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SHP1 Phosphatase-Dependent T Cell Inhibition by CEACAM1 Adhesion Molecule Isoforms

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Summary

T cell activation through the T cell receptor (TCR) is subsequently modified by secondary signals that are either stimulatory or inhibitory. We show that CEACAM1 adhesion molecule isoforms containing a long cytoplasmic domain inhibited multiple T cell functions as a consequence of TCR ligation. Overexpression of CEACAM1 resulted in decreased proliferation, allogeneic reactivity, and cytokine production in vitro and delayed type hypersensitivity and inflammatory bowel disease in mouse models in vivo. Conditioned deletion of CEACAM1 in T cells caused increased TCR-CD3 complex signaling. This T cell regulation was dependent upon the presence of immunoreceptor tyrosine-based inhibition motifs (ITIM) within the cytoplasmic domain of CEACAM1 and the *Src* homology 2 domain-containing protein tyrosine-phosphatase 1 (SHP1) in the T cell. Thus, CEACAM1 overexpression or deletion in T cells resulted in T cell inhibition or activation, respectively, revealing a role for CEACAM1 as a class of inhibitory receptors potentially amenable to therapeutic manipulation.

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Introduction

The activation of a T cell through the antigen-specific T cell receptor (TCR)-CD3 complex by a major histocompatibility complex (MHC)-peptide complex is dependent upon, and modified by, secondary signals that are either stimulatory or inhibitory (Greenfield et al., 1998). The major costimulatory and coinhibitory signals for the majority of naive T cells are those delivered by either CD28 or cytotoxic lymphocyte antigen (CTLA)-4, respectively (Walunas et al., 1996). However, it has been increasingly recognized that other CD28-related secondary regulatory molecules exist that likely serve similar functions (Ling et al., 2000). Non-CD28-related molecules may have analogous positive and negative regulation of T cells, but their in vivo importance remains to be determined. One such candidate molecule is carcinoembryonic antigen (CEA)-related cell adhesion molecule 1 (CEACAM1).

CEACAM1 is a type I-transmembrane glycoprotein that is a member of the CEA superfamily and was originally identified in epithelial cells (Gray-Owen and Blumberg, 2006). The human CEA superfamily consists of 19 genes and pseudogenes on chromosome 19q13.3, which encode proteins that are linked to the membrane either by a glycophosphatidylinositol anchor, as is the case for CEA (CEACAM5), or by a transmembrane region, as is the case for CEACAM1 (Obrink, 1997; Thompson et al., 1991). CEACAM1 is the only superfamily member expressed in rodents and humans, suggesting that it may be the original CEACAM gene family member (Beauchemin et al., 1999). Mouse and human CEACAM1 transcripts undergo extensive alternative splicing that generates 11 splice products in humans and 4 splice products in mice. These isoforms are named for the number of extracellular domains and the type of cytoplasmic (cyt) domain expressed (Beauchemin et al., 1999). Each splice variant of CEACAM1 consists of a membrane-distal IgV-like N-region, followed by variable numbers of alternating IgC1 and IgC2 set regions, a common transmembrane domain, and either a long (-L, 73 aa) or short (-S, 10 aa) cyt domain (Beauchemin et al., 1999). The L domain of CEACAM1 contains immune receptor tyrosine-based inhibitory motifs (ITIM) and has been linked to inhibitory signaling in epithelial cells (Daeron and Vivier, 1999; Isakov, 1997). The proposed inhibitory function of CEACAM1 in epithelial cells is triggered by the phosphorylation of ITIM tyrosine residues by *Src*-related kinases, resulting in recruitment of the *Src* homology 2 (SH2) domain-containing protein-tyrosine phosphatases (SHP)-1 and -2 (Beauchemin et al., 1997; Huber et al., 1999). Mice exhibit two allelic forms (a and b) of CEACAM1 because of differences in the N-domain with most mouse strains, including C57BL/6J (B6), expressing the CEACAM1[a] allele (Beauchemin et al., 1999).

CEACAM1 is expressed by a variety of epithelial (Watt et al., 1995), endothelial (Muenzner et al., 2000), and hematopoietic cell types including granulocytes (Watt et al., 1991), monocytes, dendritic cells, natural killer

cells (Moller et al., 1996), and B lymphocytes (Khan et al., 1993). We and others have recently observed that CEACAM1, but not other CEACAM-related proteins, is also expressed on the surface of human and mouse $CD4^+$ or $CD8^+$ T cells immediately after activation through the TCR-CD3 complex ex vivo (Kammerer et al., 1998; Moller et al., 1996; Morales et al., 1999; Nakajima et al., 2002). Although specific monoclonal antibodies (mAb) do not exist to distinguish the various proteins generated by alternate splicing, RNA analysis has shown that human T cells express transcripts for CEACAM1-4L, -4S, -3L, and -3S isoforms (Donda et al., 2000; Singer et al., 2002) and mouse T cells express CEACAM1-4L, -4S, -2L, and -2S isoforms (Beauchemin et al., 1999; Tan et al., 2002). Semiquantitative analysis in mouse and human T cells suggest that CEACAM1-L isoforms predominate over CEACAM1-S isoforms (Singer et al., 2002).

CEACAM1 exhibits homophilic ligation of the N-domain with the N-domains of another CEACAM1 molecule or a CEACAM-related molecule, mainly CEACAM5 (CEA), CEACAM6, or CEACAM8 (Oikawa et al., 1992; Watt et al., 2001). Heterophilic ligands also exist for CEACAM1 and include E-selectin (CD62E) through expression of Le^x or sLe^x on CEACAM1 expressed on granulocytes (Stocks et al., 1996), galectin-3 (Feuk-Lagerstedt et al., 1999), bacterial products (Boulton and Gray-Owen, 2002), or viral proteins (Gagneten et al., 1995). Several independent in vitro studies have shown that this heterophilic ligation of CEACAM1 on T cells with bacterial products of *Neisseriae gonorrhea* (Boulton and Gray-Owen, 2002), the spike glycoprotein of mouse hepatitis virus (Gagneten et al., 1995), or CEACAM1-specific mAbs can inhibit different T cell functions, consistent with the ability of the CEACAM1-L domain to associate with SHP1 (Boulton and Gray-Owen, 2002; Chen and Shively, 2004; Markel et al., 2002; Morales et al., 1999). These studies suggest that CEACAM1-L isoforms may be inhibitory to T cells. Consistent with this, we have observed that heterophilic (mAb) or homophilic (CEACAM1-Fc fusion protein) ligation of mouse CEACAM1 in vivo results in protection from hapten-mediated colitis (Iijima et al., 2004). However, because mouse T cells express multiple different CEACAM1 transcripts and because multiple different cell types other than T cells express CEACAM1 in vivo, the inhibition observed in these colitis models could also have resulted from inhibition of CEACAM1-activating functions on T cells or blockade of CEACAM1 functions on non-T cells. Therefore, assignment of a coinhibitory function to CEACAM1 remains unproven.

We have, therefore, generated model systems to study the function of specific isoforms of CEACAM1 on T cells in vivo. Specifically, we established a retroviral transfection system of primary T cells with the mouse CEACAM1-2L splice variant together with conditional deletion of SHP1 specifically in T cells and a transgenic mouse that directs specific overexpression of the CEACAM-4L isoform in T cells. The studies with these models show multiple different CEACAM1 isoforms that contain a long cyt domain possess the general property of inhibiting T cell function in a pathway that depends on the ITIM domains and SHP1.

Results

Definition of CEACAM1-4L and -2L Function in Primary T Cells

Because the CEACAM1-L isoforms are predicted to be inhibitory and dominantly expressed in mouse and human T cells (Singer et al., 2002), we sought to determine their function in primary mouse T cells. The *Ceacam1^a-4L* or *-2L* cDNA, cloned into GFP-RV (see Figure S1, top, in the Supplemental Data available online; GFP-4L or 2L, respectively), were packaged into retroviruses and transduced into primary $CD4^+$ splenocytes (SPL) from B6 WT mice or NIH3T3 cells (Figure 1A). The efficiency of GFP-4L and GFP-2L infection into NIH3T3 was greater than 90%, similar to the efficiency of infection by GFP-RV alone (VA, Figure 1A, top). Transfection of CEACAM1-2L and -4L into primary T cells was confirmed by flow cytometry (Figure 1A, bottom) and immunofluorescence (Figure 1B). However, the infection efficiency of GFP-4L into primary T cells was lower than that observed with GFP-2L (2L) or VA (Figure 1A, bottom). Consistent with this, the ability of the GFP-4L-transduced T cells to propagate was substantially lower than that of the GFP-2L-infected T cells, which was similar to the VA-transfected cells (data not shown).

After transfection of $CD4^+$ T cells, GFP^+ cells were sorted and IL-2 production determined after stimulation with anti-CD3 and anti-CD28 (Figure 1C) or PMA and ionomycin (Figure 1D and Figure S1, middle). IL-2 production from the CEACAM1-2L-transfected T cells was suppressed in comparison to the VA transfectant after anti-CD3-CD28 stimulation with less suppression observed after PMA and ionomycin stimulation. The decreased IL-2 production by CEACAM1-2L was not due to induction of apoptosis (Figure 1E). The CEACAM1-4L isoform was profoundly inhibitory after anti-CD3 and anti-CD28 stimulation with persistent, but less, inhibition observed after PMA and ionomycin stimulation. This suggests that CEACAM1-mediated inhibition mainly affects signals that are proximal to ligation of the TCR-CD3 complex.

Since overexpression of the CEACAM1-2L and -4L splice variants in primary mouse $CD4^+$ T cells caused substantial inhibition of T cell function, it would be predicted that deletion of CEACAM1 would result in T cell hyperresponsiveness to TCR-CD3 complex signaling. We therefore created a T cell-specific *Ceacam1* null mouse and examined the response of T cells to anti-CD3 and -CD28 stimulation. *Ceacam1$^{flox/flox}$* mice containing lox-P sites flanking exons 7–9 that encode the cyt tail of CEACAM1 (see Experimental Procedures and Figure S2) were crossed with mice expressing *Cre* recombinase under the control of the proximal *Lck* promoter, which is active in T cells within the thymus and periphery. *Ceacam1$^{flox/flox}$* mice expressed lower amounts of CEACAM1 on B cells and hepatocytes (Figure S2), consistent with the fact that insertion of the neo cassette in intron 6 caused a slight decrease in CEACAM1 protein expression prior to *Cre*-mediated deletion, so *Ceacam1$^{flox/flox}$* mice were used as controls. Purified $CD4^+$ T cells from the spleens of *Ceacam1$^{flox/flox}$* X *Lck-Cre* mice exhibited extinction of CEACAM1 protein expression as defined by immunoblotting with the CC1 mAb in comparison to *Ceacam1$^{flox/flox}$* mice

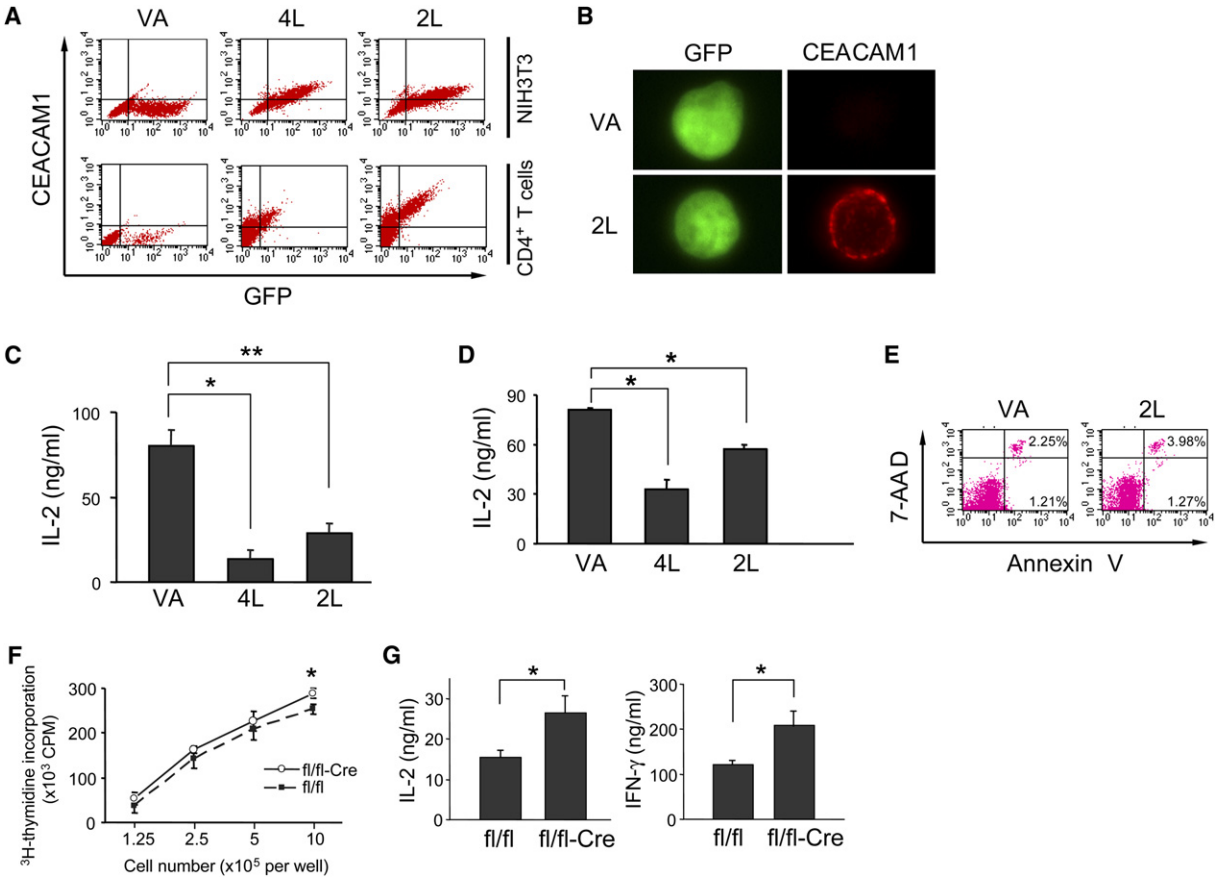

Figure 1. CEACAM1[a]-4L and -2L Transduction into Mouse CD4+ T Cells

(A) Retroviral infections. NIH3T3 cells (top) or primary CD4+ T cells (bottom) were infected with GFP-RV vector alone (VA), GFP-4L (4L), or GFP-2L (2L). 36 hr after infection, cells were stained with CC1 and anti-mouse IgG1-PE and analyzed by flow cytometry.

(B) GFP and CEACAM1 expression of CD4+ T cell transfectants with GFP-RV (VA) and GFP-2L (2L). Cells were stained with CC1 and Alexa[568]-conjugated anti-mouse IgG.

(C) IL-2 production by CD4+ GFP-RV infectants. Retrovirally infected cells were stained with anti-CD4 CyChrome, and CD4+GFP+ cells were sorted by FACS and stimulated with anti-CD3 and anti-CD28. IL-2 production was determined by ELISA in triplicate after 48 hr of stimulation. Bars indicate SEM. *p < 0.01; **p < 0.05.

(D) IL-2 production by CD4+ GFP-RV infectants after stimulation with phorbol ester (PMA) and ionomycin. Bars indicate SEM. *p < 0.05.

(E) Analysis of apoptosis after CEACAM1-2L transfection. CD4+ T cells were infected with either GFP-RV vector alone (VA) or GFP-2L (2L), and sorted GFP+ cells were subjected to 7-AAD and Annexin-V staining. Numbers in quadrants indicate percent of cells.

(F) Proliferation of CEACAM1-deficient T cells. CD4+ T cells from 12-week-old littermate control *Ceacam1*$^{flox/flox}$ X *Lck-Cre* (fl/fl-Cre) or *Ceacam1*$^{flox/flox}$ (fl/fl) mice were incubated with anti-CD3 and anti-CD28 for 48 hr and proliferation assessed by [^{3}H]thymidine uptake in triplicate. Data shown are representative of two experiments. Bars indicate SEM. *p < 0.05.

(G) Cytokine secretion by CEACAM1-deficient T cells. CD4+ T cells from *Ceacam1*$^{flox/flox}$ X *Lck-Cre* or *Ceacam1*$^{flox/flox}$ mice were incubated with anti-CD3 and anti-CD28 for 48 hr and supernatants analyzed for IL-2 and IFN-γ production by ELISA in triplicate. Data shown are representative of two experiments. Bars indicate SEM. *p < 0.05.

(Figure S2). In association with this, *Ceacam1*$^{flox/flox}$ X *Lck-Cre* mice displayed enhanced proliferation (Figure 1F) and cytokine production as assessed by IL-2 and IFN-γ secretion (Figure 1G) in response to anti-CD3 and anti-CD28 stimulation in comparison to *Ceacam1*$^{flox/flox}$ mice. The degree of enhancement of T cell activation observed after CEACAM1 deletion was limited relative to the inhibition observed with CEACAM1 overexpression resulting from the decreased expression of CEACAM1 in the *Ceacam1*$^{flox/flox}$ mice (see Figure S2). Nonetheless, these studies show that CEACAM1 normally functions as a coinhibitory molecule for T cells after stimulation through the TCR.

ITIM-Dependent Suppression of T Helper 1 and T Helper 2 Cytokine Production by CEACAM1-2L

We next investigated whether the suppression of IL-2 production from CEACAM1-2L-transfected T cells was ITIM dependent given previous observations that human CEACAM1 functions in this manner (Chen et al., 2004). The tyrosine residues of the two ITIMs in the *Ceacam1-2L* cDNA were mutated by making conservative phenylalanine substitutions (*Ceacam1-2L* mu, Figure S1, bottom), and then cloned into GFP-RV (GFP-2L mu). After infection of primary CD4+ T cells with VA, GFP-2L, or GFP-2L mu, CD4+GFP+ cells were isolated by FACS and incubated with CD3 and CD28

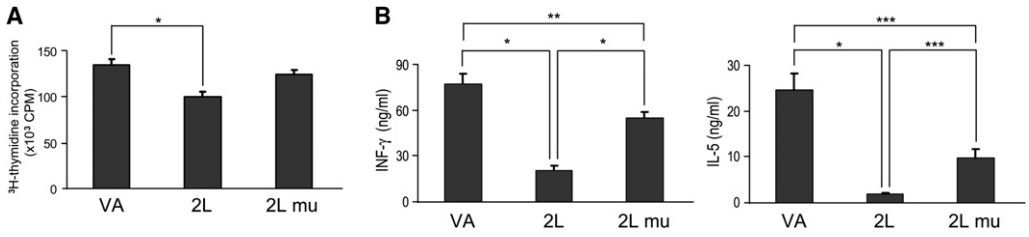

Figure 2. ITIM-Dependent Suppression of CD4$^+$ T Cell Growth by CEACAM1-2L

(A) After retrovirus infection, CD4$^+$ GFP$^+$ cells with GFP-RV vector alone (VA), GFP-2L (2L), or GFP-2Lmu (2Lmu) were cultivated in the presence of anti-CD3 and anti-CD28 for 48 hr and proliferation assessed by [^{3}H]thymidine uptake. Bars indicate SEM. *p < 0.01.

(B) Ex vivo Th1 and Th2 differentiation assays. Primary CD4$^+$CD62L$^+$CD44^{Lo} naive T cells infected with GFP-RV (VA), GFP-2L (2L), or GFP-2L mutant (2Lmu) were differentiated under Th1- or Th2-inducing conditions. Culture supernatants from Th1 (IFN-γ) or Th2 (IL-5) conditions were subjected to ELISA. Each condition was tested in replicates of six. Data are expressed as the mean + SEM. *p < 0.001; **p < 0.05; ***p < 0.005.

antibodies. Whereas the proliferation of the CEACAM1-2L transfectant was substantially suppressed, no suppression was observed with CEACAM1-2Lmu relative to the VA control (Figure 2A). This indicates that CEACAM1-2L negatively regulates T cell activity in primary T cells, and this function depends on the presence of at least one of the two ITIMs.

Ligation of endogenously expressed CEACAM1 on primary mouse T cells with MHV glycoprotein leads to specific suppression of T helper 1 (Th1) but not Th2 differentiation (Iijima et al., 2004). However, in transfected Jurkat cells, overexpression of human CEACAM1-3L inhibits secretion of Th1 and Th2 cytokines (Chen et al., 2004). We therefore investigated the effects of CEACAM1 overexpression in primary mouse T cells. CD4$^+$CD62L$^+$CD44L^{Lo} naive T cells derived from WT B6 mice were infected individually with GFP-RV VA, GFP-2L, or GFP-2Lmu, and CD4$^+$CD62L$^+$GFP$^+$ populations were resorted and then cultivated under Th1- or Th2-inducing conditions. Although the VA-infected CD4$^+$CD62L$^+$ T cells were able to be differentiated to secrete Th1 (IFN-γ) and Th2 (IL-5) cytokines after stimulation with anti-CD3, anti-CD28, and rIL-2, such a capacity was substantially suppressed by overexpression of CEACAM1-2L (Figure 2B). This inhibition of Th1 and Th2 cytokine secretion was substantially reduced, but not completely reversed, by mutation of the two ITIM domains. These studies indicate that CEACAM1-2L inhibits both Th1 and Th2 differentiation of primary mouse T cells.

CEACAM1-2L Inhibits T Cell Function In Vivo in a Pathway that Is Dependent on a Functional cyt Tail and SHP1

To assess the in vivo significance of T cell inhibition by CEACAM1-2L overexpression, we established a modification of the naive T cell adoptive transfer model into *Rag2*$^{-/-}$ recipients (Powrie et al., 1991, 1996). This modification was required because primary T cells require prestimulation with anti-CD3 or anti-CD28 and rIL-2 to render them permissive to retroviral infection. Stimulated, but uninfected, control naive T cells transferred into recipients induced severe colitis (Figure S3, top left and right) based upon secretion of IL-2 and IFN-γ from mesenteric lymphocytes (ML, data not shown) and lamina propria lymphocytes (LPL; Figure S3, bottom left and right). The clinical severity of colitis and Th1 cytokine production induced by the activated "naive" T cells were inhibited by CD4$^+$CD25$^+$ regulatory T cells (Treg, Figure S3), confirming the validity of this model (Powrie, 1995; Powrie et al., 1994a; Read et al., 2000).

CEACAM1-2L-mediated suppression of T cells ex vivo (Figure 2) was dependent upon the presence of the two ITIMs. In addition, the L domain has been previously shown to associate with SHP1 in mouse (Nakajima et al., 2002) and human (Chen and Shively, 2004) T cells ex vivo. We, therefore, next determined whether CEACAM1-2L-based negative regulation occurs in vivo and requires the ITIMs and SHP1. *Ceacam1-2L* or its ITIM-mutated counterpart was retrovirally transduced into WT or SHP1-deficient splenic naive T cells. SHP1-deficient CD4$^+$ T cells were obtained from T cell-specific SHP1-deficient mice (L.P. and B.G.N., unpublished data). Transfected T cells were injected into *Rag2*$^{-/-}$ mice to generate the modified naive T cell adoptive transfer colitis model, as described above (Figure S3). 12 weeks after injection, mice were assessed for the presence of the adoptively transferred, retrovirally transfected T cells and clinical and pathological features of colitis. LPLs were isolated from *Rag2*$^{-/-}$ mice, which received naive T cells infected with GFP-RV VA or GFP-2L and analyzed by FACS. As shown in Figure 3A, approximately one-half of the CD4$^+$GFP$^+$ LPLs were CEACAM1$^+$ and thus derived from the naive T cells adoptively transferred 12 weeks prior to the analysis. Notably, mice receiving WT naive T cells transfected with CEACAM1-2L exhibited substantially less colitis based upon weight scores (Figure 3B), macroscopic pathology (Figure S4), and microscopic pathology (Figure 3C) in comparison to the mice that received T cells infected with VA that exhibited severe colitis (Figures 3B and 3C). Absence of SHP1 in the transferred T cells substantially reduced the inhibitory effect of CEACAM1-2L as indicated by worsened severity of colitis, but interestingly late in the course of the development of colitis (Figures 3B and 3C). Likewise, the absence of the ITIMs reversed the ability of CEACAM1-2L to inhibit the severity of colitis (Figure 3B). These observations were confirmed by a quantitative assessment of microscopic pathology (Figure 3D). These studies indicate that overexpression of CEACAM1-2L in T cells directly inhibits the pathogenic abilities of the T cells in vivo. In addition, this inhibitory function of CEACAM1-2L is dependent upon the two functional ITIM domains and the presence of SHP1 in the transduced T cells.

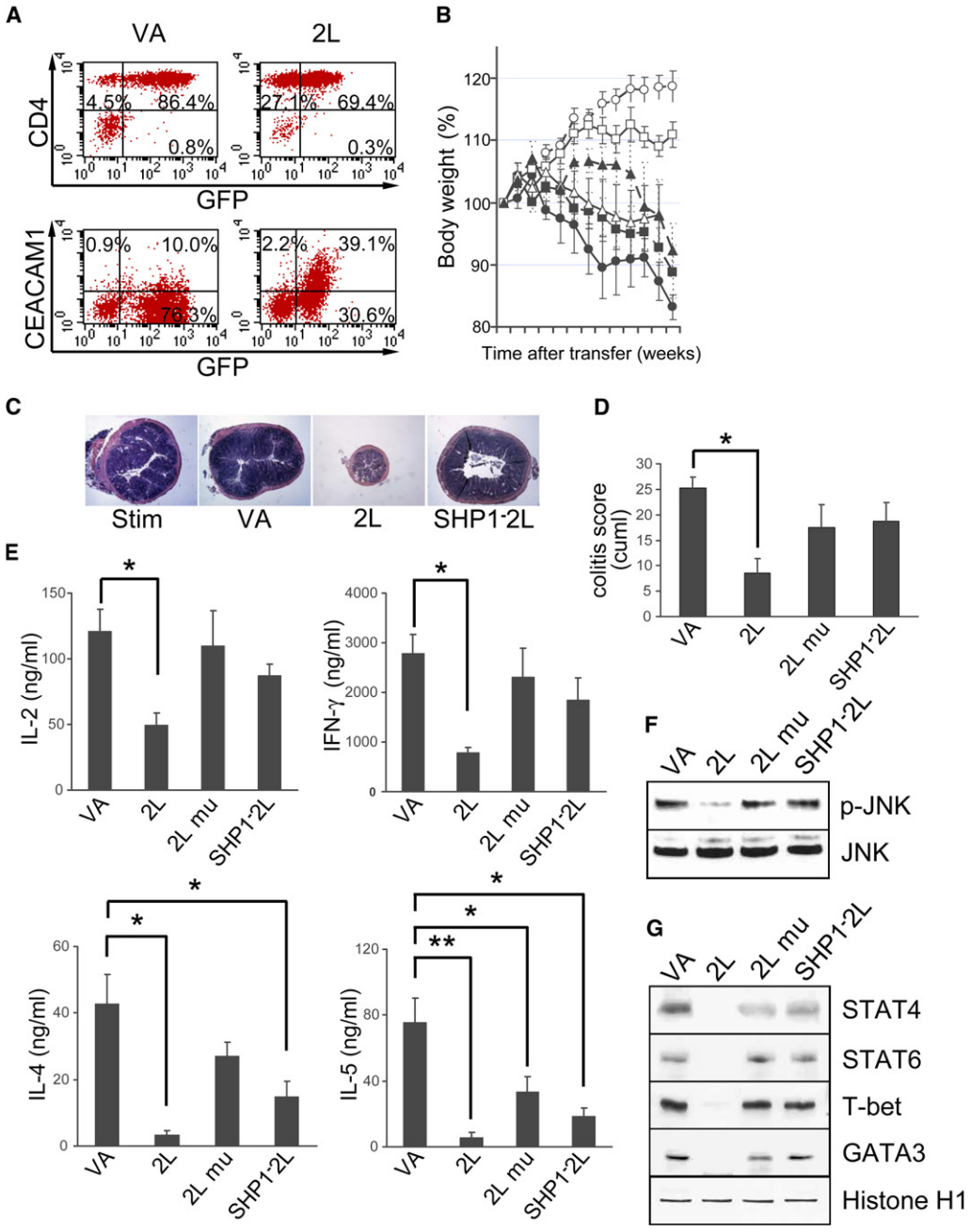

Figure 3. Effect of CEACAM1-L Overexpression on T Cells Is Dependent on ITIM Domains and SHP1 in Naive T Cell Adoptive Transfer Colitis Model

(A) 12 weeks after transfer, purified LPL were stained with anti-CD4 PE or CC1 and anti-mouse IgG1 PE, followed by flow cytometry.

(B) *Rag2*$^{-/-}$ mice injected with WT naive T cells that were not infected with virus (noninfected control, closed circles) or infected with GFP-RV VA (closed squares), GFP-2L (open squares), GFP-2Lmu (open triangles), or injected with SHP1-deficient naive T cells infected with GFP-2L (SHP1-deficient-2L, closed triangles), and noninjected control group (open circles) were monitored for wasting. Data are shown as mean ± SEM and represent eight mice per group.

(C) Histology (H&E stain) from colitic tissues with noninfected WT naive T cells (Stim), GFP-RV alone (VA), GFP-2L (2L), and SHP1-deficient naive T cells with GFP-2L (SHP1^{-}2L) are shown (magnification ×20). One representative feature from each group of eight is shown.

(D) Quantitative histopathological assessments of colitis severity caused by WT naive T cells with VA, 2L, 2Lmu, or SHP1-deficient-2L naive T cells are shown. Data are provided as mean + SEM and represent eight mice per group. *$p < 0.01$.

(E) Culture supernatants from colitic LPL were harvested for determination of IL-2 (top left), IFN-γ (top right), IL-4 (bottom left), and IL-5 (bottom right) by ELISA. Cytokines produced by LPL in colitis caused by VA, 2L, 2Lmu, and SHP1-deficient-2L naive T cells or *Rag2*$^{-/-}$ mice that received stimulated wild-type CD4^{+}CD62L^{+} T cells with or without Treg (Figure S3) are shown. Data are expressed as the mean ± SEM. *$p < 0.05$ and **$p < 0.01$.

(F) Lysates of splenic CD4^{+} T cells from the naive T cell adoptive transfer colitis models that received either VA, 2L, 2Lmu, or SHP1-deficient-2L naive T cells were subjected to immunoblotting performed with either anti-phosphorylated JNK or anti-JNK.

(G) Nuclear extracts of splenic CD4^{+} T cells from the colitis models with either VA, 2L, 2Lmu, or SHP1-deficient-2L were subjected to western blotting performed with either anti-STAT4, anti-STAT6, anti-T-bet, anti-GATA3, or anti-histone H1.

Overexpression of CEACAM1-2L Suppresses Th1 and Th2 Cytokine Production in a Naive T Cell Adoptive Transfer Colitis Model

To determine whether the suppression of colitis by overexpression of CEACAM1-2L in T cells was associated with alterations in production of Th1 cytokines, such as IL-2, IFN-γ (Figure 3E, top), and TNF-α (data not shown), and for Th2 cytokines, such as IL-4 and IL-5 (Figure 3E, bottom), the amounts of cytokines produced by SPL (data not shown), ML (data not shown), and LPL (Figure 3E) were assessed. Activated naive T cells expressing VA that were transferred into *Rag2*$^{-/-}$ mice induced high quantities of Th1 cytokine production from SPL (data not shown), ML (data not shown), and LPL (Figure 3E) that were similar to the amounts of Th1 cytokines observed in *Rag2*$^{-/-}$ mice that received nontransfected, activated CD4$^+$CD62L$^+$ T cells (Figure S3). Elevated Th2 cytokine production was also observed in LPL derived from activated T cells infected with VA but at substantially lower quantities than Th1 cytokine production. Mice receiving naive T cells overexpressing CEACAM1-2L exhibited reduced Th1 and Th2 cytokine production by SPL (data not shown), ML (data not shown), and LPL, together with less severe colitis. Although expression of CEACAM1-2L with mutated ITIMs (2Lmu) or WT CEACAM1-2L in SHP1-deficient T cells (SHP1$^-$2L) substantially reversed the CEACAM1-2L-mediated inhibition of Th1 cytokine production by LPLs, there was only limited reversal of the inhibition of Th2 cytokine production by CEACAM1-2L. Given that severe colitis was observed in *Rag2*$^{-/-}$ recipients of 2Lmu and SHP1-deficient-2L T cells (Figure 3D) in the context of suppressed Th2 cytokine production, these studies suggest that Th1 cytokines are the major pathologic effectors in this model, consistent with previous studies also in this adoptive transfer model (Powrie et al., 1994b). These studies further suggest that, consistent with the in vitro studies of Th1 and Th2 differentiation (Figure 2B), overexpression of CEACAM1-2L can cause suppression of Th1 and Th2 cytokine production in vivo.

ITIM and SHP1-Dependent Suppression of Th1 and Th2 Signal Transduction by CEACAM1-2L

Next, we examined whether CEACAM1-2L exerts a negative effect on Th1 and Th2 cytokine secretion by modulating MAPK pathways (Chen et al., 2004). *Rag2*$^{-/-}$ mice that received either WT T cells infected with the GFP-RV VA, 2L, or 2Lmu retroviruses or SHP1-deficient naive T cells infected with 2L (SHP1$^-$2L) were examined for mitogen-activated protein kinase (MAPK) activity in association with colitis by subjecting splenic CD4$^+$ T cells from these recipient groups to immunoblotting. As seen in Figure 3F, downregulation of phosphorylated JNK was observed in T cells that overexpressed CEACAM1-2L in comparison to the VA group. In comparison, the inhibition of C-Jun NH_2-terminal Kinase (JNK) phosphorylation by CEACAM1-2L was reversed by mutation of the ITIMs or overexpression of CEACAM1-2L in an SHP1-deficient background within T cells. The expression of phosphorylated JNK correlated with the severity of colitis in this model (Figures 3A–3D) and cytokine production by SPL (data not shown), MLN (data not shown), and LPL from the colitic tissues (Figure 3E).

In addition, nuclear extracts from splenic CD4$^+$ T cells in the mice that had received T cells infected with VA, 2L, and 2Lmu and 2L in SHP1-deficient T cells (SHP1$^-$2L) were examined for transcription factors known to be associated with Th1 and Th2 differentiation. As can be seen in Figure 3G, whereas substantial quantities of nuclear translocation of STAT4, STAT6, T-bet, and GATA-3 were observed in T cells that received the VA, these amounts were substantially reduced in the 2L infectants with reversal in the inhibition of STAT6, T-bet, and GATA3, and partial reversal of STAT4 by mutation of the ITIM (2Lmu) and expression of 2L in SHP1-deficient T cells (SHP1$^-$2L).

Generation and Characterization of CEACAM1-4L Transgenic Mice

Given the limitations in using retroviral transduction to examine the in vivo function of the CEACAM1-4L variant (Figure 1), we sought an alternative experimental approach to address this issue. We created transgenic mice in which CEACAM1-4L was expressed under the control of the human (h) CD2 promoter (Figure S5) in order to generate T cells that overexpressed CEACAM1-4L (4L Tg). As seen in Figures 4A and 4B, WT (CD3$^+$) T cells express low amounts of endogenous CEACAM1 on the cell surface (Figure 4A), with most of this likely derived from the CEACAM1-4L splice product based upon the apparent molecular mass of CEACAM1 as detected by immunoblotting (Figure 4B). Introduction of the *Ceacam1*^{a}-4L transgene increased the surface expression of CEACAM1 on 4L Tg T cells in thymus (data not shown), spleen (Figure 4A), and peripheral lymph nodes (data not shown) but, interestingly, not on intestinal intraepithelial lymphocytes (iIEL, data not shown). The latter is consistent with the tight control of cell-surface CEACAM1 expression on IEL (Morales et al., 1999). Consistent with the increased cell-surface expression of CEACAM1, 4L Tg T cells expressed larger amounts of CEACAM1-4L protein by immunoblotting (Figure 4B). Overexpression of CEACAM1-4L in T cells caused a decrease in the CD3$^+$ T cell population in total splenocytes from the 4L Tg mice in comparison to WT mice (Figure 4C), but not in the thymus (Figure S5), suggesting that the effects of CEACAM1 overexpression are mainly due to effects in the periphery. The relative ratio of CD4$^+$ and CD8$^+$ T cells in spleen and the proportion of CD4$^+$CD25$^+$ T cells in lamina propria in 4L Tg mice were similar to WT mice, suggesting that such an effect was evident among all subsets of T cells (data not shown and Figure S5).

Because overexpression of CEACAM1-4L was expected to regulate primary T cell function, we examined the proliferation of T cells in 4L Tg mice in comparison to WT mice. 4L Tg T cells exhibited decreased proliferation in comparison to WT T cells (Figure 4D) as well as decreased IL-2 production (Figure 4E) in response to CD3 and CD28 antibodies. T cells from 4L Tg mice were also less responsive than WT T cells in a MLR (Figure 4F). Moreover, 4L Tg mice exhibited decreased contact hypersensitivity response to oxazolone (Figure 4G).

To further examine the function of CEACAM1-4L function to a specific nominal antigen, CEACAM1-4L Tg mice were crossed with OT-II mice that express a transgenic TCR specific for ovalbumin (OVA) and restricted to I-A^b (Barnden et al., 1998). CD4$^+$ T cells from spleen from

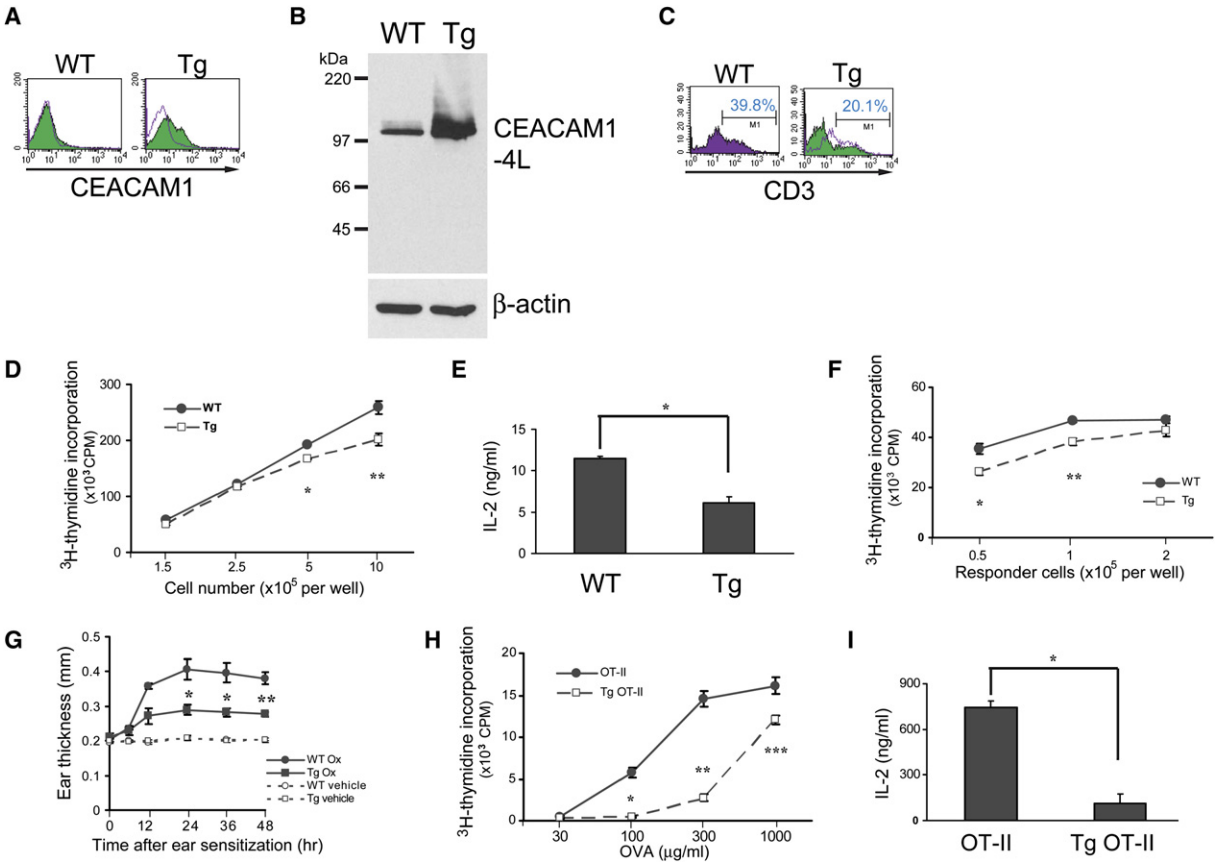

Figure 4. Generation of CEACAM1-4L Transgenic Mice

(A) Total splenocytes were stained with either control IgG1 or CC1, anti-mouse IgG1 FITC, or anti-CD3 PE. Data show IgG1 (open histogram)- and CC1 (closed histogram)-stained populations of $CD3^+$ T cells from WT (left) and 4L Tg (right) mice.

(B) Protein lysates of $CD4^+$ splenocytes from WT or 4L Tg mice were subjected to immunoblotting with either the CC1 mAb or a β-actin Ab.

(C) Reduction of $CD3^+$ T cells in 4L Tg mice. Total splenocytes from WT (left) and CEACAM1-4L Tg (right) mice were stained with anti-CD3 FITC (closed histogram). Tg panel shows overlay of $CD3^+$ staining in WT (open histogram) and 4L Tg mice (closed histogram).

(D) Splenic $CD4^+$ T cells isolated from B6 WT or 4L Tg mice were stimulated with anti-CD3 and anti-CD28 for 48 hr and proliferation assessed by [^{3}H]thymidine uptake. Data are expressed as mean ± SEM. *$p < 0.001$; **$p < 0.005$.

(E) Splenic $CD4^+$ T cells isolated from B6 WT or 4L Tg mice were stimulated with anti-CD3 and anti-CD28 and IL-2 production assessed by ELISA. Bars indicate +SEM. *$p < 0.001$.

(F) Indicated numbers of spleen $CD4^+$ T cells isolated from B6 WT or 4L Tg mice were subjected to MLR with mitomycin C-treated stimulator cells derived from BALB/c mice and proliferation examined by [^{3}H]thymidine uptake. Data are expressed as mean ± SEM. *$p < 0.005$; **$p < 0.001$.

(G) DTH responses were measured at the indicated time points. Data are expressed in units of 0.02 mm ± SEM and represent six mice per group. *$p < 0.01$, **$p < 0.005$.

(H) $CD4^+$ T cells were isolated from OT-II and OT-II X 4L Tg (Tg OT-II) mice and proliferation assessed by [^{3}H]thymidine uptake after incubation with various concentrations of OVA protein as indicated. Data are expressed as mean ± SEM. *$p < 0.005$; **$p < 0.001$; ***$p < 0.05$.

(I) $CD4^+$ T cells were isolated from OT-II and OT-II X 4L Tg (Tg OT-II) mice and IL-2 secretion assessed by ELISA after incubation with 300 μg/ml OVA protein. Bars indicate +SEM. *$p < 0.005$.

OT-II and CEACAM1-4L Tg X OT-II mice were examined for their response to OVA (Figures 4H and 4I) and $OVA_{323-339}$ (data not shown), the peptide of OVA to which OT-II T cells are specific, when presented by SPL as antigen-presenting cells from C57BL/6 mice. $CD4^+$ T cells from CEACAM1-4L Tg X OT-II mice exhibited decreased proliferation (Figure 4H) and IL-2 production (Figure 4I) in response to OVA and OVA peptide (data not shown) in comparison to OT-II mice. These studies show that CEACAM1-4L regulates the response of T cells to a specific nominal antigen when presented by MHC class II.

The functionality of 4L Tg T cells allowed for the opportunity to assess the role of CEACAM1-4L in primary T cells in the conventional naive T cell adoptive transfer colitis model. Splenic $CD4^+CD62L^+CD44^{Lo}$ naive T cells were isolated from WT or 4L Tg mice and injected into *Rag2*$^{-/-}$ mice. *Rag2*$^{-/-}$ mice that received 4L Tg naive T cells exhibited less severe wasting, compared to the recipients of WT T cells (Figure 5A). This was associated with less severe macroscopic (Figure S5) and microscopic (Figures 5B and 5C) colitis. Moreover, lessened pathological injury was associated with decreased Th1 (IFN-γ and IL-2) cytokine production by colonic LPL (Figure 5D). Taken together, these studies with the 4L Tg mice indicate that overexpression of CEACAM1-4L in primary T cells inhibits TCR-CD3 complex-stimulated T cell functions and the ability of these cells to secrete cytokines and cause inflammation in vivo.

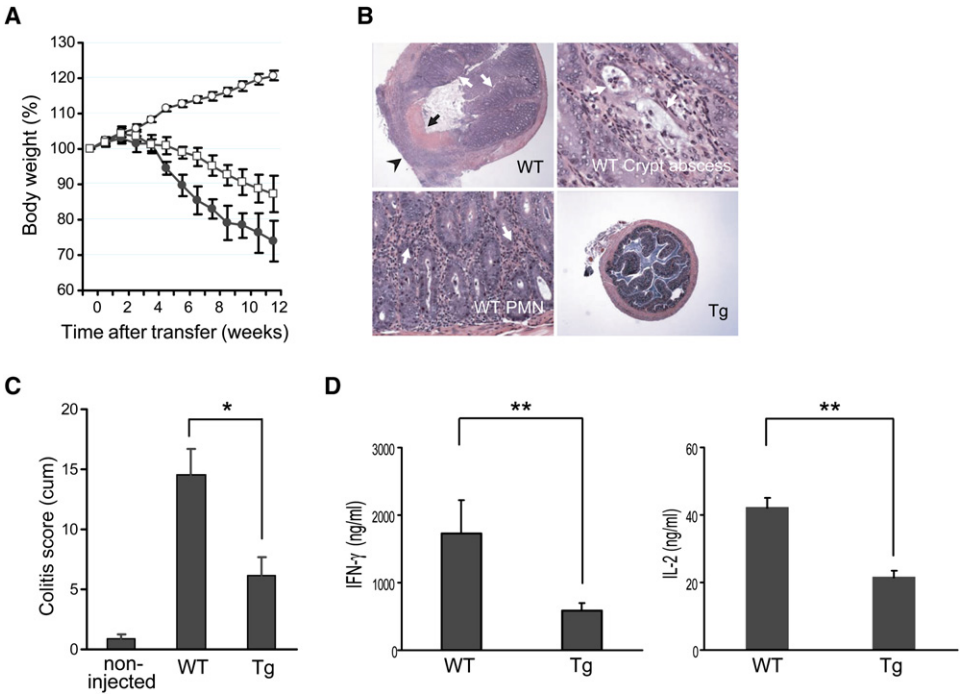

Figure 5. CEACAM1-4L Tg Naive T Cells Suppresses Conventional Transfer Colitis

(A) *Rag2*$^{-/-}$ mice injected with WT or Tg naive T cells were monitored for wasting. Data are expressed as mean ± SEM and represent 12 mice per group. Mice that did not receive T cells are shown in open circles, adoptively transferred WT cells in closed circles, and Tg T cells in open squares.

(B) H&E-stained histological features from colitic tissue with WT (top and bottom left) and Tg naive (bottom right) T cells are shown. Black arrow indicates ulcer and black arrowhead indicates mononuclear cell infiltration. White arrows indicate epithelial hyperplasia in top left panel and crypt abscesses or polymorphonuclear infiltration in the top right and bottom left panels.

(C) Quantitative histopathological assessments of colitis severity by WT or Tg naive T cells are shown. Data are provided as mean + SEM. *$p < 0.01$.

(D) Th1 cytokine (IFN-γ and IL-2) production from LPL in colitis tissues caused by WT or Tg naive T cells is shown. Data are expressed as mean + SEM. **$p < 0.05$.

Discussion

Understanding the regulation of T cell activation is central to unraveling the mechanisms underlying autoimmunity and inflammation. We here show that the two known isoforms of mouse CEACAM1 that contain a long cyt tail domain with ITIMs, CEACAM1-2L and -4L, are able to inhibit a broad array of T cell activities as a consequence of TCR ligation, including proliferation and alloactivation and Th1 and Th2 cytokine production associated with tissue injury. These properties of CEACAM1 were shown to depend on the presence of the ITIMs and SHP1. Because CEACAM1-L isoforms are normally expressed to a greater extent than the isoforms containing a short cyt tail domain lacking ITIMs, our studies indicate that CEACAM1 is an inhibitory coreceptor in T cells. This is consistent with prior in vitro functional studies of CEACAM1 in T cells that demonstrate an ability of CEACAM1 to co-cap with CD3 (Chen and Shively, 2004).

In the current report, we observed that overexpression of CEACAM1-2L on naive T cells led to inhibition of in vitro T cell differentiation to both Th1 and Th2 cells and to in vivo inhibition of Th1 and Th2 cytokine secretion by lamina propria T cells in an adoptive transfer colitis model. Consistent with this, overexpression of CEACAM1-2L also inhibited nuclear translocation of T-bet, GATA-3, STAT-4, and STAT-6 in adoptively transferred splenic T cells. The inhibition of STAT-4 and STAT-6 suggests that CEACAM1 inhibits not only TCR-CD3 signaling in T cells but also likely IL-4 and IL-12 receptor signaling. The latter is consistent with previous studies showing CEACAM1 inhibition of IL-2R signaling (Chen and Shively, 2004). Moreover, this inhibition of Th1 cytokines by CEACAM1 was dependent on the ability of CEACAM1 to elicit an inhibitory signal through the ITIMs and SHP1 in the T cell, because individual deletion of either factor abrogated the inhibitory effect of CEACAM1-2L. These studies suggest that CEACAM1 is capable of inhibiting both pathways of cytokine secretion when the quantities of cell-surface CEACAM1 expression achieve a critical amount. This is consistent with studies with human CEACAM1-3L, which, when overexpressed in Jurkat cells, was similarly able to inhibit both Th1 and Th2 cytokine secretion in an ITIM-dependent pathway (Chen et al., 2004). These studies also suggest that Th1 pathways may be more sensitive to CEACAM1-mediated suppression than Th2 pathways, a result that is similar to previous observations with PD-1 and PD-1 ligand interactions (Latchman et al., 2001). In addition, deletion of the ITIMs or SHP-1 only partially abrogated the inhibitory functions of CEACAM1 on Th2 differentiation and cytokine secretion in vitro and in vivo and

occurred independently of GATA-3 in contrast to Th1 inhibition. Whether this represents differing sensitivities of Th1 and Th2 cytokine pathways to TCR signal strength when modulated by CEACAM1 (Takato-Kaji et al., 2005) or mechanisms by which CEACAM1-L regulates Th2 cytokines independently of ITIMs and SHP-1 (such as regulation of IL-2 per se, which plays an important role in Th2 differentiation) (Yamane et al., 2005) remains to be defined.

CEACAM1-2L also suppressed the proliferation of primary T cells in vitro in a pathway that was also dependent upon the presence of the two ITIMs. Consistent with this, JNK activation was inhibited in vivo after adoptive transfer of naive T cells overexpressing CEACAM1-2L. These results are consistent with and extend our previous observation and the observation of others that the human CEACAM1-3L and CEACAM1-4L isoforms inhibited the activation of the NFAT-AP-1 elements of the IL-2 promoter and IL-2 receptor expression, respectively, in Jurkat transfectants in a pathway that involves SHP1 regulation of MAPK activity (Chen and Shively, 2004; Chen et al., 2004). These studies also indicate the ability of CEACAM1-2L to inhibit multiple different T cell functions that are downstream of the TCR-CD3 complex, suggesting a proximal effect of CEACAM1 in TCR-CD3 complex signaling events.

In this study, we have also shown that overexpression of CEACAM1-2L by retroviral transfection and CEACAM1-4L by generation of a T cell-specific transgenic animal model inhibits the ability of primary naive T cells to induce colitis. This indicates that regulation of T cells in vivo is imposed not only by cytokines from regulatory populations but also by the expression of inhibitory coreceptors on the T cell. In our studies with the CEACAM1-2L, the original model as described by Powrie and colleagues (Powrie et al., 1994b) was modified slightly in that the adoptively transferred T cells required activation. It should be noted that the in vitro activation as used here did not alter the cell-surface expression of CD62L (data not shown). In addition, the ability of the stimulated naive T cells to cause colitis could be similarly suppressed by the cotransfer of $CD4^+CD25^+$ Treg. This inhibitory property of CEACAM1-2L was not related to an alteration induced by activation because similar transfer of $CD4^+CD62L^+CD44^{Lo}$ naive T cells from the 4L Tg mice was also able to suppress colitis without prior ex vivo activation.

Importantly, inhibition of the ability of naive T cells to cause colitis by CEACAM1 overexpression occurred in the absence of cotransferred Treg. Analysis of lamina propria mononuclear cells showed suppressed production of IL-10 (data not shown) as well as the other Th2 cytokines, but no substantial alteration in TGF-β production (data not shown). This suggests that CEACAM1-mediated inhibition occurs independently of these inhibitory cytokines. It is thus predicted that CEACAM1-driven regulation of pathogenic T cells differs from that imposed by regulatory cytokines from Treg and may involve either homotypic T-T interactions between CEACAM1 on opposing T cells or heterotypic interactions between the CEACAM1 expressed on effector T cells with a variety of sources of potential CEACAM1 ligands, including those expressed by dendritic cells, monocytes, and intestinal epithelial cells. Such a model of T-T interaction for regulation of effector T cells has been suggested by others (Fuss et al., 2002; Powrie et al., 1994a).

Retroviral overexpression of CEACAM1-4L also inhibited the proliferative capability of the expanded T cells and their ability to secrete IL-2 to a larger extent than the CEACAM1-2L isoform. Consistent with this, CEACAM1-4L retroviral infectants propagated less well than did CEACAM1-2L infectants. This suggests that CEACAM1 with more extracellular Ig domains may be a more potent regulator of T cells. This notion is supported by homophilic adhesion between a four than a two Ig domain-containing isoform of CEACAM1 (Watt et al., 2001). However, the potent inhibition of T cell function by the CEACAM1-4L isoform limited in vivo manipulation of retrovirally transfected T cells. To circumvent this limitation, a transgenic mouse was generated to assess the biologic function of CEACAM1-4L. As predicted, the overexpression of CEACAM1-4L was observed in T cells from thymus (data not shown), spleen (Figure 4), mesenteric lymph node, and lamina propria (data not shown). Given that the 4L Tg mice exhibited decreased numbers of $CD3^+$ cells, it is possible that overexpression of CEACAM1-4L encoded by the hCD2 promoter-driven transgene leads to suppression of T cell development in the thymus, antigen-driven expansion, and/or increased sensitivity to activation-induced cell death. However, we observed no overt abnormalities in the proportion of CD3-positive cells within the thymus. Given the diminished responsiveness of the 4L Tg T cells to an allo-MLR and the inability to cause colitis and generate contact hypersensitivity, as shown here, such effects of CEACAM1 on T cells are likely to be important for regulating the T cell functions that lead to their aggressive behavior. In a corollary fashion, conditional deletion of CEACAM1 signaling function in T cells led to T cell hyperactivity. These studies with 4L Tg mice and mice in which CEACAM1 is conditionally deleted in T cells provide a direct demonstration that CEACAM1 on T cells is a coinhibitory receptor for TCR-CD3 complex signaling.

In summary, we have shown that CEACAM1 isoforms expressing a long cyt tail have the general property of negatively regulating T cell responses that are initiated by antigen-MHC complex-mediated signals. Moreover, we have demonstrated that this is clinically relevant, because CEACAM1 functions in the suppression of colitis in an adoptive transfer model. Defining the mechanisms of CEACAM1-mediated T cell regulation may provide insights into therapeutic methods for the treatment of IBD and other immune-mediated disorders.

Experimental Procedures

Constructs for Retrovirus Transfection

EcoRV and XhoI restriction sites were introduced at the 5′ and 3′ ends, respectively, of each of the *Ceacam1^a-4L* or *-2L* cDNAs, which were cloned into the pBluescript (pBS)-SK(+) and kindly provided by N. Beauchemin (McGill University, Montreal, Quebec, Canada), by PCR utilizing the following primers: 5′-CCTGATATCGAGACATGGAGCTGGCCT-3′ and 5′-CCGCTCGAGCTCACTTCTTTTTTACTTCTG-3′. The retrovirus vector GFP-RV (Neurath et al., 2002; Ouyang et al., 1998, 2000; Szabo et al., 2000), kindly provided by K. Murphy (Washington University School of Medicine, St. Louis, MO), was digested with BglII, modified with Klenow fragment to create blunt

ends, and digested with XhoI. EcoRV-XhoI-digested *Ceacam1-4L* or *-2L* fragments were then cloned into the digested GFP-RV, resulting in GFP-4L or -2L, respectively.

Generation of ITIM Mutations of CEACAM1-2L

The pBS-*Ceacam1-2L* Y308F mutant containing a phenylalanine substitution at aa308 was generated by PCR-directed mutagenesis of the pBS-*Ceacam1-2L* with the following primers: 5′-GTGGAT GACGTCGCATTCACTGTCCTGAAC-3′ (forward primer) and 5′-GAAGTTCAGGACAGTGAATGCGACGTCATC-3′ (reverse primer) with Platinum *Pfx* polymerase (GIBCO Invitrogen). The vector encoding *Ceacam1-2L* Y308F Y335F (pBS-*Ceacam1-2L* mu) containing two phenylalanine substitutions was generated by PCR of the pBS-*Ceacam1-2L* Y308F with the following primers: 5′-GCCACA GAAACAGTTTTTTCAGAAGTAAAAAAG-3′ (forward primer) and 5′-CTTCTTTTTTACTTCTGAAAAAACTGTTTCTGTG-3′ (reverse primer). PCR products were digested with DpnI to remove prokaryotic template DNA. The *Ceacam1-2L* mu cDNA was cloned into the GFP-RV vector as described above.

Cell Lines, Cell Culture, and Retrovirus Transfection

The ecotrophic packaging cell line, Phoenix-E (Hofmann et al., 1996), derived from human embryonic kidney line 293 was kindly provided by G. Nolan (Stanford University, Stanford, CA). NIH3T3 cells were purchased from American Type Culture Collection (Manassas, VA). High-titer retrovirus supernatants were prepared by lipofection (Effectene, Qiagen, Valencia, CA) of Phoenix-E cells with the GFP-RV constructs for the retrovirus transfection as previously described (Neurath et al., 2002; Szabo et al., 2000). Subsequently, cells were analyzed by FACS and GFP$^+$ cells sorted.

Animals

Wild-type (WT) C57BL/6 (B6) or RAG2-deficient (*Rag2*$^{-/-}$, B6) mice were purchased from Charles River Breeding Laboratories (Wilmington, MA) or Taconic Farms Inc. (Germantown, NY), respectively. T cell-specific SHP1-deficient mice were generated by crossing mice with "floxed" alleles of SHP1 (L.P., K.P. Lam, M.L. Thomas, and K. Rajewsky, unpublished data) with transgenic mice expressing the *Cre* DNA recombinase under the control of CD4 promoter (Lee et al., 2001). All mice used were in mixed B6/129 background. Mice were maintained under specific pathogen-free conditions at the Animal Facilities of Harvard Medical School. All animal experimentations were performed in accordance with institutional guidelines and the Animal Review Board of Harvard Medical School which granted permission for this study.

Antibodies and Reagents

A monoclonal antibody (mAb) specific for mouse CEACAM1, CC1, was previously established by K.V. Holmes (University of Colorado Health Sciences Center, Denver, CO) (Dveksler et al., 1993). CD4 CyChrome, CD4 PE, L-selectin (CD62L) PE, CD44 FITC, and CD25 PE Abs were purchased from BD Biosciences (San Jose, CA). JNK, phosphorylated JNK, STAT4, STAT6, T-bet, GATA3, and histone H1 Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Human rIL-2 for the stimulation of mouse primary T cells was purchased from Roche Diagnostics Corporation (Indianapolis, IN).

Immunoblotting and Flow Cytometry

The expression of CEACAM1, JNK, p-JNK, T-bet, GATA-3, STAT4, and STAT6 were assessed by immunoblotting by standard protocols as previously described (Chen et al., 2004). Flow cytometry (FACS) was performed with a FACSort (BD Bioscienses) after GFP-RV infection and/or staining by standard methods as previously described (Nakajima et al., 2002).

Preparation of CD4$^+$ T Cells and Proliferation Assays

Splenic CD4$^+$ T cells were isolated from mice by magnetic immunobeads (MACS, Miltenyi Biotech, Germany) according to the manufacturer's instructions, and retroviral infection was performed. For proliferation assays, 2×10^5 sorted GFP$^+$CD4$^+$ T cell were incubated for 48 hr in 96-well flat bottom microtiter plates at 37°C in 5% CO_2 in the presence of 5 μg/ml plate-bound anti-CD3 and 2 μg/ml soluble anti-CD28. Proliferation assays were performed by addition of [^{3}H]thymidine (1.0 μCi/well) for 18 hr.

Preparation of Naive T Cells and In Vitro Th1 and Th2 Differentiation Assays

Whole splenic mononuclear cells derived from either WT or SHP1$^{fl/fl}$ CD4-*Cre* mice were subjected to B cell depletion by negative selection with CD19 MACS beads (Miltenyi Biotech). Subsequently, CD4$^+$CD62L$^+$CD44^{Lo} naive T cells were sorted by BD FACSAria or a negative selection column (R&D Systems, Minneapolis, MN) as previously described (Neurath et al., 2002), and the isolated naive T cells were used for retroviral infection. After prestimulation with anti-CD3-anti-CD28 and rIL-2, followed by retroviral infection, the GFP$^+$CD4$^+$CD62L$^+$ population was resorted. 5×10^5 sorted cells were incubated in 100 μl complete RPMI-1640 with 1 μg/ml plate-bound anti-CD3 and 1 μg/ml soluble anti-CD28 and, for Th1 cells, supplemented with 5 ng/ml rIL-12 (R&D Systems) and 5 μg/ml IL-4 Ab (BD Biosciences) at 37°C in 5% CO_2 for 72 hr. For the generation of Th2 cells, 50 ng/ml rIL-4 (R&D Systems), 1 μg/ml anti-IL-12, and 10 μg/ml anti-IFN-γ (BD Biosciences) were used to supplement the culture medium. Cells were washed twice with PBS and restimulated in complete RPMI-1640 with the same concentrations of CD3 and CD28 Abs for 48 hr. Culture supernatants were harvested for cytokine ELISA (Opt EIA set, BD Biosciences) according to the manufacturer's instructions.

Generation of CEACAM1-4L Transgenic Mice

EcoRI restriction sites were introduced at the both the 5′ and 3′ ends of the *Ceacam1-4L* cDNA by PCR via the following primers: 5′-CCGGAATTCGAGACATGGAGCTGGCCT-3′ and 5′-CCGGAATTCT CACTTCTTTTTTACTTCTG-3′. The VACD2 cassette (Zhumabekov et al., 1995), which was cloned into pBS-SK(-), was kindly provided by M. Owen (Imperial Cancer Research Fund, London, UK). pBS-VACD2 DNA was digested with EcoRI followed by 5′ end dephosphorylation with calf intestinal alkaline phosphatase (NEB) to prevent self-ligation. The EcoRI-digested *Ceacam1-4L* fragment was then cloned into the digested pBS-VACD2 and double digested with KpnI and XbaI to remove prokaryotic plasmid sequences, resulting in VACD2-*Ceacam1-4L*. The microinjection of the transgene construct into 0.5 day B6 embryos was performed at the Transgenic Core Facility of the Brigham and Women's Hospital, Harvard Medical School. Genotyping for Tg mice was performed by PCR of genomic DNA purified from tail tissues with specific primer pairs: 5′-TCAATGGTGACTTCAGCAGTGGTG-3′ and 5′-CGTGGTTAAGCTCT CGGGGTG-3′ specific for the region between hCD2 promoter to the extracellular domain of *Ceacam1*, and 5′-GGAAGTACGGATATG CACATCAGG-3′ and 5′-ATAGCAGGGCTGGCATATTTCC-3′ specific for the region between L domain of *Ceacam1* to hCD2 LCR.

Generation of OT-II X CEACAM1-4L Tg Mice

OT-II mice (kindly provided by J. Ravetch, Rockefeller University, New York, NY) were crossed with CEACAM1-4L Tg mice. CD4$^+$ T cells from spleen from OT-II X CEACAM1-4L Tg mice were incubated with OVA or OVA peptide (323–339) at the indicated concentrations with splenocytes from B6 mice as antigen-presenting cells. IL-2 in the supernatant was measured after 24 hr and proliferation assessed after 48 hr by means of [^{3}H]thymidine uptake.

Generation of CEACAM1-Deficient Mice

A conditional knockout construct was designed to delete exons 7–9 encoding the cyt tail of CEACAM1. The targeting construct was designed by inserting a loxP-neo cassette at the XmnI site in intron 6 and a loxP fragment at the HindIII site in intron 9 of *Ceacam1* in a BAC clone from a 129Sv library (Figure S2, top). The targeting vector included 4.3 kb of homologous DNA upstream of the loxP-neo site and 3.4 kb of homologous DNA downstream of the second loxP site. The targeting vector was verified by restriction mapping. The targeting vector was transfected into 129Sv embryonic stem (ES) cells by electroporation. Clones that contained a hybridization signal at the size (8.1 kb), predicting for the homologous recombinant allele after SphI digestion with a 0.7 kb Apa1 and SpeI fragment of *Ceacam1* as a probe, were identified from 270 ES cell candidates. The genomic DNAs of these potential positive clones were digested with XhoI and hybridized with a 3′ probe (a 1.2 kb SacI and XhoI fragment of *Ceacam1*) and clones that exhibited a ~17.1 kb band corresponding to the recombinant allele were identified. Three positive ES cell clones were injected into mouse blastocysts according to

standard procedures. Chimeric mice were obtained and mated with B6 mice. The F1 mice having germline transmission of the loxP-targeted *Ceacam1* allele were interbred to generate F2 mice. Germline transmission was confirmed by PCR of tail DNA tissue with the following three primers that detect the WT and the *floxed* allele of *Ceacam1* (and the *Ceacam2*) gene (Han et al., 2001): primer A (5′-ACACAAGGAGGCCTCTCAGATGGCG-3′) and primer C (5′-GCGCC TCCCCTACCCGGTAGAATT-3′), containing sequences from exon 6 of *Ceacam1* and neomycin cassette, respectively, produce a 488 bp PCR product from the *floxed Ceacam1* allele. Primer A and primer B (5′-GACTTTGGCTTCCTGACTGGAGGA-3′), containing sequences from intron 6, generate a 382 bp PCR product from WT *Ceacam1*. Two founder mice from two embryonic cell clones showed germline transmission of the targeting construct containing the conditional *Ceacam1* gene (Figure S2, middle left). Mice with homozygous conditional *Ceacam1* alleles were born from these two founder mice. These results indicate that embryonic lethality does not occur in the mice carrying conditional *Ceacam1* knockout alleles.

Ceacam1$^{flox/flox}$ homozygous mice were examined for their expression of CEACAM1 protein by immunoblotting liver cell lysates with Ab669, an Ab recognizing the entire CEACAM1. Decreased CEACAM1 expression was observed in liver (Figure S2, middle center) and on the cell surface of B cells (Figure S2, bottom left) of *Ceacam1*$^{flox/flox}$ mice in comparison to WT littermates typical of many neo containing recombinantly inserted genes. The functionality of the floxed cassette was tested with recombinant adenovirus with the *Cre* gene (Ad-*Cre*). *Ceacam1*$^{flox/flox}$ mice were treated with Ad-Cre or control virus Ad-Luc (containing the firefly luciferase gene) by i.v. injection. Immunoblots were performed on liver lysates at 21 days after injection. As shown in Figure S2 (middle right), there was a substantial decrease in the amount of L cyt tail containing isoforms of CEACAM1 in the mouse treated with Ad-*Cre*. This observation shows that exons 7–9 of CEACAM1 can be removed by treatment of *Ceacam1*$^{flox/flox}$ mice with *Cre* recombinase. As a result, *Ceacam1*$^{flox/flox}$ mice were crossed with l*ck-Cre* mice (Taconic Laboratories, C57BL/6Ntac-TgN) that express *Cre* under the control of the proximal *Lck* promoter to generate *Ceacam1*$^{flox/flox}$ X l*ck-Cre* mice allowing for deletion of CEACAM1 signaling function specifically in T lymphocytes.

Mixed Leukocyte Reaction

Splenocytes (5×10^4) derived from WT BALB/c mice were treated with 100 μg/ml mitomycin C (Sigma-Aldrich, St. Louis, MO) and used in an allogeneic MLR with 5×10^4 WT (B6) or CEACAM1-4L Tg (B6) $CD4^+$ T cells as previously described (Nakajima et al., 2002).

Delayed-Type Hypersensitivity

Mice were presensitized by painting 150 μl of 3% 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one (oxazolone, Sigma-Aldrich) dissolved in 1:3 acetone-ethanol mixture onto the shaved abdomen. 4 days after the presensitization, local skin challenge via topical application to both sides of the ears with 1% oxazolone in 20 μl of 1:1 acetone-sunflower oil mixture was performed (Szczepanik et al., 1996). Ear swelling was measured with a dual thickness gauge (Mitsutoyo Mfg. Co., Tokyo, Japan).

Colitis Models

$CD4^+CD62L^+CD44^{Lo}$ naive T cells were isolated from WT or SHP1$^{flox/flox}$ CD4-*Cre* mice, and the isolated naive T cells were infected with retroviruses. After staining with anti-CD4 CyChrome and CD62L PE Abs, $GFP^+CD4^+CD62L^+$ cells were resorted. Purified 4×10^5 cells (purity; >97%) were injected intraperitoneally into 8- to 10-week-old female *Rag2*$^{-/-}$ mice. In some experiments, $CD4^+CD25^+$ regulatory T cells (Treg) were sorted with CD4 CyChrome and CD25 PE Abs, then cotransferred with naive T cells. After the injection, wasting was monitored for 12 weeks.

Histological Assessment of Colitis

Colonic tissues were fixed in 10% buffered formalin phosphate and embedded in paraffin, cut into sections, and then stained with hematoxylin and eosin and evaluated by a pathologist, J. Glickman (Brigham and Women's Hospital, Harvard Medical School), who was blinded to the treatment of the mice. Evidence and severity of colitis were defined by means of a scoring system as previously described (Brozovic et al., 2004; Iijima et al., 2004; Neurath et al., 2002).

Lymphocyte Isolation and Determination of Cytokine Production

Single-cell isolations from spleen and mesenteric lymph nodes were performed with a 40 μm cell strainer (BD) and ACK solution for depletion of red blood cells. Colonic lamina propria lymphocytes (LPL) were isolated as previously described (Iijima et al., 2004; Neurath et al., 2002).

Isolated splenocytes (SPL), mesenteric lymphocytes (ML), or LPL (5×10^5 each) were cultured in complete RPMI-1640 supplemented with 50 μM β-mercaptoethanol (Sigma-Aldrich) and 10 μg/ml gentamycin (BioWhittaker) for LPL, and 5 μg/ml plate-bound anti-CD3 and 2 μg/ml soluble anti-CD28 in flat bottom 96-well plate at 37°C in 5% CO_2 for 48 hr. Culture supernatants were harvested and cytokine production determined (IL-2, IFN-γ, TNF-α, IL-4, IL-5, IL-10, and TGF-β) by ELISA (BD Biosciences) according to the manufacturer's instructions.

Statistical Analysis

Data were expressed as the mean ± SEM and statistical significance determined by the Student's t test. For some experiments, statistical analyses were conducted by means of the analysis of variance followed by an evaluation of pairwise differences among the means with Bonferroni tests. p values <0.05 were considered significant.

Supplemental Data

Five Supplemental Figures can be found with this article online at http://www.immunity.com/cgi/content/full/25/5/769/DC1/.

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