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CEACAM1 structure and function in immunity and its therapeutic implications

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Abstract

The type I membrane protein receptor carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) distinctively exhibits significant alternative splicing that allows for tunable functions upon homophilic binding. CEACAM1 is highly expressed in the tumor environment and is strictly regulated on lymphocytes such that its expression is restricted to activated cells where it is now recognized to function in tolerance pathways. CEACAM1 is also an important target for microbes which have co-opted these attributes of CEACAM1 for the purposes of invading the host and evading the immune system. These properties, among others, have focused attention on CEACAM1 as a unique target for immunotherapy in autoimmunity and cancer. This review examines recent structural information derived from the characterization of CEACAM1:CEACAM1 interactions and heterophilic modes of binding especially to microbes and how this relates to CEACAM1 function. Through this, we aim to provide insights into targeting CEACAM1 for therapeutic intervention.

Keywords

CEACAM1; co-receptor; interaction; immunity

1. Introduction

Carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1), also known as cluster of differentiation 66a (CD66a) and biliary glycoprotein (BGP), is the primordial member of the carcinoembryonic antigen cell adhesion molecule (CEACAM) family of glycosylated immunoglobulin (Ig) molecules [1]. Its initial discovery was as a biliary glycoprotein that is present in the bile in association with expression on the hepatocyte membrane [2–4]. CEACAM1 expression occurs as early as embryonic development where it is found on trophoblasts of both the pre-implanting embryo, placenta and infiltrating leukocytes [5–7]. CEACAM1 also exhibits either constitutive (e.g. epithelial cells) or regulated (e.g. endothelial cells and lymphocytes) expression on a wide variety of cell types

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[8]. As such, CEACAM1 has been demonstrated to play a role in morphogenesis [9], apoptosis [10], angiogenesis [11], cell proliferation [12], cell motility [13], fibrosis [14], and most recently in immune T cell tolerance [15].

The fundamental function of CEACAM1 is to transduce extracellular signals across the cell membrane and into the cytosolic compartment. This process is triggered by intercellular (or *trans*) binding of CEACAM1 on two opposing cells. The major mode of CEACAM1 binding is homophilic although several other host and microbial ligands also exist. The binding of cell surface CEACAM1 by these ligands induces higher order multimerization (Figure 1) and clustering of the signaling machinery associated with the cytoplasmic tail and modulation of membrane proximal signaling events associated with other receptors. Depending on the presence of a full length or alternatively spliced short cytoplasmic tail, CEACAM1 ligation will impart inhibitory or non-inhibitory signals, respectively. At basal steady state, CEACAM1 exists as monomers and *cis* dimers on the cell surface [16,17] with the monomer serving as the primary receptive form for ligand binding [18]. The *cis* CEACAM1 dimer interface buries the critical binding epitope targeted by CEACAM1's homophilic or heterophilic ligands and therefore must be disrupted in order for CEACAM1 to participate in *trans* interactions which orient the cytoplasmic tails of CEACAM1 in a manner that elicits intracellular signaling [8]. The need to disrupt *cis* dimers to generate *trans* dimers poses a unique biophysical challenge to CEACAM1's ligands or therapeutic strategies aimed at defusing or co-opting CEACAM1 inhibitory function for promoting anti-cancer immunity or inhibiting autoimmunity, respectively. In this review, we describe the molecular mechanisms that determine CEACAM1 interactions and consequently how CEACAM1 modulates innate and adaptive immune functions thereby enabling an understanding of CEACAM1 as an appealing immunotherapeutic target.

2. CEACAM1 structure and function

Human CEACAM1 (hCEACAM1) is a single pass type I transmembrane protein expressed as 12 alternatively spliced isoforms that all contain the critical N-terminal V set fold of the immunoglobulin superfamily (IgV) ectodomain followed by up to three type 2 constant immunoglobulin (IgC2) ectodomains (A1, B, A2), a transmembrane sequence, and a signaling cytoplasmic domain comprised of either a long (L) immunoreceptor tyrosine-based inhibitory motif (ITIM)-containing domain or short (S) domain devoid of ITIMs (Figure 1A). Each membrane-integrated isoform is designated by the number of extracellular domains and the presence of an L or S cytoplasmic tail depending on the inclusion or absence of exon 7, respectively [19]. For example, the longest isoform contains 4 extracellular Ig domains (IgV domain and three IgC2 domains), the transmembrane sequence and the ITIM-containing long cytoplasmic domain and is designated as CEACAM1-4L. In contrast, the most truncated membrane form of CEACAM1 only contains the extracellular IgV domain, transmembrane sequence and short cytoplasmic tail and is therefore denoted as CEACAM1-1S (summarized in Figure 1A). Two splice isoforms are uniquely expressed as secreted proteins containing three Ig domains (IgV and two IgC2 domains) due to the presence of a premature stop codon proximal to the transmembrane sequence and are designated CEACAM1-3 and CEACAM1-3C2. Notably, CEACAM1 is the only CEACAM family member that has homologous murine orthologs. Mouse

CEACAM1 (mCEACAM1) is classically described to exist as 4 membrane-bound isoforms (mCEACAM1–4L, –4S, –2L, –2S) (Figure 1A) [19] however recent data suggests the presence of additional secreted variants [20] as found in humans. Although the differences in function are not well elucidated for each isoform while of great importance, the roles of the IgV domain in ligand interactions and the cytoplasmic domain in signaling have been the foci of considerable investigation.

2.1 The N-terminal IgV domain

The N-terminal IgV domain functions as the extracellular binding element that is responsible for determining CEACAM1's unique homophilic and heterophilic binding properties. The hCEACAM1 IgV domain contains 108 amino acids arranged in 9 beta strands (ABCC'C''DEFG) that fold into the conserved IgV anti-parallel beta-sandwich tertiary structure [15,21] adopted by other IgV-containing proteins including CD2 [22], T cell receptor (TCR) [23], T cell inhibitory and mucin domain containing protein 3 (TIM-3) [24,25], programmed cell death protein 1 (PD-1) and programmed death-ligand 1 (PD-L1) [26] and its murine ortholog mCEACAM1 [21]. The opposing ABED and GFCC'C'' faces of the CEACAM1 beta-sandwich are tethered by an internal salt bridge (R64 : D82) that mimics a stabilizing covalent disulfide linkage found in most Ig domains [15,27]. Also notable is the prominent protrusion of the CC' loop on hCEACAM1 that differs significantly from the ordered β -hairpin observed in other described IgV structures and which forms a cleft that exposes key residue side chains important for ligand binding such as F29, Y34, V39, D40, R43, Q44 [15,21,27] (Figure 1B, C). Although the IgV domain of hCEACAM1 and mCEACAM1 only share 41% sequence identity, their global structure overlaps closely with a main chain root square deviation (r.m.s.d.) of 1.6 Å [15,27,28]. The majority of the sequence diversity between the human and mouse orthologs lie on the C'C'' and FG loops where the majority of homophilic and heterophilic ligands bind and are interestingly analogous to the hypervariable CDR2 and CDR3 loops, respectively, found on antibodies and the TCR that determine their unique ligand specificities. Both the hCEACAM1 and mCEACAM1 IgV domains are glycosylated exclusively on the ABED surface, leaving the GFCC'C'' surface devoid of any post-translational modifications and accessible to participate in protein-protein interactions.

2.1.1 CEACAM1 IgV domain mediates *cis* and *trans* oligomerization—The first structural information on CEACAM1 was achieved through a low resolution crystal structure (3.32 Å) of a two domain, glycosylated mCEACAM1 construct containing the IgV domain and the membrane proximal IgC2 domain (PDB ID 1L6Z) representing the extracellular domain of the mCEACAM1–2L and mCEACAM1–2S isoforms [28]. Although the crystal structure was determined as a mCEACAM1 monomer, the packing of the mCEACAM1 crystal suggested the possibility of a mCEACAM1 dimer mediated by hydrophilic interactions at the GFCC'C'' surface. Independent mutagenesis analysis of hCEACAM1 corroborated these latter results by showing the critical role of the GFCC'C'' interface in determining the high affinity homodimerization of the hCEACAM1 IgV domain (Kd ~ 450 nM) [29], and specifically highlighting the contribution of the CC' (residues Y34, V39, D40, R43, Q44) and FG (residues Q89, I91) loops [30–32]. Our recent high resolution crystal structure (2.04 Å) of the unglycosylated hCEACAM1 IgV domain (PDB ID 4QXW)

[15,27] confirmed this by featuring two IgV molecules in the crystallographic asymmetric unit anchored at their respective GFCC'C'' surfaces and confirmed at atomic level resolution the participation of critical CC' and FG loop residues determined previously to be necessary for hCEACAM1 homodimerization [30–32]. Nuclear magnetic resonance studies (NMR) on unglycosylated hCEACAM1 IgV additionally demonstrated the presence of an exclusive GFCC'C''-mediated IgV dimer in solution [31] thus further reinforcing the significance of the GFCC'C'' face in mediating hCEACAM1 IgV homo-oligomerization.

Curiously, the first reported crystal structure of hCEACAM1 (PDB ID 2GK2) was solved from an asymmetric unit comprised of two unglycosylated IgV domains docked through hydrophobic interactions on the unmodified ABED face [21] thus raising the possibility that the CEACAM1 IgV domain could adopt two distinct dimeric conformations that centered on either the GFCC'C'' or ABED face. The involvement of the ABED surface in mediating CEACAM1 homo-oligomerization was surprising due to the presence of N-linked glycosylation on the endogenously expressed protein at three different sites (N70, N77, N81) that would be predicted to introduce steric clash at a potential ABED-mediated dimerization interface. As additional support for a role of the ABED face in CEACAM1 interactions, low resolution (20 Å) molecular tomography studies of liposome-immobilized, glycosylated, four domain rat CEACAM1 protein (IgV and 3 IgC2 domains) representing the full, post-translational modified extracellular domain revealed multiple homo-oligomeric species including two discernible dimers and a trimeric complex [33]. These were all linked at the IgV domain which could be predicted to accommodate GFCC'C'' and ABED-mediated homodimerization [33]. In addition, surface plasmon resonance (SPR) data has demonstrated that two different sites of the IgV domain could simultaneously participate in homophilic binding however with markedly different affinities [33]. Considering that oligosaccharides are flexible units and CEACAM1 undergoes variable glycosylation [34], it is likely the ABED surface is transiently exposed to participate in low affinity homo-oligomerization [33]. These observations collectively suggest that the ABED face only modestly contributes to CEACAM1 multimerization and that the major mode of CEACAM1 homophilic binding and higher order oligomerization is dominated by GFCC'C'' face interactions (Figure 1B, C).

2.1.2 CEACAM1 IgV heterophilic interactions with host ligands—The IgV domains expressed by members of the CEACAM family, specifically CEACAM1, CEACAM3, CEACAM5, CEACAM6 and CEACAM8 exhibit > 90% similarity along the GFCC'C'' face and might be predicted to interchangeably interact with each other to form an assortment of GFCC'C''-mediated CEACAM family heterodimers [29]. However, only CEACAM5, commonly referred to as CEA, appreciably binds to CEACAM1 with high specificity and affinity as determined by biochemical studies on purified proteins [29]. CEACAM5 IgV shares the highest sequence homology with CEACAM1 IgV among the CEACAM family members. CEACAM1 and CEACAM5 contain key conserved residues in the CC' (F29, S32, V39, R43, Q44) and FG (I91, E99) loops that mediate independent high affinity CEACAM1 ($K_D = 450$ nM) and CEACAM5 ($K_D = 800$ nM) homophilic interactions [17,29]. In contrast, CEACAM3 and CEACAM6 differ from CEACAM1 at two specific CC' loop sites where substitutions at position 43 (R43S) and more importantly position 44

(Q44L) severely weaken potential heterophilic binding to CEACAM1 [32]. CEACAM8 shares an arginine at position 43 with CEACAM1 but has a unique substitution at position 44 (Q44R) that results in a di-arginine motif with R43 which shifts its specificity away from CEACAM1 and towards CEACAM6 [29]. As a consequence of the sequence variability in the CC' and FG loops, both CEACAM1-CEACAM6 [35] and CEACAM1-CEACAM8 heterophilic interactions [36,37] are weak with binding constants that are at least 100-fold weaker than CEACAM1 homophilic interactions [29]. Unsurprisingly, CEACAM6 is also a weak homodimer in view of R43S and Q44L substitutions and CEACAM8 exists predominantly as a monomer in solution [29].

More recently, T cell immunoglobulin and mucin-domain containing-3 (TIM-3) was identified as a ligand for CEACAM1 [15], representing the first non-CEACAM family, IgV-containing host binding partner. TIM-3 is also a member of the immunoglobulin superfamily and is expressed as a type I membrane protein consisting of an N-terminal IgV domain, heavily O-linked glycosylated mucin domain stalk, transmembrane sequence and SH2-binding motif-containing cytoplasmic domain. The CEACAM1 and TIM-3 IgV domains share a similar beta-sandwich fold [24] and exhibit a calculated main chain r.m.s.d. of 1.31 Å indicative of high structural homology. That said, the TIM-3 IgV domain, in contrast to CEACAM1, is heavily stabilized by three interchain disulfide linkages in mouse and human. Both biochemical [15,24,27] and NMR studies [27] demonstrated calcium-dependent binding of CEACAM1 IgV to the TIM-3 IgV and biochemically localized the interaction to the GFCC'C'' surface where CEACAM1 binds homophilically. CEACAM1 has also been demonstrated to interact with α_v and β_1 -integrins [13,38] and dendritic cell (DC)-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) [39]. The later interactions however occur through post-translational modification of CEACAM1 by non-sialylated Lewis glycans [39].

2.1.3 CEACAM1 IgV heterophilic interactions with foreign ligands—Despite the complicated kinetics of CEACAM1 IgV monomer-dimer transition and requirement of GFCC'C'' surface accessibility for participation in *trans* interactions, several bacteria, fungi, and a virus that include *E. coli* [17], *Neisseria sp.* [40], *Moraxella catarrhalis* [41], *Haemophilus influenza* [42], *Helicobacter pylori* [43], *Fusobacterium sp.* [44], *Candida sp.* [45], and murine hepatitis virus [28] have evolved structurally distinct microbial receptors that converge on binding shared residues on the GFCC'C'' surface of CEACAM1 IgV critical to homophilic interactions as a universal target [46]. By docking on CEACAM1 specifically at the homodimerization interface, microbes have developed clever strategies of not only exploiting CEACAM1 as an attachment site needed for colonization and invasion but additionally benefiting from CEACAM1 oligomerization-induced inhibitory functions for evading the immune response [47–49].

Among the most heavily investigated microbes demonstrated to bind hCEACAM1 are the *Neisseria* species. *Neisseria gonorrhoeae*, *Neisseria meningitidis* and commensal *Neisseria* species express adhesion molecules belonging to the opacity-associated (Opa) family of variable outer membrane protein receptors that are subdivided into a group that bind heparin sulfate proteoglycans (Opa_{HS}) or members of the CEACAM family (Opa_{CEA}) including CEACAM1, CEACAM3, CEACAM5, and CEACAM6 [50]. Opa_{CEA} proteins adopt an

eight-stranded β -barrel structure (PDB ID 2MLH) [51] that arranges its extracellularly exposed hypervariable (HV) loops HV1 and HV2 to form a dynamic binding surface specific for the GFCC'C'' surface on CEACAM1. All Opa_{CEA} proteins target the critical CC' and FG loops on CEACAM1 IgV necessary for homophilic interactions and specifically require residues Y34 (CC' loop) and I91 (FG loop) on CEACAM1 for heterophilic binding [52]. Opa_{CEA}-CEACAM1 binding-mediated attachment of *Neisseria* on mucosal epithelium and immune cells influences a series of processes that include bacterial colonization, engulfment and host-pathogen induced immune inhibition that benefits microbial survival. *Neisseria* species bind to mucosal CEACAM1 found primarily on the apical membrane of polarized epithelial cells, resulting in phosphatidylinositol-3' kinase (PI3K)-mediated internalization [53] and transcytosis through actin-mediated cytoskeletal rearrangement for advantageous access to subepithelial spaces [54]. Once exposed to the immune compartment, *N. gonorrhoeae* exploits the negative regulatory function of CEACAM1 on T cells. Upon attachment to T cells through Opa_{CEA}-CEACAM1 interactions, *N. gonorrhoeae* recruits and retains CEACAM1 molecules at the cell surface where high order clustering and oligomerization of CEACAM1 disengages TCR-mediated T cell activation at the most membrane proximal signaling events thus diminishing immune-mediated bacterial clearance [18,47,55,56]. In a similar manner, the MHV spike glycoprotein binds murine CEACAM1 within the vicinity of the homophilic docking site of the GFCC' face [28] and inhibits T cell receptor signaling [57] suggesting that this is a common mechanism of microbial regulation of the immune response.

Similar to *Neisseria* species, the gram-negative coccobacillus *Haemophilus influenzae* also expresses a specific outer membrane protein, namely OMP P1, that targets the IgV domain of CEACAM1 in a grossly isolate-independent manner [58]. In contrast, the CEACAM3 IgV and CEACAM5 IgV also bind OMP P1 but only in fraction (~25%) of studied isolates suggesting that OMP P1 has co-evolved with CEACAM1 as its primary binding partner [58]. This reflects the significance of the OMP P1-CEACAM1 IgV interaction but more importantly emphasizes the highly specific nature of OMP P1 by which CEACAM1 is the major preferred CEACAM family member. Based on structural modeling from the *E. coli* outer membrane protein FadL that shares 39% sequence identity with OMP P1, the latter has been proposed to adopt a 14-stranded beta-barrel structure that contains 7 extracellular loops of which four (L1, L3, L4, L7) are surface exposed and potentially able to interact with the CEACAM1 IgV domain. Interestingly, although certain isolates of OMP P1 share a GxI/V/LxQ motif in HV2 that is suggested to be critical for Opa_{CEA} specificity for CEACAM1 [50], not all isolates contain this motif further suggesting that OMP P1 has a unique binding mode that is highly discriminatory for CEACAM1 but which remains to be established by formal biophysical assessments.

Diffusely adhering *Escherichia coli* (DAEC) express members of the Afa/Dr family of adhesins (e.g. AfaE-I, AfaE-III, DraE, and DaaE) that mediate attachment to epithelial cells through binding of CD55 (decay accelerating factor, DAF) and several members of the CEACAM family including CEACAM1, CEACAM5 and CEACAM6 [59,60]. Despite high sequence variation between the phylogenetically distinct groups of Afa/Dr adhesins, they adopt a similar Ig-like six stranded beta-sandwich [17] that can bind DAF and CEACAM1 simultaneously on mutually exclusive binding surfaces. The NMR solution structure of a

CEACAM5 IgV-AfaE-dsc heterodimer (PDB ID code 2VER) [17] in combination with site-directed mutagenesis studies biochemical analysis [61] revealed the critical role of the GFCC'C'' surface on CEACAM5 in mediating Afa/Dr adhesion binding, specifically highlighting the residues F29, D40, Q44 (CC' loop) and I91A, E99 (FG loop) that are conserved in CEACAM1 and thus likely to also contribute to CEACAM1 specificity for Afa/Dr adhesins. Owing to the shared binding surface, Dr adhesions must compete with CEACAM1 homophilic binding to successfully form a heterodimeric complex. Indeed, kinetic studies suggest that Dr adhesions can disrupt CEA homodimers and thus facilitate binding to free CEA monomers [17]. It is unclear if Dr adhesins affect CEACAM1 function upon ligation however it is noteworthy that Dr adhesions are highly oligomeric and therefore may induce CEACAM1 *cis* clustering following *trans* interactions resulting in activation of CEACAM1 inhibitory function.

Recently, the first high resolution structures (2.68–2.8 Å) of the hCEACAM1 IgV domain in complex with a bacterial receptor were solved with the *H. pylori* outer membrane adhesin HopQ (PDB ID 6AW2, 6GBG) [62,63]. Although HopQ adopts an alpha-beta folded structure that diverges significantly from Opa_{CEA}, its mode of binding to the GFCC'C'' surface on CEACAM1 is conserved with other microbial ligands. Four loops (α 3- β 1, β 2- α 4, α 5- α 6, α 7- α 8) form a binding surface that allows for the β 2- α 4 loop to participate in an extensive van der Waals and hydrogen bonding network with the CEACAM1 IgV GFCC'C'' surface. This specifically involves interactions with CEACAM1 residues on the CC' loop (F29, V39, Q44) and FG loop (Q89, I91, V96) [63]. HopQ binds to CEACAM1 through a distinct coupled folding and binding mechanism [63] that exhibits high affinity for CEACAM1 IgV with a binding constant that exceeds CEACAM1 homodimerization (K_D = 23–279 nM) [43,63]. Importantly, small-angle X-ray scattering (SAXS) studies demonstrated that HopQ is able to disassociate CEACAM1 IgV homodimers into monomeric species that allow for heterophilic interactions with HopQ. Through this structural mechanism, *H. pylori* takes advantage of HopQ-CEACAM1 interactions to attach to the gastric epithelium and enable translocation of the oncoprotein CagA [43,64].

Together, these studies on diverse types of microbial interactions with CEACAM1 reveal how microbes have co-opted the homophilic GFCC'C''-mediated binding interface for docking and the transmission of inhibitory signals and other contextually specific purposes.

2.2 The IgC2 domains of CEACAM1 allow for accessibility of the IgV domain

The IgV domain is distanced from the cellular membrane by the presence of up to 3 heavily glycosylated IgC2 domains that each share sequence similarity to the CEACAM1 IgV domain but are smaller, ranging from 81 to 91 amino acids, consistent with an IgC domain. The anti-parallel beta sandwich formed by the IgC2 sequences are stabilized by a core covalent disulfide bridge and are more extensively glycosylated than the IgV domain. Although the function of the CEACAM1 IgC2 domains are not fully understood, limited studies suggest that they likely serve as molecular spacers that allow for increasing accessibility of the IgV domain to participate in *trans*-interactions [30]. In cell attachment studies with CEACAM1-expressing CHO cells and immobilized CEACAM1-Fc fusion proteins, only extended CEACAM1-Fc immobilized proteins containing 2 or 3 IgC2

domains significantly bound CEACAM1 expressed on the surface of the CHO cells. CEACAM1 is also significantly handicapped in its ability to facilitate PI3K-mediated uptake of Opa_{CEA}-expressing *N. gonorrhea* when expressed in transfected cells as a single IgV domain associated with the CEACAM1–1L isoform as compared to the full length CEACAM–4L protein [65]. Taken together, these results indicate that CEACAM1 homophilic and heterophilic interactions are both affected by the degree of IgV domain extrusion away from the cellular membrane through inclusion of the IgC2 domain in the CEACAM1 tertiary structure.

2.3 The transmembrane domain regulates *cis* dimerization

The human CEACAM1 transmembrane domain spans 24 residues (A429-L452) and contains both a canonical GxxxG/tetrad motif [66] and heptad repeat [67] that mediate right-handed and left-handed helix packing angles, respectively. The GxxxG motif in CEACAM1 (G432-G436) in particular is expected to participate in transmembrane helix-helix dimerization based on computational modeling [18] similar to other GxxxG motif-containing transmembrane proteins such as glycoporphin A [68] where the short glycine side chains are positioned on the same helix face and allow for tight packing of the putative transmembrane helix-helix dimer. Replacement of the glycine residues with bulkier side chain-containing leucine residues (G432L G436L) resulted in a striking increase in CEACAM1-dependent cellular aggregation [18] consistent with an increase in surface CEACAM1 monomers available to participate in *trans* CEACAM1-CEACAM1 interactions. Fluorescence anisotropy measurements of CEACAM1–4L LxxxL mutant-transformed HeLa cells further demonstrated that leucine substitution in the GxxxG motif shifts CEACAM1 oligomerization exclusively towards a monomeric state [18] thus establishing the fundamental requirement of the GxxxG/tetrad motif for CEACAM1 *cis* dimerization at the cell surface. This connotes the transmembrane domain as a second source of homophilic interactions.

2.4 The cytoplasmic domain determines inhibitory or non-inhibitory signal transduction

The cytoplasmic domain of CEACAM1 exists in either an extended long (L) form or alternatively spliced short (S) form based on the inclusion or exclusion of exon 7, respectively (Figure 1A). Although there is no secondary structural information on the fold of the two cytoplasmic isoforms, primary sequence analysis and extensive mutagenesis studies have revealed a myriad of signaling motifs that distinguish each variant's function [69]. The most notable difference between the two isoforms is the inclusion of two classical immunoreceptor tyrosine based inhibitory motifs (ITIMs) (V/I/SxYxxI/L) in the L isoform that are absent in the truncated S cytoplasmic domain and define the principal inhibitory role of CEACAM1 in immune function.

Despite the vast difference in length, the 74-residue L and 12-residue S cytoplasmic tails both contain an abbreviated, membrane proximal six amino acid consensus sequence (H⁴⁴⁴FGKTG⁴⁴⁹) that imparts shared functionality. Both cytoplasmic tails bind to calmodulin in a calcium-dependent manner with near nanomolar affinity [70]; calmodulin also binds to a distal region on CEACAM1-L but with significantly reduced affinity. Association of calmodulin with both types of CEACAM1 cytoplasmic domains appears to

interfere with CEACAM1 *cis* homodimerization [70] supporting the notion that cellular activation increases the population of available CEACAM1 monomers on the cell surface to participate in *trans* intercellular interactions. Thus, elevation of intracellular calcium as a consequence of activation via a variety of cell-specific receptors provides the impetus for calmodulin binding and CEACAM1 monomerization [71]. This consensus sequence also is required for lumen formation in transformed breast epithelial cells through calmodulin kinase IID (CaMKIID)-mediated phosphorylation of the shared threonine (T448) [72,73]. Finally, this consensus motif accounts for the ability of both CEACAM1-L and CEACAM1-S to bind subdomain 3 of actin which is mediated by residues F445 and K447 [72,73].

Outside these similarities, the CEACAM1-L and CEACAM1-S isoforms have highly divergent functions. This is most clearly seen through the possession of two ITIMs in the former but not the latter. Although ubiquitous in the cytoplasmic domain of negative regulators of immune activation such as PD-1 and TIGIT, CEACAM1-L is unique among the CEACAM family as it is the only family member to contain the inhibitory ITIM domains. Depending on the cell type, CEACAM1 ITIM phosphorylation has been shown to be initiated by activation of immune receptor tyrosine-based activating motif (ITAM) containing surface receptors such as the T cell receptor [74–76] receptor tyrosine kinase receptors such as the insulin receptor (IR) [77] and epidermal growth factor receptor (EGFR) [78]. Upon extracellular engagement, these receptors both provide signals that lead to increased intracellular levels of calcium to enable calmodulin binding to the CEACAM1 cytoplasmic tail which facilitates disruption of CEACAM1 *cis* dimers [16], activation of cell type specific Src-family kinases (SFK) [79,80] such as p56^{lck} in T cells [74] and association with the cytoskeleton [81]. SFK-mediated phosphorylation of the intracellular ITIM tyrosine residues (Y493, Y520) encourages interactions of CEACAM1 with N-terminal Src-homology 2 (SH2) protein domain containing proteins. This includes protein tyrosine phosphatases SHP-1 (PTPN6) found predominately in hematopoietic cells or SHP-2 (PTPN11) that is more ubiquitously expressed [82]. As CEACAM1 associates with a variety of activating receptors, this functions to bring inhibitory phosphatases into close proximity of the signaling cascade [74].

Interestingly, SHP-1 and SHP-2 discriminate in their binding of CEACAM1 based upon its oligomeric state. Recent data from Muller and colleagues suggest that these phosphatases prefer oligomerized CEACAM1 and, consistent with their possession of two SH2 binding domains, bind two adjacent phosphorylated CEACAM1 molecules [83]. SH2-mediated SHP binding releases the PTP phosphatase domain for downstream dephosphorylation activity of its various target molecules associated with an array of activating receptors.

Both CEACAM1-L and -S isoforms are typically expressed on the same cell and in varying ratios based on the cell type, functional state, cell cycle state and tumorigenic potential [34,84,85] and work in concert as a tunable supramolecular system to affect collective CEACAM1 signaling function. CEACAM1-L and CEACAM1-S isoforms are present on the cell surface as a mixture of monomeric species and *cis* homo-oligomeric species [83] that likely includes CEACAM1-S homodimers, CEACAM1-L homodimers and CEACAM1-L-CEACAM1-S heterodimers that are collectively non-functional in the absence of *trans*-ligation. Recent *in vitro* studies demonstrated that *trans* homophilic interactions not only

engage surface CEACAM1 monomers but also augment *cis* dimerization [83] through allosteric mechanisms potentially involving increased transmembrane interactions. As SHP-1 and SHP-2 prefer CEACAM1-L *cis* dimers, the *trans* homophilic induction of CEACAM1-S homodimers or mixed heterodimers may serve to blunt the inhibitory functions of CEACAM1-L homodimers through diminished recruitment and activation of these inhibitory phosphatases to the activating receptor complex. A similar mechanism may regulate the function of CEACAM1-L with its other specific intracellular binding partners such as paxillin [86] that associate with phosphorylated CEACAM1-L.

3 CEACAM1 modulates the immune response

CEACAM1 is expressed as a co-receptor on a variety of immune and parenchymal cell types as well on neoplastic and developing tissues with important functional implications. There are a number of excellent reviews on these topics [87–90]. Here we will focus on the role played by CEACAM1 in the immune system where it functions in both innate and adaptive immunity.

3.1 CEACAM1 mediates innate immunity

3.1.1 CEACAM1 inhibits natural killer cell-mediated cytotoxicity—Natural killer cell (NK) cells mediate early defense against viral infections and tumor progression and play a role in the development of autoimmunity and immune tolerance [91]. NK cytotoxic function is determined by the net balance between signaling from activating surface receptors (NKG2D, CD16, NKp30, NKp44, NKp46, killer cell lectin-like receptor K1, natural cytotoxicity triggering receptors 1, 2 and 3, DNAX accessory molecule 1) and inhibitory molecules (killer-cell immunoglobulin-like receptors, KIRS) that recognize class I major histocompatibility complex (MHC) proteins on opposing cells [92]. The presence of MHC molecules or MHC mimics on infected cells or tumors results in inhibitory signaling through MHC-KIR interactions on NK cells that are lost in the absence of MHC surface expression on cells that are susceptible to cytolysis [91]. CEACAM1 was recently identified as an additional inhibitory NK co-receptor exploited by select tumor cells that does not rely on MHC-binding to impart inhibitory function [93]. In contrast, CEACAM1 and CEACAM5 expression on melanoma cells was demonstrated to participate in *trans* IgV-mediated homophilic and heterophilic interactions, respectively, with CEACAM1 expressed on NK cells to evade NK-mediated tumor killing [32,94]. Mutagenesis analysis demonstrated that key residues (R43 Q44) on the GFCC'C'' surface were critical for CEACAM1-L's inhibitory function on NK cells [32]. Upon *trans* dimerization and clustering on the NK cell surface, CEACAM1 associates with the activating NKG2D receptor resulting in SHP-1-mediated dephosphorylation of the guanine nucleotide exchange factor Vav1 and consequential disruption of cytolysis [95].

In humans who are deficient in TAP2 expression and therefore do not express surface MHC-peptide complexes, NK cells have been reported to express abnormally high levels of inhibitory surface CEACAM1 as a compensatory inhibitory mechanism resulting in protection against NK-mediated cytotoxicity and autoimmune disease in early life [96,97]. Moreover, TAP2-deficient patients compared to healthy individuals have been described to

exhibit decreased levels of circulating soluble CEACAM1 isoforms that potentially disable *trans* homophilic interactions on opposing cells to occur [97].

In addition to a direct inhibition of NK cell signaling, CEACAM1 further dampens NK-mediated cytotoxicity through the downregulation of surface expression of NKG2D ligands (NKG2DL) on tumor cells [98]. When CEACAM1 was silenced in both mouse tumor cells, there was notably no change in NKG2DL transcription but rather increased surface presentation of specific NKG2DLs such as RAE-1 coincident with decreased intracellular stores. Furthermore, surface RAE-1 was significantly more heavily glycosylated compared to intracellularly retained RAE-1 suggesting that CEACAM1 negatively affects NKG2DL membrane trafficking through regulation of NKG2DL post-translational modification. Together, these studies suggest that CEACAM1 normally serves to inhibit NK cell cytotoxicity and expression of certain NKG2DL which distills into diminishing the activity of NK cells in tumors and potentially other processes.

3.1.2 CEACAM1 affects monocyte development and function—CEACAM1 promotes the survival of murine monocytes by suppressing intrinsic (e.g. mitochondrial-mediated apoptosis) and extrinsic (e.g. CD95 ligand induced apoptosis) apoptosis likely through *trans* homophilic interactions [99]. Interestingly, the CEACAM1-induced survival associated with the intrinsic pathway was through upregulation of Bcl-2 mediated by phosphatidylinositol 3-kinase (PI3K) and Akt-dependent signaling pathways that result in downregulation of caspase 3 activation. Interestingly, CEACAM1's inhibitory function is mitigated late in apoptosis when the long cytoplasmic tail is subject to caspase 3-mediated proteolytic cleavage at a membrane proximal cytoplasmic DQRD motif rendering it completely devoid of its ITIM domains and thus inhibitory activity [100]. This suggests that CEACAM1 promotes survival in monocytes and in a self-regulating manner.

CEACAM1 also plays a critical role in monocyte and macrophage development and function. Using an experimental model of cutaneous *Leishmaniasis* to study inflammatory lymphangiogenesis and hemangiogenesis, CEACAM1 was demonstrated to be critical for myeloid differentiation and development of a Ceacam1⁺Ly6C⁺CD11b⁺ monocyte population responsible for mediating local neovascularization and angiogenesis [101]. More recent studies have defined a new subset of Ceacam1⁺Msr1⁺Ly6C⁻F4/80⁻Mac1⁺ monocytes coined segregated-nucleus-containing atypical monocytes (SatM) that derive from Ly6C⁻FcεRI⁺ granulocyte/macrophage progenitors and not macrophage/dendritic-cell progenitors that is under the tight regulation of CCAAT/enhancer binding protein β (C/EBPβ) [14]. As such, SatM share granulocyte characteristics and are crucial to the development of fibrosis in tumor models. Although the mechanisms of CEACAM1 affecting angiogenesis and fibrosis are unknown, they likely follow a similar adhesion-activation process demonstrated in other cell types.

3.1.3 CEACAM1 regulates neutrophil development and function—CEACAM1 is rapidly trafficked to the membrane from intracellular granule stores during neutrophil activation [102–104] where it functions as a cellular receptor for bacterial pathogens [105,106] and negative regulator of neutrophil function. During infection with *Neisseria gonorrhoeae*, CEACAM3, an immunoreceptor activation motif (ITAM)-containing

CEACAM family member that is constitutively expressed on neutrophils, is first engaged by Opa_{CEA} resulting in initiation of a Syk-, PKC δ - and Tak1-dependent signaling cascade that triggers an NF- κ B-dependent transcriptional response and ultimately phagocytic engulfment and oxidative killing of the invading bacteria [106,107]. CEACAM1 is upregulated on the cell surface following initiation of neutrophil activation by CEACAM3 where it is accessible for Opa_{CEA} binding and rapid phosphorylation of its ITIM domains through the tyrosine kinase activity of Lyn and Hck [79]. Notably, CEACAM1-Opa_{CEA} binding and subsequent bacterial internalization does not potentiate neutrophil activation [108] consistent with a negative regulatory role of CEACAM1 on CEACAM3 function.

In addition to its functional role, CEACAM1 also plays an essential role in granulopoiesis where genetic deletion of *Ceacam1* in mice (*Ceacam1*^{-/-}) results in profound neutrophilia [109]. CEACAM1 affects granulocyte differentiation through its ITIM domains that mediate SHP-1-dependent inhibition of the granulocyte colony-stimulating factor receptor (G-CSFR)-signal transducer and activator of transcription (Stat3) pathway. Reconstitution of CEACAM1 into the bone marrow of *Ceacam1*^{-/-} mice restored granulopoiesis thus substantiating CEACAM1 as a co-inhibitory receptor for G-CSFR.

3.2 CEACAM1 mediates adaptive immunity

3.2.1 CEACAM1 regulates T cell activation and mediates tolerance—

CEACAM1 is largely an activation-induced cell surface molecule on mouse and human T cells. It is essentially absent from the surface of naïve T cells and only significantly upregulated following stimulation of the TCR or stimulation with IL-2 or mitogens such as phytohemagglutinin [110,111]. CEACAM1 is the only CEACAM family member that is expressed on activated T cells [112]. Small quantities of CD4⁺ T cells can be detected in the peripheral blood which express CEACAM1, but their identity and function is not yet clarified. Single-cell PCR analysis of CEACAM1 isoform expression has yet to be described but it is anticipated that a single T cell expresses variable mixtures of CEACAM-L and CEACAM-S variants. When present on the cellular membrane, CEACAM1 exerts considerable influence on T cell function depending on the expressed isoform. CEACAM1-L isoforms exert a negative inhibitory effect on TCR-mediated signaling whereas CEACAM1-S isoforms counteract CEACAM1-L function in an opposing anti-inhibitory role that allows for CEACAM1 global function to be modulated in a tunable fashion. As such, variable ratios of CEACAM1-L:CEACAM1-S expression would be expected to differentially regulate T cell responses to downstream signals provided by receptors which CEACAM1 modulates such as the TCR or cytokine receptors (e.g. IL-2 receptor).

The majority of activated T cells express increased levels of inhibitory CEACAM1-L relative to CEACAM-S variants in peripheral blood [111]. CEACAM1 localizes on the cell surface within the immune synapse [75,113] and biochemically associates with the TCR/CD3 complex [74]. Following T cell activation, hCEACAM1-L is phosphorylated by p56^{lck} at its two ITIM tyrosine residues (Y493, Y520) and activates SHP-1 through release of its phosphatase domain. SHP-1 subsequently dephosphorylates the signaling chain of the TCR/CD3 complex (CD3 ζ) and ZAP-70 thereby disrupting the membrane proximal TCR signaling machinery including inhibition of mitogen activated protein kinases [74]. Site-

directed mutagenesis studies have further shown that both ITIM domains are required for proper CEACAM1-mediated inhibitory function [76]. Abrogation of TCR-mediated T cell signaling by CEACAM1-L results in downregulation of Tbet-mediated Type 1 T helper (Th1) cytokine production (IFN- γ , IL-2) [57], T cell proliferation and T cell-mediated cytotoxicity [76], consistent with its targeting of proximal signaling pathways. Notably, interference of CEACAM1 homophilic binding by an anti-CEACAM1 F_{ab} relieves CEACAM1-L-mediated TCR inhibition and restores TCR-mediated cytolytic function supporting *trans* homodimerization as being important in these processes [74]. Direct evidence that CEACAM1-L is an inhibitory receptor *in vivo* derives from studies in which CEACAM1-4L expression has been conditionally forced in T cells either transgenically or through the use of transduction of T cells with CEACAM1 containing retroviruses. These studies have directly shown that CEACAM1 inhibits primary T cells in a pathway that requires the ITIM domains in CEACAM1 and SHP-1 in the T cell [74]. Collectively, this suggests that CEACAM1-L functions as an activation-induced inhibitor of T cell signaling that protects T cells from prolonged or unrestrained activation.

The pattern of CEACAM1 expression in the bloodstream and other peripheral tissues may be different from those found in mucosal sites. In the intestine, T cells constitutively express CEACAM1 consistent with their activated phenotype and are enriched in the transcriptional levels of CEACAM1-S compared to CEACAM1-L isoforms [76]. The dominant physiologic expression of CEACAM1-S isoforms in intestinal tissues has been yet to be examined with reagents that detect CEACAM1 protein as the tools available for this remain limited. That said, they crucially suggest that CEACAM1-S isoforms possess a physiologic activity in T cells and the splicing machinery which determines deletion of exon 7. Further, these studies suggest that the generation of CEACAM1-L and CEACAM1-S isoforms is a highly regulated tissue-specific response [74,76,114].

The specific function of CEACAM1-S isoforms has been addressed by forced expression of mouse CEACAM1-4S in the presence and absence of other CEACAM isoforms conditionally in T cells to examine the potential functions of the CEACAM1-S containing isoforms. These studies suggest that CEACAM1-S and CEACAM1-L isoforms can act as independent signaling units in a T cell. Using forced conditional expression of CEACAM1-4S in T cells, these studies have shown that CEACAM1-4S upregulates the nuclear factor of activation (NFAT) signaling pathway, induces expression of pro-inflammatory cytokines such as IFN- γ , IL-2 and IL-4, cell surface expression of activating proteins such as CD40L and finally promotes activation induced cell death of the CEACAM1-4S expressing cell [114]. At a population level, these properties of CEACAM1-4S expression were associated with the enrichment of both T follicular helper cells (Tfh) in association with mucosal IgA production and CD4⁺CD25⁻LAP (latency associated peptide)⁺ regulatory T cells (Treg) [114]. Similarly, forced expression of CEACAM-4L in the absence of CEACAM1-S isoforms promotes inhibition of T cell function [114]. These studies suggest that CEACAM1-S isoforms in T cells when in isolation can promote activation and activation-induced cell death and the deviation of T cells to unique regulatory phenotypes which together may represent an alternative means to delimit T cell activity. This may be particularly important to the intestines which are dominated by effector-memory T cells that are continuously activated by foreign antigens [114].

These observations suggest that global CEACAM1 function in T cells is likely an orchestrated response that involves three inter-related modes of signal transduction. These include inhibitory mechanisms involving CEACAM1-L dimers that effectively recruit phosphatases (SHP-1 and SHP-2) to inhibit activating receptor function, non-inhibitory modules involving CEACAM1-L/CEACAM1-S heterodimers that are incapable of phosphatase recruitment and thus disabled in their inhibitory activity or activating mechanisms involving CEACAM1-S homodimers which use NFAT and serve to delimit T cell survival and promote T cell regulatory function.

CEACAM1 may thus serve as a molecular rheostat in determining the consequences of an activating T cell receptor signal. This may be particularly important in pathways of tolerance. In a model using ovalbumin-specific TCR transgenic mice, an absence of CEACAM1 on the T cells abrogated the development of tolerance *in vivo* [15]. CEACAM1 expression is upregulated upon *Staphylococcal* enterotoxin-B (SEB) administration and an absence of CEACAM1 prevents both SEB-induced deletional (potentially associated with CEACAM1-S isoforms) and non-deletional (potentially associated with CEACAM1-L isoforms) mechanisms of tolerance [15]. Together with the role played by CEACAM1-S isoforms in promoting the development of distinct types of regulatory T cells [115,116], these observations implicate a broad role for CEACAM1 in multiple pathways involved in extrathymic tolerance [15]

An extreme form of tolerance is that associated with so-called T cell “exhaustion” [15]. The progressive dysfunction of T cells in the context of exhaustion is accompanied by the expression of several receptors (e.g. CEACAM1, CTLA-4, PD-1, LAG-3, TIM-3, TIGIT, 2B4) that transduce inhibitory signals upon interactions with their ligands on target cells [117–121]. In contrast to T cell anergy that results from T cell priming in the absence of costimulatory signals [122], exhausted T cells arise from gradual modulation of the T cell’s ability to elicit pro-inflammatory activities in the presence of continuous TCR stimulation during chronic infection, autoimmunity or malignancy [118]. In the case of malignancy, exhaustion of tumor specific T cells is suggested to impede the ability of the immune system to eradicate the tumor thus allowing tumor escape [118]. Exhausted T cells have also been observed in response to several chronic persistent viral infections such as human immunodeficiency virus (HIV), hepatitis B virus (HBV), hepatitis C virus (HCV) and human T lymphotropic virus 1 (HTLV1) [118,123]. In contrast, mice exhibiting deficient T cell exhaustion develop severe spontaneous or induced autoimmune diseases [15,124,125].

Recent studies implicate CEACAM1 as an important participant in establishing the exhausted phenotype through its ability to promote tolerance. A genetic absence of CEACAM1 results in increased resistance to inflammation-induced colorectal cancer or colorectal cancer xenografts in mouse models [15,126]. In addition, *Ceacam1*^{-/-} mice demonstrate exacerbated intestinal inflammation when challenged with dextran sodium sulfate (DSS) [15]. Consistent with this, adoptive transfer of CEACAM1-deficient T cells into immune-deficient mice causes severe colitis which is reversed by forced expression of mouse CEACAM1–4L in the T cell [127], together suggesting an absence of CEACAM1 promotes anti-tumor immunity and inflammation which is prevented by the expression of CEACAM1 in the T cell. CEACAM1 expression on peripheral blood T cells during chronic

HIV infection in humans is associated with decreased production of IFN- γ in response to SEB or HIV-gag protein [15]. In addition, expression of CEACAM1 on CD8⁺ and CD4⁺ tumor infiltrating lymphocytes in human glioma [128] and colorectal cancer [126] is inversely correlated with the ability of the T cells to produce IFN- γ [15]. The latter is consistent with the accumulation of CEACAM1 expression together with other exhaustion markers such as PD1 and TIM-3 on TILs in mouse models of cancer in association with decreased production of IFN- γ relative to that produced by CEACAM1-negative T cells [15]. Interestingly, in both mouse colorectal cancer xenografts [15] and humans with colorectal cancer [126], the most functionally exhausted T cells were in fact characterized by co-expression of CEACAM1 and TIM-3. Like other molecules associated with exhaustion such as PD1 [129], CEACAM1 is upregulated by IFN- γ [130,131] and interleukin-27 [15]. These studies support CEACAM1 as part of the exhausted T cell phenotype.

The mechanism by which CEACAM1 functions in tolerance and the exhausted phenotype is likely through trans-cellular homophilic binding in view of the high affinity nature of this interaction. This presumably involves an activated, CEACAM1 expressing T cell and a source of CEACAM1 from another cell type (e.g. antigen presenting cell or tumor). Indeed, the singular deletion of CEACAM1 in a T cell even when other potential inhibitory molecules are retained disables tolerance induction [15]. CEACAM1 also enables TIM-3 to function as an inhibitory molecule as an absence of CEACAM1 disables TIM-3 inhibitory activity even when TIM-3 expression is enforced in a T cell [15]. Furthermore, reduced expression of CEACAM1 and TIM-3 on circulating CD4⁺ and CD8⁺ T lymphocytes in individuals with multiple sclerosis correlates with disinhibited T cell activation and consequently increased disease severity [132]. TIM-3 has been suggested to also possess activating functionality [133–141]. This raises the possibility that CEACAM1 associates with TIM-3 as it does other activating receptors and serves to inhibit its function or that CEACAM1 association with TIM-3 enables other novel inhibitory signaling mechanisms that remain to be determined. CEACAM1-TIM-3 interactions have been biochemically mapped to the GFCC'C" interface of both molecules that overlaps with those involved in homophilic binding of CEACAM1 and the phosphatidylserine binding sites on TIM-3 [15,24,142]. Interestingly, the binding epitope of anti-TIM-3 antibodies that function as effective anti-cancer agents map to the CEACAM1 binding site on TIM-3 suggesting that targeting TIM-3 requires disabling TIM-3 interactions with CEACAM1 and/or phosphatidylserine [143]. Notably, a single nucleotide polymorphism in TIM-3 that causes a substitution at residue 101 (T101I) maps outside the CEACAM1-TIM-3 binding interface but is associated with an increased risk of inflammatory bowel disease [15]. Although the mechanism is unknown, the T101I substitution may induce allosteric inhibition of CEACAM1-TIM-3 interactions or other consequences of protein misfolding such as induction of endoplasmic stress responses. The T101I allele is also recently reported to be associated with the subcutaneous panniculitis and cutaneous lymphoma (SPCTL) syndrome suggesting a broad relevance to human disease [144].

These observations are interesting and suggest that T cell expression of CEACAM1 funnels into inhibition of activated T cells through either non-deletional (CEACAM1-L) or deletional (CEACAM1-S) mechanisms and may additionally promote and/or affect the function of regulatory populations of cells that provide inhibitory signals.

4 Implications for therapeutic CEACAM1 blockade

The aforementioned discussion supports CEACAM1 as an appealing target for immunotherapy both for autoimmune, oncologic and potentially infectious disorders but poses a set of equally difficult challenges in therapeutic development. First, CEACAM1 is unique among the majority of inhibitory co-receptors as its primary ligand is itself. This is in stark contrast to PD-1, CTLA-4, LAG-3, 2B4, TIM-3 and TIGIT that participate in heterophilic binding to their ligands, namely PD-L1/PD-L2, CD80, MHC class II, CD48, CEACAM1/galectin-9 and CD112/CD155, respectively [120]. Moreover, the affinity of the GFCC'C''-mediated CEACAM1 homophilic interaction from whence inhibitory signals commence far surpasses that of the other inhibitory co-receptors for their respective ligands. Whereas most inhibitory receptors function heterophilically and exhibit affinities that are in the micromolar range [26,145], CEACAM1 homophilic interactions occur with nanomolar affinity [29]. Finally, the CEACAM1 IgV GFCC'C'' interface involved in homophilic interactions are highly homologous to other CEACAM family members (especially CEACAM3, 5, 6 and 8) making specific targeting challenging.

As opposed to enzymatic proteins that have a targetable active site, the most rational therapeutic target on CEACAM1 is its high affinity *cis* and *trans* homophilic GFCC'C'' interaction surface that initiates the CEACAM1-mediated inhibitory signaling cascade upon self-ligation. The tremendous benefit of an equivalent interaction surface on opposing receptors is that any therapeutic molecule will antagonize both donor and recipient receptors on opposing cells thus providing dual blockade. However, the major consequence of this potential advantage is that any multivalent antagonistic molecule may effectively cross-link CEACAM1 receptors on opposing cells and thus potentially mimic a *trans* interaction or alternatively cross-link *cis* receptors with appropriate spatial organization that elicit undesired, functionally agonistic activation of CEACAM1-mediated inhibition. Microbes have developed clever strategies of activating CEACAM1 inhibitory function by exploiting the CEACAM1 IgV GFCC'C'' surface through evolution of high affinity receptors [46] that outcompete CEACAM1 for binding and thus induce aggregation for purposes of attachment resulting in deliberate suppression of NK and T cell activity that allows for immune evasion by *Neisseria*, *Fusobacterium* and *Helicobacter species* [44,47,48,108] and suppression of anti-tumor immunity by *Fusobacterium* [49]. An unfortunate byproduct of this activity may be microbe-induced tumor initiation and/or promotion of tumor progression. Consistent with this, exogenous delivery of multivalent microbial receptors exhibits a similar inhibitory profile as demonstrated by purified recombinant murine hepatitis virus receptor (MHV) that forms constitutive oligomers [146] and were demonstrated to inhibit T cell activation and Th1 differentiation [57]. Although beneficial for the microbe, this concept may be amenable to being co-opted for therapeutic use as multivalent CEACAM1 molecules such as CEACAM1-Fc fusion proteins have been shown to exhibit a protective presumably agonistic effect against T-cell mediated disorders as shown in mouse models of inflammatory bowel disease (oxazole- and TNBS-induced colitis) [57] and multiple sclerosis (experimental autoimmune encephalomyelitis, EAE) [147]. Similarly, forced overexpression of intestinal CEACAM1 through trans-rectal delivery of adenovirus containing CEACAM1-4L alleviates DSS-mediated colitis [148]. These studies suggest that homophilic native ligands of

CEACAM1 that bind in an oligomeric manner may mimic physiologic *trans* interactions with the proper supramolecular organization to transmit signals associated with CEACAM1-L or CEACAM1-S isoforms.

Antibodies are also often used experimentally or therapeutically as multivalent ligands to mimic or disable the physiologic functions of the target receptor. The Anti-CD3 ϵ antibody OKT3 [149] is, for example, used to stimulate TCR-mediated signaling which is therapeutically useful in inducing tolerance for autoimmunity [150]. However the multivalent properties of an anti-CEACAM1 molecule are potentially different in that, depending upon their epitope and mode of binding, can hypothetically bridge CEACAM1 molecules in a *cis* or *trans* configuration resulting in blockade or activation of CEACAM1 function. This may explain conflicting observations with the CC1 monoclonal antibody (mAb) which has been reported to protect against the development of murine colitis [57] but exhibit varying effects in murine cancer models [15,151,152]. The CC1 antibody recognizes residues in the CC' loop of the N-terminal IgV domain of mouse CEACAM1 tangential to the crystallographically resolved homophilic binding site [28] and may therefore variably accommodate higher order *trans* oligomers or *cis* clustering that effectively activate or inhibit CEACAM1, respectively.

The issue of conflicting agonistic and antagonistic properties of anti-CEACAM1 monoclonal antibodies (mAb) was also demonstrated with two N-terminal IgV domain-specific mAbs Be9.2 and 5.4 generated from mice immunized with rat hepatocytes [153]. Although both mAbs were specific for the IgV domain, they exhibited opposite effects on CEACAM1-mediated homophilic binding and multimerization. Whereas mAb 5.4 increased *trans* homophilic interactions, mAb Be9.2 inhibited *trans* interactions and correspondingly increased microclustering suggestive of increased *cis* interactions [83].

Recently, two different classes of antibodies to target human CEACAM1 have been described for potential use in cancer immunotherapy. The first example is the MRG1 monoclonal antibody which has been humanized for therapeutic use [151]. This antibody binds the N-terminal IgV domain of CEACAM1 with high affinity and has been reported to have anti-tumor activity in pre-clinical models [151], through undefined mechanisms as the mode of binding has not been described. A second potential therapeutic agent is a single chain (sc)-Fv fragment (DIATHIS1) that has also been reported to exhibit promising behavior in pre-clinical models of melanoma [154]. As they both exhibit cross-reactivity with multiple CEACAM family members [151,154], possess different modes of binding as a bivalent (MRG1) or monovalent (DIATHIS1) ligand and likely act through different epitopes, they will provide important insights into the design of therapeutics against CEACAM1.

5 Conclusions

CEACAM1 function is initiated by *trans* intercellular homophilic engagement of its N-terminal IgV domain that induces higher order CEACAM1 multimerization and subsequent activation of downstream signaling modules. CEACAM1 signaling can either be inhibitory or non-inhibitory depending on the inclusion or exclusion of two ITIMs, respectively, in the

cytoplasmic tail through alternative splicing. All CEACAM1 isoforms contain the IgV domain that mediates nanomolar-range high affinity homophilic interactions primarily through the CC' and FG loops housed in the GFCC'C'' surface. Various microbes have co-evolved with CEACAM1 and developed a collection of structurally diverse high affinity receptors that also specifically bind the GFCC'C'' face of CEACAM1 IgV at sites that directly overlap with those associated with physiologic homophilic binding. This achieves the dual advantage for the microbe of targeted docking but also precise triggering of CEACAM1 function for enhanced survival through either increased invasion and/or immune evasion. Considering the difficulty in competing with CEACAM1 homophilic interactions to form heterophilic CEACAM1-microbial receptor complexes that are useful to the microbe and the need to precisely avoid binding highly homologous CEACAM family members that are activating (e.g. CEACAM3) [1], this achievement by the microbes is remarkable. More importantly with respect to therapeutic strategies, these unique evolutionary strategies that microbes have evolved provide meaningful lessons on the significance of CEACAM1 function, insight into key structural and potentially intervenable features that determine CEACAM1 homodimerization, and especially the value of targeted CEACAM1 intervention.

CEACAM1 is variably expressed on the surface of several cell types including different myeloid cells and lymphocytes. In the latter case, it is strictly dependent upon activation wherein it modulates immune responses with a critical role in tolerance pathways. Owing to the expression of various splice isoforms, CEACAM1 can be viewed as a molecular rheostat that features inhibitory and non-inhibitory properties depending on the CEACAM1-L:CEACAM1-S ratio. As such, CEACAM1 isoform expression has important implications for autoimmune and infectious diseases as well as anti-cancer immunity. Although not discussed in this review, CEACAM1 has equally relevant roles in directly regulating the growth and metastatic behavior of tumors and controls vascular and lymphatic neogenesis making it particularly important as a therapeutic target in cancer therapy [11,155,156].

The work to date in understanding CEACAM1 as summarized in this review has made it a ripe target for therapeutic intervention. The major challenges in the development of targeted CEACAM1 therapeutics focus on the nature of the high affinity GFCC'C''-mediated CEACAM1 homophilic interaction. Any exogenously delivered antagonistic molecule must specifically compete with the host CEACAM1 IgV domain to gain access to the GFCC'C'' surface in order to provide effective blockade of native CEACAM1 function. However, as most therapeutic agents are designed to be multivalent to enhance overall affinity through avidity, there is the risk of inadvertent induction of CEACAM1 multimerization either in *trans* or *cis* that may possess agonistic properties which must be avoided. Therefore, further careful dissection of the GFCC'C'' surface as has been performed by microbes throughout evolution [46,157], as well as the effects that this bestows upon therapeutic ligation of CEACAM1 will be critical for the development of agents that effectively and safely target CEACAM1 for immune-related disorders and cancer.

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Highlights

- CEACAM1 is expressed on the surface of a variety of cell types and plays a significant role in mediating cellular adhesion and immune function.
- CEACAM1 functions as a molecular rheostat that exerts a tuned signal determined by the net contribution of various CEACAM1 isoforms which possess inhibitory or non-inhibitory profiles dependent on the inclusion of ITIM domains in the cytoplasmic tail
- The GFCC'C'' surface of the CEACAM1 N-terminal IgV domain serves as the dominant binding region shared by its homophilic ligand CEACAM1 and its heterophilic host and microbial ligands.
- Microbes have evolved a diverse array of receptors that specifically target the GFCC'C'' surface of the CEACAM1 IgV and modulate CEACAM1 function

Figure 1. CEACAM1 structure, oligomerization, activation and targeted intervention.

A. Human and mouse CEACAM1 isoforms. The 12 unique human isoforms each contain the N-terminal IgV domain but due to alternative splicing, contain up to 3 IgC2 domains (A1,B,A2) and/or an Alu sequence, a transmembrane sequence and a short or long ITIM-containing cytoplasmic tail. The 4 mouse isoforms all contain the N-terminal IgV domain and either 3 or 1 IgC2 domain, a transmembrane domain and either a short or long ITIM-containing cytoplasmic tail. The homophilic and heterophilic binding surface on the IgV domain is denoted in red, glycosylation sites are depicted as brown circles and the ITIM tyrosines are denoted by black circles. **B. hCEACAM1 IgV homodimer.** One of the hCEACAM1 IgV binding partners is drawn in ribbon form (left) with residues involved in the hemophilic interaction surface drawn in stick model and labeled. The other hCEACAM1 binding partners is depicted in as a surface with the residues involved in hemophilic binding colored red. **C. hCEACAM1 IgV homophilic interaction surface.** The hCEACAM1 homophilic binding region on the GFCC'C'' surface is denoted in red with surface representation of the interacting residues labeled. **D. CEACAM1 oligomerization,**

activation and intervention. *Left*, CEACAM1 in monomeric and *cis* dimeric form on the membrane. *Middle*, *trans* engagement of the CEACAM1 IgV by homophilic GFCC'C''-mediated interactions (top) and heterophilic GFCC'C''-mediated microbial ligands interactions (bottom) induce CEACAM1 multimerization and activation of CEACAM1-mediated signaling pathways. *Right*, specific engagement of the CEACAM1 IgV GFCC'C'' homophilic/heterophilic binding surface by an antibody disrupts the ability of CEACAM1 to participate in *trans* interactions and undergo multimerization thereby defusing CEACAM1-mediated signaling.