**Abstract**

**Introduction**

Carcinoembryonic antigen adhesion molecule 1 (CEACAM1) in humans is a member of a large cluster genes expressed on human chromosome 19. These proteins are characterized by a membrane distal IgV-like N-domain which is coupled to a variable number of IgC2-related domains linked to the membrane by either a glycophosphatidyl or transmembrane anchor. CEACAM1 is unique in that it is the only human member of this family that is expressed by rodents, contains a cytoplasmic tail with inhibitory functions through possession of two immunoreceptor tyrosine-based inhibitory motifs (ITIM) and is displayed on the cell surface of activated T cells where it serves an important role in immune inhibition and tolerance. CEACAM1 is characterized by significant alternative splicing that generates 12 isoforms which all share an identical membrane distal N-domain primarily involved in high affinity homophilic adhesion which are linked to a long (L) ITIM containing cytoplasmic tail of 74 amino acids which is inhibitory or a 12 amino acid cytoplasmic tail that is non-inhibitory. Upon homophilic trans-dimerization, CEACAM1-L variants serve to recruit Src homology domain phosphatase (SHP)-1 or SHP-2 after ITIM phosphorylation by p56lck in T cells resulting in inhibition of proximal T cell receptor (TCR) signaling.

CEACAM1 has been increasingly recognized to play an important role in not only immune tolerance in association with autoimmunity but also in infectious pathogenesis and cancer. In the former case, a variety of microorganisms including viruses (murine hepatitis virus), bacteria (*Neiserriae sp.*, *Helicobacter pylori*, *Moraxella sp.*, *Haemophilius sp.*, *Escherichia sp.*) and fungi (*Candida sp.*) use binding to the homophilic interaction site of CEACAM1 on the GFCC’ face for host invasion and immune evasion through co-opting the inhibitory activity of CEACAM1. In some cases, the microbes that use CEACAM1 to cause infection are associated with neoplasia themselves such as *H. pylori* which uses the homophilic binding interface of CEACAM1 on epithelial cells to dock and subsequently insert proteins such as CagA which are critical to this organism’s pathogenesis. Similarly, CEACAM1 has long been recognized to potentially play a role in the pathogenesis of a wide variety of solid tumors where its expression correlates with poor survival as shown in non-small cell lung cancer, colorectal cancer, osteosarcoma, squamous cell cancer and melanoma. Here CEACAM1-L and CEACAM1-S isoforms variably contribute to the survival, growth and metastasis of tumors. In addition to these potentially direct effects on tumors, CEACAM1 may contribute to neoplasia through its role in inhibiting T cell and natural killer cell function, regulating the fibrotic activity of macrophages and promoting vascular neogenesis and lymphangiogenesis, among other activities (reviewed in X). Although targeting CEACAM1 in cancer pathogenesis can be considered a high priority, it is potentially challenging as any therapeutic must optimally engage CEACAM1 at the homophilic binding interface on the N-domain, maintain specificity for CEACAM1 and not cross-react with highly related CEACAM family members and not mimic CEACAM1 trans-ligation and inhibitory function. Here, we describe a first in class monoclonal antibody and its structural characterization that achieves these goals in eliciting anti-tumor activity as monotherapy in humanized model systems.

**Results**

**Generation and Characterization of a Humanized Affinity Matured Blocking mAb Directed against Human CEACAM1**

We have previously described a mouse monoclonal antibody (mAb) specific for the N-domain of human CEACAM1 (5F4) (REF) that does not cross-react with human CEACAM3, 5 and 6 (REF) and which has recently been recognized to possess direct anti-tumor effects on human pancreatic cancer xenografts in immune-deficient mice. In addition, we used an ELISA to show that the 5F4 mAb blocked homophilic interactions with CEACAM1 suggesting its epitope was contained within the GFCC’ face. Indeed, when we resolved a variety of site-directed mutants of CEACAM1 which contained alanine substitutions in residues critical to the crystallographic interface such as Y34, G41, Q44, G47, Q89 and D94 and V96 by SDS-PAGE and performed immunoblotting, we observed that 5F4 recognition of CEACAM1 was disabled relative to that observed with wild-type CEACAM1.

>>We humanized this 5F4 antibody by fusion with an IgG4 with a single point mutation in the Fc heavy chain to prevent the formation of half-antibodies and screened the selected humanized clones for binding to CEACAM with an affinity similar to that of the original murine mAb<<. The selected humanized clone was CP08H03/Vк8 S29A (thereafter CP08), which was further used in several cell-based and *in vivo* studies. Importantly, unlike other anti-CEACAM1 mAbs described to date (REFs), CP08 is highly specific for human CEACAM1, as it bound only on human CEACAM1+ cells, but not on CEACAM3+, CEACAM5+, CEACAM6+, CEACAM8+-transfected cells, expressing other CEACAM family members that exhibit similarity to CEACAM1 higher than 95% sequence homology (**Supplementary Figure.** ) Of note that, CEACAM3 is the activating isoform characterizing with ITAM domain located in the cytoplasmic tail belongs to this CEACAM family (REFs).

Finally, another critical feature of CP08 resides in its capacity to inhibit the binding of CEACAM1 to CEACAM1 (**Figure.** ), and CEACAM1 to TIM-3 (**Figure.** ).

**Human (h) CEACAM1 mutagenesis study and role of hCEACAM1 *C-C*’ and *F-G* loops residues for binding with humanized CEACAM1 antibodies**

During the generation of CP08H03/Vк8 S29A antibody, we first identified the hCEACAM1 residues that are involved in binding by immunoblot. To determine this, we transfected and expressed CEACAM1-FLAG proteins human embryonic kidney (HEK) cells with mutations in hCEACAM1 *C-C*’ and *F-G* loops and proteins were resolved by SDS-PAGE and then immunoblotted. The wild-type (WT) or mutant CEACAM1 proteins were detected using the indicated chimeric (VH0/Vκ0) and humanized CEACAM antibodies (**Figure.** ). The blots showed that mutation of hCEACAM1 *C-C*’ and *F-G* loops residues affect binding and mutation of Y34, Q44, G47, and Q89 residues lead to more decreased binding to humanized CEACAM antibodies. Also, taking advantage of understanding at the single amino acid level of how CEACAM1 interacts with its ligands such as CEACAM1 (**Figure.** ), and TIM-3 (**Figure.** ), (REFs), we also include the screening processes to select the leads antibodies with CEACAM1 mutants such as Y34A, G44L, Q89H transient Hela cell line (**Supplementary Figure.** ).

**Selective binding of CP08\_H03 antibody to hCEACAM1**

Human CEACAM1 is very homologous to other hCEACAM family members, and shares sequence identity (%) of 87.96, 88.89, and 89.81 with hCEACAM3, hCEACAM5, and hCEACAM6, respectively (**Figure 3**). To maintain hCEACAM1 binding selectivity during the generation of homogenized CP08\_H03 antibody, we have characterized the binding selectivity of CEACAM1 antibodies VH0/Vκ0, CP08H03/Vк8 S29A, CP08H03/CP08F05 CEACAM1 by Surface plasmon resonance (SPR) and ELISA. We accessed binding affinities to hCEACAM1, hCEACAM3, hCEACAM5, and hCEACAM6 using single-cycle kinetics SPR analysis. No significant binding of the three CEACAM1 antibodies, CP08H03/Vк8 S29A, CP08H03/CP08F05, and VH0/Vκ0, was observed for hCEACAM3, hCEACAM5, and hCEACAM6 (**Figure 3A**). These SPR results were consistent with data obtained by measuring antibody specificity with an ELISA. Essentially no binding of the three CEACAM1 antibodies, CP08H03/Vκ8 S29A, CP08H03/CP08F05, and VH0/Vκ0 to CEACAM3, CEACAM5, or CEACAM6 was observed (**Figure 3B-2C**).

**Crystal structures of hCEACAM1:CP08\_H03 Fab complex and hCEACAM1-A49V/Q89H natural allelic variants**

To obtain the structural basis of hCEACAM1 binding to CP08\_H03 antibody, we first crystallized the hCEACAM1:CP08\_H03 Fab complex and confirmed the presence CP08\_H03 antibody fab and hCEACAM1 in the crystal by SDS-PAGE/silver stain analysis under reducing and non-reducing conditions (**Figure 4**). Next, we determined the crystal structure of the hCEACAM1:CP08\_H03 Fab complex at 3.3 Å resolution by x-ray crystallography (**Figure 5-7**). This crystal structure revealed two copies of the hCEACAM1:CP08\_H03 Fab complex in the asymmetric unit in the centered tetragonal space group I4122. A Cα trace of all protein chains in the asymmetric unit is shown in Fig. 6A. Chains A and D correspond to Fab light chains, chains B and E correspond to Fab heavy chains, and chains C and F correspond to molecules of CEACAM1.

The CP08\_H03 Fab binds to hCEACAM1 in a 1:1 stoichiometric ratio and the complex formed from chains A, B, and C is better ordered than the other complex, and therefore the A, B, C complex is the focus of analysis in this discussion. With reference to an existing structure (PDB ID 4QXW) of the hCEACAM1 dimer (Figure 6B) that involves hCEACAM1 *C-C*’ and *F-G* loops residues F29, Y34, Q44, Q89, and N97 for dimer formation, this CEACAM1:CP08\_H03 Fab complex crystal structure revealed that the Fab also binds to this *GF-CC’* dimeric interface of hCEACAM1, and presumably this competitive interaction leads to dissociation of the hCEACAM1 dimer in solution. The binding epitopes are shown in a molecular surface representation of CEACAM1 in Fig. 6C. The interacting surfaces have a shape complementarity of 0.5, and complex formation buries 1607 A2 of total solvent accessible surface. In the Fab heavy chain, residues of CDR2 and CDR3 interact mainly with hCEACAM1 residues F29, S32, Q44, T56, Q89, and I91. In the Fab light chain, residues in CDR1, CDR2, and CDR3 (Figure 6D, and Figure 7) interact mainly with hCEACM1 residues D40, N42, L95, V96, N97 and E99. In addition to interactions that are hydrophobic/van der Waals in nature such as interactions between F29 of hCEACAM1 and F104 of heavy chain CDR3, some of the above mentioned interactions also involve hydrogen bonding between Q89 of hCEACAM1 and Y103 of heavy chain CDR3, T56 of hCEACAM1 and D102 of heavy chain CDR3, and N97 of hCEACAM1 and Y31 of CDR1 of light chain. The interaction between Q89 of hCEACAM1 and Y103 of heavy chain CDR3 is at the center of CP08\_H03: hCEACAM1 binding interface. As observed in crystal structure (PDB ID 4QXW) of the hCEACAM1 dimer, F29 of one CEACAM1 monomer binds F29 of a second monomer at the CEACAM1:CEACAM1 homodimeric interface. Thus, observed binding of CDR3H residue F104 of CP08\_H03 antibody blocks F29-F29 CEACAM1 interactions (Figure 6D). In addition, the observed binding interface of hCEACAM1:CP08\_H03 Fab complex crystal structure is consistent with the results of a hCEACAM1 mutagenesis study described above (Figure 1), which was aimed at identifying residues in CEACAM1 that are involved in binding to the indicated CEACAM1 antibodies.

Based on the selective binding of CP08\_H03 antibody to hCEACAM1 only, but not to hCEACAM3, hCEACAM5 or hCEACAM6 as reported here, the crystal structure also provides insights and structural basis of this selectivity. Of above mentioned residues important for hCEACAM1 and CP08\_H03 interactions, residue Q44 is not conserved in hCEACAM3, residue Q89 is not conserved in hCEACAM5, and residues F29, Q44 are not conserved in hCEACAM6 (Figure 8) . In addition, our crystal structure predicts that A49 residue could also contribute to CP08\_H03 antibody selectivity for hCEACAM1. This A49 residue is adjacent to CP08\_H03: hCEACAM1 binding interface and alignments of human CEACAM family members indicated that CEACAM3, 5, 6, 7, and 8 all contain a valine residue at this position (**Figure 8**). Thus, it was anticipated that hCEACAM1 A49 residue could play an important role in CP08\_H03: hCEACAM1 binding due to its least steric hindrance and less hydrophobicity property compared to valine. Interestingly, the polymorphisms in hCEACAM-1 at these A49 and Q89 residues include Ala49Val (rs8110904) and Gln89His (rs8111468). Further, the CEACAM1 Ala49Val polymorphism (rs8110904) is linked to lymphedema caused by *Wuchereria bancrofti* (Debrah L, B. et. al Hum Genomics. 2017 Nov 9;11(1):26) – a filaria worm that invades the lymphatic system.

To test the role of A49 for CP08\_H03: hCEACAM1 binding selectivity, a human CEACAM1 A49V/Q89H mutant was expressed and purified as described before. Next, crystal structure of the CEACAM1 A49V/Q89H mutant was determined to 1.7 Å resolution and compared to the CP08\_H03: hCEACAM1 Fab complex. As discussed above, CDR3H residue F104 of CP08\_H03 antibody makes contact with residue F29 in wildtype CEACAM1 (**Figure 9**, left panel). CEACAM1 residue A49 is located close to the F104/F29 interaction site. Due to the increase in hydrophobicity of valine in non-CEACAM1 family members as compared to alanine in human CEACAM1, a mutation of human CEACAM1 residue A49 to valine causes hydrophobic CEACAM1 residue F29 to move closer to CEACAM1 V49 residue. This rotameric shift of F29 was also observed in human CEACAM5 (PDB code 2QSQ) and human CEACAM3 (PDB code 6AW1) crystal structures and is predicted to clash with CDR3H residue F104 (**Figure 9**, right panel). This is illustrated by the change in orientation displayed by the CEACAM1 F29 ring, which moves closer to the space previously occupied by CDR3H residue F104 (**Figure 9**, right panel). These data indicate that this steric hindrance caused by the A49V mutation interferes with binding of CEACAM1 antibody CP08H03/Vκ8 S29A to other CEACAM1 family members containing a valine at position 49 and is, as such, a major contribution to the selectivity of the antibody. It is predicted that this rotameric shift of F29 ring could also affect the interaction between CDR2H residue Y57 and F29. Development of lymphedema disease caused by *Wuchereria bancrofti* is linked to the Ala49Val polymorphism, which marks the alanine 49 residue found to be involved in binding to CP08\_H03/Vκ8 S29A antibody. As such, it is expected that CP08\_H03/Vκ8 S29A may also interfere with *Wucheria bancrofti* and other related pathogens or cancer processes that phenocopy worm interactions with lymphatics such as tumor invasion.

To further validate hCEACAM1-A49V/Q89H natural allelic variants crystal structure and role of A49 to determined CP08\_H03 selectivity, we tested the binding of hCEACAM1-A49V/Q89H natural allelic variant protein with CP08\_H03 antibody, CM24-hCEACAM1 antibody and control IgG4 antibody by ELISA. Consistent with predictions of CP08\_H03: hCEACAM1 Fab complex and hCEACAM1-A49V/Q89H natural allelic variants crystal structures, the CP08\_H03 and control IgG4 antibodies did not show any binding to hCEACAM1-A49V/Q89H natural allelic variant protein. Interestingly, the concentration dependent binding was detected with CM24-hCEACAM1 antibody that could suggest non-specific binding away from GF-CC’ interface of hCEACAM1 (**Figure 10**).

**Anti-human CEACAM1 mAb promotes CEACAM1 monomer on the cell surface**

Functional studies showed that anti-human CEACAM1 mAb substantially resulted in CEACAM1 monomer on surface of the tumor cell lines (**Figure**).

**Targeting human CEACAM1 by CP08 reinvigorate effector T cell events**

The lack of CP08 cross-reactivity with murine Ceacam1 required the development of novel small animal models for the *in vivo* functional assessment of CP08. Due to merely 41% sequences similarity between mouse and human CEACAM1 (**Supplementary Figure**), especially the cross-species discrepancy lies majority within CC’ and FG loop (**Supplementary Figure**), which is critical for human CEACAM1 homodimerization (REFs) and the interaction with heterophilic ligands such as pathogens, or other CEACEA family such as CEACAM1 itself and CEACAM5, or TIM-3 (**Supplementary Figure**) (REFs), we then sought for evaluating the biological activity of CP08 in mice with the NOD genetic background carrying the severe combined immunodeficient (SCID) mutation and deficiency in the IL-2 receptor common gamma chain (commonly termed NSG) adoptively transferred with human peripheral mononuclear cells (PBMCs) (REFs). >>DISCUSSION<< We attempted to access the blocking activity of CP08 on homeostatic effector CD45+, a pan-leukocytes proliferative capacity and on human CD4+ and CD8+ T effector cells by monitoring the cell surface expression of PD1. Frist, we compared two published engraftment regimes with minor modifications (REFs). As shown in Figure a and b, the protocols differed in the length of 5 weeks (9-wk) (**Figure a**) or 3 weeks (4- wk) (**Figure b**). The mice were evaluated for engraftment using 3-weeks and 5-weeks schedules (**Supplementary Figure**), we found that in the 3-weeks regime, the mice reached higher humanization degree reflected by higher human CD4+ and CD8+ T cell implantation in the spleens together with up-regulations of activation induced T cell markers (e.g. CEACAM1, PD1 and TIM-3) (**Supplementary Figure**) which is consistent with other reports suggesting that T cells rapidly acquire an activated phenotype following the engraftment of human cells (REF). Thus, we used this humanized NSG model to assess the functional effect of *in vivo* systemic administration of CP08 on human immune cells.

With single dosage of CP08 administration (regime showed in **Supplementary Figure**) was found to be able to enhance homeostatic expansion of human CD45+ leukocytes in a dose-dependent manner as an increase in the proportion of human CD45+ cells of mice receiving 2mg/kg compared to relative isotype control, however, the mice receiving 10mg/kg showed similar response as 2mg/kg thus lead us to titrate down CP08 started from 2mg/kg for the following studies. Of note, there is no evidences in depletion of human CD45+ leukocytes when mice receiving CP08 (**Supplementary Figure**). We observed that PD1 up-regulation on CD4 T cells (**Supplementary Figure**) and CD8 T cells (**Supplementary Figure**.) with CP08 administration, demonstrating T cells reinvigorate events occurred when targeting CEACAM1 by CP08. >>SEB humanized NSG mice PD1 data<<.

**Targeting human CEACAM1 by CP08 promotes the anti-tumor cell activities**

*CP08 dose dependent figure -Fibrosis figure -Survival figure- Focus on tumor environments especially for CD4 TILs, CD8 TILs, CEACAM1+CD4+TILs, CEACAM1+CD8+TILs. PD1+CD4+TILs, PD1+CD8+TILs figures- ICCS data in humanized mice-RNAseq figure*

To determine whether CP08 exhibits anti-tumor therapeutic properties in an immune competent environment, we investigated this preclinical testing possibility by using this antibody to treat i.e. the subcutaneously injected MALME-3M (HLA-A2+) (REFs) in NSG adoptively transferred with HLA-A2 matched human peripheral mononuclear cells (PBMCs), as CP08 doesn’t cross-react with mouse CEACAM1. In light of the biophysical and biochemical studies of CP08 (**Supplementary Figure**), we then test for the potential *in vivo* effective dosages of CP08 with 2mg/kg (13nM), 0.4mg/kg (6.5nM), 0.08mg/kg (3.25nM) (**Figure**). Administration of 0.08mg/kg (3.25nM) CP08 has reached the *in vivo* effectiveness and is enhanced with 0.4mg/kg (6.5nM) and gradually with 2mg/kg (13nM) (**Figure**). Signs of liveness had been significantly improved in CP08 treated mice (**Supplementary Figure**). Of note, injected MALME-3M by a subcutaneous route resulted in lung metastases (**Supplementary Figure**) and fewer lung fibrosis was present in mice treated with CP08 compared to the mice treated with control IgG4 (**Supplementary Figure**).

When monitored the survival of these experimental mice, we found the mice received significant survival benefits with 2mg/kg CP08 treatment (**Figure**) and the mice live averagely 100 days post-tumor injection compared to the control mice which lived averagely 25 days (**Figure**).

We then dissected the effect of CP08 in targeting immune responses to eradicate cancer cells in the tumor microenvironment by analyzing tumor-infiltrating lymphocytes (TILs).

Tumors from control IgG4 treated mice were found to be infiltrated by human CD4+ and CD8+ T cells, in which, ~45% of tumor-infiltrating CD4+ and ~15% of tumor-infiltrating CD8+ T cells expressed the CEACAM1 whereas tumors from CP08 treated mice were found to be infiltrated by human CD4+ and CD8+ T cells, in which, ~xx% of tumor-infiltrating CD4+ and ~xx% of tumor-infiltrating CD8+ T cells expressed the CEACAM1 (**Figure**). Of note, we barely observe TIM-3 expression on the surface of human CD4+ and CD8+ T cells from the TILs (**Supplementary Figure**). Purified human CD4+ and CD8+ T cells from mice receiving CP08 recovered their proliferative capacities *in vitro* compared to the control mice (**Figure**); Tumors from mice receiving CP08 barely detected and the less proliferative potential compared to the tumor from the control mice were observed (**Figure**). We next sought for changes in transcriptional profiling that might provide insights into the mechanisms that account for the significant survival benefit of CP08 treatment in this humanized melanoma model (Fig. ) >>RNAseq data<<

**Combined blockade of CEACAM1 and PD1 has beneficial outcome for the hosts in a pre-clinical model and from clinical patient samples.**

*CP08 PD1 comb NSG figure CD4 CD8 tumor proliferation figure – melanoma patient phenotype figure - in vitro patient comb TNF-a, IFN-g figure-*

Pre-treatment tumour samples from with PD-1 blockade in metastatic melanoma cancer were used

for an integrated evaluation of biomarkers (REFs). We categorized patients who responded to treatment with a complete or partial response as responders and compared them with non-responders, who displayed stable or progressive disease. In all of our cases, post-immune treatment metastatic samples represented immune treatment resistance (acquired resistance), as the tumors were resected for progression of disease. Pre-immune treatment samples represented a treatment-naïve state.

Floating cells represents T cells which have been deprived of the cell-cell interactions of the tumor microenvironment by depleting of (or separating from) the stroma cells whereas the digested T cells reflects the state of immune cells naïve to the intact metastatic lesion (i.e. intact tumor microenvironment).

CEACAM1 expression on CD4+ and CD8+ T cells was significantly associated with response (Fig. ).

By contrast, CEACAM1 expression on tumour cells was not associated with response (Supplementary Figure).

We isolated and conducted immune profiling of the tumor infiltrating lymphocytes (TILs) in metastatic lesions from pre- and post-immune treated melanoma patients (metastatic melanoma: Stage III/IV) (Table. 1).

Our preliminary data showed CEACAM1 up-regulation on CD3+CD4+ and CD3+CD8+ TILs from the patients who are resistant to targeted PD1 therapy (Fig. 1& 2).When we isolated peripheral blood mononuclear cells (PBMC) from the pre- and post-immune treated melanoma patients (metastatic melanoma: Stage III/IV) (Table. 2), we found CEACAM1 up-regulation on CD3+CD8+ circulating T cells from the patients who are resistant to targeted PD1 therapy (Fig. 3). Culture supernatants from *in vitro*-incubation of CP08 with tumor dissociated cells from targeted PD1 therapy failure melanoma patients were shown to enhance the IFN-g and TNF-a production from ELISA (Fig. x).

Treatment with a combination of mAbs targeting CEACAM1 and PD1 resulted in tumor control (Fig.) in xx % of tumor regression and enhanced CD4+ and CD8+ T cells infiltrations (Fig. ) and proliferations (Fig. ). We have previous demonstrated that targeting CEACAM1 together with PD1 generates clinical additive benefits and is likely to function in different but agonistic pathways (REF). In that setting, CEACAM1 expression can also be identified in the most exhausted T cells which lack the capacity in producing IFN-g (REF).

We next performed transcriptome RNA sequencing (RNA-seq) in these tissue samples and assessed correlations with effector immune responses with CP08 treatment (Supplementary Figure).

**Reinvigorate effector T cells from immunotherapy treatment failure melanoma patients**

**The generation of protective anti-tumor memory**

**Discussion**

**Methods**

**CyTOF Sample Preparation**

Antibodies were either purchased pre-conjugated from Fluidigm or purchased purified and conjugated in-house using MaxPar X8 Polymer Kits (Fluidigm) according to the manufacturer’s instructions.

For the first cohort of 15 patients (10 with immunotherapy resistant), cells from each tissue (blood, and tumor) were was stained for viability with 5mM cisplatin in PBS (Fluidigm) for 10 minutes at RT. Cells were then washed and split into three equal aliquots. One aliquot was stained with a Immune-cell-focused panel of antibodies (Panel 1, see below, Table) and the other aliquot was stained with a T-cell cytokines focused panel of antibodies (Panel 2) for 30 min RT. For the later, samples were washed, fixed, and permeabilized (eBiosciences) at RT for 30 min before being stained with intracellular antibodies (see below). They were washed and incubated in 0.125nM Ir intercalator (Fluidigm) diluted in PBS containing 2% formaldehyde and stored at 4 oC until acquisition. The third aliquot of the cells were subjected to function analyses (see later section).

For the second cohort of 10 patients (8 with immunotherapy resistant), cells from each tissue (blood, and tumor) were was stained for viability with 5mM cisplatin in PBS (Fluidigm) for 10 minutes at RT. Cells were then washed and split into two equal aliquots. One aliquot was stained with a T-cell co-inhibitory and co-stimulatory molecules focused panel of antibodies (Panel 3, see below, Table) and the other aliquot was stained with a myeloid focused panel of antibodies (Panel 4) for 30 min RT. Samples were washed, fixed, and permeabilized (eBiosciences) at RT for 30 min before being stained with intracellular antibodies (see below). They were washed and incubated in 0.125nM Ir intercalator (Fluidigm) diluted in PBS containing 2% formaldehyde, and stored at 4 oC until acquisition

***In vivo* human lymphocytes expansion using the Humanized NSG mice**

Freshly isolated human PBMCs (5x10^6) were transfer via intraperitoneal (i.p.) injection into NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG) mice. 21-days post PBMCs injections, NSG animals were examined for human immune cells implantations by the tail-bleeding. 24- , and 31-days post PBMCs injections, the humanized NSG mice were administered the first and the second doses of the indicated concentration of CEACAM1 antibody or isotype control antibody via i.p. injection.

Upon study termination (34-days post PBMCs injection), mice were sacrifice and surgical dissected the spleens for further analyses. Single cell suspension from the engraft mice were stained with cell proliferation dye and cultured in-vitro for additional 2-days in the presence of soluble form of anti-human CD3 stimulation (2ug/ml, OKT3 clone) and rIL-2 (40U/ml) in completed RPMI medium. Cells were maintained at 10^7 cell/ml concentration. After in-vitro stimulation, cells were stained with antibodies to human CD45 pan leukocyte marker and assessed by flow cytometer.

The human melanoma cell lines MALME-3M were obtained at the ATCC. MALME-3M were established in year of 1975 from a metastatic site (lung) in a 43-year-old Caucasian male with metastatic melanoma with BRAFV600E. 2x10^7 MALME-3M were subcutaneously (s.c.) injection into NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG) mice. After a 30 min settle down of the mice, freshly isolated human PBMCs (1x10^8) were then transfer via intraperitoneal (i.p.) injections onto the tumor-bearing NSG mice. 7- to 9-days post PBMCs injections, NSG animals were examined for human immune cells implantations by the tail-bleeding. 10- , 13- , 17- , 20- , and 24-days post human cells injections, the tumor-bearing humanized NSG mice were administered the in total five doses of the indicated concentration of CEACAM1 antibody or isotype control antibody via i.p. injection. Upon study termination (34-days post human cells injection), mice were sacrifice and surgical dissected local grew as well as metastatic tumor together with the spleens and lung and liver were saved for further analyses.

Tumour cells were stained with the following antibodies according to standard procedures: fluorochrome-conjugated monoclonal antibody specific for human CD44, CD133 and CEACAM1. Data were acquired with a Cytoflex flow cytometer (Invitrogen) and analyzed with FlowJo software (TreeStar, V7.6.5 for Windows). Sorting of local and metastatic cancer cells was performed with a FACS DiVa cell sorter (BD Biosciences).

[389] Tumour biopsies were subjected to either an enzymatic digest or to a commercial mechanical/enzymatic dissociation system (GentleMACS dissociator, Miltenyi Biotec). The enzymatic digest was based upon methodology previously established for the generation of melanoma TILs (Dudley et al, 2003, 2008). In brief, tumour biopsies were cut into small fragments ∼2–3 mm in length and put in an enzyme digest mix consisting of 100U ml−1 DNAse, 10 mg ml−1 collagenase VIII (Sigma-Aldrich) and incubated 45 min at 37oC temperature under continuous rotation. GentleMACS dissociation was performed according to the manufacturer’s protocol. Briefly, the tumour was cut into small fragments about 2–3 mm in length and put in a C-tube (Miltenyi Biotech) with RPMI 1640 (Lonza, Slough, UK) and solutions 1, 2 and 3 (all from Miltenyi Biotec) according to the manufacturer’s recommendation; the digest mix containing the tumour was then subjected to three 36-second mechanical disaggregation steps (programs h\_tumor\_01.01, 02.01 and 03.01) in the GentleMACS dissociator interspersed by two 30-min incubations at 37 °C performed after the first and the second disaggregation steps, respectively. After disaggregation, TILs from the enzymatic digest and the GentleMACS dissociation were passed through 100-μm strainers for the further analyses. Dissociated tumor cells and the autologous PBMCs were stained with the following antibodies according to standard procedures: fluorochrome-conjugated monoclonal antibody specific for human CD3, CD4, CD8, TIM-3, PD1, CEACAM1, CD45 and viable dye. Data were acquired with a Cytoflex flow cytometer (Invitrogen) and analyzed with FlowJo software (TreeStar, V7.6.5 for Windows).

In vitro assay for T cell function in tumor milieu: Dissociated tumor cells and the autologous PBMCs were cultured in complete media (RPMI 1640 (Lonza) supplemented with 10% fetal calf serum (FCS), 1% glutamine, 100 IU ml−1 penicillin, 100 μg ml−1 streptomycin (Life Technologies), 25 mM HEPES (Sigma-Aldrich) in 96-well plates at a concentration of 0.5 × 10^5 ml−1 with 100 IU ml−1 recombinant IL-2 (NIH) and soluble CD3 (1g/ml) in the presence of various concentrations of antibodies or the relevant isotype controls. After 96 hors with such T cells activation condition cultivated, 180 ul of 0.5 × 10^5 total cell-cultured supernatants were collected for further TNF- and IFN- ELISA (BD) analyses following the manufacture procedures.

The cervical adenocarcinoma cell line HeLa (ATCC No CCL-2) as well as transfected cell lines HeLaCEACAM1, HeLaCEACAM3, HeLaCEACAM5, HeLaCEACAM6 and HeLaCEACAM8 used for flow cytometry experiments were were cultured at 37 °C, 5.0% CO2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml) and dihydrostreptomycin (100 μg/ml). Cell lines were stained with the indicative antibodies followed by fluorochrome-conjugated monoclonal antibody specific for the indicative antibodies isotypes such as human IgG4 and muse IgG1 together with viable dye (DAPI). Data were acquired with a Cytoflex flow cytometer (Invitrogen) and analyzed with FlowJo software (TreeStar, V7.6.5 for Windows).

Merkel cell carcinoma biopsies were subjected to a commercial mechanical/enzymatic dissociation system (GentleMACS dissociator, Miltenyi Biotec), according to the manufacturer’s protocol. Briefly, the tumour was cut into small fragments about 2–3 mm in length and put in a C-tube (Miltenyi Biotech) with RPMI 1640 (Lonza, Slough, UK) and solutions 1, 2 and 3 (all from Miltenyi Biotec) according to the manufacturer’s recommendation; the digest mix containing the tumour was then subjected to three 36-second mechanical disaggregation steps (programs h\_tumor\_01.01, 02.01 and 03.01) in the GentleMACS dissociator interspersed by two 30-min incubations at 37 °C performed after the first and the second disaggregation steps, respectively. After disaggregation, TILs from the enzymatic digest and the GentleMACS dissociation were passed through 100-μm strainers for the further analyses. In vitro assay for T cell function in tumor milieu: Dissociated tumor cells and the autologous PBMCs were cultured in complete media (RPMI 1640 (Lonza) supplemented with 10% fetal calf serum (FCS), 1% glutamine, 100 IU ml−1 penicillin, 100 μg ml−1 streptomycin (Life Technologies), 25 mM HEPES (Sigma-Aldrich) in 96-well plates at a concentration of 0.5 × 10^5 ml−1 with 100 IU ml−1 recombinant IL-2 (NIH) and soluble CD3 (1g/ml) in the presence of various concentrations of antibodies or the relevant isotype controls. After 96 hors with such T cells activation condition cultivated, 180 ul of 0.5 × 10^5 total cell-cultured supernatants were collected for further TNF- and IFN- ELISA (BD) analyses following the manufacture procedures.

The human melanoma cell lines MALME-3M were obtained at the ATCC. MALME-3M were established in year of 1975 from a metastatic site (lung) in a 43-year-old Caucasian male with metastatic melanoma with BRAFV600E. 2x10^7 MALME-3M were subcutaneously (s.c.) injection into NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG) mice. After a 30 min settle down of the mice, freshly isolated human PBMCs (1x10^8) were then transfer via intraperitoneal (i.p.) injections onto the tumor-bearing NSG mice. 7- to 9-days post PBMCs injections, NSG animals were examined for human immune cells implantations by the tail-bleeding. 10- , 13- , 17- , 20- , and 24-days post human cells injections, the tumor-bearing humanized NSG mice were administered the in total five doses of CP08 antibody (2mg/kg), CM-24 (2mg/kg) or isotype control antibody (2mg/kg) via i.p. injection. Upon study termination (34-days post human cells injection), mice were sacrifice and surgical dissected local grew as well as metastatic tumor together with the spleens and lung and liver were saved for further analyses. Specifically in this setting of the experiment, total cells counts as well as the frequency of the CD4, CD8 and tumor cells characterized by high in FSC and high in SSC with negative for human pan-leukocytes, CD45 marker expression were documented.

**Humanized NSG tumor implantation model**

**Cell lines**

The human melanoma cell lines MALME-3M were kindly provided by NB (Institute). The cervical adenocarcinoma cell line HeLa (ATCC No CCL-2) as well as transfected cell lines HeLaCEACAM1, HeLaCEACAM3, HeLaCEACAM5, HeLaCEACAM6 and HeLaCEACAM8 used for flow cytometry experiments were a gift from Dr. Scott Gray-Owen (University of Toronto, Toronto, Canada). All cell lines were cultured at 37 °C, 5.0% CO2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml) and dihydrostreptomycin (100 μg/ml).

MALME-3M were established in year of 1975 from a metastatic site (lung) in a 43-year-old Caucasian male with metastatic melanoma with BRAFV600E

**ELISA for hemophilic and heterophilic interaction (REFs)**

**Patients and samples**

All tissue and blood samples were collected with informed patient consent from BWH. Blood was collected directly into a 50-ml polypropylene tube containing 100 IU of heparin or into vacutainers (BD Biosciences). Tumour samples were collected into serum-free DMEM media and all samples transported directly to the laboratory for processing.

**Tissue banking**

The tissue bank protocol used for this study was developed and approved BWH. The protocol was developed to avoid any compromise in patient care, pathologic diagnosis, tumor staging or treatment. Patient confidentiality was maintained by password and firewall protected access to all pertinent databases. Melanoma specimens were obtained with informed consent from all patients according to protocols approved by BWH. All patients included in this study had clinically apparent melanoma disease (biopsy-proven stage II, III, or IV, or obvious clinical stage IV) from which a small (typically 2–5 mm) tissue sample not required for standard-of-care pathology assessment was obtained. Most of the melanomas in this study were regional stage III lymph node or skin/soft tissue disease with palpable, clinically enlarged node(s) or soft tissues, undergoing definitive surgical resection, with biopsy-proven (most often needle core) diagnosis confirmed before surgery.

**Isolation of Tumor infiltrating lymphocytes**

Tumour biopsies were subjected to either an enzymatic digest or to a commercial mechanical/enzymatic dissociation system (GentleMACS dissociator, Miltenyi Biotec). The enzymatic digest was based upon methodology previously established for the generation of melanoma TILs (Dudley et al, 2003, 2008). In brief, tumour biopsies were cut into small fragments ∼2–3 mm in length and put in an enzyme digest mix consisting of 100U ml−1 DNAse, 10 mg ml−1 collagenase VIII (Sigma-Aldrich) and incubated 45 min at 37oC temperature under continuous rotation. GentleMACS dissociation was performed according to the manufacturer’s protocol. Briefly, the tumour was cut into small fragments about 2–3 mm in length and put in a C-tube (Miltenyi Biotech) with RPMI 1640 (Lonza, Slough, UK) and solutions 1, 2 and 3 (all from Miltenyi Biotec) according to the manufacturer’s recommendation; the digest mix containing the tumour was then subjected to three 36-second mechanical disaggregation steps (programs h\_tumor\_01.01, 02.01 and 03.01) in the GentleMACS dissociator interspersed by two 30-min incubations at 37 °C performed after the first and the second disaggregation steps, respectively. After disaggregation, TILs from the enzymatic digest and the GentleMACS dissociation were passed through 100-μm strainers for the further analyses.

***In vitro* assay for T cell function in tumor milieu**

Dissociated tumor cells were cultured in complete media (RPMI 1640 (Lonza) supplemented with 10% fetal calf serum (FCS), 1% glutamine, 100 IU ml−1 penicillin, 100 μg ml−1 streptomycin (Life Technologies), 25 mM HEPES (Sigma-Aldrich) in 96-well plates at a concentration of 0.5 × 105 ml−1 with 100 IU ml−1 recombinant IL-2 (NIH) and soluble CD3 (1g/ml) in the presence of various concentrations of antibodies or the relevant isotype controls.

**Flow cytometry-based analysis of immune and tumor cells in melanoma**

Antibody used in the study: Anti-hCEACAM1, 26H7; anti-PD1, EH12 also known as EH12.1; anti-hTIM-3 : 2E2;

***In vitro* assay for T cell function in tumor milieu**

Dissociated tumor cells were cultured in complete media (RPMI 1640 (Lonza) supplemented with 10% fetal calf serum (FCS), 1% glutamine, 100 IU ml−1 penicillin, 100 μg ml−1 streptomycin (Life Technologies), 25 mM HEPES (Sigma-Aldrich) in 96-well plates at a concentration of 0.5 × 105 ml−1 with 100 IU ml−1 recombinant IL-2 (NIH) and soluble CD3 (1g/ml) in the presence of various concentrations of antibodies or the relevant isotype controls.

**Expression of recombinant CEACAM1 antibody**

**Humanization of CEACAM1 antibody**

**Affinity maturation of humanized CEACAM1 antibody**

**Selection of humanized, affinity matured CEACAM1 antibody**

**Statistical**

Data sets derived from individual groups of mice were compared using Student-t-test and grouped data sets were analysed by ANOVA. Statistical analyses and graphs were assembled using GraphPad PRISM (version 5.01, GraphPad software for Science, San Diego, CA). P values ≤ 0.05 were considered significant unless otherwise indicated.

**CEACAM1 Expression in Human Tumor**

In order to monitor the expression of CEACAM1 at the surface of several human tumors, to identify the indications of which anti-CEACAM1 therapeutic blocking mAbs might promote anti-tumor immunity in cancer patients. In consistent with the literature, CEACAM1 was found to be widely expressed on the surface of several human tumors. >We observed CEACAM1 expression in melanoma, lung, pancreas, stomach, colon, head and neck, and liver tumor tissues (Figure x; Table S1). Other studies have showed that PD-L1 expression was restricted to a fraction of lung, stomach, and colon tumors (REFs). CEACAM1 was strongly expressed by *type of the cancer* (Figure x), in which we also detected TIM-3-positive cells (ligands?). CEACAM1-positive cells and TIM-3 expression were also found in *type of the cancer* (Figures x). CEACAM1+CD4+ and CEACAM1+CD8+ TILs were also present in *type of the cancer* (Figures x).

Given the important outcome in respond to immune therapy of the spatial tissue evaluation of the tumor infiltrating lymphocytes comprising the tumor microenvironment (REFs); we analyzed the percentage of CD4+ and CD8+ for CEACAM1, PD1 and TIM-3+ among floating cells/ stromal TILs (sTILs) and percentage of CD4+ and CD8+ for CEACAM1, PD1 and TIM-3+ among digested/ intra-epithelial lymphocytes (iTILs). Digested/ intra-epithelial lymphocytes (iTILs) reflect the state of immune cells naïve to the intact metastatic lesion (i.e. intact tumor microenvironment), whereas, floating cells/ stromal TILs (sTILs) represents T cells which have been activated via the cell-cell interactions of the tumor environment by the stromal cells; Hence, tumor-stromal embedded T cells floating cells/ stromal TILs (sTILs) will show differences in activation potentials than iTIL.

> (invasive margin and tumor center& intra-epithelial and stromal; iTILs located within carcinoma nests whereas sTILs were those not in direct contact with the carcinoma nest.).

We investigated melanoma more closely by flow cytometry and detected significantly higher frequencies of CD8+ sTILs expressing CEACAM1 and co-expressing both PD-1 and CEACAM1, TIM-3 CEACAM1, TIM-3 PD1? phenotypes in the tumor of secondary resistant to immune checkpoint inhibitor (ICB) patients compared to treatment naïve patients (Figure x). CEACAM1-expressing CD4+ sTILs were also present at higher frequency in the tumor of secondary resistant to immune checkpoint inhibitor (ICB) patients compared to treatment naïve patients (Figure x), and some of these CD4+ sTIL had and co-expressing both PD-1 and CEACAM1, TIM-3 CEACAM1, TIM-3 PD1 ? phenotype (Figure x). Similar frequencies of CD4+ or CD8+ iTILs expressing CEACAM1 in the tumor of secondary resistant to immune checkpoint inhibitor (ICB) patients compared to treatment naïve patients (Figure x). One possible explanations if that tumor-stromal embedded T cells floating cells/ stromal TILs (sTILs) exhibits differences in activation potentials from iTIL. Thus, several melanoma tumors expressed CEACAM1 and were infiltrated with CD4+ and CD8+ TILs expressing CEACAM1. Therefore, we reasoned that CEACAM1 blockade might improve the anti-tumor efficacy of CD4+ and CD8+ TILs in cancer patients. Given the major histocompatibility complex class I antigen presentation pathways, beta-2-microglobulin (2m) loss is likely a common mechanism of resistance to therapies targeting CTLA4 or PD1 (REFs), we also examined whether in the secondary resistant patients pool displayed this defect. Indeed, we observed that 2m related molecules (HLA-A, HLA-B, HLA-C) are down-regulated in secondary resistant patients, interestingly, this down-regulation population exhibits up-regulation of CEACAM1 on the cell surface (Figure). (Should we check the cytoplasm compartment?)

Central/effector memory results

*These results validate CEACAM1 as a potentially useful target.*

**Generation and Characterization of a Chimeric Blocking mAb Directed against Human CEACAM1**

A murine anti-human CEACAM1 IgG1 mAb clone, 5F4, was generated in a previous study with careful evaluation (Morales et al., 1999). We confirmed that the blockade of CEACAM1 *in vitro* (promoted ? inhibits?) in a CD3 redirected assay etc. (REF). Others and us have confirmed 5F4 blocks NETosis (REF), prevents neutrophils activation (data not shown) and mastocytosis (Figure Sx). We humanized this antibody by fusion with an IgG4 with a single point mutation in the Fc heavy chain to prevent the formation of half-antibodies and screened the selected humanized clones for binding to CEACAM1 with an affinity similar to that of the original murine mAb.

Humanization process & affinity maturation process.

The selected humanized clone was named CP08. Importantly, unlike other anti-CEACAM1 mAbs described to date, CP08 is specific for human CEACAM1, as it bound human CEACAM1+ cells, but not HELA-transfected cells expressing human CEACAM3, the activating isoform, or CEACAM5, or CEACAM6 or CEACAM8 by flow cytometry (Figure x), ELISA (Figure) and SPR (Figure). The IC50 calculated by ELISA was xx ng/mL for tag-less CEACAM1 IgV domain protein derived from E-coli and xx ng/mL for glycosylated CEACAM1 with forming monomer as potential (3sol REF) and xx ng/mL for (sino material) and xx ng/mL for CEACAM1 HELA transfectant (Figure S), and xx ng/mL for CEACAM1 (Figure S) by SPR. Another critical feature of CP08 resides in its capacity to inhibit the binding of CEACAM1 homodimer and CEACAM1:TIM-3 hetrodimer (REF, Figure x). We also found that the epitopes remains during the humanization process (Figure x), deglycosylation process (Figure x) and affinity maturation process (Figure xx).

When used as single agents *in vitro*, 5F4 modestly improved *ex vivo* tumor-infiltrating CD8+ T cell effector activities after re-stimulation with soluble CD3 (Figure S). By contrast, the use of anti-CEACAM1 and anti-PD-1 mAbs in combination increased the IFNg production (Figure Sx).

**Anti-human CEACAM1 mAb promotes CEACAM1 monomer on the cell surface**

Functional studies showed that anti-human CEACAM1 mAb substantially resulted in CEACAM1 monomer on surface of the tumor cell lines (Figure x).

**Generation and characterization of crystal structure of CP08**

**CP08 induces the expansion of the human CD45+ cells *in vivo***

We next sought the effect of CP08 on immune cells *in vivo*. We utilized humanized PBMC-NSG model to see this effect. Anti-CEACAM1 treatment, CP08 led to an expansion of human CD45+CD3+CD4+ and CD45+CD3+CD8+ T cells (Figure x). Surprisingly, a phenotypically express TIM3high population expanded the most among CD8 populations (Figure x). To address whether T cell expansion results from increased proliferation**,** we assessed short-term incorporation ofthe proliferation fluorescent dye binds to any cellular protein containing primary amines, and as cells divide, the dye is distributed equally between daughter cells. Anti-hCEACAM1 mAb-responsive CD8 incorporated the dye, suggesting that these cells are proliferating within thetumor microenvironment (TME) (Figure Sx). Given the timing ofdye treatment and retention of dye in daughter cells, this approachmay detect extratumoral-blasting T cells that subsequentlyinfiltrate the tumor, in addition to cells proliferating within theTME. Nonetheless, these observations indicate that anti-hCEACAM1-sensitiveT cells retain proliferative capacity even after multiple rounds ofCP08 promotes the anti-tumor cell activities of human CD4 and CD8 T cells. The use of superantigen can be accompanied or followed by a state of immunosuppression, which in turn jeopardizes the host's ability to combat and clear infections (REFs). We use another model to investigate the effect of CP08 in vivo.

We further investigated the effects of immunotherapy with anti-CEACAM1 by treating MALME-3M tumor-bearing NSG mice with anti-CEACAM1 mAb, or hIgG4 (Figure x). In this experimental setting, anti-CEACAM1 mAb rescued ∼40% of tumor-bearing mice from death, as shown by comparison with untreated mice (Figure x). Interestingly, a combination of anti-CEACAM1 and anti-PD-1 mAbs had a synergistic effect, improving the control of tumor growth and rescuing ∼xx% of the mice from death (Figure x).

Thus, the combination of a blocking anti-CEACAM1 mAb with a blocking anti-PD-1 mAb had a therapeutic anti-tumor effect, because it unleashed CD4+ and CD8+ T cells in the humanized melanoma model.

> We then dissected the immune response to MALME-3M in the tumor microenvirment by analyzing tumor-infiltrating lymphocytes (TILs). MALME-3M tumors were found to be infiltrated by CD4+ and CD8+ T cells. ∼60% of tumor-infiltrating CD4+ T cells expressed the CEACAM1 (Figure x). We also monitored PD-1 expression, because the immune control of MALME-3M tumors has been reported to be partially dependent on TIM-3 or PD-1 (REFs). The expression of TIM-3, either alone or together with CEACAM1, was barely detectable on the surface of tumor-infiltrating CD4+ or CD8+ T cells. The expression of PD1, either alone or together with CEACAM1, was detectable on the surface of tumor-infiltrating CD4+ or CD8+ T cells.

>> We then dissected the human acute immune response in order to engraft onto the murine host. at week of 3, the ratio of CD4+ and CD8+ T cells; however at > 5 weeks, the ratio of CD4+ and CD8+ T cells dropped dramatically (Figure x) so that we chose the protocol adapt with early time point (Figure x); ∼60% of CD4+ T cells expressed the CEACAM1 (Figure x). We also monitored PD-1 and TIM-3 expression, because both the molecules have been reported to be partially relayed to CEACAM1 (REFs). The expression of TIM-3, either alone or together with CEACAM1, was barely detectable on the surface of engrafting CD4+ or CD8+ T cells. The expression of PD1, either alone or together with CEACAM1, was detectable on the surface of engrafting CD4+ or CD8+ T cells.

**Methods**

**Crystallization of CEACAM1:CP08\_H03 Fab complex**

CEACAM1 was expressed from *E. coli* transformed based on our published protocols (tagless construct in pET21D), and was refolded in an arginine-containing buffer and purified. The CP08\_H03 Fab was prepared by digestion of the antibody, after concentrating to ~18mg/ml, using immobilized papain resin and then purified by protein A affinity and gel filtration chromatography. Purified CEACAM1 and Fab were mixed at a 1:1 molar ratio prior to crystallization screening. Initial crystallization hits of the CEACAM1:CP08\_H03 Fab complex were identified and subsequently optimized. Diffraction quality crystals (Fig. 1) were grown at room temperature in a condition containing 18 - 20% PEG 6000, 50mM potassium dihydrogen phosphate, 20mM Tris pH 7.0, and 1% β-octylglucoside. SDS-PAGE analysis and silver staining of a washed crystal was used to verify crystallization of the complex.

X-ray data from numerous crystals were collected from beamline NE-CAT 24-ID-E at the Advanced Photon Source of Argonne National Laboratory, and processed at NYSBC. In general, the crystals diffracted weakly and subsequent analysis of X-ray data revealed that most crystals were twinned, potentially compromising final model

quality; however, a small subset of data were not twinned and the best data from two non-twinned isomorphous crystals were merged to produce a highly redundant dataset at 3.3 A for structure determination and refinement. The structure of the complex was solved by molecular replacement and refined to final *R* and *Rfree* values of 24.9% and

32.8%, respectively. Data collection and refinement statistics are listed in Table 1.

**Crystallization of hCEACAM1 A49V/Q89H allelic variant**

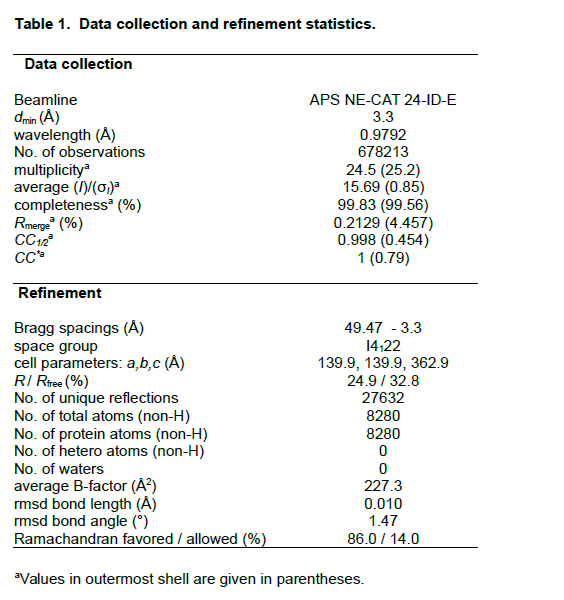
Cell pellets containing expressed hCEACAM1 IgV domain double mutant

were expressed, purified based on our previously published protocols for hCEACAM1 wild type Ig-V domain protein. Purified protein was concentrated to 10 mg/ml prior to crystallization screening. After the initial crystallization hits and subsequently optimization, diffraction quality crystals were grown at 4°C in a condition containing 18 - 20% PEG 6000, 50mM potassium dihydrogen phosphate, 20mM Tris pH 7.0, and 1% β-octylglucoside. SDS-PAGE analysis and silver staining of a washed crystal was used to verify crystallization of the complex.

**SPR and ELISA**.

Single cycle SPR kinetics was conducted using protein concentrations from 280 nM to 70 nM. Antibodies were loaded onto the chip at the following concentrations (taking into account the varying analyte MWs):100 RU for CEACAM1, 375 RU for CEACAM3, 71.4 RU for CEACAM5, and 150 RU for CEACAM6.

For the ELISA experiments, a 96 well plate was coated with CEACAM1 at either 0.5 or 1.0 μg/ml. Non-specific binding was blocked with 2% BSA/Dulbecco’s PBS. A 1:3 dilution series of CP08H03/Vκ8 S29A, CP08H03/CP08F05, or VH0/Vк0 (50 µg/mL starting concentration) was prepared in 2% BSA/PBS. 100 µL of the sample was added to the pre-coated plate and incubated for 1 h at RT. Anti-human Igκ chain – Peroxidase secondary antibody (AP502P) used to detect the CEACAM antibodies. Plates were developed with TMB and stopped with 3M HCl. Results were analyzed by subtracting the background.

****

**Table 2: Crystal information, data collection and refinement parameters of hCEACAM1 A49V/Q89H allelic variant**

|  |  |
| --- | --- |
|  |  |
| **Data collection** |  |
| Beamline | APS-LSCAT |
| dmin (Å) | 1.7 |
| wavelength (Å) | 0.97872 |
| No. of observations | 220881 |
| Multiplicity a | 4.6 (4.5) |
| average (I)/(σI)a | 14.2 (7.3) |
| Completeness (%)a | 100 (100) |
| *Rmerge* (%)a | 7.5 (27.7) |
| *CC*1/2 a | 0.996 (0.886) |
| **Structure refinement** |  |
| Bragg spacings (Å) | 82.14-1.70 |
| Space group | P 1 21 1 |
| cell parameters: *a,b,c* | 63.88 41.96 85.93 90.00 107.08 90.00 |
| *R* / *R*free (%) | 17.8/20.8 |
| No. of unique reflections | 45803 |
| No. of total atoms (non-H) | 3698 |
| No. of protein atoms (non-H) |  |
| No. of hetero atoms (non-H) | 0 |
| No. of waters |  |
| average B-factor (Å2) | 12.796 |
| rmsd bond length (Å) | 0.020 |
| rmsd bond angle (°) | 2.062 |
| Ramachandran favored / allowed (%) |  |

aValues in outermost shell are given in parentheses.

**Figure Legends**

**Figure 1** shows the results of a CEACAM1 mutagenesis study aimed at identifying residues in CEACAM1 that are involved in binding to the indicated CEACAM1 antibodies. CEACAM1-FLAG was expressed in transfected human embryonic kidney (HEK) cells with CEACAM1 containing the indicated mutations, the proteins resolved by SDS-PAGE and then immunoblotted. The wild-type (WT) or mutant CEACAM1 proteins were detected using the indicated chimeric (VH0/Vκ0) and humanized CEACAM antibodies. Decreased detection indicates that the mutated residue is involved in binding to the respective antibody used for detection.

**Figure 2:** The sequence identity between human CEACAM family members.

**Figures 3A, 3B, and 3C** illustrate thatCEACAM antibodies CP08H03/Vκ8 S29A (labeld “CP08\_H03/Parent VL”), CP08H03/CP08\_F05, and VH0/Vк0 are selective for CEACAM1. CP08H03/Vκ8 S29A and CP08H03/CP08F05 contain a S29A mutation in CDR1L (Kabat numbering scheme, corresponding to a S28A mutation in the primary amino acid sequence of the variable light chain). **Figure 3A** shows single-cycle kinetics sensorgrams and fitted curves for the purified lead humanized and affinity-matured IgG4 variants. Increasing concentrations on different CEACAM family members were injected and a single off-rate was determined by single cycle kinetics (surface plasmon resonance, SPR). **Figure 3B** shows three-point binding ELISA data for the binding of the chimeric antibody VH0/Vκ0(labeled “chimeric’), purified lead humanized and affinity-matured IgG4 variants to CEACAM1 and CEACAM3 family members. A three-point (high, medium and low, with concentrations based on the binding of the chimeric antibody VH0/Vκ0to CEACAM1) titration was performed and binding was detected using an anti-human kappa chain antibody and TMB substrate. **Figure 3C** shows three-point binding ELISA data for the binding of the chimeric antibody VH0/Vκ0 (labeled “chimeric’) and purified lead humanized and affinity-matured IgG4 variants to CEACAM1, 5 and 6 family members. A three-point (high, medium and low, with concentrations based on the binding of the chimeric antibody VH0/Vκ0to CEACAM-1) titration was performed and binding was detected using an anti-human kappa chain antibody and TMB substrate.

**Figure 4:** Crystallization of the complex. A) A crystal of the CEACAM1:CP08\_H03 Fab complex. B) SDS-PAGE/silver stain analysis of a crystal in non-reducing (NR) and reducing (R) conditions.

**Figure 5:** Crystal structure of CEACAM1:CP08\_H03 Fab complex. A) The overall binding of CEACAM1:CP08\_H03 Fab complex as observed in the crystal structure. The hCEACAM1 binds in a 1:1 stoichiometric ratio to the CP08\_H03. The hCEACAM1 (pink) and CP08\_H03 Fab (blue) are shown by a ribbon diagram. B) The binding interface formed by heavy and light chain of CP08\_H03 antibody with hCEACAM1. The selected residues that are involved in binding are shown by stick representation. The heavy chain and light residues of CP08\_H03 Fab are labeled in blue, and the CEACAM1 residues are labeled in pink.

**Figure 6:** Human CEACAM1 *C-C*’ and *F-G* loops residues are involved in the binding with CP08\_H03 antibody. The CP08\_H03 antibody fab is shown by the ribbon diagram, where heavy chain is shown in teal, and light chain is shown in green. Human CEACAM1 is shown in by surface view and selected CEACAM1 *C-C*’ and *F-G* loops residues that are involved in binding are highlighted in yellow and labeled.The central interaction involving residues hCEACAM1-Q89 and Y103 of CDR3 of heavy chain is highlighted by stick representation of Y103 of CDR3 residue.

**Figure 7:** **Structure of the CEACAM1:CP08\_H03 Fab complex. A)** All protein chains of each complex in the asymmetric unit of the crystal lattice are drawn as Cα traces and labeled. **B)** Above, in the structure of the complex (A, B, and C chains) the Fab is shown as a Cα trace and the antigen is shown as a ribbon. Below, the dimeric structure of CEACAM1 is shown as a ribbon. **C)** A view of the antigen towards the dimer interface. The molecular surface drawn is drawn in semi-transparent green, the protein backbone is drawn as a green ribbon, and relevant side chains are drawn as sticks. Regions of the surface interacting with the Fab light and heavy chains are colored teal and magenta, respectively. **D)** A stereo image of a close up view of the Fab-CEACAM1 interaction.

CEACAM1 is drawn as a green ribbon and Fab chains (light chain in light gray and heavy chain in dark gray) are drawn as Cα traces. Residues of interacting Fab CDR regions are colored and labeled, following previous color convention. Relevant side chains are drawn as sticks with oxygens and nitrogens colored red and blue, respectively. Carbons are colored by respective chain and hydrogen bonds are drawn as gray dashes.

**Figure 8:** The alignment and residues homology between human CEACAM family members.

**Figure 9** shows a comparison of CEACAM1 F29 and V49 or A49 residues in CEACAM1 WT: CP08H03/Vκ8 S29A antibody crystal structure (left) or CEACAM1 A49V/Q89H mutant crystal structure (right).

**Figure 10** shows hCEACAM1 A49/Q89H natural allelic variant binding study by ELISA. The hCEACAM1 A49/Q89H natural allelic variant protein does not bind to CP08\_H03 antibody and IgG4 control antibody (0-400 nM), but shows concentration dependent binding to anti-hCEACAM1 CM24 antibody (0-400 nM).

**Potential anti-tumor activity controlled by CP08 in patients with metastatic melanoma**

**Figure** illustrates the binding selectivity of different affinity-matured CEACAM1 antibodies. Affinity matured antibodies CP08H03/Vк8 S29A (labeled “CP08\_H03/Parent VL”), CP08H03/CP08F05, 8H3\_9B3/CP08F05, and CP08H03/CP08E05 contain a phenylalanine (F) at CDR3H residue 104. Affinity matured antibodies CP09B03/CP08E05, CP09C02/CP08E05, CP09C02/CP08F05, and 9B3\_9E5/CP08E05 contain an aspartic acid residue (D) at CDR3H residue 104. HELA cells were transfected with vector alone (Hela-Neo) or vectors expressing CEACAM1, CEACAM3, CEACAM5, or CEACAM6, respectively, and stained with the indicated antibodies. The y-axis shows % staining of each antibody with the transfected panel of cells. hIgG4 = control antibody with identical stabilizing hinge mutation. MOPC = mouse IgG1 control antibody. Mouse antibodies as positive control for transfected CEACAM isoforms: Col-1 = CEACAM3 and 5 antibody. 9A6 = CEACAM6 antibody. T84.1 = CEACAM cross-reactive antibody and T84.66 = CEACAM5. Only 2nd FITC = No primary antibody, only secondary FITC conjugated antibody. Col-1 and 9A6 are commercial antibodies (Dako) and T84.1 and T84.66 have been previously described (Neumaier M, J Immunol 1985;135:3604-9). Data for affinity-matured antibodies 9B3\_8H3/ Vк8 S29A, 8H3\_9B3/CP08\_E05, and 8H3\_9C2/CPO08\_F05, as well as data for CEACAM8 antibody 80H3 were omitted from the figure due to an unusually high background signal.

***ELISA assays showed CP08 interferes homophilic and heterophilic of CEACAM1***

**Figures 12A, 12B, and 12C** illustrate thatCEACAM antibodies CP08H03/Vκ8 S29A (labeld “CP08\_H03/Parent VL”), CP08H03/CP08\_F05, and VH0/Vк0 are selective for CEACAM1. CP08H03/Vκ8 S29A and CP08H03/CP08F05 contain a S29A mutation in CDR1L (Kabat numbering scheme, corresponding to a S28A mutation in the primary amino acid sequence of the variable light chain). **Figure 12A** shows single-cycle kinetics sensorgrams and fitted curves for the purified lead humanized and affinity-matured IgG4 variants. Increasing concentrations on different CEACAM family members were injected and a single off-rate was determined by single cycle kinetics (SPR). **Figure 12B** shows three-point binding ELISA data for the binding of the chimeric antibody VH0/Vκ0and purified lead humanized and affinity-matured IgG4 variants to CEACAM-1 and -3 family members. A three-point (high, medium and low, with concentrations based on the binding of the chimeric antibody VH0/Vκ0to CEACAM-1) titration was performed and binding was detected using an anti-human kappa chain antibody and TMB substrate. **Figure 12C** shows three-point binding ELISA data for the binding of the chimeric antibody VH0/Vκ0and purified lead humanized and affinity-matured IgG4 variants to CEACAM-1, -5 and -6 family members. A three-point (high, medium and low, with concentrations based on the binding of the chimeric antibody VH0/Vκ0to CEACAM-1) titration was performed and binding was detected using an anti-human kappa chain antibody and TMB substrate.

**Figure X** shows the sequence homology among the N- domains of different CEACAM family members. CEACAM1 (C1, UniProtKB accession number P13688), CEACAM3 (C3, UniProtKB accession number P40198), CEACAM4 (C4, UniProtKB accession number O75871), CEACAM5 (C5, UniProtKB accession number P06731), CEACAM6 (C6, UniProtKB accession number P40199), CEACAM7 (C7, UniProtKB accession number Q14002), and CEACAM8 (C8, UniProtKB accession number P31997). The shown percent identity matrix was created using Clustal2.1. The specific residues analyzed for each CEACAM family member are indicated.

***Cross reactivity assays showed CP08 is only specific for CEACAM1***

**Figure 14** illustrates that CEACAM1 antibody CP08H03/Vκ8 S29A (labeld “CP08”) has substantially enhanced selectivity as compared to CEACAM1 antibodies T84.1, T84.66, and CM-24. HELA cells were transfected with vectors expressing CEACAM1, CEACAM3, CEACAM5, CEACAM6, or CEACAM8, respectively, and stained with the indicated antibodies. The y-axis shows % staining of each antibody with transfected panel of cells. Col-1 = Collagen 1 antibody. 9A6 = CEACAM6 antibody. 80H3 = CEACAM8 antibody.

**Figure 15** shows the results of a CEACAM1 mutagenesis study aimed at identifying residues in CEACAM1 that are involved in binding to the indicated CEACAM1 antibodies. CEACAM1-FLAG was expressed in transfected human embryonic kidney (HEK) cells with CEACAM1 containing the indicated mutations, the proteins resolved by SDS-PAGE and then immunoblotted. The wild-type (WT) or mutant CEACAM1 proteins were detected using the indicated chimeric (VH0/Vκ0) and humanized CEACAM antibodies. Decreased detection indicates that the mutated residue is involved in binding to the respective antibody used for detection.

***ELISA assays showed CP08 interferes homophilic and heterophilic of CEACAM1***

**Figure 20** shows a comparison of CEACAM1 F29 and V49 or A49 residues in CEACAM1 WT: CP08H03/Vκ8 S29A antibody crystal structure (left) or CEACAM1 A49V/Q89H mutant crystal structure (right).

**Figures 21A and 21B** illustrate that the CEACAM1 antibody CP08H03/Vκ8 S29A (labeled “CP08”) blocks human CEACAM1:CEACAM1 (**Figure 21A**) and CEACAM: human TIM-3 interactions (**Figure 21B**). CP08 = CEACAM1 antibody CP08H03/Vκ8 S29A. IgG4 = control antibody.

***Humanized mice data showed the ability of CP08 that induced the expansion of lymphocytes***

**Figure 22** shows the experimental setup for testing the ability of CEACAM1 antibodies to induce CD45+ cell proliferation in humanized NOD scid gamma mice (NSG mice). Engraftment of human PBMC adoptively transferred via intraperitoneal injection to NSG host mice was analyzed by FACS for human CD45 and proliferation dye staining 38 days following injection. On day 24 following PBMC injection, mice were treated with a single injection of human IgG4 isotype control or the indicated concentration of CP08H03/Vκ8 S29A (labeled “CP08\_H03/Parent VL”) antibody. On day 31, mice were treated with the second injection. On day 38, mice were sacrificed for data acquisition.

**Figure 23** shows that CEACAM1 antibodies CP08H03/Vκ8 S29A(labeled “CP08\_H03/Parental”) and CP08H03/CP08F05 do not deplete the transplanted human cells in humanized NOD scid gamma mice (NSG mice). Mean percentage of human CD4 and CD8 T lymphocytes assessed at day 38. CP08H03/CP08F05 contains a S29A mutation in CDR1L (Kabat numbering scheme, corresponding to a S28A mutation in the primary amino acid sequence of the variable light chain).

***Humanized mice data showed the therapeutic benefits of CP08***

**Figure 24** illustrates that the administration of CEACAM1 antibody CP08H03/Vκ8 S29A (labeled “CP08\_H03/Parent VL”) or CP08H03/CP08F05, respectively, leads to an increase in antibody induced human CD45+ immune cell expansion in humanized NOD scid gamma mice. CP08H03/Vκ8 S29A induces expansion of human CD45 PBMC *in vivo*. On day 38, isotype hIgG4 control (10 mg/kg), CP08H03/Vκ8 S29A (2 and 10 mg/kg) and CP08H03/CP08F05 (2 and 10 mg/kg) treated mice were sacrificed and solenocytes were isolated and collected for proliferation analyses. Proliferation ex vivo were carried out under T-cell-stimulation condition wherein cells were cultured under the soluble anti-CD3 (OKT3) (at indicated concentrations 10, 5, 2.5 µg/ml) and rIL-2 (40 units/ml) for 120 hours as the dilution of the proliferation dyes represented dividing cell/proliferation (when cell proliferated, duplex DNA were analyzed as diluted signal). CP08H03/CP08F05 contains a S29A mutation in CDR1L (Kabat numbering scheme, corresponding to a S28A mutation in the primary amino acid sequence of the variable light chain).

**Figures 25A, 25B,** and **25C** illustrate that CEACAM1 antibody CP08H03/Vκ8 S29A (labeled “CP08\_H03/Parent VL”) reduces tumor growth in humanized mice. **Figure 25A** provides a schematic for the experimental protocol resulting in **Figure 25B**. **Figure 25B** shows average tumor size after 1x106 MALME-3M (human melanoma) cells were injected subcutaneously into NSG, along with 5 X 106 human PBMC. After 10 days, palpable tumors were documented and the mice were randomized mice to treatment on days 10, 13, 17, 20 and 24 with the respective antibodies intraperitoneally. **Figure 25C** shows statistical comparisons by linear regression of the hIgG4 control treated group to the three different CP08\_H03/Parent VL groups (2 mg/kg, 0.4 mg/kg and 0.08 mg/kg).

**Figure 26** illustrates that CEACAM1 antibody CP08H03/Vκ8 S29A does not deplete T cell populations in the experimental setup shown in **Figure 25A**.

**Figure 27** illustrates that T cells from humanized mice engrafted with human melanoma cell line MALME-3M treated with CEACAM1 antibody CP08H03/Vκ8 S29A as described in **Figures 24-26** exhibit decreased quantitites of tumor cells with decreased proliferation and increased quantities of intratumoral CD8 and CD4 positive T cells that exhibit increased proliferation when examined *ex vivo* after stimulation with anti-CD3. On the day of sacrifice, isotype hIgG4 control (2 mg/kg), CP08H03/Vκ8 S29A (labeled “CP08\_H03/Parent VL”, 0.08 and 2 mg/kg) treated humanized NSG mice bearing melanoma tumors were sacrificed. Tumor cells as well as CD4+ and CD8+ tumor infiltrating lymphocytes were isolated and collected for proliferation analyses. Tumor cells were identified by being FSCHiSSCHi cells which were human CD45-negative and proliferation quantified by dilution of a commercial dye that assesses proliferation (Becton-Dickinson). Human CD45+CD4+ and CD45+CD8+ T cells were identified by flow cytometry. Measurements of T cell proliferation ex vivo were carried out under T-cell-stimulation conditions wherein cells were cultured under the soluble anti-CD3 (1 g/ml) and rIL-2 (50 units/ml) for 6 days.

**Figure 28** illustrates phenotypic changes in intratumoral memory CD8 T cells upon blocking CEACAM1 with CEACAM1 antibody CP08H03/Vκ8 S29A (labeled “CP08\_H03/Parent VL”) as described in **Figures 25-27**. Flow cytometry analyses of tumor infiltrated CD3+ CD8+ T cells populations from humanized NSG mice bearing melanoma using CD62L and CD44 cell markers for the characterizations of the central memory (CD62L+CD44+) and effector memory (CD62L- CD44+) CD3+ CD8+ T cells populations from different treatment: isotype hIgG4 control (2 mg/kg) and CP08H03/Vκ8 S29A (0.08, 0,4 and 2 mg/kg).

***Melanoma patients showed CP08 and memory T cell status***

**Figure 29** shows that CEACAM1 is expressed on primary CD4+ T cells in TILs from naïve (closed circles) and PD-1/PD-L1 resistant (closed triangles) melanoma patients. A similar characterization of PD1 and TIM-3 expression on the CD8+ T cells is shown on the right.

**Figure 30** shows that tumor associated cells (TACs) from patients that had acquired resistance to anti-PD-1 therapy show significantly higher CEACAM1 expression as compared to TACs from patients that had no previous exposure to anti-PD-1 therapy. TACs were obtained from melanoma patients who were naïve (no previous exposure to anti-PD1 therapy) or those that acquired resistance to anti-PD1 therapy (acquired resistance). Tumor associated cells (TAC) were obtained by culturing tumor tissue in DMEM medium and the floating cells removed from the supernatant. The cells were stained for CD3, CD4 and CD8 and CEACAM1 expression on the CD3+ CD4+ and CD3+ CD8+ assessed. \*, *P* = 0.05; \*, *P* < 0.01.

***Melanoma patients showed CP08 and memory T cell status***

**Figure 31** illustrates a relative decrease in central memory (Tcm) relative to effector memory (Tem) cells among CD8+ T cells isolated from patients resistant to PD-1 treatment as compared to CD8+ T cells isolated from naïve patients. Tumor associated cells from naïve patients and those with acquired resistance were stained for central memory (CCR7+ CD62L+) and effector memory (CCR7-CD62L-) markers in TACs derived from naïve and resistant patients.

***Melanoma patients COMB efficiency***

**Figure 32** illustrates that CEACAM1 antibody CP08H03/Vκ8 S29A reverses T cell exhaustion in PD1/PDL-1 resistant tumors. Tumor associated cells and PBMC were isolated from a melanoma patient with secondary resistance to Pembrolizumab, Ipilimumab + Nivolumab and Dabrafenib + Trametinib and Stage IV disease. Tumor associated cells and PBMC were stained for CEACAM1, PD1 or TIM-3 and the proportion of CD8+ and CD4+ T cells denoted that express these markers (left). PBMC or tumor-associated cells (“tumor”) cultured with soluble anti-CD3 (2 g/ml) and rIL-2 (40 units/ml) in the presence of CP08H03/Vκ8 S29A or hIgG4 control antibody are shown on the right and release of IFNγ or TNFα was determined by ELISA. Note CP08H03/Vκ8 S29A (labeled “CP08”) reversal of exhaustion.

***Comparisons of CP08 and CM-24***

**Figures 33A and 33B** show flow cytometry analyses of stable Hela CEACAM1 (Hela C1) transfectant, stable Hela CEACAM3 transfectant (Hela C3), stable Hela CEACAM5 transfectant (Hela C5), stable Hela CEACAM6 transfectant (Hela C6), and stable Hela CEACAM8 transfectant (Hela C8). 5x10^4 indicated Hela transfectants were washed with staining buffer and CP08H03/Vκ8 S29A (labeled “CP08”, left) or CEACAM1 antibody CM-24 was incubated at room temperature for 30 min, washed twice with staining buffer, and stained for anti-human IgG4 Fluorescein isothiocyanate (FITC) conjugated secondary antibody in room temperature for 20 min. Fluorescence intensities were determined by flow cytometry**.** Live cells were determined by DAPI staining as shown on the y-axis and staining with the CP08H03/Vκ8 S29A antibody is shown on the x-axis. Note a positive signal in the gates shown in only the Hela CEACAM1 (C1) transfectants (left).In contrast, CM-24 (right panel) is not selective and cross-reacts with CEACAM1, CEACAM3 and CEACAM5.

**Figure 34** illustrates that CEACAM1 antibody CP08H03/Vκ8 S29A (labeled “CP08”) is more effective than CEACAM1 antibody CM-24 in reversing T-cell tolerance in tumor associated cells. Tumor associated cells derived from a naïve Merkel cell carcinoma tumor were stained for CEACAM1, PD1 or TIM-3 and proportion of CD8+ and CD4+ T cell denoted (left). Tumor associated cells cultured with soluble anti-CD3 (2 g/ml) and rIL-2 (40 units/ml) in presence of CP08H03/Vκ8 S29A, CM-24 or hIgG4 control. IFN-γ release, a measure for reversal of T-cell tolerance, was determined.\*, P=0.0138 comparing CP08 to hIgG4.

**Figures 35A, 35B, and 35C** shows that CEACAM1 antibody CM-24 is an agonistic drug in a metastatic melanoma model**.** Shown are the absolute cell counts of tumor-infiltrating CD4+ T lymphocytes (**Figure 35A**), CD8+ T lymphocytes (**Figure 35B**) and tumor cells characterized by forward/side scatter high (FSC/SCC Hi) and isolated tumor cells (**Figure 35C**) from metastatic melanomas. The values depicted from each of individual of experimental mice from each group (n=9 for IgG4; n=8 for CP08; n=6 for CM-24) are shown. \*P<0.05; \*\*P<0.001. Note the increased TILs (**Figure 35A** and **Figure 35B**) and decreased tumor cells (**Figure 35C**) in the CP08H03/Vk8 S29A (labeled ‘CP08”) treated mice vs. in the CM-24 treated mice. This data indicates that CP08H03/Vk8 S29A is an antagonistic and that CM-24 is an agonistic antibody.

**Figures 36A, 36B, 36C, and 36D**

illustrate that CM-24 treated metastatic melanoma in NSG mice exhibits deceased TILs and increased tumor cells in comparison to CP08H03/Vκ8 S29A (labeled “CP08”) treated metastatic melanoma. **Figure 36A** shows the experimental setup using a therapeutic tumor model in humanized NSG mice with human melanoma xenografts using four doses at 2 mg/kg of the respective antibodies including a hIgG4 control ciontaining the identical stabilizing hinge mutation. **Figure 36B** shows a pie chart display of the percentages of tumor-infiltrating CD4+ T lymphocytes (gray), CD8+ T lymphocytes (black) and tumor cells (white) characterized by FSC/SCC High (FSC/SCCHi) and lack of the pan-leukocyte marker human CD45 (Left: control antibody. Middle: CEACAM1 antibody CP08H03/Vκ8 S29A. Right: CEACAM1 antibody CM-24). **Figure 36C** shows tumor cell proliferation for the IgG4 control, CP08H03/Vκ8 S29A, and CM-24, indicating inhibition of tumor proliferation by CP08H03/Vκ8 S29A but not CM-24. **Figure 36D** illustrates increased proliferation of splenic CD4+ T cells in CP08H03/Vκ8 S29A treated mice and decreased proliferation of splenic CD4+ T cells in CM-24 treated mice.

***Composition of CP08 and HopQ docking on CEACAM1***

**Figures 37A and 37B** illustrate that CEACAM1 antibody CP08H03/Vκ8 S29A covers the CEACAM1:HopQ binding interface and is expected to block CEACAM1:HopQ or CEACAM1:Opa proteins interactions. **Figure 37A** shows CEACAM1:HopQ binding interface based on the analysis of three crystal structures (PDB ID 6AW2, 6GBH and 6GBG). The CEACAM1 GFCC’ face, which is formed by interactions of the CEACAM1 CC’ and FG loops’ (see Huang et al., Nature. 2015 Jan 15;517(7534):386-90) is involved in HopQ binding at CEACAM1 residues F29, Y34, N42, Q89 and N97 and makes various hydrogen bonded and hydrophobic interactions (Bonsor D,A. et. al. The Helicobacter pylori adhesin protein HopQ exploits the dimer interface of human CEACAMs to facilitate translocation of the oncoprotein CagA. EMBO J. 2018 Jul 2;37(13). pii: e98664;. Moonens K et. al. Helicobacter pylori adhesin HopQ disrupts trans dimerization in human CEACAMs. EMBO J. 2018 Jul 2;37(13). pii: e98665.).

**Figure 37B** shows superimposition of CP08H03/Vκ8 S29A:CEACAM1 crystal structure on CEACAM1:HopQ crystal structures. CP08H03/Vκ8 S29A antibody light chain and heavy chain are shown in a surface representation. HopQ chains (three different crystal structures PDB ID 6AW2, 6GBH and 6GBG) and CEACAM1 from three different co-crystal structures with HopQ (PDB ID 6AW2, 6GBH, 6GBG) and CEACAM1 from co-crystal structure with CP08H03/Vκ8 S29A are shown in ribbon diagram to highlight superimposition of the CP08H03/Vκ8 S29A and HopQ binding epitopes.