CEACAM1 specifically suppresses B cell receptor signaling-mediated activation

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Abstract

Carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) expressed in T cells may regulate immune responses in the gut. In addition to T cells, B cells are also an important population in the gut-associated lymphoid tissues that orchestrate mucosal homeostasis. However, the role of CEACAM1 in B cells has not been elucidated. We herein analyzed mature B cells to determine the functions of CEACAM1. Flow cytometry revealed high expression of CEACAM1 on B cells in secondary lymphoid tissues. Cytokine production induced by activation of B cell receptor (BCR) signaling was suppressed by CEACAM1 signaling in contrast to that associated with either Toll-like receptor 4 or CD40 signaling. Confocal microscopy revealed co-localization of CEACAM1 and BCR when activated with anti-Ig\textsubscript{μ} F(ab')\textsubscript{2} fragment. Overexpression of CEACAM1 in a murine B cell line, A20, resulted in reduced expressions of activation surface markers with decreased Ca\textsuperscript{2+} influx after BCR signal activation. Overexpression...
of CEACAM1 suppressed BCR signal cascade in A20 cells in association with decreased spontaneous proliferation. Our results suggest that CEACAM1 can regulate BCR-mediated mature B cell activation in lymphoid tissues. Therefore, further studies of this molecule may lead to greater insights into the mechanisms of immune responses within peripheral tissues and the potential treatment of inflammatory diseases.

**Keywords**
CEACAM1; B cells; BCR signaling; Spontaneous proliferation; Lymphoid tissues

1. **Introduction**

Carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) is a type I transmembrane glycoprotein and a member of the CEA family. The gene encoding CEACAM1 is known to be preserved in both rodents and humans, and its transcripts undergo alternative splicing [1,2]. Thus, CEACAM1 has several splice variants whose names are derived from the number of the extracellular domains and the type of cytoplasmic domain. The extracellular domain of CEACAM1 consists of a membrane-distal immunoglobulin (Ig)V-like N-region and variable numbers of alternating IgC1- and IgC2-like proximal regions. Therefore this molecule is also classified as a member of the Ig supergene family. Several heterophilic ligands for the N-domain of CEACAM1 have been reported [3]. However, homophilic ligation of the N-domains of CEACAM1 with another N-domain of CEACAM1 is the predominant means of physiologic interaction [4]. On the other hand, CEACAM1 possesses two types of cytoplasmic domains. This includes the long (-L) form, which contains two immunoreceptor tyrosine-based inhibition motifs (ITIMs) and the short (-S) form which lacks these. Semi-quantitative analysis of lymphoid tissues in mouse and human T and B cells has revealed that CEACAM1-L forms typically predominate over the -S forms [5,6], and the -4L isoform is the dominant variant in these cells. In addition, the function of the -2L isoform is known to be equivalent to that of -4L in T cells [7].

CEACAM1 has been reported to be involved in several functions such as tumor growth, angiogenesis, endocrine function and immunology in humans and rodents [1,2]. CEACAM1 has been reported to be expressed on activated, but not resting, T cells [8,9]. Moreover, it has been revealed that CEACAM1 expressed in T cells located in the mucosa can regulate intestinal immune responses [7,10,11]. It is known that the gut-associated lymphoid tissues (GALT) is involved in the immunological homeostasis within intestinal mucosa, and that disruption of such homeostasis may result in inflammatory bowel diseases (IBD). Therefore, it has been suggested that the functions of CEACAM1 in T cells may contribute to the role of the GALT in the maintenance of gut homeostasis.

On the other hand, we have observed that an agonistic monoclonal antibody (mAb) or the natural ligands bound to the extracellular N-domain of CEACAM1 can suppress animal models of IBD as found in one of our previous studies [10]. In this *in vivo* study, however, it was possible that the effects of these CEACAM1 stimuli might reflect the functions of CEACAM1 expressed not only on T cells but also other cell types. We know that B cells,
in addition to T cells, are a major component of the GALT. Even though several previous studies by others have reported CEACAM1 expression in B cells [6,12], the role of such expression in mature B cells in secondary lymphoid tissues has not yet been elucidated. Therefore, we focused on the role of CEACAM1 in mature B cells.

2. Materials and methods

2.1. Mice

Wild-type female C57BL/6 mice (B6, 6–8 week-old), purchased from CLEA Japan (Tokyo, Japan), were maintained in the animal facility of Tokyo Medical and Dental University (TMDU) under specific pathogen free conditions in accordance with guidelines of the institutional animal care and use committee. All experimental procedures on animals were approved by the committee, and all experiments were carried out in accordance with approved guidelines.

2.2. Flow cytometric analysis (FACS)

A20 cell line, derived from mouse B cell lymphoma on BALB/c background [13], was obtained from American Type Culture Collection (Manassas, VA). Primary cells were isolated from bone marrow (BM), spleen (SPL) or peripheral blood (PB) of B6 by standard methods. The A20 transfectants or primary cells were stained with the following antibodies (Abs): anti-mouse CEACAM1 mAb, CC1 (kindly provided by Dr. Kathryn Holmes, University of Colorado, CO), anti-IgG1, FITC-anti-B220, PE-anti-IgM, FITC-anti-CD4, PE-anti-CD3e, PerCP-anti-B220, PE-anti-CD43, PE-anti-CD25, PE-anti-Igκ, PE-anti-CD138, PE-anti-CD5, PE-anti-H-2Kd, PE-anti-I-A^d, PE-anti-CD69, PE-anti-CD80 and PE-anti-CD86 Abs (BD Biosciences, San Jose, CA). Data acquisition was performed using FACS Calibur™ and analysis software Cell Quest™ (BD Biosciences).

In some experiments, the A20 transfectants were treated with Fluo-4® (Dojindo, Kumamoto, Japan), and applied for FACS to analyze intracellular Ca^{2+} influx. During the acquisition, cells were treated with anti-Igκ (Southern Biotech, Birmingham, AL) to stimulate BCR signaling, and kinetics for Ca^{2+} influx were measured at FL2.

2.3. Determination of cytokine production

Splenic B cells were isolated by negative selection methods with magnetic beads (MACS®, Miltenyi, Bergisch Gladbach, Germany), and then 5×10^5 cells/well were incubated with either lipopolysaccharide (LPS, Sigma, St. Louis, MO), anti-CD40 (BD Biosciences) or anti-Igκ F(ab')2 fragment (Rockland, Limerick, PA) in the presence or absence of CC1 for 48 h. The culture supernatants were harvested to determine cytokine production by enzyme-linked immunosorbent assay (ELISA). Concentrations of interleukin (IL)-4, IL-5 and IL-10 were measured with OptEIA® ELISA set (BD Biosciences).

2.4. Immunofluorescence microscopy (IFM)

The isolated splenic B cells, as described above, were stimulated/stained with Alexa™568-conjugated agonistic anti-Igμ F(ab’)2 fragment (Abcam, Cambridge, UK) and the agonistic CC1 mAb, which was labeled with Alexa488 (Molecular Probe, Eugene, OR), and then
fixed with ice-cold acetone as previously described [14]. The localization of BCR and CEACAM1 expressions in the cells at 0min and 3min after the stimulation/staining were observed under a confocal microscopy, Fluoview™ 10i (Olympus, Tokyo, Japan).

2.5. Construct and transfection

NheI and NotI restriction sites were generated at the 5′ and 3′ ends, respectively, of the Ceacam1-2L cDNA (kindly provided by Dr. Nicole Beauchemin, McGill University, Montreal, Canada), by PCR utilizing the following primers: 5′ CTAGCTAGCTAGGAGACATGGAGCTGGC 3′ and 5′ ATAGTTTAGCGGCGGCTCACTTTTTTTTATTTCCTCT 3′. The Cumate switch inducible vector (PB-Cuo-MCS-IRES-GFP-EF1α-CymR-Puro, System Biosciences, Palo Alto, CA) was digested with NheI and NotI, and NheI-NotI-digested Ceacam1-2L fragment was cloned into it. The transfectants were generated by electroporation of A20 cells with this construct. Overexpression of CEACAM1 in the cells was induced by addition of Cumate (System Biosciences).

2.6. Immunoblotting

SDS-PAGE was performed as previously described [7,15,16]. Protein expressions were analyzed with the following primary and secondary Abs: anti-CEACAM1 mAb, CC1, anti-phosphorylated (p)-Syk, anti-Syk, anti-p-ERK1/2, anti-ERK1/2 (Cell Signaling, Beverly, MA), anti-β-actin (Sigma), anti-mouse IgG1-HRP (Southern Biotech), anti-rabbit IgG-HRP (GE Healthcare, Chicago, IL) and anti-goat IgG-HRP (Santa Cruz, Dallas, TX). Signals were generated with ECL (GE Healthcare).

2.7. Cell proliferation assay

Indicated numbers of A20 transfectants were incubated in the presence or absence of anti-Igκ for 48 h in 96-well microplate at 37 °C in 5% CO₂. Cell proliferation of the transfectants were analyzed by addition of [3H]-thymidine (1.0μCi/well, PerkinElmer, Waltham, MA) for 6 h, as previously described [7]. Incorporation of [3H] in the harvested cells were measured by a β-ray scintillation counter (MicroBeta™, PerkinElmer).

2.8. Statistical analysis

The results were expressed as the means ± standard error of the mean (SEM). Statistical analysis was performed with unpaired Student’s t-test. Differences were considered to be statistically significant when p value < 0.05.

3. Results

3.1. CEACAM1 expression is increased during the maturation of B cells

To first assess whether CEACAM1 expressions in major lymphoid subsets of secondary lymphoid tissues are significant, primary T and B cells were isolated from SPL (Fig. 1a), mesenteric lymph nodes and Peyer’s patches (MLN, PP, both data not shown) of B6 mice and subjected to FACS. We observed significantly more CEACAM1 expression on B cells compared to T cells in secondary lymphoid tissues (Fig. 1a).
Knowing that mature B cells in secondary lymphoid tissues highly express CEACAM1, we next assessed the expression of this molecule in B cell lineages within BM and PB. FACS analysis showed that B220^CD43^ pro-B cells express CEACAM1, and that B220^CD25^ pre-B cells and B220^Igκ^ immature B cells express even higher level of CEACAM1 (Fig. 1b left panel). This suggests that CEACAM1 expression is increased during the maturation of B cells in BM. On the other hand, B220^{high} circulating B cells in PB showed almost the same level of CEACAM1 expression as in immature B cells and splenic B cells, and B220^CD138^ plasma cells seemed to slightly downregulate such expression (Fig. 1b right panel). This suggests that more CEACAM1 expression is not required for the differentiation into plasma cells.

3.2. CEACAM1 signaling suppresses B cell receptor-mediated cytokine productions

Given that the maximum expression of CEACAM1 is in mature B cells, we next assessed the role of CEACAM1 in B cell function. Primary B cells isolated from B6 SPL were stimulated with either LPS, agonistic anti-CD40 or anti-Igμ F(ab’)2 fragment, to activate Toll-like receptor (TLR) 4, CD40, or B cell receptor (BCR) signaling, respectively, in the presence or absence of a agonistic CEACAM1 mAb, CC1. Interestingly, production of IL-4, IL-5 and IL-10 from primary B cells were suppressed by CEACAM1 signaling when cells were activated specifically via BCR. We were unable to observe such significant suppression when cells were activated via TLR4 or CD40 (Fig. 2a).

3.3. CEACAM1 aggregates and co-localizes with BCR expression when primary B cells are stimulated

Given that CEACAM1 suppressed BCR-mediated cytokine production, it was suggested that BCR signaling may be affected by the function of CEACAM1. Therefore, primary B cells were stained with anti-Igμ F(ab’)2 fragment and CC1 before and after the BCR stimulation in order to observe expression of these molecules in the cells. IFM revealed BCR aggregation, which showed the ‘capping’ phenomenon as expected [14], on a part of the cell surface upon activation by BCR stimulation (Fig. 2b, bottom left). On the other hand, CEACAM1 expression was also found to be aggregated at one location of the surface upon BCR stimulation. Interestingly, the distribution pattern of such expression was similar to that of the BCR molecules (Fig. 2b, bottom middle). Moreover, a certain amount of such CEACAM1 expression, in fact, coincided with the capping BCR localization on the surface (Fig. 2b, bottom right). These results also imply that the effect of CEACAM1 may be associated with BCR signaling.

3.4. Overexpression of CEACAM1 suppresses BCR-mediated activation in A20 cells

Since the analysis using primary B cells was limited, we established a cell line which allowed us to control the overexpression of CEACAM1. A20 is a mouse B cell-derived cell line that does not express endogenous CEACAM1 (Fig. 3a) but still retains substantial BCR signaling when stimulated with anti-Igκ. Therefore, A20 cells were transfected with either the vector alone (A20/VA) or Ceacam1-2L cDNA (A20/2L). Overexpression of CEACAM1-2L can be induced in A20/2L using Cumate (Fig. 3a) [17]. Thus, we used these transfectants to study the role of CEACAM1 in B cells.
Using these transfectants, we first assessed the Ca\(^{2+}\) influx induced by BCR signaling. After BCR stimulation, the intensity of Fluo-4®, which reflects intracellular Ca\(^{2+}\) concentration, was found to be elevated in A20/VA. However, such increase of Ca\(^{2+}\) concentration was suppressed in A20/2L (Fig. 3b). Therefore, overexpression of CEACAM1 may suppress BCR-mediated B cell activation. We also analyzed the activation surface markers in these cells by FACS. As expected, the activation markers, such as MHC class I and II, CD80, CD86 and CD69 were downregulated in A20/2L compared to those of A20/VA. It should be noted that the level of CD5, which is constitutively expressed regardless of activity, were not affected in A20 cells by the overexpression of CEACAM1 (Fig. 3c). These results indicate that the BCR signaling-specific activation of B cells may be suppressed by the overexpression of CEACAM1.

### 3.5. The overexpression of CEACAM1-2L inhibits the BCR signal cascade in B cells

Knowing that the overexpression of CEACAM1-2L suppresses the activity in A20 cells, we analyzed the effect of CEACAM1 on the BCR signal cascade in these cells. The A20 transfectants were incubated with anti-Ig\(\kappa\) to stimulate BCR signaling. As expected, phosphorylation of Syk and ERK1/2 were induced by the BCR stimulation in A20/VA cells. However, such phosphorylation of Syk and ERK1/2 were both suppressed even in the presence of anti-Ig\(\kappa\) when CEACAM1-2L was overexpressed (Fig. 4a). This provides direct evidence showing the effect of CEACAM1 on BCR signaling-specific activation in B cells.

Given CEACAM1 suppression of BCR signaling activation, the transfectants were subjected to cell proliferation assay to evaluate whether such effect was substantial. Interestingly, A20/2L cells without BCR stimulation proliferated more compared to that of A20/VA (Fig. 4b). When the BCR on these cell were stimulated with anti-Ig\(\kappa\), however, the proliferation was rather significantly suppressed by the overexpression of CEACAM1 (Fig. 4c). Thus, this proves that BCR signaling is involved in CEACAM1-associated B cell suppression.

### 4. Discussion

Previous studies had reported that CEACAM1 is expressed by several different cell types such as intestinal epithelial cells and endothelial cells as well as several hematopoietic cells including natural killer cells and T cells [1]. CEACAM1 has been also reported to be expressed more in granulocytes, monocytes and B cells than T cells in PB [6]. However, acquired immune responses through the T cell receptor- and BCR-signaling activations induced by antigen presenting cells at peripheral tissues are mainly regulated in secondary lymphoid organs. Therefore, we first assessed CEACAM1 expression in the lymphoid tissues. Our results showed that CEACAM1 is more highly expressed in B cells compared to T cells within secondary lymphoid tissues, which is similar to observations in PB by others [6]. Moreover, the same study also showed CEACAM1 expression on the B cell lineage in BM. Our observations are consistent with this. However, our results also indicate that the expression of CEACAM1 increases during B cell development from pro-B cells to immature B cells, suggesting that this expression may be required more in matured B cells.

To investigate the role of CEACAM1 expression in mature B cells, we employed an agonistic anti-CEACAM1 mAb, CC1 [10], in our study. When splenic B cells were
stimulated in the presence of CC1, the usual increased cytokine production by activation via BCR signaling was specifically suppressed by CEACAM1 signaling rather than other B cell activations via either TLR4 or CD40 signaling. Therefore, we focused on the association between BCR and CEACAM1 expressions under immunofluorescent studies. We observed the ‘capping’ of BCR expression in B cells treated with anti-Igμ F(ab’)2 fragment. This is consistent with previous reports showing this phenomenon when the BCR is stimulated [14,18]. More interestingly, the expression of CEACAM1 aggregated and co-localized with such capping BCR expression, which is similar to observation by others in a human B cell line [12]. These results imply that CEACAM1 in mature B cells may be associated with BCR signaling.

We have previously reported that CEACAM1 in T cells may negatively regulate immune responses in the gut. Previous studies by others have suggested activating or inhibitory functions of CEACAM1 on B cells however [6,12]. To address this question, we applied an inducible overexpression system for expressing CEACAM1 in transfectants. In our experiments, the inducible overexpression of CEACAM1-2L suppressed the Ca^{2+} influx in A20 cells compared to VA. Furthermore, such overexpression of CEACAM1 resulted in the downregulation of cell surface activation markers. These results suggest that CEACAM1 may suppress BCR signaling-induced B cell activation. This was supported by our biochemical analysis of the same A20 transfectants. Thus, the phosphorylated Syk and ERK1/2 induced by the BCR signaling in the cells were downregulated by the overexpression of CEACAM1. Our data imply that, when BCR is activated, the ITIMs in the cytoplasmic domain of CEACAM1-L is associated with the SH2 domain-containing protein tyrosine phosphatase-1, which may suppress BCR signaling [19]. Different observation by others showing CEACAM1-induced B cell activation [6] may be explained by the fact that the BCR was not stimulated at the same time as CEACAM1 stimulation in their study, and consequently the suppression of BCR signaling was not observed. Another interpretation of the difference from ours would be that the B cells of the conventional CEACAM1 deficient mice may have redundant functions compared to wild-type B cells. Our results are however consistent with studies of Lobo et al. using a human B cell line [12].

Finally, we assessed the effect of CEACAM1 on proliferation of A20 cells. It should be noted that neither agonistic mAb nor ligands were required for the induction of CEACAM1 signaling in this experiment, because the N-domains of overexpressing CEACAM1 may sufficiently function as the homophilic ligands for the N-domains of other CEACAM1 molecules [4,7]. Unexpectedly, the A20/2L showed accelerated cell proliferation compared to that of A20/VA when cells were not treated with anti-Igκ. However, A20/2L showed suppressed proliferation compared to that of A20/VA when stimulated via BCR signaling. These results in our study seem contradictory. The former observation in this experiment is consistent with that of various cell types observed by other groups [6,20], and the latter is consistent with our previous observations in T cells [7,21] and data in Figs. 2, 3 and 4a in this current study. Our interpretation of such conflicting results is that CEACAM1 has two distinct functions on B cell proliferation. Thus, CEACAM1 may support the homeostatic proliferation of B cells, which does not require BCR signaling, when they are immature. On the other hand, CEACAM1 shows the opposite behavior during the spontaneous proliferation that requires BCR signaling in matured B cells. Such suppression
of BCR-mediated spontaneous proliferation by CEACAM1 in the latter mechanism may contribute to the regulation of immune reactions in lymphoid organs and peripheral tissues including the intestinal mucosa. The mechanisms by which these distinct CEACAM1 functions are induced in B cells need to be clarified. Therefore, further studies of this molecule may lead to greater insights into the mechanisms of immune responses within peripheral tissues and the potential treatment of inflammatory diseases including IBD.

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Abbreviations:

Abs           antibodies
B6            C57BL/6
BCR           B cell receptor
BM            bone marrow
CEACAM1       carcinoembryonic antigen-related cell adhesion molecule 1
Ctrl          control
ELISA         enzyme-linked immunosorbent assay
GALT          gut-associated lymphoid tissues
IBD           inflammatory bowel diseases
Ig            immunoglobulin
IL            interleukin
ITIM          immunoreceptor tyrosine-based inhibition motif
-L            long isoform
LPS           lipopolysaccharide
mAb           monoclonal antibody
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Fig. 1. CEACAM1 is expressed in the B cell lineage. (a) CEACAM1 expression in splenic lymphocytes. Total splenocytes stained with Alexa488-conjugated isotype-matched Ctrl (mIgG1), splenic T cells and B cells both stained with Alexa488-CC1 are shown. Representative data from three experiments are shown. (b) CEACAM1 expression in the B cell lineage. B220^+CD43^+ pro-B cells, B220^+CD25^+ pre-B cells, B220^+Igκ^+ immature B cells in BM (left), B220^high circulating B cells, B220^+CD138^+ plasma cells in PB (right) were stained with Alexa488-CC1. Representative data from three experiments are shown. Numbers in each histogram indicate the mean fluorescence intensity.
Fig. 2. CEACAM1 may be associated with BCR.
(a) Cytokine productions such as IL-4, IL-5 and IL-10 from primary B cells stimulated with either LPS (left), anti-CD40 (middle) or anti-\( \mu \) F(ab’)_2 fragment (right) in the presence (closed) or absence (opened) of CC1 are shown. Data are indicated as means ± SEM. n = 4. n.s., not significant. *p < 0.01. (b) Distribution of BCR and CEACAM1 expressions in primary B cells 0min (top) or 5min (bottom) after BCR stimulation. Cells were stimulated/stained with Alexa568-anti-\( \mu \) F(ab’)_2 and Alexa488-CC1. Representative observations from three experiments under the confocal microscopy of BCR (left), CEACAM1 (middle) and merged images (right) are shown. Scale bar indicates 10 μm.
Fig. 3. Overexpression of CEACAM1 suppresses BCR-mediated B cell activation.
(a) Inducible CEACAM1 expression in A20 cells. CEACAM1 expressions in the A20 cells transfected with either the vector alone (VA) or Ceacam1-2L (2L) incubated with (+) or without (−) Cumate are shown. (b) Ca²⁺ influx via BCR signaling in the A20 transfectants. Arrows indicate the time point of BCR stimulation with anti-Igκ. Representative data from three experiments are shown. (c) Expressions of the activation surface markers on the A20 transfectants. Histogram shows expressions of A20 transfectants with VA and Ceacam1-2L stained with anti-H-2Kᵈ, anti-I-Aᵈ, anti-CD80, anti-CD86, anti-CD69, anti-CD5 or VA with each isotype-matched Ctrl.
Fig. 4. Spontaneous proliferation via BCR signaling is suppressed by CEACAM1. (a) Expressions of BCR signaling in the transfectants. A20 transfectants with either VA or 2L with (+) or without (−) BCR stimulation with anti-Igκ were applied for immunoblotting. Representative data from three experiments were shown. (b) Homeostatic proliferation of A20 transfectants. Indicated numbers of A20 cells transfected with either VA (triangle) or 2L (square) without the BCR stimulation were applied for proliferation assay. Representative data from three experiments were shown. Data are indicated as means ± SEM. n = 3. *p < 0.0005. (c) Spontaneous proliferation of A20 transfectants. Indicated numbers of the transfectants with either VA (triangle) or 2L (square) with the BCR stimulation by anti-Igκ were applied for proliferation assay. Representative data from three experiments are shown. Data are indicated as means ± SEM. n = 3. *p < 0.0005.