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 mAb (MRG1) is a promising strategy for cancer immunotherapy. MRG1, a murine IgG1 mAb, was raised against human CEACAM1. It recognizes the CEACAM1-specific N-domain with high affinity (KD~2nM). Further, MRG1 is a potent inhibitor of CEACAM1 homophilic binding, and doesn't 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 anti-tumor effect of adoptively transferred melanoma-reactive human lymphocytes using human melanoma xenograft models in 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, ~90% of melanoma specimens are CEACAM1+, implying that the majority of melanoma patients 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 anti-cancer antibodies in clinical use. Importantly, MRG1 doesn't directly affect CEACAM1+ cells. CEACAM1-blockade is different from other immune-modulatory 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.
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). Immune-modulation with IL-2 yields an objective clinical response in ~20% of the patients, with 5% of them exhibiting a durable complete response (5). Very recently, the US Food and Drug Administration approved the anti-CTLA4 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 comprised of sequentially ordered immunoglobulin-like domain(s). It is subjected to alternative splicing that raises two forms of cytosolic tail, a long form containing immunodominant tyrosine-based 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, e.g. some epithelial cells, melanoma and activated lymphocytes (10).

Many different functions have been attributed to the CEACAM1 protein, including anti-proliferative properties in carcinomas of the colon and prostate, central involvement of CEACAM1 in angiogenesis metastasis and insulin clearance, as well as in immune-modulation (reviewed in (11)). T-cell inhibition through engagement of CEACAM1 has been demonstrated by direct T-cell receptor (TCR) cross-linking (14) and via binding of Neisseria opacity associated proteins (15). We have previously shown that CEACAM1 homophilic interactions inhibit NK cell-mediated killing independently of major
histocompatibility complex (MHC) class I recognition (16-18). We have further demonstrated that CEACAM1 inhibits effector functions of tumor infiltrating lymphocytes (TIL), such as cytotoxicity and IFNγ release (19). Moreover, we found that an IFNγ-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 et al demonstrated 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 demonstrated an unusually high percentage of CEACAM1+ circulating lymphocytes in the peripheral blood of melanoma patients, as compared to 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 metastatic melanoma patients could be amenable to CEACAM1-targeted therapy.
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 TIL were established and grown as previously described (25). Cells from the synovial fluids of pediatric patients with acute exacerbation of Juvenile Rheumatic Arthritis were obtained following 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 comprised 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). 50,000 BW cells were co-incubated with 25,000 721.221/CEACAM1 cells for 5h in humidified incubator. The concentration of mouse IL-2 in the supernatant was quantified with standardized sandwich ELISA (R&D Systems, Minneapolis, MN, USA).

Generation of MRG1 mAb

Balb/c mice were immunized with 5mg 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, Bet-Haemek, Israel) on a protein G column (GE Healthcare, Munich, Germany). SDS-PAGE confirmed the presence of light and heavy chains, as well as routinely performed 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 0.2μg of antibody diluted in PBS/0.5% BSA/0.02% NaN3 (FACS medium) for 1h on ice. Cells were centrifuged 400g/5min and supernatant was removed. The cells were incubated on 30mins on ice with secondary antibodies, washed with FACS medium and analyzed with FACS calibur instrument (BD, Franklin Lakes, NJ, USA) and FlowJo or CellQuest software. When fluorochrome-conjugated antibodies were used, cells were not further stained with secondary reagents.

Surface plasmon resonance

Experiments were performed with Biacore3000 following preparation as detailed in supplementary methods (Institute of Life Sciences, Hebrew University, 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 25nM) in PBS-ET buffer. Regeneration was done by injecting 10ul of 10mM NaOH. Flow rate during kinetics was 50ul/min. Data was analyzed with BIAEvaluation software 4.1

Knockdown of CEACAM1

Generation of CEACAM1-silenced melanoma cells was performed using commercially available target sequences cloned in the MISSION short hairpin RNA system (lentiviral plasmids pLKO.1-puro) (Sigma-Aldrich, Rehovot, Israel) and lentiviral expression system, as previously described (20). Scrambled non-target sequence was used as negative control.

Cytotoxicity assay

Cytotoxicity measurements were based on CFSE labeling of target cells and co-staining with PI after 6h of incubation with the effector cells, as previously described (19). Blocking with MRG1 was performed by pre-incubation of either effectors or targets for 1h on ice at the indicated concentrations. Background level didn't exceed 20% in all experiments.

Net proliferation assay
Melanoma cells (3x10^3) 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-FITC and PI (BD), according to appropriate specific calibrations. Apoptosis was demonstrated *in-situ* in histopathological slides with TUNEL staining (Millipore, Billerica, MA, USA), according to manufacturer’s instructions.

**Melanoma xenograft model**

2x10^6 melanoma cells were injected subcutaneously to the thigh of 7-8w old 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 (small diameter)^2×(large diameter)/2. When tumors reached a volume of 100mm^3, mice were randomized into experimental treatment groups, which included 8-10 mice each. Upon experiment termination, tumor masses were extracted, halved and processed for histology or homogenized. All animal work was performed following approval of Sheba Medical Center 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, Grand Island, NY, USA). 1x10^6 cells of each melanoma type were mixed, together with 25μg of IgG1 control antibody or MRG1. 20x10^6 TIL14 cells or carrier only were further added to a final volume of 200μl. The mixture was immediately injected intravenously to Balb/c mice. Each group consisted of three animals. After 6h, the mice were sacrificed; their lungs were removed and rendered into single cell suspension with enzymatic digestion using Collagenase IV and DNAse I (Sigma-Aldrich) for 1h in 37h. 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 \textit{in-vivo}.

\textit{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 $4^\circ\text{C}$ with MRG1 mAb. Detection was performed 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 (glycergel).

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 $60^\circ\text{C}$ for 1 hour and further processed with a fully automated protocol. Detection was performed 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 over-express CEACAM1

Fifty five primary cultures of metastatic melanoma were stained for CEACAM1 expression with the in-house anti-CEACAM1 mAb MRG1. Importantly, 49/55 cultures (89%) were significantly stained (Figure 1A), while it is known that CEACAM1 is not expressed by normal melanocytes (27). Moreover, the presence of CEACAM1+ lymphocytes was demonstrated in the vicinity of a representative CEACAM1+ melanoma metastasis to a lymph node (Figure 1B). Double staining for MRG1 and CD8 demonstrated that almost all CD8-positive lymphocytes in the tumor and its close vicinity were MRG1-positive (Figure 1C). Interestingly, MRG1-positive lymphocytes were more common within the tumor and in juxta-tumoral areas, as compared to other areas distant from tumor edge (Figure 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 doesn't 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 1.46-2.83 nM, as determined with Surface Plasmon Resonance (Figure 1S).

A cellular system reporting on homophilic intercellular CEACAM1 interactions was used (16). Co-incubation 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 40ng/ml of MRG1 (Figure 2A). BW/Mock cells didn't secrete mIL-2 (Figure 2A). 1h pre-incubation of MRG1 either with the BW/CEACAM1-zeta or with the 721.221/CEACAM1 cells
similarly abrogated the secretion of mIL-2 (Figure 2B). Remarkably, reduction of 50% in
the secretion of mIL-2 occurs with ~5ng/ml and nearly complete abolishment was
achieved with 20ng/ml (Figure 2B). MRG1 exhibited a substantially stronger CEACAM1
blocking activity than 5F4 mAb, Kat4C mAb and polyclonal antibodies against
CEACAM1,5,6, which were previously used for functional blocking of CEACAM1
(13,16-19,24,28) (Figure 2C). Finally, MRG1 didn't induce secretion of mIL-2 from
BW/CEACAM1-zeta cells, indicating that it doesn't have an agonistic role (Figure 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
shRNA (Figure 2S). MRG1 enhanced the killing of 526mel cells by TIL14 in a dose-
dependent manner, when the lymphocytes were pre-incubated with the antibody (Figure
3A). 624mel, another CEACAM1⁺ melanoma line, was also rendered susceptible to
TIL14 cells (Figure 3B) or to other T-cell cultures (data not shown). MRG1 didn't
influence the killing of CEACAM1-negative 09mel cells, and didn't enhance the killing
of CEACAM1⁺ 938mel cells, which were not recognized by the T-cell cultures. IgG1
isotype control didn't exert any significant effect on T-cell killing (Figure 3B). Pre-
incubation 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 (0.1μg/ml-10μg/ml). The net proliferation
of melanoma cultures was determined on days 2 and 4 of cultivation. MRG1 didn't affect
the proliferation of 526mel (CEACAM1⁺) or 003mel (CEACAM1⁻) cells in all
concentrations tested (representative concentration of 2μg/ml, Figure 3C). Further,
MRG1 didn't induce apoptosis in 526mel cells when it was added into the culture
medium or cross-linked with goat anti-mouse IgG (Figure 3D). IgG1 isotype control had
no significant effect on melanoma cell proliferation or apoptosis rates (Figure 3C-D).
Similarly, MRG1 didn't affect the proliferation or apoptosis rate of a variety of

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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 subcutaneously injected into SCID-NOD mice. Mice bearing tumors of estimated volume of 100mm³ were randomized to one of the following four treatment combinations: a single intravenous injection of PBS or 20x10⁶ tumor-specific human T-cells, combined with weekly intraperitoneal injections of PBS or 0.5mg MRG1. In addition, mice were injected intraperitoneally with 6,000IU 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 (Figure 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 (Figure 4A), which concurs with the in-vitro experiments (Figure 3A-B). Similar results were observed with another CEACAM1+ melanoma cell line SK-MEL-05 (Figure 3S). Intraperitoneal and intravenous administration of MRG1 yielded similar results (data not shown). Isotype IgG1 control had no significant effect (Figure 4B). Furthermore, when inert CEACAM1-positive T cells, which don't mediate any in-vivo effect on 526mel tumor growth (Figure 4C), were used, MRG1 had no significant effect (Figure 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 anti-tumor effect. A strong TUNEL staining was observed only in TIL+MRG1 group, directly indicative for enhanced tumor cell death by the combined treatment (Figure 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 demonstrated 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 (Figure 3B) were mixed with or without TIL14 cells in the presence of 25μg of MRG1 or of IgG1 control. Both melanoma cell lines strongly express CEACAM1...
The various mixtures were immediately injected intravenously into mice. After 6h, 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 co-administered with the TIL14 cells, but not when used in the absence of TIL14 (Figure 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 \textit{in-vivo}. Noteworthy, adoptively transferred human T-cells stably expressed CEACAM1 \textit{in-vivo} for at least 14 days (Figure 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 (Figure 4F). No ADCC activity of MRG1 could be detected in higher effector-to-target ratios (Figure 5S). Other monoclonal and polyclonal antibodies did induce a significant ADCC response in a dose-dependent manner (Figure 4F). This confirms that the tumor growth inhibition by MRG1 is most probably ADCC-independent.

\textit{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 don't produce endogenous immunoglobulins. Only cell suspensions derived from mice treated with MRG1 (either alone or with TIL) displayed staining (Figure 5A). Tumor cell suspensions from mice treated with TIL only or with control treatment remained unstained (Figure 5A). All the tumors within each treatment group displayed consistent staining patterns. CEACAM1 was similarly expressed in all treatment groups according to Kat4C-FITC staining (Figure 5A). Kat4C and MRG1 mAbs don't recognize the same domain (Figure 1S) and don't 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 intraperitoneally with 0.5mg of MRG1. One, four and seven days post-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 one day post injection, which decreased by 50% on day four and completely disappeared by day seven (Figure 5B). MRG1 doesn't cross-react with the murine CEACAM1, as liver cells, which are known to express CEACAM1 (29), were not stained (Figure 5C). These results were further confirmed in a Tissue Species Array (data not shown). These results point that the antibody is reach the tumor and is retained for several days to exert its effects.

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 (Figure 6 and 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 (Figure 6 and data not shown). The only cells of the immune system that found positive were neutrophils within capillaries (Figure 6). Lymphocytes in tissues and in lymphatic organs were negative (Figure 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 (Figure 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 (Figure 6).
Discussion

Recently, there have been substantial advances in the field of melanoma immunotherapy, mainly by targeting immune-suppressive mechanisms like 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 (30)). Adoptive transfer of tumor infiltrating lymphocytes 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 biological evidence highlight CEACAM1 as a rational target for immunotherapy: CEACAM1 is not found on normal melanocytes (27) but undergoes neo-expression and is widely expressed on the vast majority of metastatic melanoma specimens (Figure 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 (Figures 1S, 2-4). 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 (Figure 4). The absence of direct MRG1 effects on melanoma cells in-vitro (Figure 3) or in-vivo (Figure 4), combined with the negligible ADCC effect (Figure 4, 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). Further, CEACAM1 was reported as a tumor suppressor, which suppresses cell proliferation of colon and prostate cancer cells (35) and as a regulator of insulin clearance. These points could raise two safety concerns: a) direct effect of MRG1 on normal CEACAM1\(^+\) cells; b) erroneous attack of normal CEACAM1\(^+\) cells by activated lymphocytes. MRG1 showed strong staining of melanoma cells (Figure 1), as compared to 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 (Figure 6). However, MRG1 didn't directly affect CEACAM1\(^+\) cells, or induced non-specific T-cell activation or significant ADCC (Figures 3-4). 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 EGFR (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 post-receptorial 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 (Figure 1S), without any agonistic effect (Figure 2). MRG1 had no direct effects on cell proliferation or death in CEACAM1\(^+\) melanoma cells, either in solution or following cross-linking with secondary antibodies (Figure 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 based on the CEACAM1 expression profile on melanoma cells and normal tissues (Figures 1A, 6), as well as due to the presence of CEACAM1+ CD8+ lymphocytes in the vicinity of CEACAM1+ tumor metastases in melanoma patients (Figure 1B-D). Quantification of surface CEACAM1 molecules showed that melanoma cells express CEACAM1 >20-fold stronger than normal CEACAM1-positive, such as prostate cells (Figure 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 didn't induce non-specific T-cell killing activity and had no direct effect on CEACAM1+ cells (Figures 3-4). 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 didn't stain lymphocytes obtained from the synovial fluids of patients with acute event of autoimmune arthritis (Figure 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 performed in order to characterize potential adverse effects.

Melanoma cells employ various immune-evasion strategies, such as reduced expression of MHC class I and B7 co-stimulation, metabolic dysregulation through the activity of indoleamine 2,3-dioxygenase and via over-expression of inhibitory molecules such as PD-L1 and CEACAM1 (reviewed in (3)). Targeting CEACAM1 offers several potentially important advantages: CEACAM1 inhibits both T-cells and NK cells, thus anti-CEACAM1 would have an effect regardless of MHC class I expression status; Due to 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 \textit{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 anti-tumor 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 induces 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 CEACAM1-targeted therapy, because a similar prognostic link between expression of CEACAM1 by tumor cells and poor prognosis was previously reported (44,45).
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References


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Figure legends

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 point on exemplar melanoma cells. Black triangles point on CEACAM1+ lymphocytes; (C) Double staining for CEACAM1 (brown pigmentation) and CD8 (pink pigmentation) on slide from the same patient material. White arrows point on CEACAM1+CD8+ cells. Black arrows and dashed circle point on CEACAM1−CD8+ cells; (D) Quantification of cells per high power field in different tissue regions, as indicated in the figure. Data was obtained from 5 fields in 3 slides.

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 co-incubation with .221/Mock or .221/CCM1 cells, in the absence or presence of 40ng/ml of MRG1; (B) Dose-dependent blocking of CEACAM1 activity with MRG1. 100% of CEACAM1 activity was determined as the mIL-2 concentration in the absence of MRG1. "Targets" refers to .221/CCM1 cells and "Effectors" to 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; (D) No agonistic effect of MRG1 on BW/CCM1-zeta cells. ** denotes P value <0.01; *** denotes P value < 0.001. Figure shows a representative experiment out of four performed.

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\(^{\text{dim}}\) (09mel) and HLA-irrelevant CEACAM1\(^+\) (938mel). * denotes P value <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 5µg/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 18h 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 co-staining in flow cytometry. Percent of apoptotic cells in each treatment are indicated in the figure. Figure shows a representative experiment out of four performed.

Figure 4: MRG1 synergizes with adoptively transferred human TIL in-vivo

(A) SCID-NOD mice bearing human \(~100\text{mm}^3\) 526mel xenografts were randomized according to the four treatment groups indicated in the figure. Black arrows indicate on 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 panels 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 6h post their intravenous injection with 25µg of either MRG1 or IgG1 control (Y-axis). Results were determined from three pooled mice of each treatment group; (F) Spleens of SCID-NOD were used in E:T ratios of 10:1 to test ADCC against 526mel cells pre-incubated with various concentrations of MRG1 or of other antibodies, as indicated in the figure. Y-axis denotes the difference between specific lysis in the presence and absence of each antibody. Abbreviations: IV- intravenous, IP- intraperitoneal, *, ** and *** denote P value <0.05, <0.01 and < 0.001, respectively.
Figure 5: MRG1 reaches xenografts in-vivo

(A) Single cell suspensions generated from tumors excised from animals of all four treatment groups described in Figure 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.

Figure 6: Staining of normal human tissues and autoreactive human lymphocytes

(Left) MRG1 immuno-reactivity of representative normal human tissues out of three similar specimens; (Right) Double staining of CD3 and CEACAM1 on synovial cells derived from patients with Juvenile Rheumatoid Arthritis.
Figure 1

A

CEACAM1 (MFI)

Metastatic melanoma specimens

B

Metastatic melanoma infiltrating a lymph node

C

D

Number of cells in HIF

CEACAM1^+ CD8^+ CEACAM1^+ CD8^−

Intra Juxta Distant Intra Juxta Distant
Figure 2

(A) Analysis of mL-2 (pg/ml) production in the presence of MRG1.

(B) Effect of MRG1 on % CEACAM1 activity.

(C) Concentration (ng/ml) dependence of MRG1 on mL-2 production.

(D) MRG1 concentration (ng/ml) dependence of % CEACAM1 activity.
Figure 3

A

% Specific lysis

Concentration (μg/ml)

0 1 2 3 4 5 6 7 8 9 10

B

% Specific lysis

[Bar graphs showing different concentrations and specific lysis percentages for No Ab, Control Ab, and MRG1]

C

Cells (x1,000)

No Ab
Control Ab
MRG1

0d 2d 4d 0d 2d 4d

003mel 526mel

D

Annexin

PI

Control Ab
MRG1 only

2nd Ab only
MRG1+2nd Ab

003mel 526mel
Figure 4

(A) Tumor volume (mm$^3$) over time (days) for Control, MRG1, TIL, and MRG1+TIL groups. Significant differences are indicated by *, **, and ***.

(B) Tumor volume (mm$^3$) over time (days) for TIL+IgG1, TIL, IgG1, and irTIL groups. Significant differences are indicated by *, **, and ***.

(C) Tumor volume (mm$^3$) over time (days) for irTIL+MRG1, irTIL, and IgG1 groups. Significant differences are indicated by *, **, and ***.

(D) Images showing cells IV and antibody IP.

(E) Ratio plots for IgG1 and MRG1 with and without TIL.

(F) Percentage of DCC for MRG1, HC10, Poly CEA, and Anti-MICA at concentrations of 2.5, 5, and 25 μg/ml.
Figure 5

A. Goat α mouse-FITC

- PBS
- TIL
- MRG1
- MRG1+T

Kat4c-FITC

- Isotype control

B. Counts (FITC)

- 1d
- 4d
- 7d

C. FITC (log scale)

- 10^0
- 10^1
- 10^2
- 10^3
- 10^4
Figure 6

Normal human tissues

Cerebrum  Bone Marrow  Small Intestine  Large Intestine

Spleen  Thymus  Liver  Kidney

Heart  Lung  Ovary  Adrenal

JRA patients

Patient 2

Patient 3

Patient 6

CD3  CEACAM1
Molecular Cancer Therapeutics

Novel immunotherapy for malignant melanoma with a monoclonal antibody that blocks CEACAM1 homophilic interactions

Rona Ortenberg, Yair Sapir, Lee Raz, et al.

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