that anti-CEACAM Abs were able to activate ERK (37). Moreover, they are likely to be physiologically relevant because we have observed similar inhibition of JNK and ERK activation by ligation of CEACAM1 either homophilically or heterophilically in an adoptive transfer model of colitis and a hapten-mediated colitis model, respectively, in association with diminished immunopathology and a specific decrease in Th1 cytokine production (Ref. 41, and unpublished observations). The inhibition of both Th1 and Th2 cytokines by CEACAM1 as shown here in this in vitro model system suggests that overexpression of CEACAM1 can inhibit secretion of both types of cytokines.

Ligation of CEACAM1 on human CD4+ T cells by the Opa52 Ag of Neisseria gonorrhoea leads to association of SHP-1 and SHP-2 with CEACAM1 (25). In addition, the long cyt tail of mouse CEACAM1 can associate with SHP-1 in T cells when phosphorylated but not when nonphosphorylated using CEACAM1 fusion proteins in pull-down assays (27). Together with this and the observation that deletion of the two ITIMs within the cyt tail of the CEACAM1–3L isoform abrogated the inhibition of MAPK pathways, we assessed the role of SHP-1 in mediating the inhibitory effects of the CEACAM1–3L isoform using siRNA mediated gene silencing. siRNA represents double stranded RNA containing 21–23 nucleotides which induces mRNA degradation in a sequence-specific fashion (42). While it is increasingly evident that siRNA acts as a potent method for posttranscriptional gene silencing in mammalian cells, its effect on Src homology 2-containing phosphatases has not been previously explored. As there are no specific chemical inhibitors of SHP-1 function, the proven efficacy of siRNA as reported herein indicates that it may be an important tool for manipulation of downstream signaling pathways in T cells. It was specifically observed that cotransfection with SHP-1 specific siRNA significantly decreased SHP-1 gene expression in human T cells and in turn dramatically abrogated the inhibitory action of the CEACAM1–3L isoform on the activation of JNK and ERK as induced by the TCR/CD3 complex. These results are consistent with reports that JNK is significantly increased in SHP-1 deficient B cells (43) and that SHP-1 positively regulates B cell apoptosis by negatively modulating JNK activation (44). Taken together, these studies predict that in contrast to B cells, both tyrosine residues of CEACAM1 are involved in the inhibitory function as mediated by SHP-1 independently of any contribution of the short cyt tail containing isoforms.

In summary, by an examination of CEACAM1 isoforms in Jurkat cells, which do not normally express CEACAM1, we have shown that distinct stimulatory and inhibitory functions can be assigned to a short cyt domain- and long cyt domain-containing isoforms of CEACAM1, respectively. Although the mechanism for stimulation by CEACAM1–3S remain to be defined, the inhibitory function of CEACAM1–3L in T cells appears to be mediated by the participation of the two ITIMs contained within the long cyt tail which direct the SHP-1 mediated inhibition of the JNK and ERK MAPKs. These studies draw clear functional similarities between CEACAM1 splice variants and KIRs and KARs. This is further supported by evidence that CEACAM1 expression on activated human T cells, NK and decidual NKT cells is associated with inhibition of cytotoxic T cell and NK activity, respectively (45). Defining the regulation of CEACAM1 splice product generation and the mechanisms that underlie these functional attributes of CEACAM1 will be crucial future areas worthy of investigation.

Acknowledgments

We thank Steven Cornwall and Dan Bailey for expert technical assistance.

References


