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Novel Immunotherapy for Malignant Melanoma with a Monoclonal Antibody That Blocks CEACAM1 Homophilic Interactions

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Abstract

CEACAM1 (biliary glycoprotein-1, CD66a) was reported as a strong clinical predictor of poor prognosis in melanoma. We have previously identified CEACAM1 as a tumor escape mechanism from cytotoxic lymphocytes. Here, we present substantial evidence *in vitro* and *in vivo* that blocking of CEACAM1 function with a novel monoclonal antibody (MRG1) is a promising strategy for cancer immunotherapy. MRG1, a murine IgG1 monoclonal antibody, was raised against human CEACAM1. It recognizes the CEACAM1-specific N-domain with high affinity ($K_D \sim 2$ nmol/L). Furthermore, MRG1 is a potent inhibitor of CEACAM1 homophilic binding and does not induce any agonistic effect. We show using cytotoxicity assays that MRG1 renders multiple melanoma cell lines more vulnerable to T cells in a dose-dependent manner, only following antigen-restricted recognition. Accordingly, MRG1 significantly enhances the antitumor effect of adoptively transferred, melanoma-reactive human lymphocytes using human melanoma xenograft models in severe combined immunodeficient/nonobese diabetic (SCID/NOD) mice. A significant antibody-dependent cell cytotoxicity response was excluded. It is shown that MRG1 reaches the tumor and is cleared within a week. Importantly, approximately 90% of melanoma specimens are CEACAM1⁺, implying that the majority of patients with melanoma could be amenable to MRG1-based therapy. Normal human tissue microarray displays limited binding to luminal epithelial cells on some secretory ducts, which was weaker than the broad normal cell binding of other anticancer antibodies in clinical use. Importantly, MRG1 does not directly affect CEACAM1⁺ cells. CEACAM1 blockade is different from other immunomodulatory approaches, as MRG1 targets inhibitory interactions between tumor cells and late effector lymphocytes, which is thus a more specific and compartmentalized immune stimulation with potentially superior safety profile. *Mol Cancer Ther*; 11(6); 1300–10. ©2012 AACR.

Introduction

Melanoma accounts for nearly 4% of all skin cancers and it causes 75% of skin cancer-related deaths worldwide (1). Disease progression and development of metastasis require stepwise acquisition of aggressive characteristics (2), including resistance to the immune system (3), even though melanoma cells are frequently immunogenic (4). Immunomodulation with interleukin (IL)-2 yields an

objective clinical response in approximately 20% of the patients, with 5% of them exhibiting a durable complete response (5). Very recently, the U.S. Food and Drug Administration approved the anti-CTLA4 monoclonal antibody (mAb; ipilimumab), which alleviates the inhibitory effects of CTLA4 on T-cell activation (6), for the indication of metastatic melanoma. Another beneficial form of immunotherapy for metastatic melanoma is cell-based therapy, especially adoptive cell transfer of tumor-infiltrating lymphocytes (TIL), which yields up to 50% response rate (7–9). Nonetheless, immunotherapy for melanoma is still far from its full potential in terms of efficacy and safety, which mandates the development of improved, alternative, or complementary approaches.

CEACAM1 is a transmembrane glycoprotein that belongs to the carcinoembryonic antigen family, which encompasses several forms of proteins with different biochemical properties, all encoded on chromosome 19 (10). CEACAM1 is composed of sequentially ordered immunoglobulin (Ig)-like domain(s). It is subjected to alternative splicing that raises 2 forms of cytosolic tail, a long form containing immunodominant tyrosine-based

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inhibitory motif (ITIM) and a short form devoid of ITIM (11). CEACAM1 interacts homophilically with CEACAM1 (12) and heterophilically with CEACAM5 but not with other CEACAM proteins (13). CEACAM1 is expressed on a variety of cells, for example, some epithelial cells, melanoma, and activated lymphocytes (10).

Many different functions have been attributed to the CEACAM1 protein, including antiproliferative properties in carcinomas of the colon and prostate, central involvement of CEACAM1 in angiogenesis metastasis and insulin clearance, as well as in immunomodulation (reviewed in ref. 11). T-cell inhibition through engagement of CEACAM1 has been shown by direct T-cell receptor cross-linking (14) and via binding of *Neisseria* opacity-associated proteins (15). We have previously shown that CEACAM1 homophilic interactions inhibit natural killer (NK) cell-mediated killing, independently of MHC class I recognition (16–18). We have further shown that CEACAM1 inhibits effector functions of TILs, such as cytotoxicity and IFN γ release (19). Moreover, we found that an IFN γ -driven upregulation of CEACAM1 on melanoma cells surviving TIL-mediated attack renders them even more resistant (20). The inhibitory effect of CEACAM1 is exerted by the recruitment of SHP-1 phosphatase to the cytosolic ITIM sequences (21). Lymphocytes express only the CEACAM1 isoform that bears a long cytosolic tail (22), and there is a similar dominance of the long isoform in melanoma cells (20).

Thies and colleagues showed that the presence of CEACAM1 on primary cutaneous melanoma lesions strongly predicted the development of metastatic disease (23). This was in line with our findings that CEACAM1 protects melanoma cells and inhibits both activated NK cells (17) and activated T cells (19, 20, 24). Furthermore, we have recently shown an unusually high percentage of CEACAM1⁺ circulating lymphocytes in the peripheral blood of patients with melanoma, as compared with healthy donors (24). This emphasizes the potential role played by CEACAM1 in the pathogenesis of metastatic melanoma. These collective observations provide a strong justification for the development of a therapeutic approach that targets the function of CEACAM1.

Here, we present substantial evidence *in vitro* and *in vivo* that blocking of CEACAM1 function with a newly generated mAb (MRG1) and thereby rendering melanoma cells more vulnerable to reactive lymphocytes is a promising strategy for cancer immunotherapy. We show that 89% of the metastatic melanoma specimens tested are CEACAM1-positive and that CEACAM1⁺ lymphocytes are present in the vicinity of melanoma metastases. This implies that the majority of patients with metastatic melanoma could be amenable to CEACAM1-targeted therapy.

Materials and Methods

Cells and antibodies

A detailed list of the cells and antibodies used in this work appears in the Supplementary Methods. Primary

cultures of melanoma and TILs were established and grown as previously described (25). Cells from the synovial fluids of pediatric patients with acute exacerbation of juvenile rheumatoid arthritis were obtained following Institutional Review Board (IRB) approval no. 920070593.

Melanoma cell lines were routinely stained for melanoma antigens such as GP100, MART1, and MCSP. 721.221 and BW cell lines were not routinely authenticated.

Functional BW reporting system of CEACAM1 homophilic interactions

BW cells stably transfected with a chimeric molecule composed of the extracellular portion of human CEACAM1, fused in frame to mouse zeta chain (BW/CCM1-zeta), or with an empty vector (BW/mock) were previously described (16). Fifty thousand BW cells were coincubated with 25,000 721.221/CEACAM1 cells for 5 hours in humidified incubator. The concentration of mouse IL-2 in the supernatant was quantified with standardized sandwich ELISA (R&D Systems).

Generation of MRG1 mAb

Balb/c mice were immunized with 5 μ g of human CEACAM1 (R&D Systems) every 2 weeks for a total of 4 injections. Preparation of hybridoma library and assays used for clone selection are described in Supplementary Methods. The MRG1 mAb was produced from supernatant of hybridoma cultured in Low Protein Medium (Biological Industries) on a Protein G Column (GE Healthcare). SDS-PAGE confirmed the presence of light and heavy chains, as well as was routinely carried out to evaluate degradation. N-terminal protein sequencing (Weizmann Institute, Rehovot, Israel) and DNA sequence genotyping confirmed it is a single clone.

Flow cytometry

For staining with purified antibodies, 100,000 cells were incubated with 0.2 μ g of antibody diluted in PBS/0.5% bovine serum albumin (BSA)/0.02% NaN₃ [fluorescence-activated cell-sorting (FACS) medium] for 1 hour on ice. Cells were centrifuged 400 \times g for 5 minutes and supernatant was removed. The cells were incubated for 30 minutes on ice with secondary antibodies, washed with FACS medium, and analyzed with FACSCalibur instrument (BD Biosciences) and FlowJo or CellQuest software. When fluorochrome-conjugated antibodies were used, cells were not further stained with secondary reagents.

Surface plasmon resonance

Experiments were carried out with Biacore3000 following preparation as detailed in Supplementary Methods (Institute of Life Sciences, Hebrew University, Jerusalem, Israel). For kinetics experiments, hCEACAM1 was injected in various concentrations (0.19, 0.39, 0.78, 1.56, 3.12, 6.25, 12.5, and 25 nmol/L) in PBS-ET buffer. Regeneration was done by injecting 10 μ L of 10 mmol/L NaOH.

Flow rate during kinetics was 50 $\mu\text{L}/\text{min}$. Data were analyzed with BIAevaluation software 4.1

Knockdown of CEACAM1

Generation of CEACAM1-silenced melanoma cells was carried out with commercially available target sequences cloned in the MISSION short hairpin RNA system (lenti-viral plasmids pLKO.1-puro; Sigma-Aldrich) and lenti-viral expression system, as previously described (20). Scrambled nontarget sequence was used as negative control.

Cytotoxicity assay

Cytotoxicity measurements were based on carboxy-fluorescein succinimidyl ester (CFSE) labeling of target cells and costaining with propidium iodide (PI) after 6 hours of incubation with the effector cells as previously described (19). Blocking with MRG1 was carried out by preincubation of either effectors or targets for 1 hour on ice at the indicated concentrations. Background level did not exceed 20% in all experiments.

Net proliferation assay

Melanoma cells (3×10^3) were seeded in triplicate wells in 96F-well microplates with or without the presence of MRG1. Net proliferation was determined by standardized XTT colorimetric assay (Biological Industries) as previously described (26).

Apoptosis tests

Apoptosis was tested with Annexin-V/fluorescein isothiocyanate (FITC) and PI (BD Biosciences), according to appropriate specific calibrations. Apoptosis was shown *in situ* in histopathologic slides with terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining (Millipore), according to manufacturer's instructions.

Melanoma xenograft model

A total of 2×10^6 melanoma cells were injected s.c. into the thigh of 7- to 8-week-old severe combined immunodeficient/nonobese diabetic (SCID/NOD) mice to create human melanoma xenografts. Mice were monitored 3 times per week for tumor volume by caliper measurement. Tumor volume was calculated as $(\text{small diameter})^2 \times (\text{large diameter})/2$. When tumors reached a volume of 100 mm^3 , mice were randomized into experimental treatment groups, which included 8 to 10 mice each. Upon experiment termination, tumor masses were extracted, halved, and processed for histology or homogenized. All animal work was done following approval of Sheba Medical Center (Ramat-Gan, Israel) IRB (562/2010).

In vivo killing assay

Melanoma cell lines 526mel (TIL14-sensitive) and 938mel (TIL14-resistant) were labeled with Vybrant DiO cell-labeling solution and Vybrant DiD cell-labeling solution, respectively (Vybrant Multicolor Cell-Labeling Kit,

Invitrogen). A total of 1×10^6 cells of each melanoma type were mixed together with 25 μg of IgG1 control antibody or MRG1. A total of 20×10^6 TIL14 cells or carrier only was further added to a final volume of 200 μL . The mixture was immediately injected intravenously to Balb/c mice. Each group consisted of 3 animals. After 6 hours, the mice were sacrificed; their lungs were removed and rendered into single-cell suspension with enzymatic digestion by collagenase IV and DNase I (Sigma-Aldrich) for 1 hour in 37°C. Lung single-cell suspensions of mice injected with the same mixture were pooled together and analyzed by flow cytometry. The ratio between 526mel and 938mel was determined in each group and it reflects the specific activity of TIL14 against 526mel cells *in vivo*.

Immunohistochemistry and normal human tissue array

Multi-normal organ tissue microarrays of human (US Biomax: FDA999) were prepared for immunostaining with standard protocols (Supplementary Methods) and incubated overnight at 4°C with MRG1 mAb. Detection was conducted with the Histostain-SP-Broad-Spectrum Kit (Invitrogen) and visualized with the substrate chromogen AEC. Sections were counterstained with hematoxylin (Ventana Medical Systems) and coverslipped with an aqueous mounting fluid (glycerol).

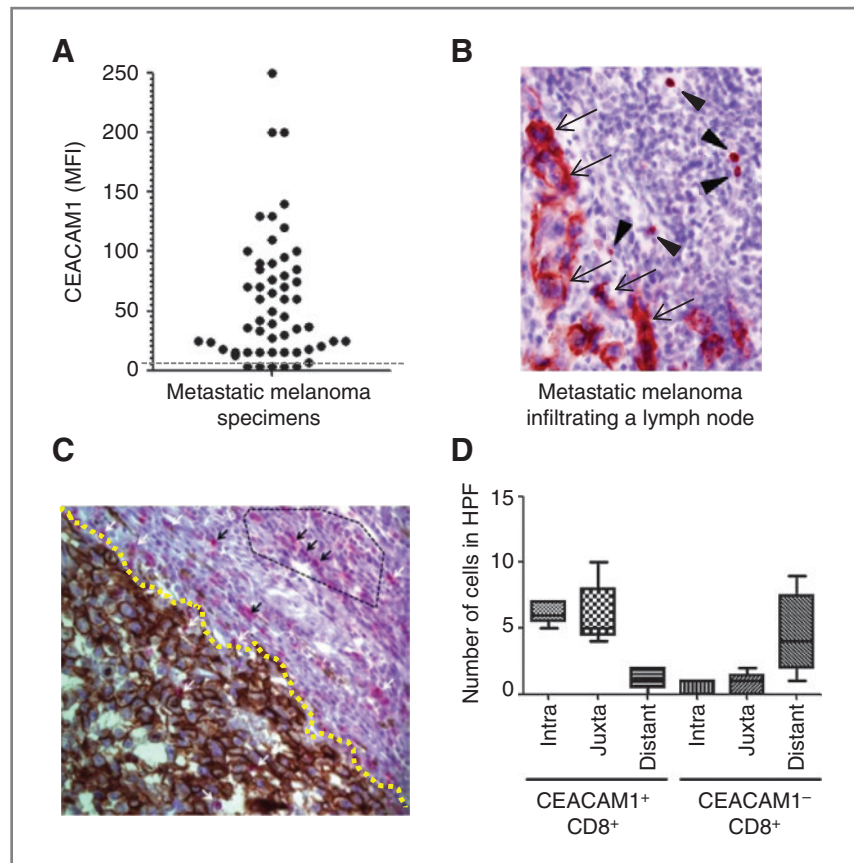
For double staining of dewaxed and rehydrated formalin-fixed tissue sections, CC1 Standard Benchmark XT pretreatment for antigen retrieval was selected (Ventana). The antibodies were diluted in antibody diluent. The slides were warmed up to 60°C for 1 hour and further processed with a fully automated protocol. Detection was carried out with Ultraview Detection Kit (Ventana) and counterstained with hematoxylin. After the run on the automated stainer, we dehydrated the slides in 70% ethanol, 95% ethanol, and 100% ethanol for 10 seconds each. Before coverslipping, sections were cleared in xylene for 10 seconds and mounted with Entellan. Stained sections were reviewed by an expert pathologist and suitable digital images were captured with Olympus BX51 microscope.

Results

The vast majority of melanoma metastases overexpress CEACAM1

Fifty-five primary cultures of metastatic melanoma were stained for CEACAM1 expression with the in-house anti-CEACAM1 mAb MRG1. Importantly, 49 of 55 cultures (89%) were significantly stained (Fig. 1A), while it is known that CEACAM1 is not expressed by normal melanocytes (27). Moreover, the presence of CEACAM1⁺ lymphocytes was shown in the vicinity of a representative CEACAM1⁺ melanoma metastasis to a lymph node (Fig. 1B). Double staining for MRG1 and CD8 showed that almost all CD8-positive lymphocytes in the tumor and its close vicinity were MRG1-positive (Fig. 1C). Interestingly, MRG1-positive lymphocytes were more

Figure 1. Frequency of CEACAM1 expression in metastatic melanoma specimens. **A**, primary cultures of metastatic melanoma ($N = 55$) were tested for CEACAM1 expression. Expression was quantified with a CEACAM1-specific mAb in flow cytometry (MFI, median fluorescence intensity). Dashed horizontal line represents the background staining. **B**, staining for CEACAM1 with MRG1 of a lymph node infiltrated with melanoma cells. Arrows indicate exemplar melanoma cells. Black arrowheads indicate CEACAM1⁺ lymphocytes. **C**, double staining for CEACAM1 (brown pigmentation) and CD8 (pink pigmentation) on slide from the same patient material. White arrows indicate CEACAM1⁺CD8⁺ cells. Black arrows and dashed circle indicate CEACAM1⁻CD8⁺ cells. **D**, quantification of cells per high-power field (HPF) in different tissue regions, as indicated in the figure. Data were obtained from 5 fields in 3 slides.



common within the tumor and in juxtatumoral areas, as compared with other areas distant from tumor edge (Fig. 1D). CD4-positive MRG1-positive cells were not detected (data not shown). It was previously reported that CEACAM1 expression is induced following lymphocyte activation (22), but MRG1 reactivity by itself does not indicate on the antigenic specificity of the T cells. This *in vivo* proximity between CEACAM1⁺ CD8⁺ lymphocytes and tumor cells suggests that inhibitory homophilic CEACAM1 interactions indeed hinder anti-melanoma immune response, which could be targeted by an anti-CEACAM1 modality.

MRG1 is a potent blocker of intercellular homophilic CEACAM1 interactions

MRG1, a mAb selected from a library of antibodies against CEACAM1, is specific to the N-domain of CEACAM1, which plays a critical role in CEACAM1 binding (12, 13), and exerts an affinity of 1.46 to 2.83 nmol/L, as determined with surface plasmon resonance (Supplementary Fig. 1S).

A cellular system reporting on homophilic intercellular CEACAM1 interactions was used (16). Coincubation of BW cells expressing CEACAM1-zeta fusion protein with 721.221/CEACAM1 cells, but not with 721.221/mock cells, elicited the secretion of mouse IL-2 (mIL-2) to the supernatant, which was completely abolished with 40 ng/mL

of MRG1 (Fig. 2A). BW/mock cells did not secrete mIL-2 (Fig. 2A). One-hour preincubation of MRG1 either with the BW/CEACAM1-zeta or with the 721.221/CEACAM1 cells similarly abrogated the secretion of mIL-2 (Fig. 2B). Remarkably, reduction of 50% in the secretion of mIL-2 occurs with approximately 5 ng/mL, and nearly complete abolishment was achieved with 20 ng/mL (Fig. 2B). MRG1 exhibited a substantially stronger CEACAM1 blocking activity than 5F4 mAb, Kat4C mAb, and polyclonal antibodies against CEACAM1, 5, and 6, which were previously used for functional blocking of CEACAM1 (refs. 13, 16–19, 24, 28; Fig. 2C). Finally, MRG1 did not induce secretion of mIL-2 from BW/CEACAM1-zeta cells, indicating that it does not have an agonistic role (Fig. 2D). In summary, MRG1 is selective for CEACAM1, has high affinity, and a potent blocking activity.

MRG1 facilitates antigen-restricted killing of melanoma cells by specific T cells

The immune-protective effect of CEACAM1 was validated by selective knockdown with short hairpin RNA (shRNA; Supplementary Fig. 2S). MRG1 enhanced the killing of 526mel cells by TIL14 in a dose-dependent manner, when the lymphocytes were preincubated with the antibody (Fig. 3A). 624mel, another CEACAM1⁺ melanoma line, was also rendered susceptible to TIL14 cells (Fig. 3B) or to other T-cell cultures (data not shown).

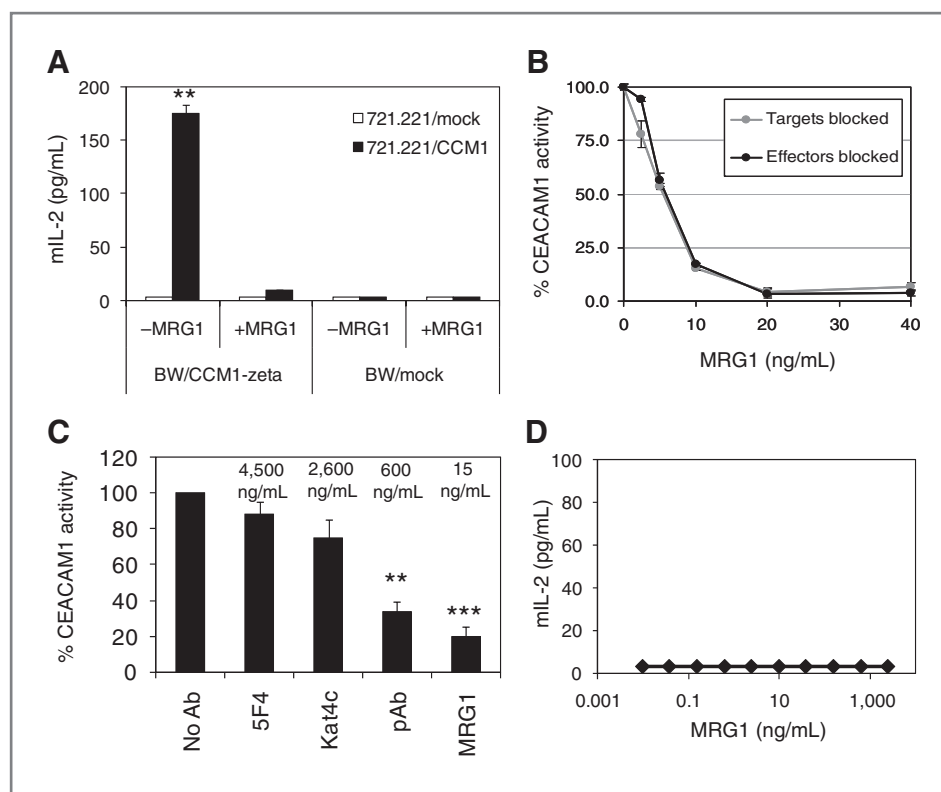


Figure 2. Efficient CEACAM1-blocking properties of MRG1. A, secretion of mIL-2 from BW cells transfected with CEACAM1-zeta (BW/CCM1-zeta) or with an empty vector (BW/mock) following coincubation with 721.221/mock or 721.221/CCM1 cells in the absence or presence of 40 ng/mL of MRG1. B, dose-dependent blocking of CEACAM1 activity with MRG1. One hundred percent of CEACAM1 activity was determined as the mIL-2 concentration in the absence of MRG1. Targets, 721.221/CCM1 cells; effectors, BW/CCM1-zeta cells. C, a comparative analysis of CEACAM1-blocking potency of various anti-CEACAM antibodies. The antibody concentrations included in the assay are indicated in the figure. CEACAM1 activity was determined as above. pAb, polyclonal antibody. D, no agonistic effect of MRG1 on BW/CCM1-zeta cells. **, $P < 0.01$; ***, $P < 0.001$. Figure shows a representative experiment of 4 conducted.

MRG1 did not influence the killing of CEACAM1-negative 09mel cells and did not enhance the killing of CEACAM1⁺ 938mel cells, which were not recognized by the T-cell cultures. IgG1 isotype control did not exert any significant effect on T-cell killing (Fig. 3B). Preincubation of MRG1 either with the lymphocytes or with the target cells yielded similar results (data not shown).

The direct effect of MRG1 on melanoma cells was tested. Melanoma cells were incubated with various MRG1 concentrations (0.1–10 $\mu\text{g}/\text{mL}$). The net proliferation of melanoma cultures was determined on days 2 and 4 of cultivation. MRG1 did not affect the proliferation of 526mel (CEACAM1⁺) or 003mel (CEACAM1⁻) cells in all concentrations tested (representative concentration of 2 $\mu\text{g}/\text{mL}$; Fig. 3C). Furthermore, MRG1 did not induce apoptosis in 526mel cells when it was added into the culture medium or cross-linked with goat anti-mouse IgG (Fig. 3D). IgG1 isotype control had no significant effect on melanoma cell proliferation or apoptosis rates (Fig. 3C and D). Similarly, MRG1 did not affect the proliferation or apoptosis rate of a variety of CEACAM1-positive normal cells, such as lymphocytes, kidney, and prostate epithelium, over a wide range of concentrations (data not shown).

MRG1 synergizes with adoptively transferred lymphocytes to inhibit melanoma xenograft growth

Human CEACAM1⁺ 526mel melanoma cells were s.c. injected into SCID/NOD mice. Mice bearing tumors of

estimated volume of 100 mm³ were randomized to one of the following 4 treatment combinations: a single i.v. injection of PBS or 20×10^6 tumor-specific human T cells, combined with weekly i.p. injections of PBS or 0.5 mg MRG1. In addition, mice were injected i.p. with 6,000 IU of hIL-2 twice a day for the first 5 days. A moderate inhibition of tumor growth was observed either with MRG1 alone or with T cells only, which was not statistically significant (Fig. 4A). hIL-2 only had no effect on tumor growth (data not shown). The combination of adoptive human T-cell transfer with MRG1 exhibited significant synergism and strongly inhibited xenograft growth (Fig. 4A), which concurs with the *in vitro* experiments (Fig. 3A and B). Similar results were observed with another CEACAM1⁺ melanoma cell line SK-MEL-05 (Supplementary Fig. 3S). Intraperitoneal and intravenous administration of MRG1 yielded similar results (data not shown). Isotype IgG1 control had no significant effect (Fig. 4B). Furthermore, when inert CEACAM1-positive T cells, which do not mediate any *in vivo* effect on 526mel tumor growth (Fig. 4C), were used, MRG1 had no significant effect (Fig. 4C). Moreover, histochemical TUNEL staining revealed only few TUNEL-positive cells in the tumors derived from the PBS-treated or IgG1-treated mice and some TUNEL reactivity in the MRG1, TIL, or TIL + IgG1 groups, indicative of some low antitumor effect. A strong TUNEL staining was observed only in TIL + MRG1 group, directly indicative for enhanced tumor cell death by the combined treatment

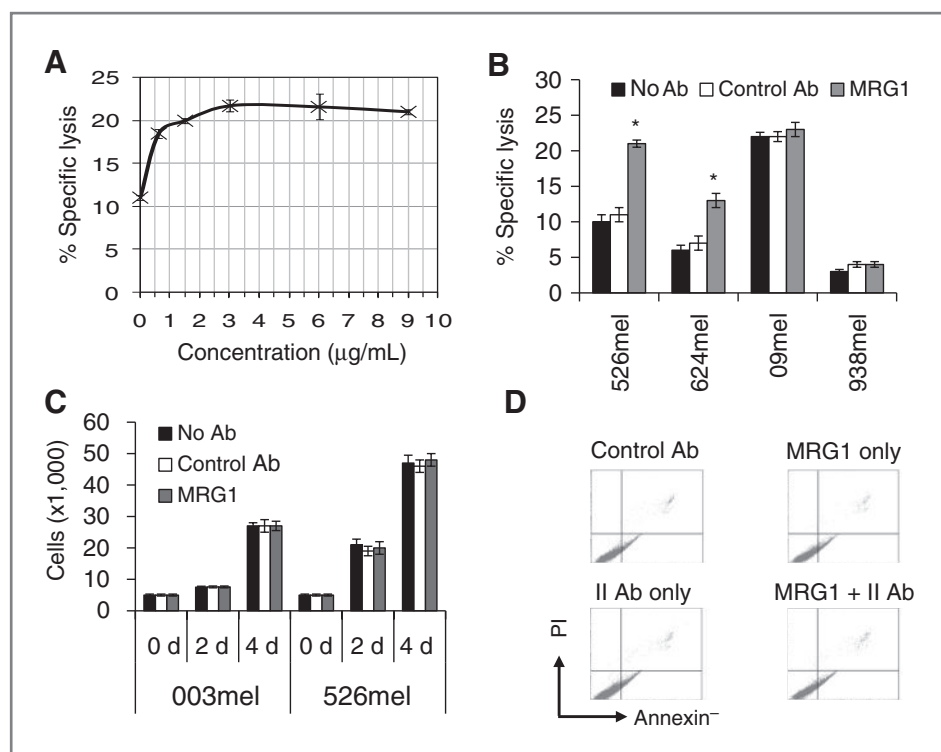


Figure 3. MRG1 renders CEACAM1⁺ melanoma cells more susceptible to T cells in a dose-dependent antigen-restricted manner. **A**, killing of 526mel cells by TIL014 in the presence of various concentrations of MRG1. **B**, killing rate of various melanoma cell lines: HLA-matched CEACAM1⁺ (526mel and 624mel), HLA-matched CEACAM1^{dim} (09mel), and HLA-irrelevant CEACAM1⁺ (938mel). *, $P < 0.05$. **C**, CEACAM1⁻ melanoma cells (003mel) or CEACAM1⁺ melanoma cells (526mel) were cultivated for 2 or 4 days in the absence or presence of 5 µg/mL of IgG1 control mAb or MRG1. At the indicated time points, cells were tested for net proliferation in a standardized XTT assay. **D**, 526mel cells were incubated for 18 hours with IgG1 control mAb, MRG1, goat anti-mouse IgG, both reagents, or with none of them, as indicated in the figure. The apoptosis rate was analyzed with Annexin-V and PI containing in flow cytometry. Percentages of apoptotic cells in each treatment are indicated in the figure. Figure shows a representative experiment of 4 conducted.

(Fig. 4D). These observations support the notion that MRG1 enhances the effect of tumor-specific T cells in an antigen-restricted manner.

In vivo killing assay showed that MRG1 enhances the ability of TIL14 cells to eliminate 526mel cells *in vivo*. The TIL14-sensitive 526mel cells and the TIL14-resistant 938mel cells (Fig. 3B) were mixed with or without TIL14 cells in the presence of 25 µg of MRG1 or of IgG1 control. Both melanoma cell lines strongly express CEACAM1 (data not shown). The various mixtures were immediately injected intravenously into mice. After 6 hours, lungs were harvested and the ratio between the 526mel and 938mel cells was determined in the recovered cells. A significant reduction in the proportion of 526mel cells was observed when MRG1 was coadministered with the TIL14 cells but not when used in the absence of TIL14 (Fig. 4E). The amount of recovered 938mel cells was constant among all groups. These combined experiments indicate that MRG1 enhances the activity of tumor-specific T cells *in vivo*. Noteworthy, adoptively transferred human T cells stably expressed CEACAM1 *in vivo* for at least 14 days (Supplementary Fig. 4S).

SCID/NOD mice lack an endogenous adaptive immune response, yet retain functional macrophages and

some NK cells capable of mediating antibody-dependent cell-cytotoxicity (ADCC). The killing of 526mel cells by SCID/NOD splenocytes was very limitedly enhanced by MRG1, and not in a dose-dependent manner, at an effector-to-target ratio of 10:1 (Fig. 4F). No ADCC activity of MRG1 could be detected in higher effector-to-target ratios (Supplementary Fig. 5S). Other monoclonal and polyclonal antibodies did induce a significant ADCC response in a dose-dependent manner (Fig. 4F). This confirms that the tumor growth inhibition by MRG1 is most probably ADCC-independent.

***In vivo* MRG1 clearance and characterization of normal human tissue staining**

All xenografts were removed, processed into single-cell suspensions, and stained with FITC-conjugated anti-mouse IgG for detection of cell-bound antibodies. SCID/NOD mice do not produce endogenous immunoglobulins. Only cell suspensions derived from mice treated with MRG1 (either alone or with TIL) displayed staining (Fig. 5A). Tumor cell suspensions from mice treated with TILs only or with control treatment remained unstained (Fig. 5A). All the tumors within each treatment group displayed consistent staining patterns. CEACAM1 was similarly expressed in all treatment groups according

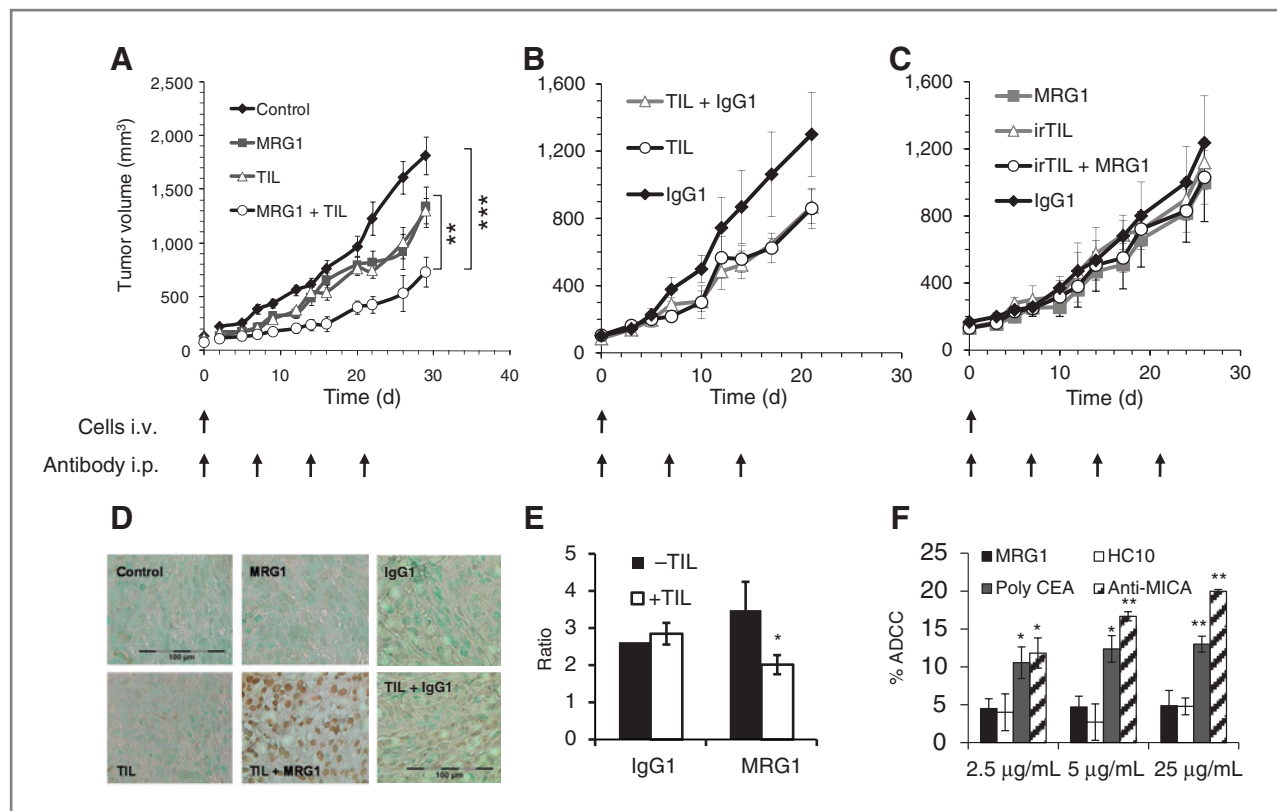


Figure 4. MRG1 synergizes with adoptively transferred human TILs *in vivo*. A, SCID/NOD mice bearing human ($\sim 100 \text{ mm}^3$) 526mel xenografts were randomized according to the 4 treatment groups indicated in the figure. Black arrows indicate time of administration. The same xenograft tumor model was used with focus on isotype control antibody (IgG1; B) or with focus on activated T cells with irrelevant antigenic specificity (irTIL; C). The different treatment groups are indicated in each figure. D, TUNEL staining of representative xenograft tumors derived from treatment groups of A and B, as indicated in the figure. E, *in vivo* killing activity of adoptively transferred anti-tumor T cells is presented as the ratio of 526mel cell (TIL-sensitive) to 938mel cells (TIL-resistant) recovered from the lungs 6 hours after their i.v. injection with 25 μg of either MRG1 or IgG1 control (y-axis). Results were determined from 3 pooled mice of each treatment group. F, spleens of SCID/NOD were used in effector-to-target ratios of 10:1 to test ADCC against 526mel cells preincubated with various concentrations of MRG1 or of other antibodies, as indicated in the figure. The y-axis denotes the difference between specific lysis in the presence and absence of each antibody. *, **, ***, $P < 0.05$, < 0.01 , and < 0.001 , respectively. CEA, carcinoembryonic antigen.

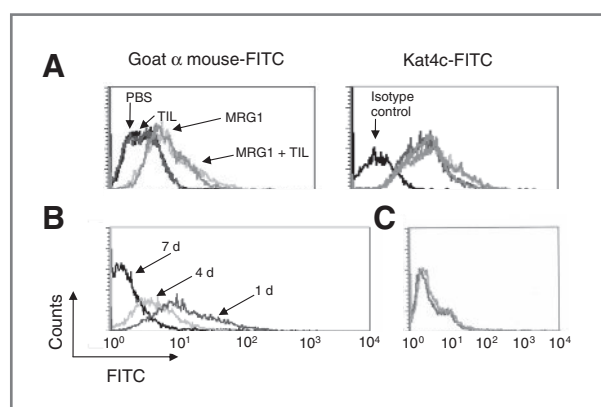


Figure 5. MRG1 reaches xenografts *in vivo*. A, single-cell suspensions generated from tumors excised from animals of all 4 treatment groups described in Fig. 4A were stained for any bound antibodies (left) or for CEACAM1 (right). B, single-cell suspensions generated from tumors excised from animals treated with MRG1 at the indicated time points after administration of MRG1. C, MRG1 staining of mouse liver cells.

to Kat4C-FITC staining (Fig. 5A). Kat4C and MRG1 mAbs do not recognize the same domain (Supplementary Fig. 1S) and do not compete with each other in CEACAM1 binding (data not shown).

The *In vivo* clearance rate of MRG1 from the tumors was evaluated. SCID/NOD mice bearing similar sized CEACAM1⁺ 526mel tumors were injected i.p. with 0.5 mg of MRG1. One, 4, and 7 days after injection, mice were sacrificed and the tumors were removed, processed into single-cell suspensions, and stained with anti-mouse IgG antibodies or with FACS medium only, which served as background. A clear staining was observed 1 day after injection, which decreased by 50% on day 4 and completely disappeared by day 7 (Fig. 5B). MRG1 does not cross-react with the murine CEACAM1, as liver cells, which are known to express CEACAM1 (29), were not stained (Fig. 5C). These results were further confirmed in a tissue species array (data not shown). These results point that the antibody has reached the tumor and is retained for several days to exert its effects.

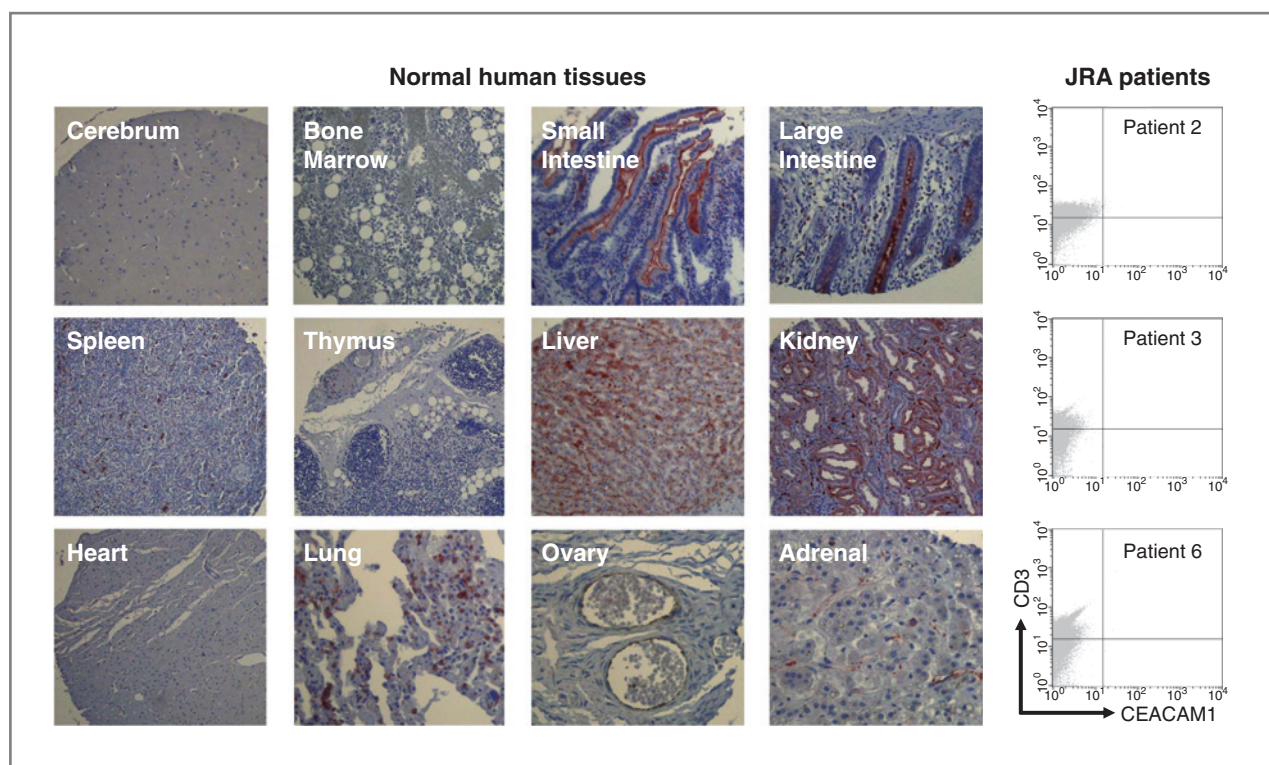


Figure 6. Staining of normal human tissues and autoreactive human lymphocytes. Left, MRG1 immunoreactivity of representative normal human tissues of 3 similar specimens. Right, double staining of CD3 and CEACAM1 on synovial cells derived from patients with juvenile rheumatoid arthritis (JRA).

Normal human organ tissue microarray included 33 types of normal organs, each type taken from 3 normal human individuals (age, 2–67 years; 43% females). The following tissues were negative for MRG1 binding: cerebrum, cerebellum, ovary, pancreas, parathyroid gland, hypophysis, thyroid gland, tonsil, bone marrow, spleen, thymus, lung, cardiac muscle, stomach, skeletal muscle, skin, peripheral nerves, mesothelium, and retina (Fig. 6, data not shown). A cell-specific staining was detected in some organs, mainly on the luminal side of epithelial cells forming ducts or glands in hollow visceral organs such as brush border of small intestine, some apical colonic glands, breast ductal epithelium, liver bile canaliculi, inner surface of renal tubules, few endometrial glands, and luminal part of salivary gland (Fig. 6, data not shown). The only cells of the immune system that were found positive were neutrophils within capillaries (Fig. 6). Lymphocytes in tissues and in lymphatic organs were negative (Fig. 6). A weak-to-moderate positive staining was found in endothelial cells of small blood vessels at selective sites, including ovary, adrenal gland, kidney, and rarely in pancreas, prostate, hypophysis, and endometrium (Fig. 6 and data not shown). Finally, cells from the synovial fluids derived from 8 patients with an acute event of juvenile rheumatoid arthritis, an autoimmune disease, were triple-stained for CD3, CD45RA, and CEACAM1. Remarkably, all inflammatory synovial cells tested from all 8 patients were CEACAM1-negative (Fig. 6).

Discussion

Recently, there have been substantial advances in the field of melanoma immunotherapy, mainly by targeting immunosuppressive mechanisms such as CTLA4 and PD1 (6, 30). However, these modalities are still largely suboptimal and carry substantial immune toxic effects in the skin, gastrointestinal tract, and other syndromes such as uveitis or hypophysitis (31–33). This occurs because anti-CTLA4 and anti-PD1 target general immune checkpoints, which are not tumor-specific, although those caused by anti-PD1 seem to be milder (reviewed in ref. 30). Adoptive transfer of TILs is efficacious and may yield clinical benefits for longer periods of time, but this modality is still not widely available and carries significant toxicity (7, 34).

The clinical and biologic evidence highlight CEACAM1 as a rational target for immunotherapy: CEACAM1 is not found on normal melanocytes (27) but undergoes neoexpression and is widely expressed on the vast majority of metastatic melanoma specimens (Fig. 1A). It has a strong prognostic value (23) implying on its clinical mechanistic importance. Mechanistically, CEACAM1 protects melanoma cells by inhibiting effector functions of NK cells (13, 17, 24) and T cells (19, 20) in a homophilic manner. Indeed, blocking of CEACAM1 homophilic interactions with polyclonal antibodies enhanced the killing of CEACAM1⁺ melanoma cells by NK and T cells (17, 19).

Here, we present MRG1, a CEACAM1-specific, high-affinity mAb that efficiently blocks CEACAM1 homophilic interactions and renders CEACAM1⁺ melanoma cells more susceptible to elimination by T cells in an antigen-restricted, dose-dependent manner (Supplementary Figs. 1S–4S). *In vivo*, MRG1 efficiently enhances the specific T-cell-mediated elimination of melanoma cells to inhibit the growth of melanoma xenografts by induction of tumor cell apoptosis (Fig. 4). The absence of direct MRG1 effects on melanoma cells *in vitro* (Fig. 3) or *in vivo* (Fig. 4), combined with the negligible ADCC effect (Fig. 4; Supplementary Fig. 5S), supports the suggested mechanism of action. It should be noted that xenograft setting is suboptimal for evaluation of recruitment of other immune components, toxicity, autoimmunity, and Ig pharmacokinetics.

Many publications reported that CEACAM1 is expressed by a wide variety of epithelial cells (11). Furthermore, CEACAM1 was reported as a tumor suppressor, which suppresses cell proliferation of colon and prostate cancer cells (35) and acts as a regulator of insulin clearance. These points could raise two safety concerns: (i) direct effect of MRG1 on normal CEACAM1⁺ cells and (ii) erroneous attack of normal CEACAM1⁺ cells by activated lymphocytes. MRG1 showed strong staining of melanoma cells (Fig. 1), as compared with no staining of most normal human tissues tested, but some selective staining was observed in the luminal aspect of epithelial cells of ducts or glands in hollow viscera (Fig. 6). However, MRG1 did not directly affect CEACAM1⁺ cells, or induced nonspecific T-cell activation or significant ADCC (Figs. 3 and 4). In addition, this cellular aspect is generally less accessible to an antibody administered via the peripheral blood. Noteworthy, MRG1 staining displays a more selective profile than the widespread expression of CEACAM1 that was reported previously (36). Finally, it should be noted that other major targets for cancer immunotherapy have a substantially wider expression pattern in normal tissues than CEACAM1, such as EpCAM (37, 38) or EGF receptor (39, 40). Importantly, various clinically tested drugs against these targets are well tolerated and have a safe toxicity profile, such as adecatumumab (41), edrecolomab (42), and cetuximab (43).

Reports on the direct function of CEACAM1 in some normal cells, such as regulation of proliferation, have established an exclusive role for the cytoplasmic tail, which is entirely independent of the extracellular part of CEACAM1. A truncated CEACAM1, which was devoid of the entire extracellular domain, was still capable of inhibiting colon cell proliferation (35). Regulation of insulin clearance by CEACAM1 was also reported to be mediated by postreceptorial interactions between CEACAM1 cytoplasmic tail and the insulin receptor. It should be emphasized that MRG1 targets the extracellular portion of CEACAM1 and blocks the N-domain (Supplementary Fig. 1S), without any agonistic effect (Fig. 2). MRG1 had no direct effects on cell proliferation or death in CEACAM1⁺ melanoma cells, either in solu-

tion or following cross-linking with secondary antibodies (Fig. 3).

In terms of hypothetical immune toxicity, a main advantage of CEACAM1 blockade over abrogation of generalized inhibitory mechanisms is the expected selectivity to the tumor vicinity. Tumor-selective effects of MRG1 are expected on the basis of the CEACAM1 expression profile on melanoma cells and normal tissues (Figs. 1A and 6), as well as due to the presence of CEACAM1⁺ CD8⁺ lymphocytes in the vicinity of CEACAM1⁺ tumor metastases in patients with melanoma (Fig. 1B–D). Quantification of surface CEACAM1 molecules showed that melanoma cells express CEACAM1 >20-fold stronger than normal CEACAM1-positive, such as prostate cells (Supplementary Fig. 6S). The tumor selectivity of MRG1 is further expected on a functional basis. Abrogation of CEACAM1 binding enhanced T-cell killing only in an antigen-restricted context. Importantly, MRG1 did not induce nonspecific T-cell killing activity and had no direct effect on CEACAM1⁺ cells (Figs. 3 and 4). Therefore, it is reasonable to speculate that normal CEACAM1⁺ cells, which are not normally recognized by endogenous T cells, would not be affected immunologically by MRG1. Furthermore, MRG1 did not stain lymphocytes obtained from the synovial fluids of patients with acute event of autoimmune arthritis (Fig. 6). This implies that autoimmune flare up by anti-CEACAM1 blockade is less probable in these cases. Nevertheless, full safety and toxicity studies must be conducted to characterize potential adverse effects.

Melanoma cells use various immune evasion strategies, such as reduced expression of MHC class I and B7 costimulation, metabolic dysregulation through the activity of indoleamine 2,3-dioxygenase, and via overexpression of inhibitory molecules, such as PD-L1 and CEACAM1 (reviewed in ref. 3). Targeting CEACAM1 offers several potentially important advantages: CEACAM1 inhibits both T and NK cells, thus anti-CEACAM1 would have an effect regardless of MHC class I expression status. Because of the homophilic nature of CEACAM1 mode of action, anti-CEACAM1 antibodies can abolish CEACAM1 intercellular interactions by binding to the lymphocytes, the tumor cells, or both. This is expected to increase the chances for successful abrogation of CEACAM1 function *in vivo*. There is a robust expression of CEACAM1 by the vast majority of the activated lymphocytes within the tumor or its close vicinity, which renders them susceptible to CEACAM1-mediated inhibition and thereby amenable to CEACAM1-targeted intervention.

In summary, CEACAM1 blocking could unleash effector functions of lymphocytes selectively against the tumor cells. It could be used as a strategy to selectively enhance the antitumor properties of the endogenous immune response. In addition, this type of modality would probably synergize with other anti-melanoma immunotherapeutics, such as IL-2, which induce CEACAM1 expression on T cells (22), anti-CTLA4, or with adoptive T-cell transfer. Finally, other types of malignancies, such as non-

small cell lung cancer, could become indicated for CEA-CAM1-targeted therapy because a similar prognostic link between expression of CEACAM1 by tumor cells and poor prognosis was previously reported (44, 45).

Disclosure of Potential Conflicts of Interest

Y. Sapir is employed by cCAM Biotherapeutics as head of research. T. Ben-Moshe is employed by cCAM Biotherapeutics as CEO. J. Schachter is a consultant/advisory board member to Roche. G. Markel has ownership interest (including patents) in and is a consultant/advisory board member of cCAM Biotherapeutics. No potential conflicts of interests were disclosed by the other authors.

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