OPEN

The Human Antibody Fragment DIATHIS1 Specific for CEACAM1 Enhances Natural Killer Cell Cytotoxicity Against Melanoma Cell Lines In Vitro

Maria L. Dupuis, Valentina Fiori,† Alessandra Soriani,‡ Biancamaria Ricci,‡ Sabrina Dominici,§ Diego Moricoli,† Alessandro Ascione,* Angela Santoni,‡ Mauro Magnani,§ and Maurizio Cianfriglia**

Summary: Several lines of evidence show that de novo expression of carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) is strongly associated with reduced disease-free survival of patients affected by metastatic melanoma. Previously published investigations report that homophilic interactions between CEACAM1 expressed on natural killer (NK) cells and tumors inhibit the NK cell-mediated killing independently of major histocompatibility complex class I recognition. This biological property can be physiologically relevant in metastatic melanoma because of the increased CEACAM1 expression observed on NK cells from some patients. Moreover, this inhibitory mechanism in many cases might hinder the efficacy of immunotherapeutic treatments of CEACAM1$^+$ malignancies because of tumor evasion by activated effector cells. In the present study, we designed an in vitro experimental model showing that the human single-chain variable fragment (scFv) DIATHIS1 specific for CEACAM1 is able to enhance the lytic machinery of NK cells against CEACAM1$^+$ melanoma cells. The coincubation of the scFv DIATHIS1 with CEACAM1$^+$ melanoma cells and NK-92 cell line significantly increases the cell-mediated cytotoxicity. Moreover, pretreatment of melanoma cells with scFv DIATHIS1 promotes the activation and the degranulation capacity of in vitro–expanded NK cells from healthy donors. It is interesting to note that the melanoma cell line MelC and the primary melanoma cells STA that respond better to DIATHIS1 treatment, express higher relative levels of CEACAM1-3L and CEACAM1-3S splice variants isoforms compared with Mel501 cells that are less responsive to DIATHIS1-induced NK cell–mediated cytotoxicity. Taken together, our results suggest that the fully human antibody fragment DIATHIS1 originated by biopanning approach from a phage antibody library may represent a relevant biotechnological platform to design and develop completely human antimelanoma therapeutics of biological origin.

Key Words: CEACAM1, melanoma, immunotherapy, scFv antibodies, NK cells

(*J Immunother* 2015;38:357–370)

Received for publication March 31, 2015; accepted September 15, 2015.

From the *Department of Therapeutic Research and Medicines Evaluation, Istituto Superiore di Sanità; ‡Department of Molecular Medicine, Istituto Pasteur-Fondazione Cenci Bolognetti, Sapienza University of Rome, Rome; †Diatheva S.R.L., Fano; and §Department of Biomolecular Science, University of Urbino "Carlo Bo," Urbino, PU, Italy.

M.L.D., V.F., and A.S. contributed equally.

Reprints: Valentina Fiori, Diatheva S.R.L., viale Piceno 137/F, Fano, PU 61032, Italy (e-mail: v.fiori@diatheva.com).

Supplemental Digital Content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's Website, www.immunotherapy-journal.com.

Carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) is a transmembrane glycoprotein belonging to the carcinoembryonic antigen (*CEA*) gene family and immunoglobulin superfamily.[1] CEA-related cell adhesion molecules (CEACAMs) are involved in various cellular functions such as cell adhesion, intracellular and intercellular signaling, and in complex biological processes such as inflammation, angiogenesis, cancer progression, and metastasis.[2] CEACAM1 has the widest tissue distribution among CEACAMs family members being present in many epithelia, in leukocytes, and endothelial cells[3] and is the only member showing a complex alternative splicing process that give rise to 12 isoforms in humans.[4] All CEACAM1 splice variants contains the distal *N*-terminal IgV-like domain that is highly conserved in all CEACAM members, and differ mainly by the number of the extracellular IgC2-like domains and by the length of the intracellular tail that can be "long" (L) or "short" (S).[5] The L isoforms contain 2 immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in the cytoplasmic domain with 2 tyrosine that can be phosphorilated by Src kinases and be target for phosphatases such as Src homology domain 2–containing phosphatase 1 and phosphatase 2 (SHP-1, SHP-2).[6] The S isoforms lack the ITIM motifs but can bind calmodulin, actin, and tropomyosin and have been implicated in the interaction with the cytoskeleton.[7] The relative ratio of long to short isoforms is dynamic and can vary according to the cell type, the phase of growth, and activation state.[8] CEACAM1 has been suggested as a tumor suppressor, as its expression has been found to be reduced in colon, endometrium, breast, and prostate cancer.[9–13] However, CEACAM1 reexpression can often occur in the advanced stages of these malignancies and in a large array of solid tumors.[14] In several cancers, including malignant melanoma, non–small cell lung cancer, and hepatocellular carcinoma, CEACAM1 is upregulated and its expression highly correlates with tumor progression, the development of metastasis, and poor survival.[15–17] This discrepancy has been in part explained by the role of CEACAM1 in tumor angiogenesis. In bladder and prostate cancer, it has been shown that epithelial downregulation but endothelial upregulation of CEACAM1 in the early phases of tumorigenesis induces angiogenesis through increased expression of vascular endothelial growth factor. This in turn is involved in the switch from noninvasive and nonvascularized to invasive and vascularized cancer.[18–23] In addition, CEACAM1 has a role in the modulation of innate and adaptive immune responses.[8,24] Furthermore, Markel et al[25] provided evidences that CEACAM1 homophilic interactions via the *N*-terminal domain inhibit

natural killer (NK) cell–mediated killing of melanoma cells independently of major histocompatibility complex class I recognition and that this mechanism may be used by melanoma cells to evade destruction by tumor-reactive lymphocytes.[26] This biological property can be particularly relevant in metastatic melanoma because of the increased CEACAM1 expression observed on NK cells from some patients.[25] NK cells are effector lymphocytes of the innate immune system that exert an important role in immune surveillance. NK cell activation is regulated by a balance between signals mediated through germline-encoded activating and inhibitory receptors. Among NK cell activating receptors, NKG2D is a Lectin-like homodimeric receptor that recognizes stress-inducible activating ligands.[27] Previous studies have demonstrated that tumor cells expressing CEACAM1 escape NK cell–mediated immune surveillance by downregulating NKG2D ligands' expression on tumor cells.[28] A second mechanism of CEACAM1-mediated inhibition of NK cells' cytolytic function has been also proposed. In particular, it has been demonstrated that the direct interaction of CEACAM1 with NKG2D leads to an inhibition of downstream signaling involved in the cytolysis of CEACAM1-bearing target cells.[29] Recently, it has been also demonstrated that CEACAM1 regulates T-cell immunoglobulin domain and mucin domain-3 (TIM-3) mediate tolerance and exhaustion of T lymphocytes.[30] Furthermore, in a murine tumor model of colorectal cancer, the coblockade of CEACAM1 and TIM-3 with specific mAbs leads to a very efficient antitumor activity, superior to the administration of the single agents and that anti-CEACAM1 treatment efficiently synergized with programmed cell death 1 inhibition.[30] Accordingly, it has been shown that human CEACAM1 function may be blocked by a specific murine mAb thereby rendering human melanoma cells susceptible to cytotoxic lymphocytes.[31] However, the use of mAbs of xenogenic origin in clinical settings may suffer from rapid formation of human anti-mouse antibodies (HAMA) that alter the pharmacokinetic profile of the mAb leading to severe toxicity and preventing repeat dosing.[32] Today, with the advances of recombinant genetic strategies, rodent mAbs have been genetically modified in chimeric and humanized version significantly reducing their immunogenicity.[33,34] Furthermore, the need for homogeneous sources of immune reagents with single-antigen specificity has promoted the search for alternative techniques to produce antibody-like proteins useful in human therapy with a robust safety profile and reduced cost for good manufacturing practice (GMP) production.[35] Phage antibody technology offers a solution as the in vitro process of display and selection of antibody fragments on filamentous phage can actually mimic the in vivo immune selection leading to antibodies with improved features.[36,37] In this context, we have previously isolated from a phage display library, a fully human single-chain variable fragment (scFv) antibody specific for CEACAM1 and cross-reacting with CEACAM3 and CEACAM5 cell adhesion molecules expressed on different types of cancers including malignant melanoma.[38] Then, a scFv variant, named DIATHIS1, has been recently produced in a GMP setting.[35] To verify if this novel fully human scFv could be used as a biotechnological platform to design an immunotherapeutic approach of CEACAM1$^+$ tumors, such as in a context of NK cell adoptive transfer and/or in new combinations of immune checkpoint inhibitors, we studied in in vitro model the ability of DIATHIS1 to enhance NK cell–mediated cytotoxicity of malignant melanoma cells.

MATERIALS AND METHODS

mAbs and Cells

The recently isolated human scFv DIATHIS1 is a genetically modified version of the scFvE8 antibody that recognizes a cell surface binding site shared by CEACAM1, CEACAM3, and CEACAM5.[38] The antibody has been recently produced by Diatheva (http://www.diatheva.com) in a GMP setting and shows high reactivity for the *N*-terminal IgV-like domain of CEACAM1.[35] The following unconjugated mAbs were also used: polyclonal anti-human CEACAM1 *N*-terminal domain (Antibodies-online GmbH), anti-MICA (MAB159227), anti-MICB (MAB236511), anti-ULBP1 (MAB170818), anti-ULBP2 (MAB165903), and anti-ULBP3 (MAB166510) from R&D Systems (Minneapolis, MN), anti-PVR (SKII.4) kindly provided by Professor M. Colonna (Washington University, St. Louis, MO); anti-Nec-2 (R2.525) from BD Pharmingen (San Diego, CA); allophycocyanin (APC)-conjugated GAM was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Anti-CD107a-APC, cIgG-APC, anti-CD3FITC, and anti-CD56PE were purchased from BD Pharmingen. NK-92 cell line was a kind gift of Professor L. Moretta, (Director of the Istitututo Gaslini, Genova, Italy) who obtained the cells from ATCC repository (ATCC; CRL-2407). The cell line NKL was obtained from Dr Robertson MJ (Department of Medicine, Indiana University, Indianapolis, IN). The following human melanoma cells have different source and cell culture characteristics: A375 melanoma cell line derived from ATCC (CRL-1619), Me30966 MelC and Mel501 are metastatic melanoma cell lines, STA and ATT are primary tumor cells derived from lesions of melanoma patients and adapted to standard cell culture conditions. All these cells were kindly supplied by Dr Licia Rivoltini (Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy). The M14 melanoma cell line was a kind gift of Dr Agnese Molinari [Italian National Institute of Health (ISS), Rome, Italy]. SKO-007(J3) multiple myeloma cell lines were provided by Professor P. Trivedi (Sapienza University of Rome, Italy). All cells used in this study were cultured in a basic medium (BM) which is constituted by Dulbecco's modified Eagle's medium (Gibco, Minneapolis, MN) supplemented with: 10% heat-inactivated fetal calf serum (FCS; HyClone, Logan, Utah); 1 mM penicillin-streptomycin; 1 mM nonessential amino acids; 1 mM L-glutamine; and 1 mM sodium pyruvate (all chemicals were purchased from Gibco). The NK-92 and NKL cell lines are dependent for their in vitro growth on the presence of recombinant IL-2 (100 IU/mL; R&D Systems) as previously reported.[39,40] Peripheral blood mononuclear cells (PBMC) were separated from buffy coats of healthy donors by Lymphoprep (Nycomed, Oslo, Norway) gradient centrifugation. Freshly isolated NK cells were then isolated from PBMC by negative selection using a magnetically activated cell sorter NK isolation kit (Miltenyi Biotec, Bologna, Italy). This purification protocol resulted in 85% to 95% purity of negatively selected NK cells. To obtain in vitro–expanded NK cells, isolated PBMC were cocultured for 10 days with irradiated (30 Gy) Epstein-Barr virus–transformed B-cell line RPMI 8866 at 37°C in a humidified 5% CO_2 atmosphere, as previously described.[41] On day 10, the cell population was routinely >90% CD56$^+$CD16$^+$CD3$^-$, as assessed by immunofluorescence and flow cytometry analysis. In some

experiment, T cells were depleted by $CD3^+$ Dynabead (Dynal Biotech ASA, Oslo, Norway) system, according to the manufacturers' instructions.

Flow Cytometry

The reactivity of scFv DIATHIS1 on intact/living cells was determined using conventional procedures to reveal the presence of the cell surface antigen of interest by flow cytometry investigations. Melanoma cells were harvested at the exponential phase of growth, washed with serum-free medium, and incubated for 1 hour at RT in the presence of 10 μg/mL of scFv DIATHIS1. To control the scFv DIATHIS targeting specificity for CEACAM1-expressing cells, the same amount of an irrelevant human scFv antibody isolated with the same biopanning procedure used for scFv DIATHIS1 selection and recognizing a cell surface determinant not present in melanoma (A. Mallano, ISS, Rome) was also tested in parallel. After incubation, the cells were extensively washed, pelleted, resuspended, and incubated for 30 minutes at 4°C with an anti-Flag M2 secondary antibody (25 μg/mL; Sigma Aldrich, St Louis, MO). Specific binding was detected by incubating the cells for 30 minutes at 4°C with a FITC-goat anti-mouse IgG (6 μg/mL, Pierce, IL). After staining, samples were washed, maintained at 4°C, and subjected to FACSCalibur flow cytometer analysis (Becton-Dickinson, NJ). To evaluate scFv DIATHIS1 reactivity on cytotoxic effector cells which include IL-2-dependent cell lines NK-92 and NKL as well as freshly isolated and/or activated NK cells, we proceeded with the identical methodologies used for melanoma cell lines. CEACAM1 expression was also assessed by a rabbit polyclonal antibody to human CEACAM1 *N*-terminal domain (Antibodies-online, Aachen, DE) or the anti-CEACAM1 mAb clone 4D1/C2 (Merk Millipore, Darmstadt, Germany). Both antibodies were used at the concentration of 1 μg/10^6 cells.

Immunofluorescence Competitive Assay

To test the scFv DIATHIS1 specificity against CEACAM1 a competitive assay was performed. For this purpose 2.5×10^5 MelC cells were grown overnight on glass coverlips, washed and fixed with 4% (vol/vol) of formaldehyde for 15 minutes at RT and incubated in PBS containing 1% (wt/vol) BSA and 0.1% (wt/vol) gelatin to saturate nonspecific binding sites. The cells were then exposed to 10 μg/mL of scFv DIATHIS1 alone or added in separated samples with different concentrations (5, 10, and 50 μg/mL) of soluble recombinant CEACAM1 fragment corresponding to the *N*-terminal IgV-like domain and first IgC2-like domain (CEACAM1 amino acid residues 35-233; Diatheva S.R.L., Fano, Italy) and then incubated for 60 minutes at RT. After washing, an anti-Flag M2 monoclonal antibody (Sigma Aldrich) 1:400 (vol/vol) diluted in blocking solution was added and incubated for 60 minutes at RT. After washing, an anti-mouse antibody FITC-conjugated 1:200 (vol/vol) diluted in blocking solution was added to the wells and maintained for 60 minutes RT. After washing, the fluorescence was highlighted using a Leica DMLB fluorescent microscope equipped with a DC300F CCD digital camera. Nuclei were stained with 4′-6-diamidino-2-phenylindole (DAPI) solution 1:10000 (vol/vol) diluted.

Cytotoxicity Assay

To determine the NK cell cytotoxicity, CytoTox 96 nonradioactive cytotoxicity assay (Promega, Madison, WI) was used, based on the colorimetric detection of the released enzyme LDH. NK-92 cells were harvested, washed, counted, and diluted at the concentration of 2×10^6 cells/mL in BM with 5% FCS. Effector cells were then seeded, serially diluted, ranging routinely from 1×10^5 to 6.25×10^3 cells, in round bottom 96-well Costar plates (Costar, Rochester, NY). Target cells were harvested, washed, resuspended to 2×10^5 cells/mL in BM with 5% FCS, alone or in the presence of 20 μg/mL of DIATHIS1 and incubated for 30 minutes at RT. The target cells were then added and incubated with effector cells for 4 hours at 37°C at increasing effector/target cell ratio ranging from 0.625:1 to 10:1 in quadruplicate. A total of 50 μL of supernatants were assayed for LDH activity according to the manufacturer's instruction. Controls for spontaneous and maximum LDH release in effector and target cells were also assayed. In some experiments serial dilutions of the scFv DIATHIS1 (from 1 to 20 μg/mL) were essayed in a unique effector/target cell ratio of 10:1. The calculation of cytotoxicity percentage was as follows: (Experimental − Effector Spontaneous − Target spontaneous)/(Target Maximum − Target Spontaneous) × 100.

Degranulation Assay

NK cell–mediated cytotoxicity was evaluated using the degranulation lysosomal marker CD107a as described in.[42,43] As source of effector cells were used NK-92 and NKL cell lines or in vitro–expanded NK cells. Target and effector cells were resuspended to 2×10^6 cells/mL in BM, and 100 μL of cell suspension was added to wells in a V-bottomed 96-well plate for 2 hours in BM at 37°C and 5% CO_2. Thereafter, cells were incubated with anti-CD107a-APC (or cIgG-APC) and in some experiments, with anti-CD3FITC/anti-CD56PE to gate NK cell population. For blocking experiment scFv DIATHIS1 (1 μg/mL) or the irrelevant scFv was added to the cell pellets for 30 minutes at RT.

Determination of Apoptosis

Apoptosis and necrosis were evaluated by Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (eBioscience, San Diego, CA) according to the manufacturer's instructions: briefly, the cells were seeded in 16-well plates (5×10^5 cells/mL) in BM, alone, or in the presence of 20 μg/mL of DIATHIS1 for 20 hours at 37°C. After incubation, the cells were harvested, washed in PBS, and resuspended in 195 μL of binding buffer and 5 μL of Annexin V for 10 minutes at RT. Cells were then washed in binding buffer and resuspended in 190 μL of binding buffer and 10 μL of propide iodide before analysis of the stained cells by FACSCalibur (Becton-Dickinson).

Cell Proliferation Assay

The $CEACAM1^+$ MelC and Mel501 cell lines in exponential phase of growth were collected, washed with warm RPMI-1640, and seeded (in triplicate) in 96-well microtiter Costar plates (Costar) at a density of 5×10^3 cells/wells in a volume of 100 μL/wells. Then, increasing concentrations of scFv DIATHIS1 ranging from 0 to 20 μg/mL in BM were added to a final volume of 200 μL/wells. For all previously described experiments, cell survival was determined by WST-1 assay (PreMix WST-1 cell

proliferation kit; Vinci Biochem, Italy) after 48 and 96 hours of treatment at 37°C in 5% CO_2. The values are calculated as percentage of appropriated controls, namely parallel conditions without antibody.

CEACAM1 Isoforms Analysis by RT-PCR

Total RNA was extracted using the Total RNA Purification Kit (Norgen, Canada) from 3 unrelated samples of 3×10^6 cells from MelC, STA, and Mel501 cultured for 11 days as described previously. Cells were maintained subconfluent and subcultures were made every 2 days. For CEACAM1 isoforms' relative quantification, 100 ng of each purified total RNA were reverse transcribed by using the M-MuLV Reverse Transcriptase KIT (Diatheva S.R.L.), with the primer human CEACAM1 (5′-GTTGTTTCTGTCCC-3′)[44] specific for the 3′ region of human CEACAM1 transcript and recognizing a sequence present in both L and the S isoforms. A total of 0.5, 1, and 2 μL of cDNAs from MelC and Mel501 cell line samples were amplified in PCR assays by using 2 primer for the CEACAM1 L or S isoforms detection or 3 primers for the relative quantity determination of the same isoforms by using the primer sequences[4] and the method already described and validated.[44] Briefly, 30 μL of reaction mix was prepared containing 3 μL of 10× PCR buffer, 0.2 mM dNTPs, 1 U of Hot Rescue DNA polymerase (all reagents are from Diatheva S.R.L.), and 10 pmol of each primer. The reaction conditions were as follows: 94°C for 60 seconds, followed by 30 cycles of 94°C for 30 seconds, 64°C for 15 seconds, 72°C for 40 seconds, and a final extension at 72°C for 7 minutes. In the case of STA primary tumor cells that express lower level of CEACAM1, 3 and 5 μL of cDNAs were amplified and the PCR protocol was modified including 35 cycles of amplification. The sense primer used in the PCR FP49 (5′-GCAACAGGACCACAGTCAAGACGA-3′) recognizes both the L and the S isoforms. The antisense primer BP60 (5′-GTGGTTGGAGACTGAGG GTTTG-3′) recognizes a sequence in exon 7 and is therefore specific for the L isoforms. The antisense primer BP59 (5′-TGGAGTGGTCCTGAGCTGCCG-3′) recognizes sequences on both sides of the splice junction between exons 6 and 8 and is therefore specific for the S isoforms under appropriate stringency conditions. In the triple-primer PCR, the cDNAs of the 2 isoforms compete for the sense primer, which results in a ratio of the products that corresponds to the input ratios of the templates. Amplification products were analyzed on 2.7% agarose gels and the relative quantity of CEACAM1 isoforms and their ratios were determined by quantitative scanning of the bands in the gels by using the Gel Doc instrument and the Quantity One software (BioRad, Hercules, CA).

Western Blotting

For Western blotting analysis, 1.5×10^6 of MelC, Mel501, or STA cells were lysed with 150 μL of lysis buffer consisting of: 50 mM Tris-HCl pH 7.8; 2% (wt/vol) SDS (sodium dodecyl sulfate); 5 mM EDTA (ethylenediaminetetracetic acid); 109 mM NEM (*N*-ethylmaleimide); protease inhibitors: 2 mg/mL Leupeptin, 2 mg/mL Pepstatin, 4 mM AEBSF [4-(2-Aminoethyl) benzenesulphonyl fluoride], and 1 mM PMSF (Phenyl-methylsulphonylfluoride); and phosphatase inhibitors: 1 mM sodium orthovanadate and 1 mM of sodium fluoride. Cells lysates were boiled immediately for 5 minutes and centrifuged at 6000*g* for 13 minutes. For the detection of CEACAM1, 50 μg of total proteins were resolved by SDS-PAGE on 7.5% polyacrilammide gels and then transferred for 60 minutes at 100 V onto 0.22 μm nitrocellulose membranes (Bio-Rad Laboratory, Germany). Membranes were saturated with blocking solution [Tris buffer saline (TBS) containing 5% (w/v) nonfat dry milk] for 1 hour at RT and then incubated ON at 4°C under agitation with the anti-CEACAM1 mAb 4D1/C2 (Merk Millipore) 1:500 (vol/vol) diluted in blocking solution. After 3 washes with TTBS (TBS added with 0.1% Tween 20), membranes were incubated with a goat anti-mouse IgG-HRP-conjugated (Bio-Rad) diluted 1:1000 (vol/vol) in blocking buffer for 1 hour at RT. An antiactin polyclonal antibody 1:1000 (vol/vol) diluted (Sigma Aldrich) and a horseradish peroxidase-conjugated anti-rabbit IgG (Bio-Rad Laboratory) were further utilized for the actin determination. The immunoreactive bands were revealed by the ECL detection system (Amersham Pharmacia Biotec, NJ) as substrate and images collected by a Chemi Doc System (BioRad).

Tissue Cross-Reactivity Studies

Immunohistochemistry study was conducted using human normal and melanomas tissues array systems (TriStar Technology Group, Washington, DC). Slides were processed according to standard protocols and binding revealed using Vectastain ABC (Vector Laboratories, Cambridgeshire, UK). Briefly, the cryostatic tissue sections were fixed for 10 minutes with acetone at −20°C and endogenous peroxidase was blocked with 0.2 % (vol/vol) HCl in ethanol for 15 minutes. After 2 washes with TBS, slides were blocked with normal horse serum and then incubated for 2 hours at RT with various amounts of scFv DIATHIS1 (from 5 to 20 μg/mL). Slides were then washed and incubated for 1 hour at RT with 10 μg/mL of anti-Flag M2 monoclonal antibody (Sigma Aldrich). After washing, slides were incubated with avidin-biotin peroxidase complex for 30 minutes. Finally, DAB substrate (Vector Laboratories) was added and the reaction was stopped after 5 minutes by washing in tap water. Counterstaining was performed with Mayer's hematoxylin (Vector Laboratories) for 10 seconds.

Statistical Analysis

The Student *t* test (2-tailed) was used to assess differences between means of data analyzed using GraphPad Prism software. The *P*-values are indicated in the figure legends. The criterion for statistical significance used was $*P < 0.05$; $**P < 0.01$; $***P < 0.0001$. Data are presented as mean ± SD or where indicated, SEM.

RESULTS

CEACAM1 Expression and DIATHIS1 Reactivity on NK and Melanoma Cells

The CEACAM1 expression levels of human melanoma cell lines, IL-2-dependent NK-92 and NKL cell lines, freshly isolated or in vitro–expanded NK cells from healthy donors determined by using the human scFv DIATHIS1 are shown in Figure 1. Melanoma cell lines MelC and Mel501 express the highest level of scFv DIATHIS1 staining. STA primary melanoma cells were also positive to DIATHIS1 but to a lesser extent. In contrast, ATT primary melanoma cells, M14, A375, and Me-30966 melanoma cell lines did not react with the scFv anti-CEACAM1 antibody (Fig. 1A). The reliability of the scFv DIATHIS1 in

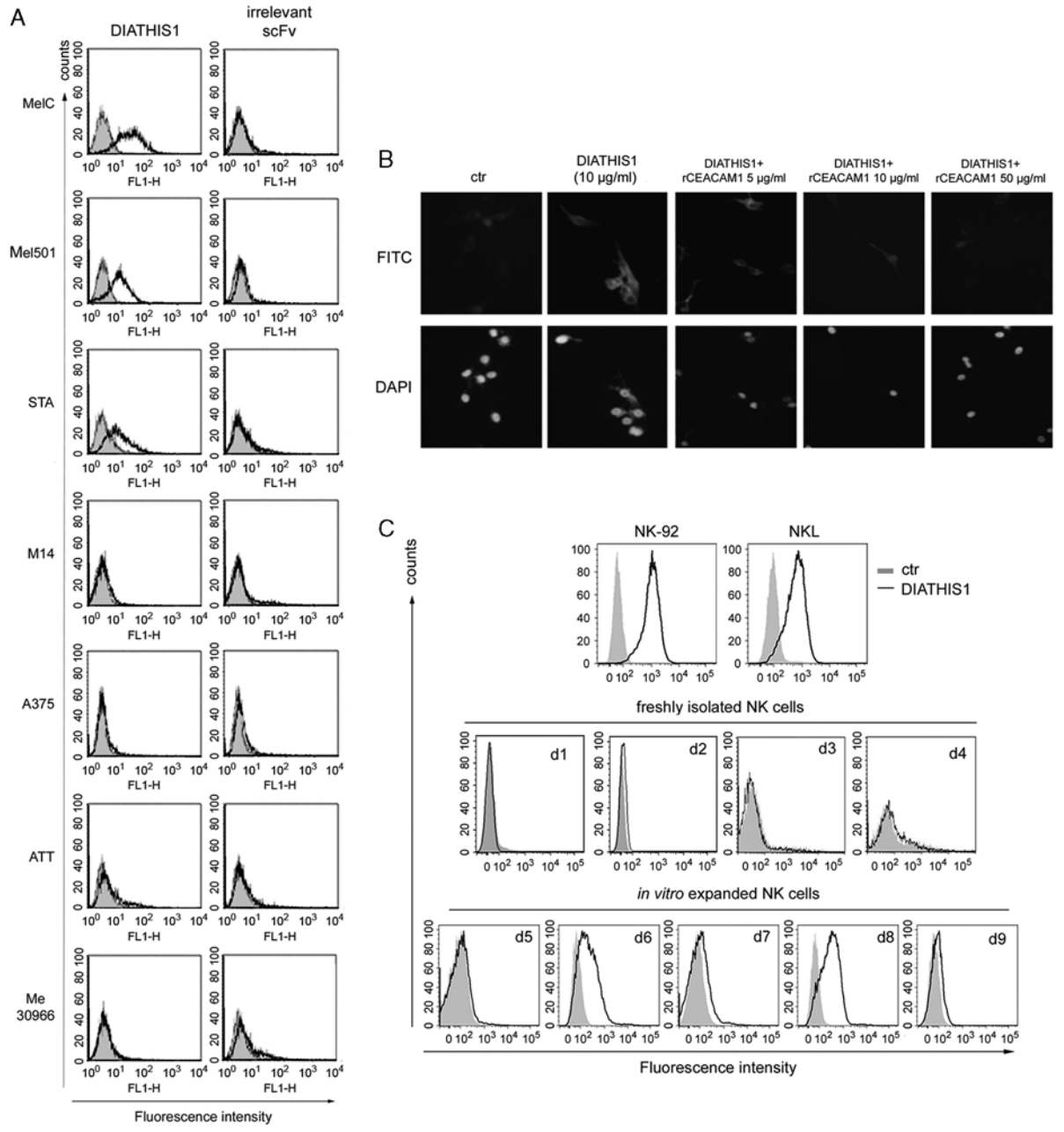


FIGURE 1. DIATHIS1 reactivity on natural killer (NK) and melanoma cells. A, Intact/living melanoma cells were stained with 10 μg/mL of single-chain variable fragment (scFv) DIATHIS1 or with the same amount of an irrelevant human scFv antibody and processed for the identification of the binding pattern profiles by flow cytometry studies. The gray histograms represent cells reacted with secondary antibodies, whereas black lines show the scFv antibodies binding. B, The specificity of the scFv DIATHIS1 for CEACAM1 was evaluated with a competitive binding assay. MelC cells were exposed to 10 μg/mL of scFv DIATHIS1 and in separated samples soluble rCEACAM1 was also added at increasing concentrations ranging from 5 to 50 μg/mL. Negative control (ctr) is represented by MelC cells reacting with secondary antibodies only. The upper panel shows the specific scFvDIATHIS1 binding revealed with the FITC fluorophore, whereas in the lower panel the counterstaining of nuclei with DAPI is shown. C, The binding pattern of scFv DIATHIS1 was also evaluated on IL-2-dependent NK-92 and NKL cell lines as well as on freshly isolated and in in vitro–activated NK cells derived from different healthy donors. Cells were stained with 10 μg/mL of scFv DIATHIS1 and processed for conventional flow cytometry studies. The gray histograms represent cells reacted with secondary antibodies (ctr), whereas black lines show the level of the scFvDIATHIS1 antibody binding. d indicates donor.

specifically recognizing the CEACAM1 expression on the cell surface of melanoma cells is confirmed by the standard controls currently used in flow cytometry studies and in particular by the parallel staining of the cell samples with an irrelevant human scFv isolated using the same biopanning approach from a phage display scFv library. This

irrelevant scFv shows identical biochemical and structural organization of DIATHIS1 but targets a different cell surface antigen not present in melanoma cells (A. Mallano, unpublished). As it shown in Figure 1A (right panel), melanoma cells are negative to the irrelevant scFv staining. To further confirm the specificity of DIATHIS1 for CEACAM1 expressed on the surface of melanoma cells, we sought as to whether the coincubation of the scFv with increasing amounts of soluble recombinant CEACAM1 correlates with a significant reduction of DIATHIS1 staining on the cell surface of MelC used as a representative CEACAM1$^+$ melanoma cell line. This study, providing evidence of a direct competition between the native and recombinant CEACAM1 proteins for DIATHIS1 confirms that this scFv is a specific immune reagent for detection of CEACAM1-expressing cells (Fig. 1B). The NK established cell lines NK-92 and NKL depending for their in in vitro growth and effector function by the presence of exogenous IL-2 express high amount of CEACAM1[26,29] as determined also by the level of scFv DIATHIS1 staining (Fig. 1C), whereas CEACAM1 was not detectable by DIATHIS1 in freshly isolated NK cells from healthy donors (Fig. 1C). As determined by DIATHIS1-binding profiles, in vitro–expanded cytokine-activated NK cells from healthy donors show different levels of CEACAM1 expression on their cell surface (Fig. 1C). Nonetheless, the binding pattern profiles in activated and freshly isolated NK cells might be differently outlined depending on the typology of antibody used for CEACAM1 detection. To this regard, in some cases, NK cells not reactive with scFv DIATHIS1 showed an evident reactivity with a polyclonal antibody raised against the *N*-terminal domain of CEACAM1 (Supplementary Fig. 1, Supplemental Digital Content 1, http://links.lww.com/JIT/A400). The differential staining of these 2 antibodies might be explained by biochemical, structural, and functional differences between an immune reagent in scFv format such as DIATHIS1 and the polyclonal antibody which is usually formed by a pool of immunoglobulins which reflect a dramatic heterogeneity in terms of isotypes, affinity, and binding sites pattern which may include similar epitopes shared by different CEACAM's family members. To this regard, the polyclonal antibody used in this study to control CEACAM1 expression on activated NK cells, cross-reacts with several other CEACAM's family members with the exception of CEACAM8. In this context, the CEACAM1-binding profiles on activated NK cells obtained using the specific anti-CEACAM1 mAb 4D1/C2 are very similar to those of DIATHIS1 confirming and extending the reliability of the scFv DIATHIS1 as immune reagent for CEACAM1 detection on the cell surface of effector and target cells (Supplementary Fig. 1, Supplemental Digital Content 1, http://links.lww.com/JIT/A400). Another possible explanation of the different staining profile of the polyclonal antibody and scFv DIATHIS1 could be represented by heterophilic interactions of CEACAM1 on the surface of activated NK cells that could mask the DIATHIS1-binding site located in a well-defined region in the *N*-terminal domain of CEACAM1 (V. Fiori et al, manuscript in preparation) impeding its binding while they could be intercepted by the polyclonal antibody recognizing multiple epitopes in the same molecule. Indeed, CEACAM1 can interact and form heterodimers via its *N*-terminal domain with several receptors including NKG2D[29] and TIM-3[30] that are highly expressed on human NK cells.

DIATHIS1 is Able to Increase the NK-mediated Cytotoxicity of Melanoma Cells

To assay the functional role of the scFv DIATHIS1 in mediating NK cells killing activity, we designed an in vitro study where melanoma cells were incubated with scFv DIATHIS1 and then NK-92 used as effector cells added to the cultures. The 2 melanoma cell lines MelC and Mel501 expressing high level of CEACAM1 were treated with increasing scFv concentrations (ranging from 1 to 20 μg/mL) at an effector:target (E:T) ratio of 10:1, whereas in a second set of experiments MelC, Mel501, STA primary tumor cells, and the CEACAM1$^-$[45] A375 melanoma cell line were incubated with a unique DIATHIS1 concentration (20 μg/mL) and then NK-92 cells added to the cultures at increasing E:T ratios. The results showed a significant increase of NK-92 cell–mediated cytotoxicity against MelC cells in parallel with the increasing of the antibody concentration (Fig. 2A) and E:T ratios (Fig. 2C). It is interesting to note that treatment of Mel501 cells with DIATHIS1 induces a lower increase of NK-92–mediated cytotoxicity in comparison to MelC cells (Figs. 2B, D). In line with the low percentage of CEACAM1$^+$ cells observed, STA cells treated with DIATHIS1 showed an increase in cytotoxicity only at high E:T ratios (Fig. 2E). However, the increase was significant and very similar to that seen in MelC (+ 45% and + 48% of cell-mediated cytotoxicity at E:T ratio of 10:1, for STA and MelC, respectively). The CEACAM1$^-$melanoma cell line A375 was not susceptible to DIATHIS1 treatment as no increase of cytotoxicity was observed confirming the role of CEACAM1 in mediating the effector function of NK-92 cell line. Taken together, these data are in good agreement with previously published observations reporting that the blocks of CEACAM1 homophilic interactions with polyclonal antibodies and/or CEACAM1-specific mAbs enhanced the killing of CEACAM1-expressing melanoma cells by NK and T cells in an antigen-restricted manner.[25,30,31] To evaluate whether pretreatment of target cells with DIATHIS1 could promote NK cell degranulation capacity, the release of granule exocytosis was evaluated analyzing CD107a expression on in vitro–expanded NK cells derived from healthy donors (Supplementary Fig. 2, Supplemental Digital Content 2, http://links.lww.com/JIT/A401) upon their interaction with MelC or Mel501 cells. Activated NK cells exhibited a significant higher expression of CD107a when interacting with MelC or Mel501 target cells preincubated with DIATHIS1 (Figs. 3A, D). In contrast, the treatment of target cells with the irrelevant scFv does not result in increased NK cell degranulation (Figs. 3A–C). As negative control, the melanoma A375 and a multiple myeloma SKO-007-J3 cell lines that not express CEACAM1 were used. In these cells the increase in NK cell degranulation was not observed further confirming the specificity of the treatment (Supplementary Fig. 3, Supplemental Digital Content 3, http://links.lww.com/JIT/A402). In line with these results, NK-92 and NKL degranulation capacity was higher after incubating MelC or Mel501 target cells with the scFv DIATHIS1 (Figs. 3B, C). In the table reported in Figure 3D the difference of the percentage of CD107a$^+$ cells upon scFv DIATHIS1 treatment minus the percentage of the CD107a$^+$ cells in the control sample is shown.

To rule out that the scFv DIATHIS1 could interfere with cellular cytotoxicity, the direct effect of the scFv on melanoma cell lines was tested analyzing apoptosis and

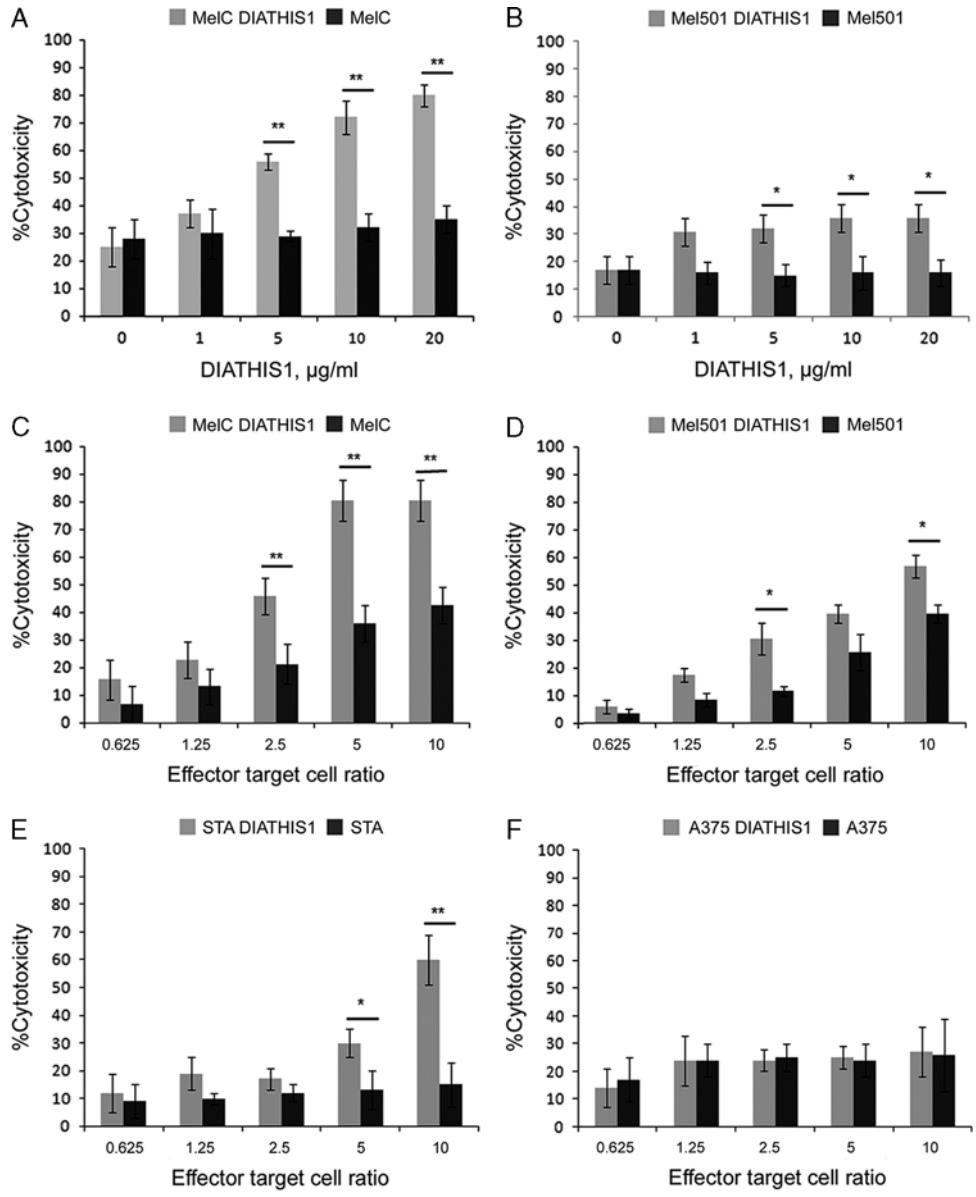
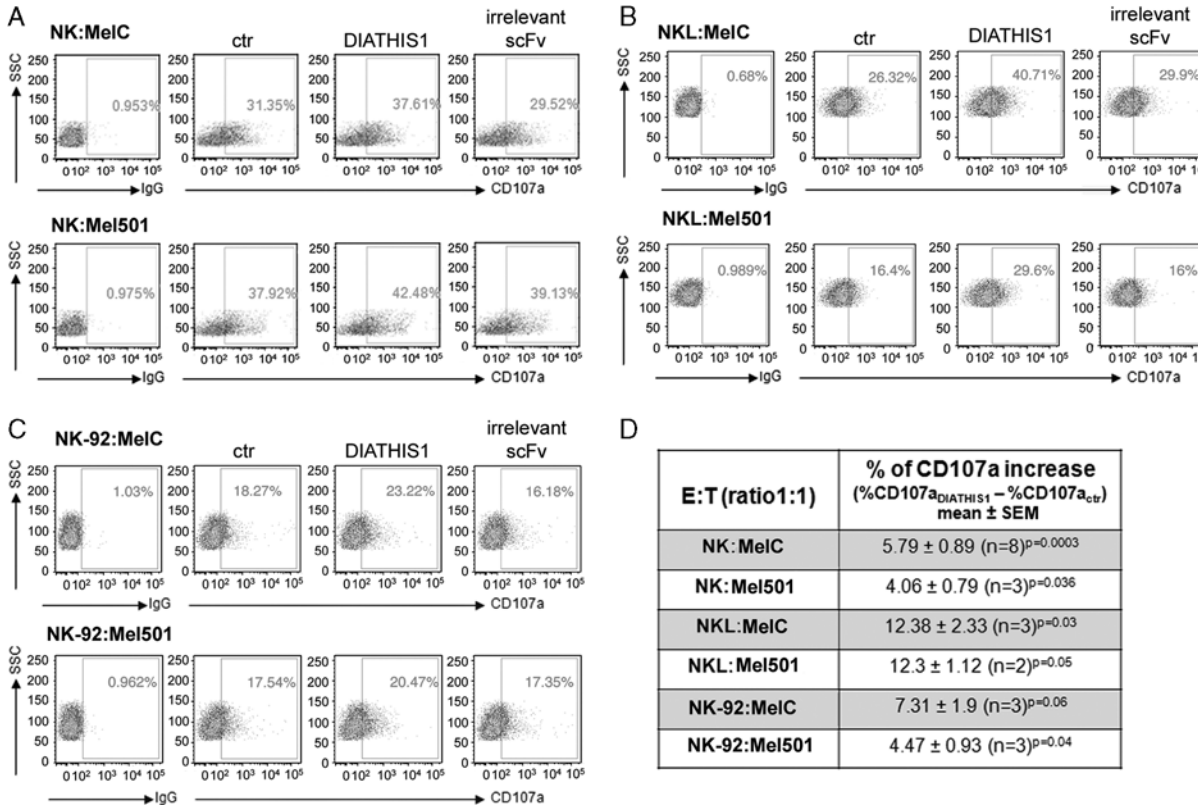


FIGURE 2. Natural killer (NK)-92 cell–mediated cytotoxicity. A and B, The killing activity of NK-92 cell line against MelC or Mel501 in the absence or in the presence of various concentrations of the single-chain variable fragment (scFv) DIATHIS1 ranging from 1 to 20 μg/mL was evaluated. In this study a unique E:T of 10:1 was used. C–F, The cytotoxic activity of NK-92 cells against MelC, Mel501, STA, and A375 melanoma cells was evaluated in the presence of 20 μg/mL of DIATHIS1 at E:T ratios ranging from 10:1 to 0.625:1. The black bars represent the intrinsic killing activity of NK-92 cell line, whereas the gray bars show the increase of NK-92 cell–mediated cytotoxicity when the cells were incubated in the presence of scFv DIATHIS1. Results are representative of 4 independent experiments. The significance was assessed by Student *t* test. All data are the mean ± SD; $*P<0.05$; $**P<0.01$.

proliferation. The incubation of MelC or Mel501 with the scFv DIATHIS1 did not induce apoptosis (Fig. 4A) nor affected the extent of net proliferation on days 2 and 4 at all concentrations evaluated (Fig. 4B).

CEACAM1 Isoforms Expression

The different response exerted by DIATHIS1 on NK-92 cell–mediated cytotoxicity of MelC, Mel501, and STA melanoma cells prompted us to analyze the CEACAM1 isoforms expression in these cells as long (L) and short (S) isoforms can exert different activities on tumor cells and their ratio has been reported to have a role in the tumorigenesis.[46] In addition, very recently it has been observed that CEACAM1-3S variant may be able to trigger melanoma cells for NK cell–mediated cytolysis by upregulating cell surface expression of MICA and ULBP2 NKG2D ligands, whereas CEACAM1-4L acts in the opposite manner inducing their shedding.[45] To gain information about the relative amount of CEACAM1 isoforms expressed in these cells, we used a competitive RT triple-primer PCR method already described and validated in which one common sense primer was used together with 2 antisense



D

E:T (ratio1:1)	% of CD107a increase ($\%CD107a_{DIATHIS1} - \%CD107a_{ctr}$) mean ± SEM
NK:MelC	5.79 ± 0.89 (n=8)$^{p=0.0003}$
NK:Mel501	4.06 ± 0.79 (n=3)$^{p=0.036}$
NKL:MelC	12.38 ± 2.33 (n=3)$^{p=0.03}$
NKL:Mel501	12.3 ± 1.12 (n=2)$^{p=0.05}$
NK-92:MelC	7.31 ± 1.9 (n=3)$^{p=0.06}$
NK-92:Mel501	4.47 ± 0.93 (n=3)$^{p=0.04}$

FIGURE 3. DIATHIS1 enhances natural killer (NK) cell degranulation capacity. NK cell–mediated cytotoxicity was evaluated using the degranulation lysosomal marker CD107a. A–C, In vitro–expanded NK cells, NKL and NK-92 cell lines were used as source of effector cells. MelC or Mel501 target cells were preincubated with or without 1 μg/mL of single-chain variable fragment (scFv) DIATHIS1 or the irrelevant scFv for 30 minutes before incubation with the effectors. Results are expressed as the percentage of $CD107a^+$ cells. D, The table summarizing the mean of percentage of CD107a increase in different experiments is shown. n = number of experiments. The significance was assessed by Student *t* test. All data are the mean × SEM; *P* values are indicated in the figure.

primers that selectively recognize the CEACAM1 L and S splice variants.[44,47] In the PCR, the cDNAs of the 2 isoforms compete for the sense primer which results in a ratio of the products that correspond to the input ratios of the templates. STA cells were confirmed to express low levels of CEACAM1 as higher amounts of cDNAs were needed for amplification compared with MelC and Mel501 and the PCR protocol was modified to include 35 cycles of amplification instead of 30 to allow the relative isoforms quantification. We found that MelC and Mel501 cell lines as well as STA primary melanoma cells express the same CEACAM1 variants that are CEACAM1-4L, CEACAM1-4S, CEACAM1-3L, and CEACAM1-3S but the relative amount of each isoform differs in the 3 cell cultures (Fig. 5A). It is interesting to note that the prevalent CEACAM1 isoform is CEACAM1-4L in MelC and Mel501 while STA expresses prevalently CEACAM1-4L and CEACAM1-3L isoforms at similar level (33.43 ± 4.18% and 32.64 ± 3.50%, respectively, relative to total CEACAM1; Figs. 5A, C). The less expressed isoform is CEACAM1-3S in all cell lines, but its relative amount is significantly higher in MelC and even more in STA compared with Mel501. The expression of both CEACAM1-3 variants (CEACAM1-3L and CEACAM1-3S) lacking the A2 extracellular domain is significantly higher in MelC and STA versus Mel501 cells which, on the contrary, express higher levels of CEACAM1-4 isoforms and in particular of CEACAM1-4L (Figs. 5A, C). In contrast, the ratio between the CEACAM1 L and S isoforms (CEACAM1-4L + CEACAM1-3L:CEACAM1-4S + CEACAM1-3S) is similar in the 2 cell lines but lower in STA primary tumor cells being 2.33 ± 0.35, 2.58 ± 0.45, and 1.96 ± 0.23 in MelC, Mel501, and STA, respectively. To validate the PCR results, we performed a Western blot analysis by using the specific anti-CEACAM1 mAb 4D1/C2 on total cell lysates obtained from MelC, Mel501, and STA cells. The results (Fig. 5B) showed a high expression of both CEACAM1-4L and S and CEACAM1-3L and S variants in MelC, whereas only a band corresponding to CEACAM1-4L and S isoforms was detected in Mel501 even if a faint signal corresponding to CEACAM1-3 isoforms appeared after a long time of exposure (Fig. 5B). We failed to observe any CEACAM1-reactive band in STA cells in Western blot probably due to the low expression as seen at the mRNA level in PCR and in the flow cytometry analysis where only a small percentage of $CEACAM^+$ cells were found.

As it has been shown that colon cancer cells with forced expression of CEACAM1 downmodulate their NKG2D-activating ligands independently from the presence of ITIM domain,[28] whereas as previously reported, it has been recently observed that CEACAM1-3S enhances the surface expression of the NKG2D ligands MICA, ULBP2, and DNAM-1 ligand PVR (CD155) on transfected melanoma cells[45] we decided to evaluate NKG2D and DNAM-1 ligands expression on MelC, Mel501 cell lines

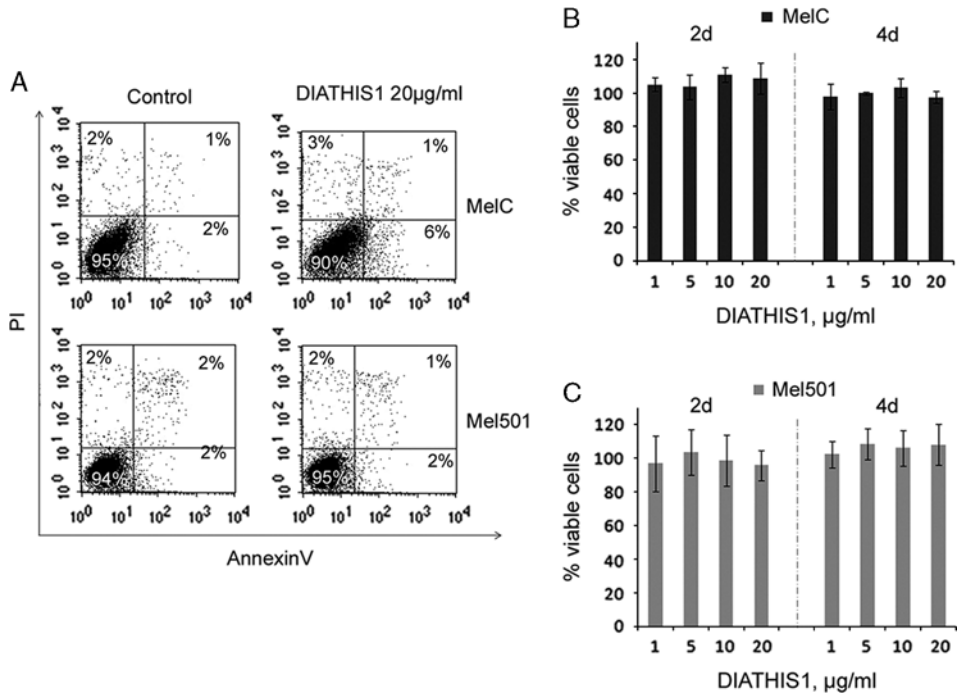


FIGURE 4. DIATHIS1 does not interfere with cellular processes. A, Apoptosis was determined by AnnexinV-FITC and propidium iodide staining in MelC and Mel501 cells after 20 hours of incubation with or without 20 µg/mL of the single-chain variable fragment (scFv) DIATHIS1. B and C, MelC and Mel501 melanoma cell lines were cultured for 2 or 4 days in the absence or presence of different amounts of scFv DIATHIS1 ranging from 1 to 20 µg/mL. At the indicated time points, cells were tested for proliferation in a WST-1 assay. The figure shows 1 representative experiment, and data are expressed as percentage of untreated control cells with each concentration tested in triplicate.

and STA primary tumor cells that naturally express different levels of CEACAM1 isoforms. As shown in Figure 6, STA cells expresses all the ligands tested and show the highest levels of ULBPs ligands compared with MelC and Mel501 cells. Also in MelC the expression of ULBPs ligand was higher with respect to Mel501. No relevant differences were found on MICA and PVR expression.

scFv DIATHIS1: Immunohystochemistry Studies

Differently from polyclonal and monoclonal antibodies originated from classical hybridoma technology, DIATHIS1 has been selected from a phage display library using the CEACAM1 antigen as a bait. This type of selection might originate a scFv with a unique CEACAM1 recognition properties.[48,49] In particular, the scFv DIATHIS1 may recognize a epitope expressed on melanoma cells (Supplementary Fig. 4A, Supplemental Digital Content 4, http://links.lww.com/JIT/A403) thus mimicking the targeting of a tumor-associated antigen. In contrast, the scFv DIATHIS1 does not react with none of the 30 normal human tissues specimen examined (Supplementary Fig. 4B, Supplemental Digital Content 4, http://links.lww.com/JIT/A403) confirming the high specificity of this antibody fragment for CEACAM1 expressed in tumor cells. In addition, the differential staining properties could be also due by the different expression level of the CEACAM1 in melanoma cells in comparison to normal tissues. Hence, the different level of affinity which generally characterize scFv and mAbs may act as discrimination parameter in CEACAM1 detection in tissues and cells.

DISCUSSION

Treatment options remain very limited for patients with metastatic melanoma. Median survival is 7–10 months and this malignancy is resistant to chemotherapy and radiotherapy.[50] New treatments employing kinase inhibitors targeting the RAS/RAF/MEK pathway are only applicable to 50% of patients with specific mutations in the BRAF kinase, and only 50% of these patients respond to target-directed treatment. In patients responding to treatment, duration is typically short.[51,52] Furthermore, patients with ocular melanoma have not generally responded to currently available therapies.[53] In contrast, new immunotherapeutic strategies employing mAbs blocking immune checkpoint molecules and able to boost the innate immunity against tumor cells have recently given important results in clinical trials leading to the approval of the anti-CTLA4 mAb ipilimumab in 2011 and of 2 anti-PD1 antibodies in 2014.[54,55] However, the response rate for ipilimumab is only around 15%, and long-term durability of response or stable disease occurs in a minority of patients. These data demands the search of new and effective immunotherapeutic options and combinations for melanoma treatment. Indeed, preclinical and ongoing clinical studies involving immune checkpoint inhibitor combinations have been shown to be more effective with respect to the administration of the single agents.[56] The adoptive cell transfer of lymphocytes is another immunotherapeutic option with the advantage of being not affected by previous treatments. In the allogenic setting, the anti-tumor activity of NK-cell or T-cell infusions has been well

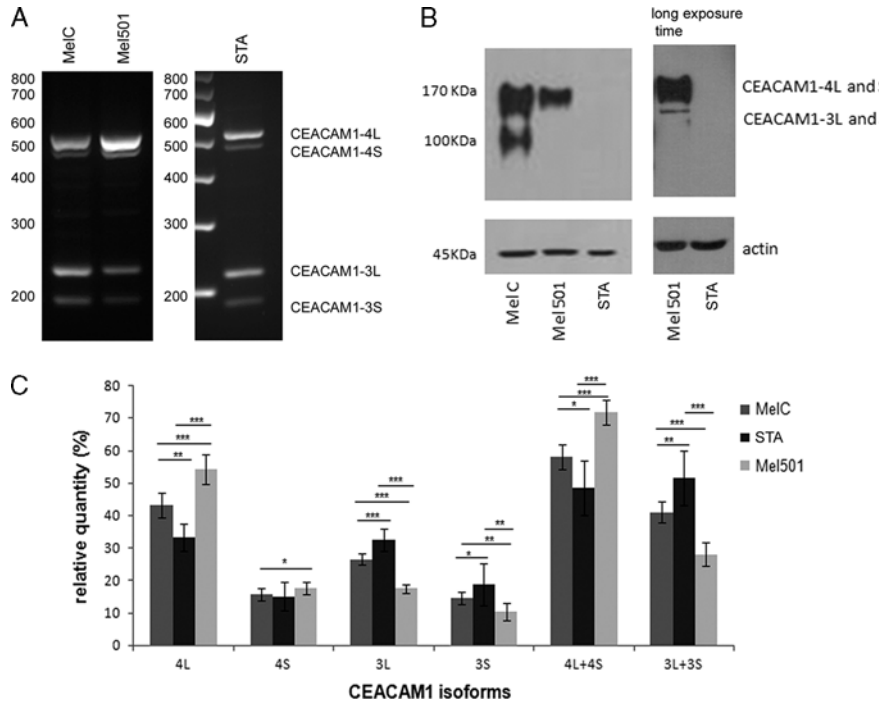


FIGURE 5. Determination of CEACAM1 isoforms. RNAs isolated from MelC and Mel501 cell lines and from STA primary melanoma cells were analyzed in RT-PCR assays for the relative quantities determination of CEACAM1 isoforms by using the primer sequences and the method described and validated in.[44] A, The gels represent a typical PCR result obtained with cDNAs from MelC, Mel501, and STA as template. The common sense primer was FP49. The antisense primers, L and S, were BP60 and BP59, respectively. PCRs were performed by using 3 primers (L + S) for the relative quantification. B, The Western blot analysis of CEACAM1 isoforms on total cellular lysates from MelC, Mel501, and STA melanoma cells reacted with the anti-CEACAM1 mAb 4D1/C2. The right panel shows the signals obtained on Mel501 and STA samples after a long time exposure. Total proteins were loaded at equal amounts and equal loading was monitored by antiactin blotting. C, The histograms reporting the relative quantities of CEACAM1 isoforms mRNA expression in percentage on the total CEACAM1 mRNA expression determined by quantitative scanning of the bands in the gels. All data are representative of 3 independent experiments. All data are the mean ± SD; $*P < 0.05$; $**P < 0.01$; $***P < 0.0001$ (2-tailed Student t test).

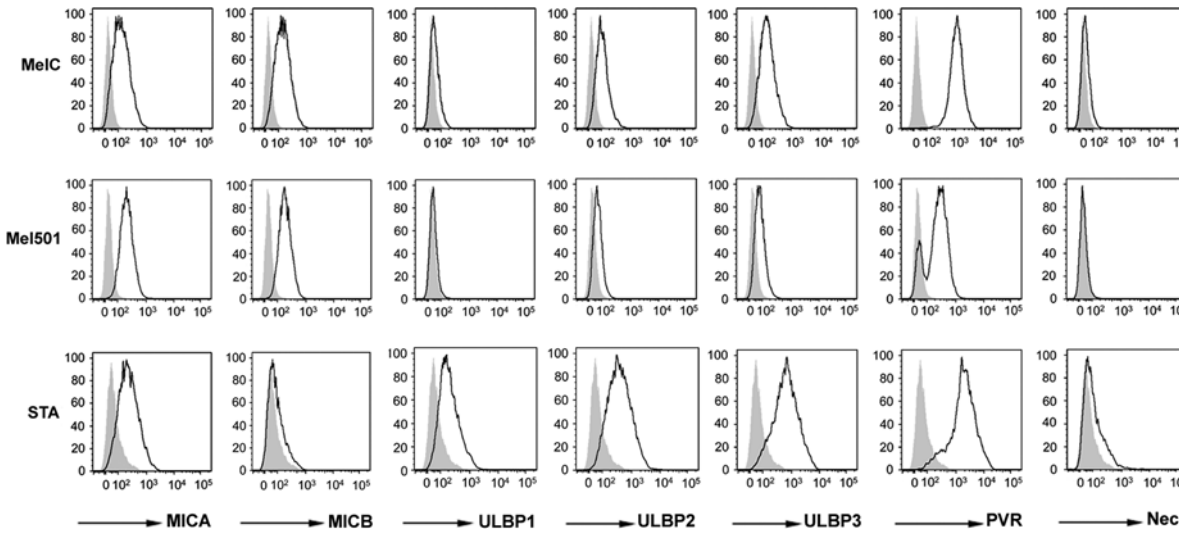


FIGURE 6. NKG2D and DNAM-1 ligands expression. NKG2D and DNAM-1 ligands staining on MelC, Mel501, and STA melanoma cells was analyzed by flow cytometry. The gray histograms represent the isotype control antibody, whereas black lines represent the specific binding.

documented in animal models and in patients with different tumors including metastatic melanoma.[57,58]

The efficacy of these immunotherapeutic treatments might be hindered in metastatic melanomas expressing CEACAM1 on their surface acting as an inhibitor of effector T-cell function.[25] Indeed, by targeting CEACAM1 with specific rodent antibodies the homophilic interactions between effector and target cells might be blocked yielding substantial anticancer effects both in vitro and in vivo,[25,30,31,59] and CEACAM1 blockade has been shown to synergize with other immune checkpoint inhibitors in mice models.[30] Of note, CEACAM1 that is not expressed on normal melanocytes was found to be highly expressed both in melanoma cell lines and primary melanoma cells from patients and found to be increased along disease progression.[16,25,45,60–62] The presence of CEACAM1 on primary cutaneous melanoma lesions strongly predicted the development of metastatic disease and is strongly associated with reduced disease-free survival of patients.[16]

In this context, by using for the first time a fully human antibody fragment in scFv format for human CEACAM1 targeting, we were able to improve the ability of NK cells to kill melanoma cells. The coincubation of melanoma cell lines expressing CEACAM1 on their surface with different NK effector cell types (NK-92, NKL, and activated NK cells from healthy donors) significantly enhance the activation of NK cells and the cell-mediated cytotoxicity against cancer cells (Figs. 2, 3) without interfering with general cellular processes such as apoptosis and proliferation (Fig. 4). It is interesting to note that the melanoma MelC cell line and STA primary tumor cells that showed an higher increase in the NK-92-mediated cytotoxicity after DIATHIS1 treatment compared with Mel501 cells, express lower levels of CEACAM1-4L and CEACAM1-4S and higher level of CEACAM1-3L and CEACAM1-3S isoforms lacking the extracellular IgC2-like A2 domain (Fig. 5; the ratio between CEACAM1-4L + 4S and CEACAM1-3L + 3S isoforms is of 0.978 ± 0.323, 1.428 ± 0.174, and 2.622 ± 0.466 for STA, MelC, and Mel501 cells). In contrast, the ratio between long and short isoforms of CEACAM1 is similar in the 3 cell types. Many studies have analyzed the role of CEACAM1 S and L isoforms in the activation or inhibition of lymphocytes and cancer cells showing that their ratio is crucial for the CEACAM1 function[15,46,63–69] but less is known about a possible different role of CEACAM1-4 and CEACAM1-3 isoforms. Our data seem to suggest that a higher expression of CEACAM1-3 isoforms to the detriment of CEACAM1-4 isoforms in melanoma cells may be correlated with a better response to NK cell–mediated cytotoxicity after blocking CEACAM1 transhomophilic interactions and that the efficacy of DIATHIS1 treatment depends not only on the CEACAM1 expression levels but also on the relative expression of the 4 isoforms. During the conduction of the experiments reported in the present article, Ullrich et al[45] published a very interesting work, describing for the first time the different function of these 4 CEACAM1 isoforms in melanoma cells motility and NK cell–mediated killing susceptibility. In particular, they found in human melanoma biopsies that CEACAM1-3S and CEACAM1-3L expression is induced during disease progression and that CEACAM1-3S expression correlate with prolonged survival. Further, they provide evidence that CEACAM1-3S triggers melanoma cells for NK cell–mediated cytolysis by upregulating cell surface expression of MICA and ULBP2, whereas CEACAM1-4L acts in the opposite manner inducing the shedding of both NKG2D ligands. In agreement with these findings, we found that STA and MelC cells, that showed an higher increase of NK cell–mediated cytolysis following DIATHIS1 treatment express higher amount of CEACAM1-3S and lower amount of CEACAM1-4L and has a higher surface expression of ULBP2 and PVR activating ligand compared with Mel501 cell line. In addition, we also found a higher expression of ULBP1 and ULBP3 in these cells. No relevant differences were found on MICA expression. It is interesting to note that we observed a positive correlation between the degree of expression of these ligands and the relative expression of CEACAM1-3 isoforms in the 3 melanoma cell cultures with the highest values of both parameters observed in STA primary tumor cells followed by MelC and with the lowest in Mel501 cell line.

The human scFv DIATHIS1 in comparison to mAbs to human CEACAM1 such as the previously published MRG1[31] originated by classic hybridoma technology and used for a novel antimelanoma immunotherapy in combination with effector NK and T cytotoxic cells shows unique structural and functional properties. In fact, DIATHIS1 is completely human and formed only by variable VH and VL domains joined by a linker peptide resulting in a 27 kDa antibody fragment, which is much smaller than a murine mAb of IgG subclass (140–150 kDa). This biochemical property is of particular medical relevance in mAb-based treatment of solid tumors where physiological parameters such as imperfect vascular supply and increased interstitial pressure, inhibit the diffusion of larger molecules.[70] Moreover, the slow rates of clearance of intact mAbs due to their large size cause significant exposure to normal organs and limit the quantities delivered to tumors.[70] In addition, the DIATHIS1 affinity is in the range of 10^{-8} M and should allow good target selectivity and a homogeneous tumor penetration in comparison to high affinity binder molecules.[70,72] CEACAM1 is expressed in a number of normal epithelial, endothelial, and immune cell compartments. Furthermore, CEACAM1 modulates a number of cellular functions such as angiogenesis, liver insulin clearance, as well as innate and adaptive immune responses, including those elicited by microbial and viral infection.[2] This distribution raises safety concerns about the direct effect of DIATHIS1 binding on these crucial biological functions. To this regard, the scFv DIATHIS1 does not bind to nonstimulated NK cells (Fig. 1 and Supplementary Fig. 1, Supplemental Digital Content 1, http://links.lww.com/JIT/A400) and, when used at the lower concentration giving the maximum staining for malignant melanoma (10 μg/mL) (Supplementary Fig. 4A, Supplemental Digital Content 4, http://links.lww.com/JIT/A403), shows in a tissue microarray investigation no evident cross-reactivity with 30 different human normal tissues (Supplementary Fig. 4B, Supplemental Digital Content 4, http://links.lww.com/JIT/A403). Different from scFv DIATHIS1, the mAb MRG1 (kd 1.46–2.83 nmol/L) which is originated by classic hybridoma technology where the commercial CEACAM1 protein was used as immunizing agent,[31] reacts with several normal human tissues and cells and some selective staining was observed in the luminal side of epithelial cells of ducts or glands in hollow viscera. The differential staining properties may be due to the different level of affinity which generally characterize scFv and mAbs and may act as discrimination parameter in CEACAM1

detection in tissues and cells. In alternative, biopanning selection by phage display that scFv library may intercept specific binding sites which do not induce a humoral immune response via animal immunization. Taken in aggregate, these findings provide evidences that scFv DIATHIS1 represents a unique reagent for targeting CEACAM1-expressing tumors supporting its medical relevance in immunotherapeutic interventions. In conclusion, the scFv DIATHIS1 meets all the properties required for a potential anticancer compound: it is human, hence poorly or not at all immunogenic, its affinity is in a range for efficient tissue penetration, capture, and retention in tumor cells while its small molecular size should facilitate rapid plasma clearance.[73] Furthermore, scFvs can be easily engineered to build up oligomeric derivatives such as diabodies or small immunoproteins and whole IgG antibodies if a longer half-life it is required in particular clinical settings. To this regard, a new diabody format of DIATHIS1 with increased efficacy in blocking homophilic interaction between CEACAM1 and NK effector cells has been already generated. The findings here reported and discussed indicate that the DIATHIS1 represents a potential platform for developing completely human anticancer therapeutics and may be used to complement the curative armamentarium of immunotherapeutic interventions against melanoma and to design novel and effective model of cancer therapy mediated by adoptive NK or T cells' transfer and/or new combinations of immune checkpoint antibodies.

CONFLICTS OF INTEREST/ FINANCIAL DISCLOSURES

V.F. and D.M. are employees of Diatheva S.R.L. M.C., V.F., D.M., S.D., and A.A. are listed as inventors in the patent application N. PCT/IB2010/055135. M.M. owns stock in Diatheva S.R.L. All the remaining authors have declared there are no financial conflicts of interest with regard to this work.

REFERENCES

1. Beauchemin N, Draber P, Dveksler G, et al. Redefined nomenclature for members of the carcinoembryonic antigen family. *Exp Cell Res*. 1999;252:243–249.
2. Beauchemin N, Arabzadeh A. Carcinoembryonic antigen-related cell adhesion molecules (CEACAMs) in cancer progression and metastasis. *Cancer Metastasis Rev*. 2013;32:643–671.
3. Kuespert K, Pils S, Hauck CR. CEACAMs: their role in physiology and pathophysiology. *Curr Opin Cell Biol*. 2006;18:565–571.
4. Barnett TR, Kretschmer A, Austen DA, et al. Carcinoembryonic antigens: alternative splicing accounts for the multiple mRNAs that code for novel members of the carcinoembryonic antigen family. *J Cell Biol*. 1989;108:267–276.
5. Obrink B. CEA adhesion molecules: multifunctional proteins with signal-regulatory properties. *Curr Opin Cell Biol*. 1997;9:616–626.
6. Beauchemin N, Kunath T, Robitaille J, et al. Association of biliary glycoprotein with protein tyrosine phosphatase SHP-1 in malignant colon epithelial cells. *Oncogene*. 1997;14: 783–790.
7. Schumann D, Chen CJ, Kaplan B, et al. Carcinoembryonic antigen cell adhesion molecule 1 directly associates with cytoskeleton proteins actin and tropomyosin. *J Biol Chem*. 2001;276:47421–47433.
8. Gray-Owen SD, Blumberg RS. CEACAM1: contact-dependent control of immunity. *Nat Rev Immunol*. 2006;6:433–446.
9. Neumaier M, Paululat S, Chan A, et al. Biliary glycoprotein, a potential human cell adhesion molecule, is down-regulated in colorectal carcinomas. *Proc Natl Acad Sci USA*. 1993;90:10744–10748.
10. Bamberger AM, Riethdorf L, Nollau P, et al. Dysregulated expression of CD66a (BGP, C-CAM), an adhesion molecule of the CEA family, in endometrial cancer. *Am J Pathol*. 1998;152:1401–1406.
11. Riethdorf L, Lisboa BW, Henkel U, et al. Differential expression of CD66a (BGP), a cell adhesion molecule of the carcinoembryonic antigen family, in benign, premalignant, and malignant lesions of the human mammary gland. *J Histochem Cytochem*. 1997;45:957–963.
12. Lin SH, Pu YS. Function and therapeutic implication of C-CAM cell-adhesion molecule in prostate cancer. *Semin Oncol*. 1999;26:227–233.
13. Luo W, Tapolsky M, Earley K, et al. Tumor-suppressive activity of CD66a in prostate cancer. *Cancer Gene Ther*. 1999;6:313–321.
14. Fiori V, Magnani M, Cianfriglia M. The expression and modulation of CEACAM1 and tumor cell transformation. *Ann Ist Super Sanita*. 2012;48:161–171.
15. Kiriyama S, Yokoyama S, Ueno M, et al. CEACAM1 long cytoplasmic domain isoform is associated with invasion and recurrence of hepatocellular carcinoma. *Ann Surg Oncol*. 2014;21(suppl 4):505–514.
16. Thies A, Moll I, Berger J, et al. CEACAM1 expression in cutaneous malignant melanoma predicts the development of metastatic disease. *J Clin Oncol*. 2002;20:2530–2536.
17. Laack E, Nikbakht H, Peters A, et al. Expression of CEACAM1 in adenocarcinoma of the lung: a factor of independent prognostic significance. *J Clin Oncol*. 2002;20: 4279–4284.
18. Ergun S, Kilik N, Ziegeler G, et al. CEA-related cell adhesion molecule 1: a potent angiogenic factor and a major effector of vascular endothelial growth factor. *Mol Cell*. 2000;5:311–320.
19. Wagener C, Ergun S. Angiogenic properties of the carcinoembryonic antigen-related cell adhesion molecule 1. *Exp Cell Res*. 2000;261:19–24.
20. Tilki D, Irmak S, Oliveira-Ferrer L, et al. CEA-related cell adhesion molecule-1 is involved in angiogenic switch in prostate cancer. *Oncogene*. 2006;25:4965–4974.
21. Oliveira-Ferrer L, Tilki D, Ziegeler G, et al. Dual role of carcinoembryonic antigen-related cell adhesion molecule 1 in angiogenesis and invasion of human urinary bladder cancer. *Cancer Res*. 2004;64:8932–8938.
22. Nouvion AL, Beauchemin N. CEACAM1 as a central modulator of metabolism, tumor progression, angiogenesis and immunity. *Med Sci (Paris)*. 2009;25:247–252.
23. Gerstel D, Wegwitz F, Jannasch K, et al. CEACAM1 creates a pro-angiogenic tumor microenvironment that supports tumor vessel maturation. *Oncogene*. 2011;30:4275–4288.
24. Moller MJ, Kammerer R, Grunert F, et al. Biliary glycoprotein (BGP) expression on T cells and on a natural-killer-cell subpopulation. *Int J Cancer*. 1996;65:740–745.
25. Markel G, Lieberman N, Katz G, et al. CD66a interactions between human melanoma and NK cells: a novel class I MHC-independent inhibitory mechanism of cytotoxicity. *J Immunol*. 2002;168:2803–2810.
26. Markel G, Seidman R, Cohen Y, et al. Dynamic expression of protective CEACAM1 on melanoma cells during specific immune attack. *Immunology*. 2009;126:186–200.
27. Raulet DH. Roles of the NKG2D immunoreceptor and its ligands. *Nat Rev Immunol*. 2003;3:781–790.
28. Chen Z, Chen L, Baker K, et al. CEACAM1 dampens antitumor immunity by down-regulating NKG2D ligand expression on tumor cells. *J Exp Med*. 2011;208:2633–2640.
29. Hosomi S, Chen Z, Baker K, et al. CEACAM1 on activated NK cells inhibits NKG2D-mediated cytolytic function and signaling. *Eur J Immunol*. 2013;43:2473–2483.

30. Huang Y, Zhu C, Kondo Y, et al. CEACAM1 regulates TIM-3-mediated tolerance and exhaustion. *Nature*. 2015;517: 386–390.
31. Ortenberg R, Sapir Y, Raz L, et al. Novel immunotherapy for malignant melanoma with a monoclonal antibody that blocks CEACAM1 homophilic interactions. *Mol Cancer Ther*. 2012;11:1300–1310.
32. Mirick GR, Bradt BM, Denardo SJ, et al. A review of human anti-globulin antibody (HAGA, HAMA, HACA, HAHA) responses to monoclonal antibodies. Not four letter words. *Q J Nucl Med Mol Imaging*. 2004;48:251–257.
33. Krebs B, Rauchenberger R, Reiffert S, et al. High-throughput generation and engineering of recombinant human antibodies. *J Immunol Methods*. 2001;254:67–84.
34. Presta LG. Selection, design, and engineering of therapeutic antibodies. *J Allergy Clin Immunol*. 2005;116:731–736.
35. Moricoli D, Laguardia ME, Carbonella DC, et al. Isolation of a new human scFv antibody recognizing a cell surface binding site to CEACAM1. Large yield production, purification and characterization in *E. coli* expression system. *Protein Expr Purif*. 2014;93:38–45.
36. Smith GP. Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. *Science*. 1985;228:1315–1317.
37. Nilsson F, Tarli L, Viti F, et al. The use of phage display for the development of tumour targeting agents. *Adv Drug Deliv Rev*. 2000;43:165–196.
38. Pavoni E, Flego M, Dupuis ML, et al. Selection, affinity maturation, and characterization of a human scFv antibody against CEA protein. *BMC Cancer*. 2006;6:41.
39. Gong JH, Maki G, Klingemann HG. Characterization of a human cell line (NK-92) with phenotypical and functional characteristics of activated natural killer cells. *Leukemia*. 1994;8:652–658.
40. Robertson MJ, Cochran KJ, Cameron C, et al. Characterization of a cell line, NKL, derived from an aggressive human natural killer cell leukemia. *Exp Hematol*. 1996;24: 406–415.
41. Mainiero F, Soriani A, Strippoli R, et al. RAC1/P38 MAPK signaling pathway controls beta1 integrin-induced interleukin-8 production in human natural killer cells. *Immunity*. 2000;12:7–16.
42. Bryceson YT, March ME, Barber DF, et al. Cytolytic granule polarization and degranulation controlled by different receptors in resting NK cells. *J Exp Med*. 2005;202:1001–1012.
43. Soriani A, Iannitto ML, Ricci B, et al. Reactive oxygen species- and DNA damage response-dependent NK cell activating ligand upregulation occurs at transcriptional levels and requires the transcriptional factor E2F1. *J Immunol*. 2014;193:950–960.
44. Singer BB, Scheffrahn I, Heymann R, et al. Carcinoembryonic antigen-related cell adhesion molecule 1 expression and signaling in human, mouse, and rat leukocytes: evidence for replacement of the short cytoplasmic domain isoform by glycosylphosphatidylinositol-linked proteins in human leukocytes. *J Immunol*. 2002;168:5139–5146.
45. Ullrich N, Heinemann A, Nilewski E, et al. CEACAM1-3S drives melanoma cells into NK cell-mediated cytolysis and enhances patient survival. *Cancer Res*. 2015;75:1897–1907.
46. Gaur S, Shively JE, Yen Y, et al. Altered splicing of CEACAM1 in breast cancer: identification of regulatory sequences that control splicing of CEACAM1 into long or short cytoplasmic domain isoforms. *Mol Cancer*. 2008;7:46.
47. Yu Q, Chow EM, Wong H, et al. CEACAM1 (CD66a) promotes human monocyte survival via a phosphatidylinositol 3-kinase- and AKT-dependent pathway. *J Biol Chem*. 2006;281:39179–39193.
48. Henderikx P, Kandilogiannaki M, Petrarca C, et al. Human single-chain Fv antibodies to MUC1 core peptide selected from phage display libraries recognize unique epitopes and predominantly bind adenocarcinoma. *Cancer Res*. 1998;58:4324–4332.
49. Labrijn AF, Poignard P, Raja A, et al. Access of antibody molecules to the conserved coreceptor binding site on glycoprotein gp120 is sterically restricted on primary human immunodeficiency virus type 1. *J Virol*. 2003;77:10557–10565.
50. Voskoboynik M, Arkenau HT. Combination therapies for the treatment of advanced melanoma: a review of current evidence. *Biochem Res Int*. 2014;2014:307059.
51. Olszanski AJ. Current and future roles of targeted therapy and immunotherapy in advanced melanoma. *J Manag Care Pharm*. 2014;20:346–356.
52. Catalanotti F, Solit DB, Pulitzer MP, et al. Phase II trial of MEK inhibitor selumetinib (AZD6244, ARRY-142886) in patients with BRAFV600E/K-mutated melanoma. *Clin Cancer Res*. 2013;19:2257–2264.
53. Carvajal RD, Sosman JA, Quevedo F, et al. Phase II study of selumetinib versus temozolomide (TMZ) in gnaq/Gna11 (Gq/11) mutant (mut) uveal melanoma (UM). *J Clin Oncol*. 2013;31(suppl):CRA9003.
54. Boasberg P, Hamid O, O'Day S. Ipilimumab: unleashing the power of the immune system through CTLA-4 blockade. *Semin Oncol*. 2010;37:440–449.
55. Ott PA, Hodi FS, Robert C. CTLA-4 and PD-1/PD-L1 blockade: new immunotherapeutic modalities with durable clinical benefit in melanoma patients. *Clin Cancer Res*. 2013;19:5300–5309.
56. Ai M, Curran MA. Immune checkpoint combinations from mouse to man. *Cancer Immunol Immunother*. 2015;64: 885–892.
57. Burke S, Lakshmikanth T, Colucci F, et al. New views on natural killer cell-based immunotherapy for melanoma treatment. *Trends Immunol*. 2010;31:339–345.
58. Weber JS. At the bedside: adoptive cell therapy for melanoma-clinical development. *J Leukoc Biol*. 2014;95:875–882.
59. Markel G, Seidman R, Stern N, et al. Inhibition of human tumor-infiltrating lymphocyte effector functions by the homophilic carcinoembryonic cell adhesion molecule 1 interactions. *J Immunol*. 2006;177:6062–6071.
60. Ebrahimnejad A, Streichert T, Nollau P, et al. CEACAM1 enhances invasion and migration of melanocytic and melanoma cells. *Am J Pathol*. 2004;165:1781–1787.
61. Ortenberg R, Galore-Haskel G, Greenberg I, et al. CEACAM1 promotes melanoma cell growth through Sox-2. *Neoplasia*. 2014;16:451–460.
62. Zippel D, Barlev H, Ortenberg R, et al. A longitudinal study of CEACAM1 expression in melanoma disease progression. *Oncol Rep*. 2015;33:1314–1318.
63. Chen Z, Chen L, Blumberg RS. Editorial: CEACAM1: fine-tuned for fine-tuning. *J Leukoc Biol*. 2009;86:195–197.
64. Chen D, Iijima H, Nagaishi T, et al. Carcinoembryonic antigen-related cellular adhesion molecule 1 isoforms alternatively inhibit and costimulate human T cell function. *J Immunol*. 2004;172:3535–3543.
65. Turbide C, Kunath T, Daniels E, et al. Optimal ratios of biliary glycoprotein isoforms required for inhibition of colonic tumor cell growth. *Cancer Res*. 1997;57:2781–2788.
66. Chen L, Chen Z, Baker K, et al. The short isoform of the CEACAM1 receptor in intestinal T cells regulates mucosal immunity and homeostasis via Tfh cell induction. *Immunity*. 2012;37:930–946.
67. Ieda J, Yokoyama S, Tamura K, et al. Re-expression of CEACAM1 long cytoplasmic domain isoform is associated with invasion and migration of colorectal cancer. *Int J Cancer*. 2011;129:1351–1361.
68. Singer BB, Scheffrahn I, Kammerer R, et al. Deregulation of the CEACAM expression pattern causes undifferentiated cell growth in human lung adenocarcinoma cells. *PLoS One*. 2010;5:e8747.
69. Lawson EL, Mills DR, Brilliant KE, et al. The transmembrane domain of CEACAM1-4S is a determinant of anchorage independent growth and tumorigenicity. *PLoS One*. 2012; 7:e29606.

70. Beckman RA, Weiner LM, Davis HM. Antibody constructs in cancer therapy: protein engineering strategies to improve exposure in solid tumors. *Cancer*. 2007;109:170–179.
71. Adams GP, Schier R, McCall AM, et al. High affinity restricts the localization and tumor penetration of single-chain fv antibody molecules. *Cancer Res*. 2001;61:4750–4755.
72. Adams GP, Schier R, Marshall K, et al. Increased affinity leads to improved selective tumor delivery of single-chain Fv antibodies. *Cancer Res*. 1998;58:485–490.
73. Lobo ED, Hansen RJ, Balthasar JP. Antibody pharmacokinetics and pharmacodynamics. *J Pharm Sci*. 2004;93:2645–2668.