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Intercellular Transfer of Carcinoembryonic Antigen from Tumor Cells to NK Cells[1]

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The inhibition of NK cell killing is mainly mediated via the interaction of NK inhibitory receptors with MHC class I proteins. In addition, we have previously demonstrated that NK cells are inhibited in a class I MHC-independent manner via homophilic carcinoembryonic Ag (CEA) cell adhesion molecules (CEACAM1)-CEACAM1 and heterophilic CEACAM1-CEA interactions. However, the cross-talk between immune effector cells and their target cells is not limited to cell interactions per se, but also involves a specific exchange of proteins. The reasons for these molecular exchanges and the functional outcome of this phenomenon are still mostly unknown. In this study, we show that NK cells rapidly and specifically acquire CEA molecules from target cells. We evaluated the role of cytotoxicity in the acquisition of CEA and demonstrated it to be mostly killing independent. We further demonstrate that CEA transfer requires a specific interaction with an unknown putative NK cell receptor and that carbohydrates are probably involved in CEA recognition and acquisition by NK cells. Functionally, the killing of bulk NK cultures was inhibited by CEA-expressing cells, suggesting that this putative receptor is an inhibitory receptor. ***The Journal of Immunology,*** **2007, 179: 4424–4434.**

Natural killer cells are a subset of lymphocytes that belong to the innate immune branch. These cells provide a first-line defense against various pathogens and transformed cells by their cytotoxic activity and cytokine secretion (1). NK cell recognition of targets involves two types of proteins: activating and inhibitory receptors. Integration of opposing signals from these two types of receptors determines the status of NK cell activation (2). Inhibitory receptors recognize molecules that are expressed on normal cells, thereby protecting healthy cells from NK cell attack. The prototypic inhibitory receptors recognize MHC class I molecules and include in humans the members of the killer Ig-like receptor (KIR),[3] the C-type lectin, and the leukocyte inhibitory receptor families (3). NK cells also express several other inhibitory receptors that recognize ligands which are not related to MHC class I molecules (4). We identified such MHC class I-independent inhibitory mechanism mediated by the carcinoembryonic Ag (CEA) cell adhesion molecule (CEACAM1) receptor. We have previously shown that CEACAM1 on NK cells interact homophilically with CEACAM1 and heterophilically with the CEA on target cells and that these two types of interactions inhibit NK cell killing (5–8).

The CEA protein is overexpressed on a wide range of carcinomas and is commonly used as a tumor marker in the prognosis and management of many types of cancer (9, 10). It consists of an Ig V-like N-terminal domain followed by three pairs of Ig C_2-like domains (11, 12). The CEA protein is heavily glycosylated and this glycosylation plays a critical role in its function (13). For example, it was demonstrated that immature dendritic cells interact with CEA through dendritic cell-specific ICAM-3-grabbing nonintegrin (DC-SIGN) (14). This recognition was based on the specificity of DC-SIGN for Lewis^x (LeX) and Lewis^y moieties on tumor-associated CEA (15).

In recent years, it became evident that an organized structure named the immunological synapse is formed between the effector cells and their targets (16). An interesting consequence of the formation of the immunological synapse in T, B, and NK cells is the observation that an effector cell acquires target cell membrane molecules and incorporates them in its own membrane, a process known as trogocytosis (17–19). NK cells have been shown, for example, to acquire MHC class I molecules from a variety of cells in vitro and in vivo (20–22).

Despite the extensive expression of CEA in many tumors, very little is known about its interaction with immune cells. In this study, we further characterize the interaction between NK cells and the CEA protein. We show that NK cells rapidly acquired CEA molecules from target cells and we demonstrate that this phenomenon is not mediated by the CEACAM1 receptor but instead requires a specific interaction with an unknown putative NK cell receptor.

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[3] Abbreviations used in this paper: KIR, killer Ig-like receptor; CEA, carcinoembryonic Ag; CEACAM, CEA cell adhesion molecule; DC-SIGN, dendritic cell-specific ICAM-3-grabbing nonintegrin; LeX, Lewis X; YFP, yellow fluorescent protein; MICA, major histocompatibility complex class I-related chain A.

Furthermore, we evaluated the role of cytotoxicity in the acquisition of CEA and demonstrated it to be mostly killing independent. Transfer of CEA to the surface of NK cells was evident either with freshly isolated NK cells or with NK clones derived from perforin-deficient patients; in both, the cytotoxic activity is drastically reduced. In addition, CEA transfer was also observed to freshly isolated T cells.

We demonstrate that the transfer is receptor specific and that carbohydrates are probably involved in the CEA acquisition by NK cells. Functionally, the killing of bulk NK cultures was inhibited by CEA-expressing cells, suggesting that the putative receptor that recognizes CEA is probably an inhibitory receptor.

Materials and Methods

Cells

The cell lines used were: the 1106mel melanoma cell line, the MHC class I-negative 721.221 (221). The generation of 221 cells stably expressing CEACAM1 (221/CEACAM1), CEA (221/CEA), and CEACAM6 (221/CEACAM6) was described previously (23). For generating 221/CEA/Cw6 and 221/major histocompatibility complex class I (MICA)-yellow fluorescent protein (YFP)/CEA cells, we used 221/Cw6 and 221/MICA-YFP cells that were described previously (24, 25) and cotransfected them with CEACAM5 cDNA subcloned into pcDNA3.1-containing hygromycin selection.

For the generation of 721.221 cells expressing the CEA protein fused to GFP, we used a GFP-GPI construct in pcDNA3.1 that was previously described (26). We amplified the extracellular portion of the CEA without the GPI-anchoring sequence using the 5′ primer GGTACCGCCACCAT GGAGTCTCCCTCGGCCC (including the *Kpn*I restriction site) and the 3′ primer CCGGAATTCAGATGCAGAGACTGTGATGCT (including the *Eco*RI restriction site). The amplified fragment was cloned into the GFP-GPI construct digested with *Kpn*I and *Eco*RI. All transfectants were periodically monitored for expression by staining with the appropriate mAb.

Primary NK cells were isolated from PBL, using the human NK isolation kit II and the autoMACS instrument according to the manufacturer's instructions (Miltenyi Biotec). NK cells were grown in culture as previously described (24).

Abs and fusion proteins

The Abs used in this work were mAb Kat4c (DakoCytomation), directed against CEACAM1,5,6,8, mAb 5F4 directed against CEACAM1 (27), mAb MCA1744 directed against CEA (Serotec). mAb 6H3 anti-LeX and mAb 4D2 anti-H type I (used as an IgM control) have been previously described (28); these mAbs are both of the IgM isotype. 12E7 an anti-CD99 mAb was used as an IgG control.

For the transfer studies, the following fluorochrome-conjugated mAbs were used: the PE-conjugated anti-human CD56 mAb (BD Pharmingen), the PE-conjugated anti-human CD8 mAb (DakoCytomation), a biotinylated anti-CD4 (OKT4; American Type Culture Collection) followed by streptavidin-Cy5 (Jackson ImmunoResearch Laboratories) as a second reagent, the FITC-conjugated anti-CEACAM (Kat4C; DakoCytomation).

For degranulation assay, FITC-conjugated anti-human CD56 mAb (Sigma-Aldrich) was used together with biotinylated anti-CD107 mAb (BD Pharmingen), followed by streptavidin-PE (Jackson ImmunoResearch Laboratories) as a second reagent.

The generation and production of CEA-Ig and CD99-Ig was previously described (8, 29). Briefly, the extracellular portion of the CEA and CD99 protein were amplified by PCR and the fragments were cloned into a mammalian expression vector containing the Fc portion of human IgG1. COS-7 cells were transiently transfected with these plasmids using FuGENE6 reagent (Roche) according to the manufacturer's instructions, and supernatants were collected and purified on a protein G column. To assay for the CEA-Ig binding, NK cells were incubated with 50 μg/ml fusion protein for 2 h on ice. The cells were washed and incubated with Fc fragment-specific (minimal cross-reaction to bovine, horse, and mouse serum proteins), PE-conjugated affinity-purified $F(ab')_2$ of goat anti-human IgG (Jackson ImmunoResearch Laboratories). Incubation was performed for 1 h and analyzed by flow cytometry.

Transfer experiments

In all transfer experiments 100,000 target cells were coincubated with 100,000 NK cells in 96U plate. Cells were coincubated for 2 h (unless indicated differently in the figure legend) at 37°C/5% CO_2 in 0.2 ml of complete RPMI 1640. Cells were then washed in 5% BSA/0.02% azide/PBS and incubated for 30 min on ice with 10% human serum (Sigma-Aldrich) to block nonspecific binding. Cells were then incubated with the various Abs for 1 h at 4°C, washed twice with 5% BSA/0.02% azide/PBS, and analyzed by flow cytometry.

Fucosidase and sodium periodate treatments

Cells were incubated with 10 mU/ml fucosidase (Calbiochem) in 50 mM sodium phosphate at 37°C for 30 min. Cells were then washed and the efficiency of the treatment was verified by FACS analysis. Cells were incubated with 30 mM sodium periodate ($NaIO_4$) at 37°C for 10 min. The cells were then washed five times with complete RPMI 1640 medium supplemented with 10% FCS, nonessential amino acids, L-glutamine, and sodium pyruvate. The cells were then used for transfer experiment.

Cytotoxicity assays

The cytotoxic activity of NK cells against the various targets was assessed in 5-h ^{35}S-labeled release assays as previously described (24). In all presented cytotoxicity assays, the spontaneous release was <25% of maximal release.

Confocal microscopy

The 221/CEA cells and bulk culture NK cells (5×10^5 of each) were coincubated, to form conjugates, for 10 min at 37°C/5% CO_2 in 0.5 ml of complete RPMI 1640, after which the medium was removed and the cells were fixed in Cytofix/Cytoperm (BD Pharmingen) for 15 min at 4°C. The fixed cells were then washed twice in 0.1% Tween 20/PBS and incubated in a blocking solution comprising 5% horse serum (Sigma-Aldrich) and 3% BSA in perm/wash buffer (BD Pharmingen) for 1 h at 4°C. Cells then were incubated with anti-CEA mAb diluted in blocking solution for 45 min at 4°C following incubation with IgG anti-Alexa Fluor 488 for 1 h at 4°C. Stained cells were then washed three times in 0.1% Tween 20/PBS and 7 μl of the pellet was placed between a microscope slide and a 24 × 24-mm coverslip. Cell conjugates were imaged under a ×63 oil immersion objective using a confocal laser scanning microscope (TCS SP2; Leica). Conjugates were scanned in the *xy* direction every 0.3 μm throughout the *z* plane.

Results

Elevation of CEACAM expression on NK cells after coincubation with CEACAM-positive target cells

We have previously demonstrated that a small percentage of NK cells derived from melanoma patients that are in direct contact with CEACAM1-positive melanoma tumors express CEACAM1, whereas no CEACAM1 expression is observed among NK cells derived from patients in which their melanoma tumors did not express the CEACAM1 protein (5). To test whether the expression of CEACAM1 on target cells might directly influence its expression on NK cells, we generated a series of transfectants that expresses high levels of different CEACAM molecules. 721.221 (221) cells were transfected with CEACAM1, CEA (CEACAM5), and CEACAM6 cDNA, thus generating 221/CEACAM1, 221/CEA, and 221/CEACAM6 cells, respectively. In addition, because our previous observations were obtained with melanoma tumors (5), we also used the melanoma cell line 1106mel, which endogenously expresses high levels of CEACAM1. The expression level of CEACAM in these cells was monitored with the Kat4c mAb that recognizes all CEACAM family proteins (Fig. 1*A*).

IL-2-activated bulk NK cultures derived from peripheral blood of healthy donors were coincubated with the various 221 transfectants and 1106mel cells, fixed, and stained for CD56 (to distinguish NK cells from the 221 cells) and CEACAM. In addition, NK cells could be easily distinguished from the target cells by the morphological characteristics (Fig. 1*B*, NK cells are gated).

As can be seen in Fig. 1*B*, weak or no staining of CEACAM expression was observed on NK cells that were incubated with

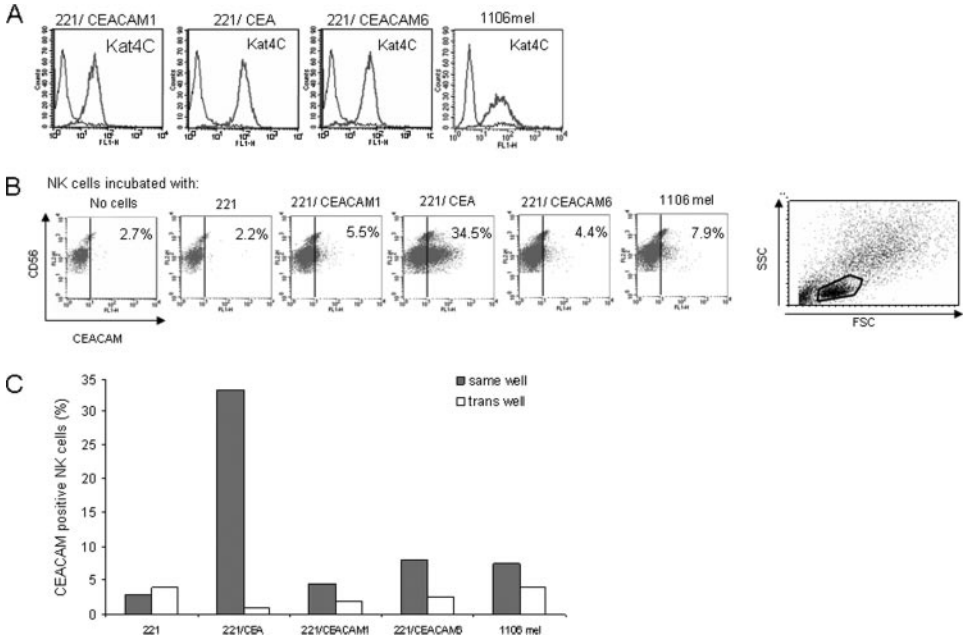

FIGURE 1. NK cells acquire CEACAM molecules after coincubation with target cells that express CEACAM proteins. *A*, Expression of various CEACAM members. The figure shows the CEACAM expression intensity on different 221 transfectants and on the melanoma cell line 1106mel, which endogenously express CEACAM1. Expression was monitored with Kat4c mAb (gray empty histogram). The background (black empty histogram) is the corresponding staining of 221 parental cells. *B*, Transfer of CEACAM proteins. NK cells were incubated with different target cells that expressed CEACAM molecules for 5 h, fixed, stained for conjugated CD56 and CEACAM, and analyzed by flow cytometry. The density plots show only NK cells which were gated according to their physical parameters as exemplified by the gate marked in the *right panel* and according to CD56-positive staining (*y*-axis). The figure shows 1 representative experiment of 10 performed. *C*, The acquisition of CEACAM molecules requires a direct contact between the cells. NK cells were incubated with the different target cells in the same well or separated by transwell filter (5-μm pore filter) and then were analyzed by flow cytometry in the same way as in *B*. The figure shows a graphical representation of the percentages of NK cells that express CEACAM after the coincubation. The figure represents the percentage of observed transfer in one single experiment of two performed.

no cells or with the parental 221 cells. Surprisingly, an elevation in the percentages of NK cells expressing CEACAM proteins was observed when NK cells were coincubated with the various CEACAM-expressing cells. The most significant elevation was observed on NK cells coincubated with the 221/CEA cells (34.5%, Fig. 1*B*). Interestingly, the increase in CEACAM expression was observed on both $CD56^{dim}$ and $CD56^{bright}$ populations demonstrating that the elevation is not dependent on a specific NK cell subpopulation.

To further test whether the observed elevation of CEACAM expression on NK cells requires direct contact between the NK and target cells, we repeated the same experiment; wherein this time the NK cells were separated from the target cell by transwell chamber (5-μm pore). No elevation in CEACAM on NK cells surface was observed when cells were separated by a transwell membrane. In contrast, a high level of CEACAM was detected when cells were incubated together in the same well (Fig. 1*C* compare □ to ■). This demonstrates that the elevation in CEACAM is not due to the presence of soluble factors but requires direct contact between the cells.

Intercellular transfer of CEA to NK cells

To further characterize the mechanism accountable for the increase in CEACAM expression, we tested the kinetics of this process. Intercellular transfer of proteins is a rapid process such that transferred molecules can be observed on the NK cell surface after 10 min (20). In agreement with these observations, when NK cells were coincubated with 221/CEACAM1, 221/CEA, or 221/CEACAM6 cells, the various proteins could be detected on the NK cells surface, as soon as 10 min after coincubation (Fig. 2*A*). The percentage of NK cells that were positive for CEACAM steadily increased and reached saturation within 1 h of incubation (Fig. 2*A*). As described above, the most efficient elevation was observed with the CEA protein.

The elevation in the CEACAM detected on NK cells can be explained by several mechanisms: 1) protein synthesis on NK resulting from exposure to cell-bound factors (30); 2) intercellular transfer between target cells and NK cells as was previously reported for MHC class I proteins (20–22, 31) and for NKG2D ligands (25, 32). The observed rapid process, and the fact that a direct contact between the cells is needed, suggested that the increase in CEACAM on NK cells results from intercellular transfer from the target cells.

To examine this hypothesis, we used a set of mAbs that recognize specifically various CEACAM molecules. The specificity of these Abs was confirmed by staining the 221, 221/CEA, and 1106mel cells. The MCA1744 detected only the 221/CEA cells; the 5F4 mAb detected only the 1106mel, while the Kat4C mAb detected both the 1106mel and the 221/CEA cells (Fig. 2*B*).

To directly demonstrate that NK cells acquire CEACAM proteins from target cells, we incubated bulk NK cultures with 221, 221/CEA, or 1106mel cells for 2 h and stained with anti-CD56 and with the various conjugated anti-CEACAM mAb (Kat4C, 5F4, MCA1744). We used the 1106mel cells in this assay and not the 221/CEACAM1 cells because CEACAM1 expression on 1106mel cell is the strongest and consequently higher percentages of CEACAM1 can be detected on NK cells after coincubation with 1106mel (Fig. 1).

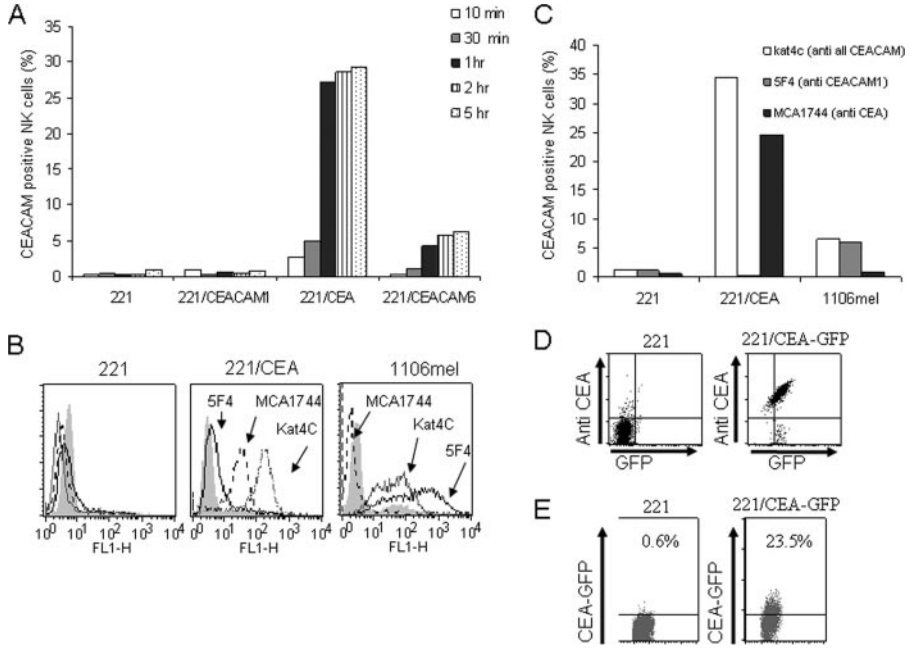

FIGURE 2. Intercellular transfer of target CEA protein to the NK cell surface. *A*, Kinetics of CEACAM acquisition by NK cells analyzed by flow cytometry. NK cells were incubated with different target cells and CEACAM expression was analyzed by flow cytometry. The figure shows the percentages of NK cells that were stained for CEACAM at the indicated time points. The figure shows one representative experiment of three performed. *B*, mAb specificity. 221, 221/CEA, and 1106mel cells were stained with three mAb: 5F4 mAb that recognize only the CEACAM1 protein (black empty histogram), MCA1744 that recognize only the CEA protein (dashed black empty histogram), and Kat4C that recognize both proteins (gray empty histogram). The background (gray filled histogram) is the staining of the corresponding cell with IgG FITC-negative control. The figure shows one representative experiment of two performed. *C*, Protein transfer to NK cells surface. NK cells were incubated for 2 h either with 221, 221/CEA, or 1106mel, fixed, and stained with anti-CD56 together with one of the three Abs that recognize different CEACAM molecules as indicated in the figure. The figure shows the percentages of NK cells that were stained with the different CEACAM Abs. The figure shows one representative experiment of three performed. *D*, CEA-GFP expression on 221. The intensity of GFP (*x*-axis) and CEA (*y*-axis) on 221 and 221/CEA-GFP are presented. The CEA levels were monitored using the Kat4C mAb following by staining with secondary CY5-conjugated $F(ab')_2$ goat anti-mouse Abs. *E*, Transfer of CEA-GFP to NK cells. NK cells were incubated for 2 h with 221 or 221/CEA-GFP, fixed, and stained with anti-CD56. The density plots show the parentages of NK cells that acquire the CEA-GFP molecule (*y*-axis) on the gated $CD56^+$ cell population. The figure shows one representative experiment of two performed.

In agreement with our hypothesis, NK cells coincubated with 221/CEA were stained with MCA1744 mAb but not with 5F4 mAb, demonstrating that NK express the CEA protein on their surface (Fig. 2*C*). In contrast, NK cells coincubated with 1106mel cells were stained with 5F4 mAb but not with MCA1744 mAb, demonstrating that NK express the CEACAM1 protein on their surface (Fig. 2*C*).

These results and the fact that CEA is not expressed on hemopoietic cells (10) led to the conclusion that the CEACAM increase on NK cells result from intercellular protein transfer.

To further verify that, indeed, the CEA appearance on NK cells resulted from intercellular transfer of CEA from target cells, we generated 221 transfectants that express CEA fused to GFP (221/CEA-GFP) (Fig. 2*D*). When these cells were coincubated with NK cells, a significant amount of CEA-GFP was detected on NK cells (Fig. 2*E*), indicating that NK cells acquire the CEA molecules from the 221/CEA target cells.

Because little transfer of other CEACAM proteins were observed, we concentrated our efforts on the CEA protein. To directly visualize the transfer of CEA to NK cells, we used confocal microscopy. The 221/CEA target cells were coincubated for 10 min with bulk NK cells culture. Cells were then fixed and stained with anti-CEA mAb and conjugates were imaged by laser scan confocal microscopy. In 20 of the 50 conjugates that were analyzed, patches of CEA were observed on the NK cell surface. In Fig. 3*A*, three peripheral blood NK cells interacting with 221/CEA cells, and patches of CEA protein that has been transferred to NK cells surface, can clearly be observed.

To investigate whether the levels of CEA expression determine the amount of protein that transfers to NK cells, we incubated bulk NK cells with 221 transfectants expressing various levels of CEA (Fig. 3*B*). A direct correlation was observed between the levels of CEA expression on target cells and the amounts of CEA protein that were transferred. High levels of CEA expression on target cells resulted in an increase in intercellular transfer of CEA to NK cells (Fig. 3*B*).

Intercellular transfer of either MHC class I proteins (20–22) or NKG2D ligands, MICA and MICB to NK cells was previously described (32).

To test whether the CEA transfer rate would be similar to that of major histocompatibility complex class I-related chain A (MICA), we generated transfectants of 221 cells expressing YFP-tagged MICA together with CEA (221/MICA-YFP/CEA). As can be seen in Fig. 3*C*, both proteins transferred to the NK cell surface concurrently, suggesting that in each intercellular contact between target cells and NK cells, both CEA and MICA transfer to the NK cell surface and that the transfer of CEA and MICA occur at similar rates.

The acquisition of CEA is not dependent on NK killing

The efficiency of CEA transfer to NK cells varied between different experiments and, frequently, when NK cells were grown for a long period of time in culture, the CEA transfer was less efficient. We therefore tested whether the efficiency of CEA transfer depended on the NK activation state. Bulk-cultured NK

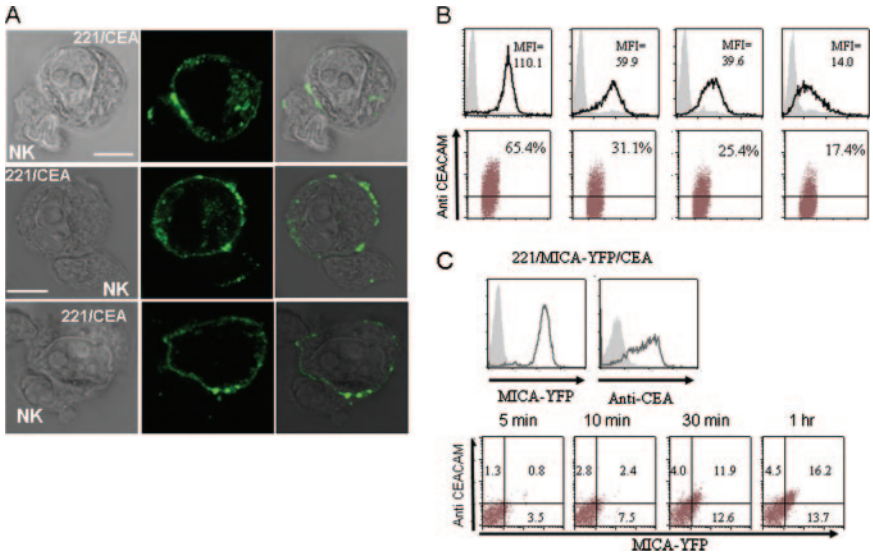

FIGURE 3. Characterization of CEA intercellular transfer. *A*, NK cells acquire CEA molecules as analyzed by confocal microscopy. Bulk NK were coincubated with 221/CEA for 10 min, fixed, stained, and imaged. Three representative fluorescence and bright-filed images of NK cells interacting with 221/CEA cell are shown. Patches of CEA stained with anti-CEA mAb on the NK cell could be observed. The figure shows 3 images of 50. Scale bar, 10 μm. *B*, CEA levels on target cell affect the amount that transfers to NK cells. Levels of CEA expression were monitored with Kat4C mAb on 221/CEA transfectants expressing various levels of CEA (*upper panel*). Bulk NK cultures were incubated for 2 h with these 221/CEA target cells, fixed, and stained with anti-CD56 together with anti-CEACAM mAb. The density plots show parentages of CEA acquisition (*y*-axis) on the gated $CD56^+$ cell population (*lower panel*). The figure shows one representative experiment of two performed. *C*, NK cells acquire CEA from target cells concomitantly to the acquisition of MICA. The *upper histograms* show the intensity of MICA-YFP and CEA on 221/MICA-YFP/CEA transfectants. The CEA levels were monitored using the Kat4C mAb followed by staining with secondary CY5-conjugated $F(ab')_2$ goat anti-mouse Abs. Bulk NK cultures were incubated for the indicated time points with the 221/MICA-YFP/CEA target cells, fixed, and stained with anti-CD56 together with anti-CEACAM mAb. The dot plots show parentages of CEA acquisition (*y*-axis) and the parentages of MICA-YFP acquisition (*x*-axis) on the gated $CD56^+$ cell population (*lower panel*). The figure shows one representative experiment of two performed.

cells were stimulated with IL-2 and the transfer of CEA to NK cells was then tested at different time points after the IL-2 stimulation. CEA transfer was maximal when NK cells were highly activated (3 days after IL-2 stimulation, Fig. 4*A*). From day 3 after IL-2 stimulation, the transfer efficiency gradually decreased reaching the lowest levels (15.9%) 10 days after IL-2 stimulation (Fig. 4*A*).

One possibility to explain the CEA transfer is that the transfer is nonspecific and is a direct consequence of the killing process, in which pieces of membranes containing CEA could be transferred from the lysed target cells to NK cells. To test this option, we examined the transfer of the CEA protein to freshly isolated NK cells that display weak killing activity at low E:T ratios. NK cells were isolated from peripheral blood of healthy donors and tested in killing assays against 221/CEA cells at different E:T ratios. At an E:T ratio of 1:1, very low levels of killing were observed (<10%, data not shown). The same NK cells were incubated with either 221 or 221/CEA for 1 h at an E:T ratio of 1:1 and stained for CD56 and CEA. Efficient transfer of CEA (40.8%) to the NK cell surface was observed (Fig. 4*B*). These results imply that most of the intercellular transfer of CEA is independent of killing.

In addition, we tested the transfer of the CEA protein to freshly isolated PBL. PBL were isolated from healthy donors, incubated either with 221 or 221/CEA for 1 h at an E:T ratio of 1:1 and stained for CD4 or CD8 together with CEA. Efficient transfer of CEA to the $CD8^+$ and to $CD4^+$ T cell surface was observed (Fig. 4*C*). Because both $CD4^+$ T and $CD8^+$ T cells do not kill the 221 or the 221/CEA cells (data not shown), these results demonstrate that the CEA transfer to T cells is killing independent and therefore further imply that intercellular transfer of CEA to NK cells might also be mostly independent of killing.

To further establish that most of the CEA transfer to NK cells is not associated with the killing, we used NK clones isolated from a perforin-deficient patient. These clones still manifest the full interactions between receptors and their appropriate ligands and the signaling machinery is intact, with only one missing element: the final killing. We tested the cytotoxicity activity of these perforin-deficient clones in a killing assay against 221/CEA cells at an E:T ratio of 2:1. As expected, killing of the 221/CEA by the perforin-deficient NK clones did not exceeded 10% (Fig. 4*D*), while the control NK clones derived from healthy donors efficiently killed these cells (>30%, data not shown). The residual NK killing observed in the perforin-deficient clones could be attributed to other killing mechanisms such as Fas/Fas-ligand interactions (33). The same perforin-deficient NK clones were then incubated with either 221 or 221/CEA at an E:T ratio of 1:1. A significant transfer of the CEA protein to the perforin-deficient NK clones was observed (Fig. 4*D*). The CEA transfer varied among the different clones but there was no correlation between the observed minimal killing and the CEA transfer. Thus, it can be concluded that CEA transfer to NK cells is largely killing independent.

The transfer of CEA to the perforin-deficient NK clones was somewhat lower than the CEA transfer to "normal" NK clones tested in the same assay (data not shown). To further investigate whether NK killing might be minimally involved in the transfer, we tested whether inhibition of the killing through MHC class I molecules affects the observed CEA transfer. For this, we generated double transfectants expressing HLA-Cw6 molecules together with the CEA protein (221/Cw6/CEA) (Fig. 5*A*). NK clones expressing the KIR2DL1 receptor were tested for their cytolytic activity against the 221/CEA and 221/Cw6/CEA

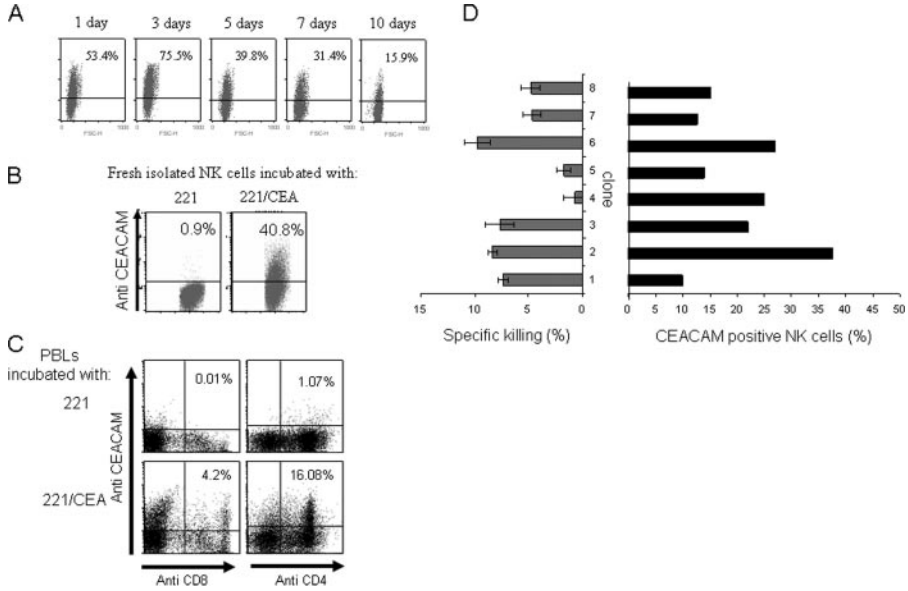

FIGURE 4. The acquisition of CEA protein by NK cells is largely killing independent. *A*, Transfer of CEA to NK cells in various time points after stimulation with IL-2, NK cells were incubated for 2 h with 221/CEA cells, fixed, and stained with anti-CD56 together with anti-CEACAM mAb. The density plots show parentages of CEA acquisition (*y*-axis) on the gated CD56$^+$ cell population. The figure shows one representative experiment of two performed. *B*, Transfer of CEA to freshly isolated NK cells. NK cells were isolated directly from PBL using the autoMACS instrument and incubated for 2 h with 221 or 221/CEA cells. Cells were fixed and stained with anti-CD56 together with anti-CEACAM mAb. The density plots show parentages of CEA expression (*y*-axis) on the gated CD56$^+$ cell population. The figure shows one representative experiment of three performed. *C*, Transfer of CEA to freshly isolated PBL. Freshly isolated PBL were incubated for 2 h with 221 or 221/CEA cells, fixed, and stained with either anti-CD4 or anti-CD8 together with anti-CEACAM mAb. The figure shows one representative experiment of two performed. *D*, Transfer of CEA is observed also in NK cells derived from perforin-deficient patient. NK clones from perforin-deficient patient were tested in killing assay against 221/CEA cells at E:T 2:1 (*left graph*, ■). The same clones were incubated for 1 h with 221/CEA cells at E:T ratio of 1:1, fixed, and stained with anti-CD56 and with anti-CEACAM mAb. The figure shows a graphical representation of the percentages of NK cells that acquired the CEA protein (*right graph*, ■).

transfectants incubated with or without anti-HLA mAb. As expected, lysis of 221/Cw6/CEA is reduced compared with lysis of 221/CEA cells due to an inhibitory interaction of the KIR2DL1 receptor with the HLA-Cw6 molecule. Indeed, preincubation of the target cells with an anti-HLA mAb restored NK killing (Fig. 5*B*). Next, to asses the impact of the protective KIR2DL1/HLA-Cw6 interaction on the intercellular CEA transfer, we compared transfer from either 221/CEA or 221/Cw6/CEA to the KIR2DL1-positive NK clone after coincubation with or without anti-HLA mAb. Although the CEA levels of 221/Cw6/CEA were similar to that of 221/CEA (Fig. 5*A*), the percentage of NK cells that acquired CEA molecules was slightly reduced when coincubated with 221/Cw6/CEA cells, compared with 221/CEA (Fig. 5*C*). Adding anti-HLA mAb restored some of the CEA transfer. This moderate restoration in CEA transfer repeated in three independent experiments and

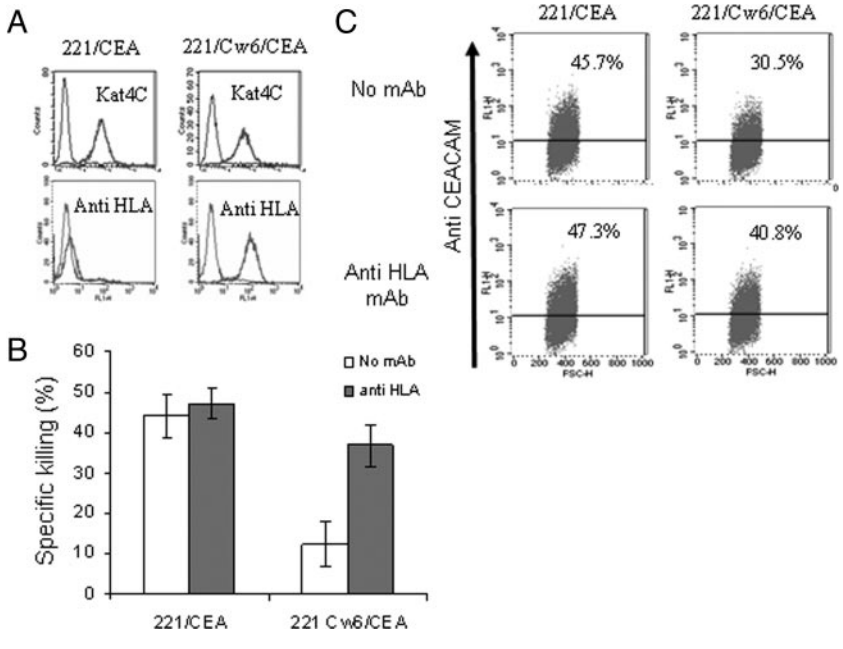

FIGURE 5. HLA-Cw6-KIR2DL1 interactions slightly reduce the transfer of the CEA protein to NK cells. *A*, Staining of transfectants used in the assays. Expression levels of either CEA or HLA were monitored by staining either 221/CEA (*left panel*) or 221/Cw6/CEA (*right panel*). The background is the corresponding staining of 221 parental cells. *B*, The KIR2DL1-positive NK clone is inhibited by HLA-Cw6. A KIR2DL1-positive NK clone was tested in killing assays against the indicated target cells. The E:T ratio was 2:1. Target cells were incubated with anti-HLA mAb in a final concentration of 5 μg/ml. *C*, Inhibition by MHC class I interactions partially inhibits the CEA transfer. NK cells were incubated for 1 h with 221/CEA or 221/Cw6/CEA in the absence (*upper panel*) or in the presence of anti-HLA mAb (*lower panel*), fixed, and stained with anti-CD56 together with anti-CEACAM mAb. The density plots show parentages of CEA acquisition (*y*-axis) on the gated CD56$^+$ cell population. In all parts of this figure, shown is one representative experiment of three performed.

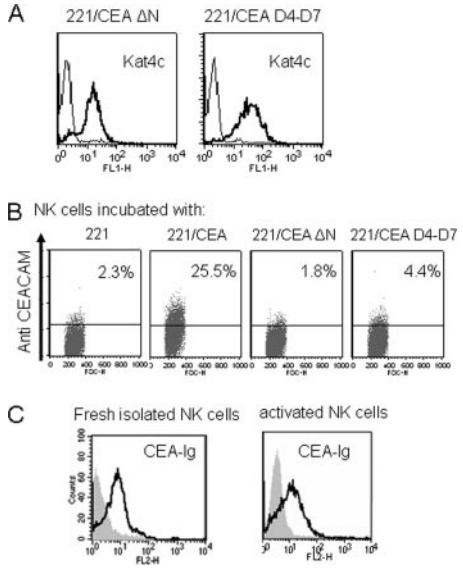

FIGURE 6. The transfer of the CEA protein requires a specific interaction. *A*, CEA expression on the truncated transfectants. The CEA expression level on 221/CEA ΔN and 221/CEA D4-D7 transfectants was monitored with Kat4c mAb (black empty histogram). The background (gray empty histogram) is the corresponding staining of 221 parental cells. *B*, The N domain is crucial for the CEA transfer. NK cells were incubated for 1 h with 221/CEA, 221/CEA ΔN, or 221/CEA D4-D7, fixed, and stained with anti-CD56 together with anti-CEACAM mAb. The density plots show parentages of CEA acquisition (*y*-axis) on the gated $CD56^+$ cell population. *C*, Binding of CEA-Ig to fresh and IL-2-activated NK cells. Binding of CEA was assessed by FACS staining using CEA-Ig (black empty histograms). The background is the staining of a control fusion protein CD99-Ig (gray histogram). The figure shows one representative experiment of four performed.

was statistically significant ($p < 0.01$ by paired *t* test), demonstrating that a limited fraction of the CEA transfer is affected by HLA-Cw6 KIR2DL1 interactions. It is therefore possible that some of the observed CEA intercellular transfer is killing dependent or that the inhibitory interaction between HLA-Cw6 and KIR2DL1 interrupt the CEA transfer by a different mechanism such as different immunological synapse structure or direct effects of the inhibitory signaling on the transfer process.

The transfer of CEA is probably receptor mediated

Intercellular transfer is dramatically augmented upon receptor recognition (34). We assumed that observed CEA transfer was also specific because the transfer of other CEACAM proteins was significantly lower then the CEA transfer (Figs. 1 and 2). We have previously demonstrated that CEA on target cells interacts with CEACAM1 on the surface of NK cells (8), however, CEACAM1 is expressed exclusively on activated CD16-negative NK cells (Ref. 27 and Fig. 1*A*), whereas the observed CEA transfer was not limited to a certain subpopulation (Fig. 1*A*). In addition, the transfer was observed with fresh NK cells that do not express CEACAM1 (Fig. 4*B*). Finally, CEACEAM1 was never expressed on >2.5% of the population of activated bulk NK cultures used in this manuscript (data not shown). Thus, we concluded that receptors other than CEACAM1 are involved in the CEA intercellular transfer.

The CEA protein consists of an Ig V-like N-terminal domain (crucial for its function; Refs. 8 and 35), followed by six Ig-C_2 like domains (11). We have previously generated mutated constructs of CEA that lacks the N domain (CEA ΔN) and a CEA protein that lacks the N domain and the first two Ig C_2-like domains (CEA D4-D7) (8). To test whether a specific recognition of CEA through its N-domain is involved in the observed intercellular transfer of CEA, 221 cells that express the truncated CEA proteins (Fig. 6*A*) were tested in transfer assays. Although the expression levels of the truncated CEA proteins and the full protein were similar (compare Figs. 1*A* to 6*A*), the percentage of NK cells that acquired the CEA molecules was significantly reduced when 221/CEA ΔN or CEA D4-D7 cells were used (Fig. 6*B*). These results indicate that the N-domain of CEA is important for the recognition and transfer to the putative unknown receptor.

To further establish that the CEA protein on the target cells can be recognized by a specific receptor on the NK cell surface, we used a fusion protein in which the extracellular portion of the CEA was fused to the Fc portion of human IgG1 thus creating CEA-Ig protein. The production and purification of the CEA-Ig and CD99-Ig (that was used as a control) was previously described (8, 29). We then tested the binding of CEA-Ig to freshly isolated and to activate bulk NK cultures which are CEACAM1 negative (data not shown); a significant binding of CEA-Ig was observed, while the control CD99-Ig did not bind to these cells (Fig. 6*C*).

The carbohydrate structures on CEA are probably essential for the transfer

It was demonstrated that the CEA protein is recognized by DC-SIGN (14) via the unique LeX glycans expressed on the CEA protein. Because we could not block the CEA transfer with any of the anti-CEA Abs we tested, such as rabbit polyclonal anti-CEA (DakoCytomation), anti-CEA MCA1744 (Serotec), Kat4C (DakoCytomation), CBL578 (Chemicon International) (data not shown), we hypothesized that the recognition of the CEA protein by NK cells might also be mediated by carbohydrates. We therefore examined whether CEA expressed on 221 cells indeed express the special LeX glycans. 221 and 221/CEA cells were stained with anti-LeX 6H3 mAb and although other proteins on 221 express LeX, a considerable staining of LeX glycans was observed on the 221/CEA cells (Fig. 7*A*) demonstrating that the CEA protein on these cells was likely to harbor the LeX glycans.

Next, we tested whether the anti-LeX 6H3 mAb could block the CEA transfer. NK cells were coincubated with 221 or 221/CEA at a ratio of 1:1 in the presence of either anti-LeX mAb, isotype-matched control mAb or without any mAb. The cells were then fixed and stained for CEA and CD56 as above. The CEA transfer to NK cells was significantly reduced when anti-LeX mAb were added while the isotype-matched control mAb had no effect (Fig. 7*B*). Because the parental 221 cells display low levels of LeX glycans independently of CEA expression, we examined whether the observed inhibition of CEA transfer is specific to CEA. It was previously reported that HLA-Cw6 is acquired by NK cells through the interactions with KIR2DL1 receptor (20). We therefore tested the effect of anti-LeX mAb on the transfer of HLA-Cw6-GFP from 221/Cw6-GFP cells. As can be seen in Fig. 7*B*, addition of anti-LeX mAb had no effect on the intercellular transfer of Cw6-GFP to NK cells, demonstrating that the observed inhibition of CEA transfer by anti-LeX is specific. Importantly, the anti-LeX mAb did not affect the staining with the anti-CEACAM, Kat4C mAb (data not shown) and thus this could not be reason for the reduced CEA transfer. We therefore concluded that carbohydrates moieties on CEA are probably important for the CEA recognition and acquisition by NK cells.

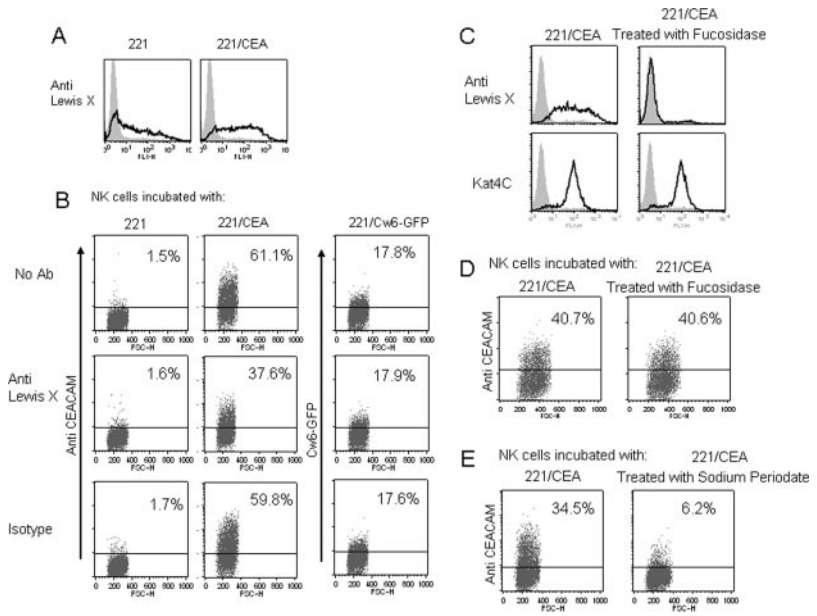

FIGURE 7. Carbohydrate structures are important for the transfer of the CEA protein to NK cells. *A*, LeX expression was determined by flow cytometry. The parental 221 and the 221/CEA were stained with anti-LeX (6H3) mAb (black histogram). The background (gray full histogram) is the staining with the secondary FITC-conjugated $F(ab')_2$ goat anti-mouse Abs. *B*, Transfer of CEA can be partially inhibited by anti-LeX mAb. 221, 221/CEA, and 221/Cw6-GFP were incubated for 20 min with anti-LeX mAb, isotype control, or medium alone. Thereafter, NK cells were added for an additional 1 h. Subsequently, cells were fixed and stained with anti-CD56 together with anti-CEACAM mAb. The density plots show parentages of CEA acquisition (*y*-axis) on the gated $CD56^+$ cell population (*six left panels*) and the parentages of Cw6-GFP acquisition (*y*-axis) on the gated $CD56^+$ cell population (*three right panels*). The figure shows one representative experiment of four performed. *C*, Staining for LeX expression after fucosidase treatment. 221/CEA cells were incubated for 1 h with or without α 1–3,4-fucosidase in sodium phosphate buffer (50 mM/L (pH 5)) at 37°C and then washed and stained with anti-LeX (6H3) mAb (black histogram). The background (gray full histogram) is the staining with the secondary FITC-conjugated $F(ab')_2$ goat anti-mouse Abs. *D*, The acquisition of CEA is not affected by the fucosidase treatment. NK cells were incubated for 1 h with 221/CEA cells that were either incubated with or without fucosidase. Cells were fixed and stained with anti-CD56 together with anti-CEACAM mAb. The density plots show parentages of CEA acquisition (*y*-axis) on the gated $CD56^+$ cell population. All panels show one representative experiment of four performed. *E*, After sodium periodate treatment, CEA transfer is diminished. NK cells were incubated for 1 h with 221/CEA cells that were treated for 10 min with 30 mM sodium periodate, fixed, and stained with anti-CD56 together with anti-CEACAM mAb. The density plots show parentages of CEA acquisition (*y*-axis) on the gated $CD56^+$ cell population. The figure shows one representative experiment of two performed.

To test whether the fucose group of the LeX glycan on CEA was directly involved in the observed transfer, we incubated the 221/CEA cells for 1 h with α1–3,4-fucosidase, an enzyme that specifically removes the fucose moiety from LeX. This treatment was very effective and the binding of the anti-LeX mAb was completely abolished (Fig. 7*C*). The enzyme treatment did not disrupt the integrity of CEA on the 221 cells as the Kat4C mAb still efficiently stained the treated and untreated cells equally well (Fig. 7*C*) and in addition, the binding of CEA-Ig and CEACAM1-Ig to the 221/CEA was not impaired (data not shown). Next, we tested whether the removal of the fucose group from the CEA affected the observed transfer. NK cells were incubated with 221/CEA cells treated or not with α1–3,4-fucosidase. Surprisingly, the fucosidase treatment did not affect the amount of CEA transferred (Fig. 7*D*), indicating that the fucose group on the CEA is not important for the CEA recognition and acquisition by NK cells. Thus, we concluded that the partial blockade of the transfer by the anti-LeX mAb was probably due to a steric hindrance exerted by the IgM anti-LeX Ab and that glycans other than fucose moiety on the LeX (which are present on CEA; Refs. 13, 36, 37) are probably involved in the CEA transfer. Unfortunately, we could not specifically test the effect of the other carbohydrate structures of CEA on its transfer as Abs against these other glycan are not commercially available. We therefore used 30 mM sodium periodate, which destroys carbohydrate structures. This treatment did not disrupt the integrity of CEA on the 221 cells as the Kat4C mAb still efficiently stained the treated cells (data not shown). This removal of carbohydrate completely diminished CEA transfer to NK cells (Fig. 7*E*), demonstrating that the carbohydrate moieties are important for the observed transfer.

Modulation of NK cells killing by CEA

We have previously demonstrated that the CEA protein on target cells inhibits NK cytotoxicity via interaction with the CEACAM1 protein on the NK cells (8). In healthy subjects, CEACAM1 is expressed only on the cell surface of the activated, CD16-negative NK cell subset (5, 27), which is present in low amounts in the peripheral blood but can be found at high percentages in secondary lymphoid tissues (38). Because, the CEA transfer was observed in the absence of CEACAM1 and was contact, carbohydrate, and N-domain dependent it indicates for the existence of an unknown, putative CEA receptor, which is broadly expressed on fresh and activated NK and T cells.

Our next aim was to determine whether this unknown putative receptor is involved in regulating NK cytotoxicity. We therefore tested bulk cultures of NK cells that do not express CEACAM1 (data not shown) in killing assays against 221 and 221/CEA, and observed a moderate but significant inhibition of NK cell killing in various E:T ratios (Fig. 8*A*).

We next compared the efficiency of the CEA-mediated inhibition to the "classical" NK inhibition mediated by MHC class I

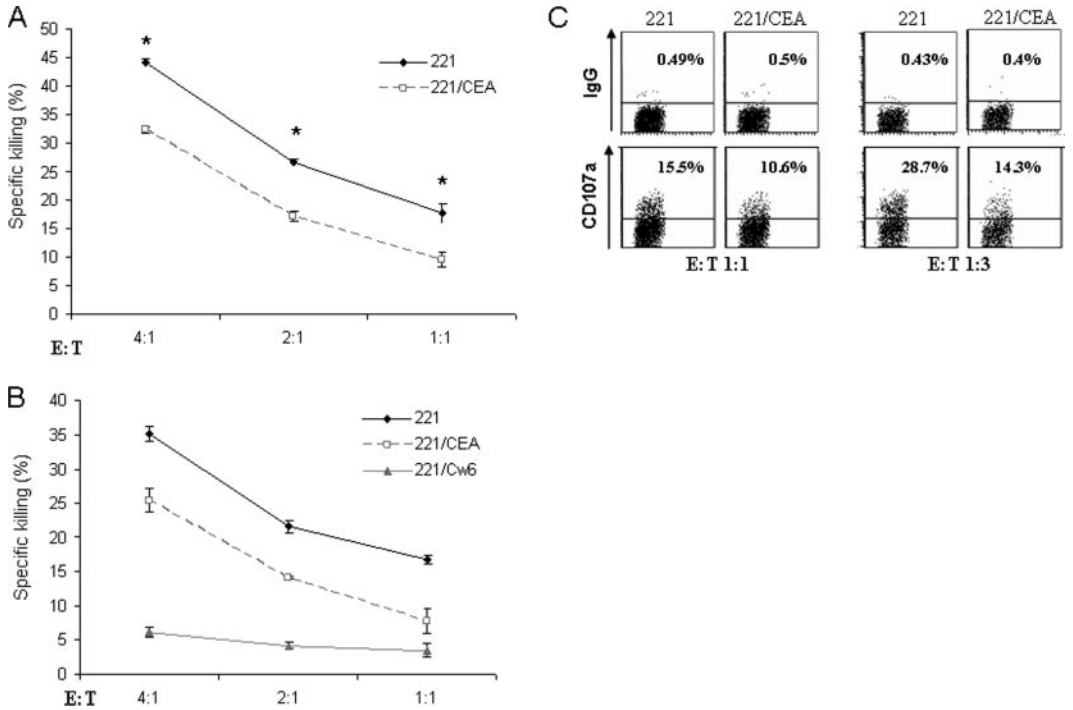

FIGURE 8. Inhibition of NK cytotoxicity by the CEA protein. *A*, CEA inhibits the killing by bulk NK cells. Activated bulk NK cultures were tested in killing assay against 221 and 221/CEA cells at various E:T ratios as indicated in the figure. ∗, Statistically significant difference between 221 and 221/CEA cells ($p < 0.01$ by *t* test). Figure shows one representative experiment of four performed. *B*, MHC class I inhibition is more prominent than the CEA-mediated inhibition. A KIR2DL1-positive NK clone was tested in killing assays against 221, 221/CEA, and 221/Cw6 cells at various E:T ratios as indicated in the figure. The figure shows one representative experiment of two performed. *C*, CEA-expressing cells inhibit NK degranulation. 221 or 221/CEA cells were incubated with NK cells for 2 h at two different E:T as indicated in the *x-axis.* Substantially, cells were stained with conjugated anti-CD56 and anti-CD107a, and analyzed by flow cytometry. The density plots show parentages of CD107a-positive cells gated on the $CD56^+$ cell population. The figure shows one representative experiment of four performed.

molecules. The killing of NK clones expressing the KIR2DL1 receptor was tested against the 221, 221/CEA, and 221/Cw6 transfectants. As expected, both the 221/CEA and 221/Cw6 cells were killed less than the parental 221 cells. The killing of 221/Cw6 was very low due to the inhibitory interaction between the KIR2DL1 receptor and the HLA-Cw6 molecule. This inhibition was more prominent than the inhibition mediated by the CEA protein (Fig. 8*B*) of 221/CEA.

To further demonstrate the inhibitory effect of CEA, we used another type of assay which examined NK degranulation, by staining for the CD107a (LAMP1) protein (39). A substantial reduction in NK degranulation was observed in NK cells when incubated with 221/CEA compared with 221 parental cells (Fig. 8*C*). The reduction in NK degranulation was more profound when more targets cells were used in the assay (Fig. 8*C*), i.e., three targets per one effector. These results further demonstrate that CEA is probably recognized by an inhibitory NK receptor.

Discussion

Different functions have been implicated for the CEA protein such as mediator of intercellular adhesion, which strengthens the metastatic potential of tumor cells (40), or as disruptor of tissue structure and inhibition of cellular differentiations (12, 41). However, only little is known about CEA interaction with the immune system. We have previously reported that CEA inhibits NK killing via interaction with the CEACAM1 protein which is expressed almost exclusively on activated CD16-negative NK cells (27). In this study, we demonstrate another novel immune function for CEA, widespread moderate inhibition of NK cytotoxicity and transfer from the target to effector cells.

Multiple previous studies have shown intercellular transfer of surface proteins from the target to effector cell. MHC molecules transfer from APCs to T cells (18), B cells acquire Ags from targets leading to enhanced presentation of these Ags to T cells (17) and NK cells acquire either MHC class I proteins (20–22) or NKG2D ligands from their targets (Refs. 32 and 25).

The CEA transfer to NK cells resembles the MHC class I and MICA transfer in several aspects. The transfer of CEA is a rapid process and CEA molecules can be detected on NK cells within 10 min of cellular interaction. Similar to MHC class I transfer, we show that CEA transfer is mediated by receptor recognition. We demonstrate that deletion of CEA domains hamper some of the observed transfer, indicating that specific recognition is required. Furthermore, we show that the recognition of CEA by NK cells is probably mediated by unique carbohydrate structures on the CEA protein.

We demonstrate that the CEA transfer can be specifically blocked by anti-LeX mAb. However, removing the fucose group did not effect on the CEA transfer. The LeX glycans consist of three different carbohydrate moieties galactose and fucose which are bound to *N*-acetyl-D-glucosamine. The fucosidase treatment removes only the fucose group from the LeX, therefore it is possible that the partial blockade of the transfer by the anti-LeX mAb was due to a steric hindrance exerted by the IgM anti-LeX Ab and that glycans other than the fucose itself are involved in the transfer. To further support the involvement of carbohydrate in CEA transfer, we show that removing all carbohydrate structures completely diminished CEA transfer. Although we cannot preclude the possibility that sodium periodate treatment has an indirect effect on the transfer,

these results strongly suggest that the carbohydrate structures are involved in the CEA recognition and transfer to NK cells. With regard to this, it was recently demonstrated that the LeX moieties on the CEA are important for the interaction with dendritic cells (14, 42), and besides LeX, several other special carbohydrate structures have been identified on the CEA (13, 36, 37).

It was reported that NK cells can capture pieces of target cell membranes (43). To exclude the possibility that the observed CEA transfer resulted from nonspecific NK scavenge of membrane fragments during killing, we examined the role of target cells lysis in the observed phenomenon. We show that perforin-deficient NK clones are still able to acquire CEA molecules from their targets (Fig. 4*B*). In addition, we show that freshly isolated NK cells and NK cells inhibited by MHC class I interactions are able to acquire CEA proteins from their target cells; finally, we show that that T cells could also acquire CEA molecules. Hence, the CEA transfer is largely independent of target cell lysis.

We show that the activation state of NK cells has an effect on the amount of CEA acquired and that highly activated NK cells acquired more CEA protein (Fig. 4*A*). The reasons for this are still unknown because it is hard to know whether this elevation in CEA transfer resulted from an intrinsic difference in the amount of protein that transfers to NK cells in a different state of activation or whether it is a secondary outcome of the difference in cell motility. It is known that activated cells move faster, thus they may make more intercellular contacts which will result in enhanced CEA transfer to these NKs.

Exploring the functional consequence of the interaction of CEA on target cells with NK cells, we demonstrate that the CEA protein can inhibit NK cytolytic activity independent of CEACAM1 expression. The observed CEA inhibition of the killing was not as prominent as the inhibition mediated by classical MHC class I proteins, but was similar to that exerted by CEA interaction with CEACAM1 (5, 8). In contrast, inhibition of NK cell degranulation by CEA was much more pronounced. This is because usual killing provides information only about the end-stage lysis of target cells while the CD107a staining provides data on the level of activation of the effector population. Thus, a moderate inhibition will be observed most effectively when effector cells are assayed directly and when more target cells are present.

Our results suggest that NK cells possess an inhibitory receptor that can recognize the CEA and that carbohydrates are probably involved in this recognition. Why do NK cells express such a receptor? An essential feature of the innate immune system is its ability to distinguish foreign from self. One way to prevent inappropriate autoreactivity against self would be for host-specific ligands to engage inhibitory receptors on effector cells, such as NK cells. It is possible that NK cells possess such an inhibitory self recognition receptor that recognizes self carbohydrate structures and that the CEA protein acquired similar structures to escape recognition by NK. One example for such lectin activity has been proposed for the inhibitory siglec receptors (44).

Disclosures

The authors have no financial conflict of interest.

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