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Carcinoembryonic Antigen (CEA) Inhibits NK Killing via Interaction with CEA-Related Cell Adhesion Molecule 1

Noam Stern,* Gal Markel,* Tal I. Arnon,* Raizy Gruda,* Henry Wong,† Scott D. Gray-Owen,† and Ofer Mandelboim2*

The NK killing activity is regulated by activating and inhibitory NK receptors. All of the activating ligands identified so far are either viral or stress-induced proteins. The class I MHC proteins are the ligands for most of the inhibitory NK receptors. However, in the past few years, several receptors have been identified that are able to inhibit NK killing independently of class I MHC recognition. We have previously demonstrated the existence of a novel inhibitory mechanism of NK cell cytotoxicity mediated by the homophilic carcioembryonic Ag (CEA)-related cell adhesion molecule 1 (CEACAM1) interactions. In this study, we demonstrate that CEACAM1 also interacts heterophilically with the CEA protein. Importantly, we show that these heterophilic interactions of CEA and CEACAM1 inhibit the killing by NK cells. Because CEA is expressed on a wide range of carcinomas and commonly used as a tumor marker, these results represent a novel role for the CEA protein enabling the escape of tumor cells from NK-mediated killing. We further characterize, for the first time, the CEACAM1-CEA interactions. Using functional and binding assays, we demonstrate that the N domains of CEACAM1 and CEA are crucial but not sufficient for both the CEACAM1-CEACAM1 homophilic and CEACAM1-CEA heterophilic interactions. Finally, we suggest that the involvement of additional domains beside the N domain in the heterophilic and homophilic interactions is important for regulating the balance between cis and trans interactions. The Journal of Immunology, 2005, 174: 6692–6701.

The human carcinoembryonic Ag (CEA) family is composed of 29 genes tandemly arranged on chromosome 19q13.2. Based on nucleotide homologies, these genes are classified into two major subfamilies, the CEACAM and the pregnancy-specific glycoprotein subgroups (1, 2). The CEACAM-encoded proteins include CEA, CEA-related cell adhesion molecule (CEACAM)1, CEACAM3, CEACAM4, CEACAM6, CEACAM7, and CEACAM8 (2). The CEACAM family belongs to the Ig superfamily. Structurally, each of the human CEACAMs contains one N-terminal domain that includes 108–110 aa and is homologous to Ig variable domains, followed by a different number (zero to six) of Ig C2-type constant-like domains (3). The CEACAM proteins can interact homophilically and heterophilically with each other (4).

CEACAM1 is a unique protein within this family because it contains an ITIM motif in its cytoplasmic domain (2). A similar ITIM is present in various inhibitory receptors that function to antagonize kinase-dependent signaling cascades initiated by lymphocyte activation. This inhibitory effect is triggered by phosphorylation of tyrosine residues within the ITIM, which results in recruitment of the Src homology 2 domain-containing tyrosine phosphatase-1 and -2 (5–7). The CEACAM1 protein is expressed on a variety of immune cells including monocytes, granulocytes, activated T cells, B cells, and NK cells (1, 2, 8).

Our laboratory has recently demonstrated that CEACAM1 homophilic interactions inhibit NK killing independently of MHC class I recognition (8). The CEACAM1 interactions are probably important in some cases of metastatic melanoma, as increased CEACAM1 expression was observed on NK cells derived from such patients compared with healthy donors (8). A major role for CEACAM1 on NK cells was also demonstrated in TAP2-deficient patients and during pregnancy (9, 10). In addition it was demonstrated that CEACAM1 engagement by the Opa proteins of Neisseria gonorrhoeae inhibits T cell activation and proliferation (5). It is well established that the CEACAM1 homophilic interactions are mediated through the N domain, and several publications have identified specific amino acids that are involved in these interactions (11, 12). It has also been previously reported that CEACAM1 interacts with other CEACAM protein family members, such as CEA (12, 13).

Our laboratory has previously demonstrated the dichotomy of CEACAM family members by showing that the recognition of CEACAM1 is determined by the presence of R and Q residues at positions 43 and 44, respectively (12). Importantly, the CEA protein contains these RQ residues, suggesting that these residues are involved in binding to CEACAM1. The CEA protein is of great interest, because it is expressed on a wide range of carcinomas and is commonly used as a tumor marker in the prognosis and management of many types of cancer (14, 15). CEA consists of an Ig V-like N-terminal domain followed by three pairs of Ig C2-like domains (denoted AxBx) which are terminated by a hydrophobic domain, that is processed to allow the addition of glycophosphatidylinositol membrane anchor (2, 16). In addition to the homophilic interaction of CEA with CEACAM1, CEA binds homophilically to itself. The CEA-CEA interactions were shown to be

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3 Abbreviations used in this paper: CEA, carcinoembryonic Ag; CEACAM, CEA-related cell adhesion molecule; mIL-2, murine IL-2.
mediated by double reciprocal bonds between the N and A3B3 domains of antiparallel CEA CEA domains on the opposed cell (17). However, until now, nothing was known about the nature of the CEA interactions with CEACAM1 or the functional consequences of these interactions.

In this work, we demonstrate that CEACAM1 binding to CEA is direct and functional. The heterophilic interactions between CEA and CEACAM1 inhibit the killing of NK cells that express the CEACAM1 protein. By using various combinations of the different CEA domains and by using functional assays, we further demonstrate that the N domains of both CEACAM1 and CEA are crucial but not sufficient for the heterophilic interactions. Furthermore, we show that each pair of A and B domains within CEA contributes to the intensity of the CEA interaction with CEACAM1. Finally, we show that the N domain of CEACAM1 plays a critical role in the functional CEACAM1 homophilic interactions.

Materials and Methods

Cells

The cell lines used in this work were: the MHC class I-negative 721.221 (221), 221 cells stably transfected with the CEACAM1 protein (221/CEACAM1), and 221 expressing the CEA protein (221/CEA) (12). The murine thymoma BW cell line which lacks expression of α- and β-chains of the TCR. The generation of the BW cell line expressing the extracellular portion of the human CEACAM1 protein fused to mouse Fc-chain (BW/CEACAM1) was previously described (10). The generation of YTS NK tumor expressing the CEACAM1 protein (YTS/CEACAM1) and the CEACAM1 in which the cytoplasmic tail of the molecule was truncated (and therefore does not include the ITIM: YTS/CEACAM1Trunc) was previously described (8). Primary NK cells were isolated from peripheral blood lymphocytes, using the human NK isolation kit and the autoMACS instrument (Miltenyi Biotec). For the enrichment of CEACAM1-negative NK cells, isolated NK cells were further purified by depletion of CD16-positive NK cells, using the anti-CD16 mAb B73.1.1 and the autoMACS instrument. NK cells were grown in culture as previously described (18).

Antibodies

The Abs used in this work were mAb Kat4c (DakoCytomation), directed against CEACAM1, 5, 6, 8, and rabbit polyclonal anti-CEACAM1 (DakoCytomation). Anti-CD99 mAb 12E7 was used as control.

Generation of Ig fusion protein and FACS staining

The generation of LIR1-Ig and CD99-Ig fusion proteins was previously described (10, 19). The sequence encoding the full extracellular portion of CEA protein was amplified by PCR using the following primers: 5′ primer GGATCCACTATCAGAGCAACCCCAAC (including HindIII restriction site) and the 3′ primer GGATCCGGCAAGGAGACTGGAATTC (including BamHI restriction site). A silent mutation, adenine TCGACGACCCCACCATTTCCC. We also amplified by PCR the CEACAM1 D2-D3 (CEACAM1 N) domains using 5′ primer CCGGATCCCTACATCACGAAAGCGCA (including EcoRI restriction site) and 3′ primer CCGTCGAGAGCTACGTTCTCTAGTGA. The various PCR fragments were then cloned into the pcDNA3 containing the leader peptide of CEA and stably transduced into the 221 cell line.

Cytotoxicity assays and ELISA

The cytotoxic activity of NK and YTS cells against the various targets was assessed in 5-h 3H release assays as previously described (18). In all presented cytotoxicity assays, the spontaneous release was <25% of maximal release. In experiments in which Abs were included, the final Abs concentration was 10 μg/ml, or 40 μg/ml in those cases where rabbit polyclonal Abs were used. The measurement of IL-2 production resulting from the BW/CEACAM1 interactions with different cells was performed as previously described (10). For ELISA, ELISA plates were coated with 0.1 μg well of purified CEA (Biodesign International) followed by incubation with 5 μg of the relevant fusion protein. Bound proteins were detected using AP-conjugated secondary mAb.

Results

The heterophilic interactions between CEACAM1 and CEA are functional

We have previously demonstrated that the entire family of the CEACAM proteins can be divided into two groups based on the presence or absence of the RQ residues in the N domain (12). In addition, we and others demonstrated that CEACAM1 interacts with CEA (12, 13). However, the functional consequences of the CEACAM1-CEA interactions were never investigated. For measuring direct binding of CEACAM1 and CEA to the purified CEA protein, the extracellular portion of the CEACAM1 and CEA were fused to the Fc portion of human IgG1, thus creating CEACAM1-Ig and CEA-Ig, respectively. Direct binding of CEACAM1-Ig and CEA-Ig to purified CEA was tested in ELISA. As demonstrated in Fig. 1A, heterophilic binding of CEACAM1-Ig and a stronger homophilic binding of CEA-Ig to the purified CEA were observed. The control LIR1-Ig fusion protein showed only weak binding (Fig. 1A).

We next examined the heterophilic and homophilic interactions between CEACAM1 and CEA in the context of mammalian cells by using cell transfectants. 221 cells were transfected with CEACAM1 cDNA (221/CEACAM1) and with the CEA cDNA (221/CEA) and the NK tumor cell line YTS was transfected with CEACAM1 cDNA (YTS/CEACAM1). Expression levels were monitored with Kat4c mAb (Fig. 1, B–D). Homophilic and heterophilic binding of the CEACAM1-Ig fusion protein was observed to 221/CEACAM1 and 221/CEA, respectively (Fig. 1, B and C). Similarly, homophilic and heterophilic binding of the CEA-Ig fusion protein was observed to 221/CEA and 221/CEACAM1, respectively (Fig. 1, B and C). A weak homophilic

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F(ab’), of goat anti-human IgG (Jackson ImmunoResearch Laboratories). Incubation was performed for 1 h and cells were analyzed by flow cytometry with a FACSscan (BD Immunocytometry Systems).

Generation of 721.221 cells expressing truncated CEA and CEACAM1 proteins

For the generation of the truncated CEACAM proteins, we amplified by PCR the leader peptide of CEA using 5′ primer GATCCCATATCAGAGCAACCCCAC (including HindIII restriction site) and the 3′ primer GAATTCGGCAAGGAGACTGGAATTC (including EcoRI restriction site) The PCR-generated fragment was cloned into pCDNA3 mammalian expression vector. Next, we amplified by PCR the relevant CEA domains using different 5′ primers (including EcoRI restriction site) and the same 3′ primer CGCGTCCGAATTCATATCGAAGCCACCCAC (including XhoI restriction site). The 5′ primer CEA D2-D7 (CEA ΔN), CCGGAATT CAAGCCCTCTATCGAAGCCACCCAC; 5′ primer CEA D4-D7, CGCGGATCTAACCTTTCATACGACACCA; 5′ primer CEA D5-D7, CCGGAATTCGACGACCCCAACATTTC. We also amplified by PCR the CEACAM1 D2-D3 (CEACAM1 ΔN) domains using 5′ primer CCGGATCCCTACATCACGAAAGCGCA (including EcoRI restriction site) and 3′ primer CCGTCGAGAGCTACGTTCTCTAGTGA. The various PCR fragments were then cloned into the pcDNA3 containing the leader peptide of CEA and stably transduced into the 221 cell line.
and heterophilic binding of CEACAM1-Ig and CEA-Ig was observed to YTS/CEACAM1, respectively, probably due to relatively low expression levels of the CEACAM1 protein (Fig. 1D). The control CD99-Ig fusion protein did not stain any of the transfectants tested (Fig. 1B–D).

We next examined the heterophilic and homophilic interactions between CEACAM1 and CEA with NK cells that endogenously express CEACAM1. NK cells were isolated from healthy donors, activated NK clones were grown and stained with Kat4c mAb (representative CEACAM1-positive clone is shown in Fig. 1E). Importantly, homophilic and heterophilic binding of the CEACAM1-Ig and CEA-Ig fusion proteins were observed to the CEACAM1-positive NK clones (Fig. 1E). Thus both the CEA and the CEACAM1 proteins are able to interact with CEACAM1 on the surface of various cells.

To test the functional relevance of the heterophilic interactions between CEACAM1 and CEA we first used the BW cell system. BW cells expressing the extracellular portion of CEACAM1 fused to mouse ζ-chain (BW/CEACAM1-ζ) were generated as previously described (10). Engagement of the CEACAM1 protein on those cells elicited the secretion of murine IL-2 (mIL-2) (10). The BW parental cells and the BW/CEACAM1-ζ were coincubated with no cells, or with irradiated 221, 221/CEACAM1, and 221/CEA cells for 48 h either with anti-CEACAM polyclonal blocking Abs or with anti-human GST (hGST) Abs as a control. Significant amounts of mIL-2 were detected in the supernatant of BW/CEACAM1-ζ cells coincubated with no cells or 221 cells in the presence of the control anti-hGST Abs. Adding the anti-CEACAM1 blocking Abs significantly reduced the mIL-2 secretion, demonstrating that the observed mIL-2 secretion was due to homophilic interaction between different BW/CEACAM1-ζ cells (Fig. 2A). Significantly higher amounts of mIL-2 were detected in the supernatant of BW/CEACAM1-ζ cells coincubated with 221/CEACAM1 and with 221/CEA cells compared with BW/CEACAM1-ζ cells coincubated with no cells or 221 cells in the presence of control Abs. Adding the anti-CEACAM1 blocking Abs partially reduced the mIL-2 secretion demonstrating that the observed elevated secretion of mIL-2 resulted from CEACAM1 interactions between the BW/CEACAM1-ζ cells and 221 cells expressing either CEACAM1 or CEA. (Fig. 2A). No secretion of mIL-2 was
observed when the parental BW cells were used (data not shown). These results clearly indicate that CEACAM1 and CEA proteins heterophilically interact with each other and that these interactions are functional.

We have previously reported that CEACAM1 expression on NK cells plays a major role in inhibition of NK cytotoxicity via the homophilic CEACAM1 interactions (8–10, 12). The CEA protein is an important tumor marker expressed in colorectal and other carcinomas (15). Because CEA is expressed on tumor cells and as shown above, is able to interact heterophilically with CEACAM1 (Fig. 1), it was of a particular importance to test whether the CEA protein can also inhibit the killing by CEACAM1-positive NK cells. NK cells were isolated from healthy donors; activated NK clones were grown and stained for CEACAM1 expression. CEACAM1-positive NK clones (representative clone is shown in Fig. 2B) were assayed for killing against 221, 221/CEACAM1, and 221/CEA cells in various E:T ratios either with anti-CEACAM blocking polyclonal Abs or with anti-CD99 Ab as a control (Fig. 2C). As was previously reported (8, 10), inhibition of NK killing was observed when 221/CEACAM1 cells were used and this inhibition could be reversed by the anti-CEACAM Abs (Fig. 2C). Importantly, a significant inhibition of CEACAM1-positive NK clones was also observed when 221/CEA cells were used (Fig. 2C). This inhibition was the result of CEACAM1 and CEA heterophilic interactions, as anti-CEACAM Abs abrogated this effect (Fig. 2C). In healthy people CEACAM1 is expressed on CD16-negative NK cells (21). Indeed, the CEACAM1-positive NK clones tested in this study did not express CD16 (data not shown).
CD16 is the major FcR on NK cells involved in ADCC (22). Nevertheless, to prove beyond any doubt that the restoration of NK killing mediated by the polyclonal anti-CEACAM Abs was not due to ADCC phenomenon; the NK clones were incubated with polyclonal anti-hGST Abs before killing to block any available FcR (Fig. 2, C and D).

The CEA-mediated inhibition was variable between the different NK clones tested (Fig. 2D) probably because the killing of target cells is determined by a balance between the inhibitory and activating signals. However, as can be seen in Fig. 2, C and D, in all clones expressing CEACAM1, the CEA-mediated inhibition was significant and was abrogated by the addition of anti-CEACAM Abs.

We demonstrated above that CEACAM1 homophilic interactions between opposing BW/CEACAM1-CEACAM Abs.

all clones expressing CEACAM1, the CEA-mediated inhibition was significant and was abrogated by the addition of anti-CEACAM Abs.

We demonstrated above that CEACAM1 homophilic interactions between opposing BW/CEACAM1-CEACAM Abs are functional, leading to the secretion of IL-2 without the presence of target cells (Fig. 2A). Therefore, it can be suggested that NK cells expressing CEACAM1 will undergo homophilic interactions with each other and that these interactions would result in a general inhibition of NK cytotoxicity. To elucidate whether homophilic interactions between CEACAM1-positive NK cells affect the overall cytotoxicity of these cells, we incubated YTS/CEACAM1 and CEACAM1-positive NK clones either with anti-CEACAM Abs or with anti-hGST Abs as a control. The cells were then tested in killing assays against 221 parental cells. No significant increase in the lysis of 221 cells by YTS/CEACAM1 cells was observed when anti-CEACAM Abs were added (Fig. 2E). In addition, in most of the CEACAM1-positive NK clones tested, the addition of the blocking CEACAM Abs had little or no significant effect on the observed killing (one representative clone is shown in Fig. 2E). Therefore, we conclude that homophilic interactions between NK cells expressing CEACAM1 do not affect the overall cytotoxicity of these cells.

The CEACAM1 protein contains two ITIM sequences located within its cytoplasmic tail. These motifs have been shown to mediate inhibitory functions in epithelial cells and T cells (5–7, 23). To demonstrate that the ITIM motifs are important for the observed inhibition of NK cell cytotoxicity, we used the YTS cells. We have previously transfected this cell line either with CEACAM1 protein (YTS/CEACAM1) or with CEACAM1 protein in which the ITIM motifs were truncated (YTS/CEACAM1 trunc) (8). The various YTS transfectants were tested in killing assays against the 221 transfectants. In agreement with the above results, moderate, but significant inhibition of YTS/CEACAM1 killing was observed when cells were incubated with 221/CEACAM1 and with 221/CEA compared with the 221 parental cells (Fig. 3A). The moderate inhibition correlated with the low CEACAM1 expression on the YTS cells (Fig. 1D). Importantly no inhibition of lysis by YTS/CEACAM1 trunc cells was observed (Fig. 3B). These results indicate that the CEACAM1 inhibitory signal is probably transduced via the ITIM sequences in its cytoplasmic tail.

The N domain of both CEACAM1 and CEA is critical but not sufficient for the heterophilic and homophilic binding

The CEACAM-related proteins share a common basic structure of several sequential Ig like domains beginning with an Ig V-like N domain and proceeding with different number of Ig C2-like domains. It was postulated before that CEACAM1 interacts homophilically in “head-to-head” N domains interactions (11). Additionally, it was reported that the CEA protein interacts homophilically through “head-to-tail” interactions between the Ig V-like N domain and the Ig C2-like domain (17). However, the nature of the CEACAM1-CEA interactions was not investigated. To determine whether the Ig V-like N domains are crucial for the binding of CEACAM1 to CEA, we generated a mutated construct of CEACAM1 and CEA that does not contain the N domain (CEACAM1 ΔN) and (CEA ΔN), respectively. The 221 cells were stably transfected with those constructs and tested for expression using the anti-CEACAM polyclonal Abs (Fig. 4, A and B). Next, we tested the binding of CEACAM1-Ig to 221/CEA and 221/CEA ΔN. Remarkably, the deletion of the N domain from CEA abolished the heterophilic binding, as CEACAM1-Ig did not bind to 221/CEA ΔN (Fig. 4A). The homophilic binding of CEA-Ig was also abolished when 221/CEA ΔN were used (data not shown). We then performed the reciprocal experiments and tested the CEA-Ig and CEACAM1-Ig for binding to 221/CEACAM1 and 221/CEACAM1 ΔN cells. Importantly, the deletion of the N domain of CEACAM1 abolished the homophilic binding of CEACAM1-Ig and the heterophilic CEA-Ig binding (Fig. 4B). These results indicated that the N domains of both CEA and CEACAM1 proteins are critically involved in their heterophilic and homophilic interactions.

Our next question was whether the N domain by itself would be sufficient for the heterophilic and homophilic interactions. Therefore, we constructed two additional fusion proteins, containing either the CEACAM1-N domain (CEACAM1-N-Ig) or the CEA-N domain (CEA-N-Ig). Surprisingly the CEACAM1-N-Ig and CEA-N-Ig did not bind 221/CEACAM1 or 221/CEA cells (Fig. 4C).
Furthermore, even when measuring direct binding of CEACAM1-N-Ig and CEA-N-Ig to purified CEA protein no binding was detected (Fig. 4D). It is unlikely that the binding was abolished due to the possible altered conformation of the N domains because we used the entire domain, which by definition can fold independently. Furthermore, when domain A and B were included in the Ig fusion protein, binding was restored (see Fig. 7B) and finally, the CEACAM3 contains only the N domain. These combined results suggest that the N domains on both CEACAM1 and CEA are critical but not sufficient for heterophilic and homophilic binding.

The functional CEACAM1-CEA heterophilic interactions and CEACAM1 homophilic interactions are crucially dependent on the N domain

As shown above (Fig. 4), the N domains of CEA and CEACAM1 are crucial, but not sufficient, for the heterophilic binding between CEA and CEACAM1 and the homophilic binding of CEACAM1. Therefore, we examined which domains are involved in the functional interactions and whether the N domain is crucial for these interactions. To test the involvement of different domains, we generated truncated constructs of CEACAM1 and CEA as illustrated in Fig. 5A. The 221 cells were stably transfected with these various constructs and tested for expression using Kat4c mAb (Fig. 5B).

The interactions between the various truncated proteins expressed on 221 cells were first examined using the BW transfected system. Significant amounts of mIL-2 were detected in the supernatant of BW/CEACAM1-ζ cells coinoculated with 221/CEACAM1 and with 221/CEA cells (Fig. 6A). Importantly, minimal amounts of mIL-2 were detected in supernatants of BW/CEACAM1-ζ coinoculated with 221/CEACAM1 ΔN, 221/CEA ΔN, 221/CEA D4-D7, and with 221/CEA D5-D7. Therefore, deletion of CEACAM1 N domain and CEA N domain was sufficient to abolish the functional homophilic and heterophilic interactions with BW/CEACAM1-ζ cells. Murine IL-2 was not detected when BW parental cells were used (data not shown).
The BW results were further confirmed using functional killing assays. CEACAM1-positive NK clones were tested in killing assays against the various 221 transfectants. Inhibition of NK killing was observed when 221/CEACAM1 cells 221/CEA cells were used (Fig. 6B). Importantly and in agreement with the above binding results (Fig. 4A), no inhibition was observed when 221/CEACAM1ΔN were used. Therefore, the deletion of the N domain is enough to abolish any functional interactions between two CEACAM1 proteins. In addition, no inhibition was observed when 221/CEAΔN, 221/CEA D4-D7, and 221/CEA D5-D7 cells were used. Therefore, although we demonstrate that other domains besides the N domain are involved in the CEACAM1 binding to CEA, the CEA N domain is crucial, because in the absence of the N domain, none of the other CEA domains were able to confer inhibition (Fig. 6B).

The heterophilic binding of CEACAM1 and CEA is dependent on the presence of the A, B, and N domains in CEA

Because we could not detect any binding between CEACAM1 and CEA when the N domains were removed (Fig. 4A), we generated truncated CEA fusion proteins containing the CEA V Ig-like N domain and different number of C2 Ig like domains as illustrated in Fig. 7A.

These fusion proteins enabled us to examine which domains, in addition to the N domain, are important for the heterophilic interaction between CEACAM1 and CEA. We tested the 221/
Figure 7. The heterophilic binding of CEACAM1 and CEA is dependent on the presence of A, B, and N domains. A. Schematic illustration of the soluble proteins used in this work. The generation of the various fusion proteins is described in Materials and Methods. Shown are the different CEA extracellular domains fused to the Fc portion of human IgG1. The names of the fusion proteins indicate which domains were left in the soluble protein. B. Staining of 221/CEACAM1 with various fusion proteins as indicated in each histogram (open histogram). The corresponding staining of 221 parental cells was used as background (gray histogram). Shown is one representative staining of four performed.

CEACAM1 for binding by the various fusion proteins. The full length CEA-Ig strongly recognized the 221/CEACAM1 with mean fluorescence intensity of 127. Deletion of D6 and D7 domains (A3, B3) in CEA-D5-D1-Ig led to a small decrease in the binding efficiency to the 221/CEACAM1 cells (Fig. 7B). The CEA-D3-D1-Ig containing only the A1, B1 domains together with the N domain still binds the 221/CEACAM1 cells, but with lower mean fluorescence intensity demonstrating that these domains are sufficient for the heterophilic binding. Remarkably, when the D3 (B1) domain was deleted as in CEA-D2-D1-Ig, the binding to 221/CEACAM1 cells was abolished (Fig. 7B). These results reveal that together with the N domain, each pair of the A and B domains of the CEA protein is probably sufficient for binding to CEACAM1 and that the presence of all A and B domains in the native form of the protein increases the intensity of the binding of CEA to CEACAM1.

Discussion
Different functions were implicated for the CEA protein on tumors; mediating intercellular adhesion strengthens the metastatic potential of the tumor cells (24), disrupting tissue structure and inhibition of cell differentiations (16, 25). However, until now, no immunological function was attributed to CEA.

We have recently demonstrated that the homophilic CEACAM1 interactions are strong enough to deliver inhibitory signals, thereby decreasing human NK-mediated cytoxicity and decidual lymphocyte functions (8, 10, 12). In this study, we demonstrate for the first time that the CEA protein can potentially protect the tumors from NK cell-mediated killing. We demonstrate by direct ELISA (Fig. 1A) and by FACS staining (Fig. 1, B–E) that CEACAM1 binds to the CEA protein. We further proved by blocking and lysis experiments that these heterophilic interactions between the CEACAM1 on NK cells and CEA on the target cells inhibit NK killing (Fig. 2). These findings suggest a new key role for the CEA, enabling tumors to evade attack by CEACAM1-positive NK cells.

In healthy people, CEACAM1 is expressed only on the surface of activated, CD16-negative NK cells (21). However, as we have demonstrated previously, in some melanoma patients, a dramatic increase in CEACAM1 expression can be observed on the surface of NK cells, independently of activation and CD16 expression (8). The reason why CEACAM1 expression is up-regulated on the surface of NK cells in these patients is not understood, but it demonstrates the possible importance of CEA-CEACAM1 interactions as a defense mechanism enabling tumors to escape NK cell attack. In addition the CEACAM1 protein is expressed on a variety of other immune cells, including monocytes, granulocytes, activated T cells, and B cells (1, 2, 8). Therefore, it is possible that the CEACAM1-CEA interactions might be important for tumor escape not only from NK killing but also from other immune cells.

It can be suggested that NK cells expressing CEACAM1 will undergo homophilic interactions with each other and that these interactions would result in general inhibition of NK cytoxicity. Similarly it can be argued that interactions between different killer cell Ig-like receptors expressed on a particular NK cell with MHC class I molecules expressed on opposed NK cell would result in inhibition of NK killing. It was previously demonstrated that in the case of killer cell Ig-like receptors and MHC class I interactions there is no inhibition of NK cytoxicity in trans (26). In agreement with these findings, in most of the NK clones tested in this study, no significant increase in the killing of 221 cells was observed when anti-CEACAM Abs were added (Fig. 2E). Therefore we propose that in NK clones, homophilic interactions between different NK cells expressing CEACAM1 do not significantly affect the overall cytotoxicity of these NK cells. However, it is clear that homophilic interactions between CEACAM1 proteins on effector cells are formed as secretion of mIL-2 was observed from BW/CEACAM1-ζ cells even without the presence of target cells (Fig. 2A). Thus the homophilic CEACAM1 interactions on NK cells do exist and might be effective in long term assays. Indeed, we often observed that CEACAM1-positive NK clones are quite difficult to grow and that the proliferation of such clones is often slower than that of CEACAM1-negative clones. Alternatively, it is possible that the CEACAM1 homophilic interactions on NK cells would affect the overall NK cytoxicity in rare situations when CEACAM1 is expressed in high levels on NK cells. Indeed, we could observe a significant increase in the secretion of mIL-2 when targets cells expression CEACAM1 or CEA in high levels (Fig. 1, B and C) were included in the assay (Fig. 2A).

The N-domains have an important role in mediating interactions between the various CEACAM proteins (27, 28). It was previously demonstrated that CEACAM1 interacts homophilically through interactions between the N-domains of the two molecules (11). Additionally, it was reported that the CEA protein interacts homophilically through reciprocal interactions between the Ig V-like
N domain and a Ig C2-like domain on opposite molecules (17). We show in this study that the N domain of both CEACAM1 and CEA is crucial but not sufficient for heterophilic interactions between CEACAM1 and CEA (Fig. 4). Furthermore, the N domain is required for functional interactions between CEA and CEACAM1 (Fig. 5). In addition, we show that deletions of each pair of A and B domains (Ig C2-like domains) reduce the intensity of the CEA binding to CEACAM1 (Fig. 6). These results indicate that the N domains and the Ig C2-like domains participate in the heterophilic interactions between CEACAM1 and CEA. Therefore, it is likely that the heterophilic interactions between CEACAM1 and CEA are similar to those reported between CEA and CEA, and are also mediated by the reciprocal binding of the N domain on one molecule with the Ig C2-like domain on the opposite molecule.

Because we show that deletions of each pair of A and B domains (Ig C2-like domains) reduce the intensity of the CEA binding to CEACAM1 (Fig. 6), we assume that the CEACAM1 N domain can bind to each pair of A and B domains and, therefore, the avidity of CEACAM1 binding to intact CEA protein, containing three pairs of A and B domains, is the strongest.

When considering the binding of CEA to CEACAM1, we must bear in mind that the CEA protein is four domains longer than the CEACAM1 isoform used in this work (illustrated in Fig. 4A). For this reason, we assume that the CEA protein is able to bend and, therefore, enable the binding of the N domain of CEACAM1 to any of the CEA Ig C2-like domains reciprocally to the binding of the bended CEA N domain to the Ig C2-like domains of CEACAM1. Although we suggest reciprocal head-to-tail binding between CEACAM1 and CEA, we demonstrate that the deletion of the N domain of CEA was sufficient to abolish the inhibitory effect. Therefore, to deliver inhibitory signals, both N domains of CEA and CEACAM1 have to participate in the binding (Fig. 5B).

We also present some new insights regarding the CEACAM1 homophilic interactions. It has been argued that the CEACAM1 interactions are mediated through interactions of the two N domains (11). However, we demonstrate that the N domain by itself is not sufficient for homophilic interactions between CEACAM1 molecules (Fig. 3C). These results suggest that even in the case of homophilic CEACAM1 interactions the Ig C2-like domains are involved in the binding. Previous studies have shown similar results as deletions of the N domain alone (11, 29) or of all the Ig C2-like domains together (29) abrogated the adhesion of CEACAM1. Importantly, deletion of the CEACAM1 N domain completely abolished the inhibition of NK cytotoxicity mediated via CEACAM1 homophilic interactions. Therefore, similar to the CEA and the CEACAM1 heterophilic interactions the CEACAM1 homophilic interactions are fully dependent on the N domains. Nevertheless, this does not preclude that in addition to the binding between the N domains of CEACAM1 there is a lower affinity reciprocal binding of the N domains to the Ig C2-like domains on the opposite molecule (similar to the CEA homophilic binding) which increases the avidity of the binding. Another possible mode of interaction that can explain the involvement of the Ig C2-like domains in the homophilic CEACAM1 binding may be similar to the suggested interactions between ICAM-1 and LFA-1. The crystal structure of the two N-terminal domains of ICAM-1 has revealed that both domains function in LFA-1 binding, the first interacting with residues on LFA-1 and the second is involved in orienting the recognition surface of the first domain (30). It is possible that in the CEACAM1 interactions residues in the N domains interact with each other whereas the Ig C2-like domains are responsible for the correct orientation.

It might be speculated that the involvement of the Ig C2-like domains in the homophilic binding of CEACAM1 and in the heterophilic binding between CEACAM1 and CEA is important for regulating the balance between cis- (interaction between molecules on the same cell) and trans (interaction between molecules on opposed cells) interactions. It is possible that through binding of the N domain of one molecule to the Ig C2-like domain on the opposing cell the counter receptors can achieve optimal contact in trans while the cis-recognition of the receptors on the same cell is prevented.

The regulation of various immune responses is achieved by many different ways. One of which is the distribution of the ligands on target cells and the receptors on the effector cells. This regulation can be hampered when the proteins interact homophilically, because a particular molecule can interact on the same cell with another protein of the same kind. Therefore a different type of regulation is needed to prevent such scenario. The results presented in this study demonstrate for the first time that CEA can inhibit the killing of NK cells by interacting with CEACAM1. Furthermore, we demonstrated a unique molecular basis for the CEACAM1 and CEA interactions that involves a critical binding of the N domain, but additionally includes binding of the other domains of the proteins.

Disclosures

The authors have no financial conflict of interest.

References


