Potent immunomodulatory effects of the trifunctional antibody catumaxomab

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L.Z, D.G, C.F, N.C, D.S wrote the manuscript.

I.M, O.K ran the CRT experiments.

A.E, supervised the work.

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ABBREVIATIONS:

CatmAb : catumaxomab
EpCAM : epithelial cell adhesion molecule
TILs : ascites- infiltrating lymphocyte
CTL : cytotoxic T lymphocyte
HMGB1 : High mobility group 1 protein
ATP : adenosine triphosphate
CRT : calreticulin
TRAIL : TNF-related apoptosis-inducing ligand
IFNg : interferon gamma
PBMC : peripheral blood mononuclear cells
MA : malignant ascites
TNF : tumor necrosis factor
CDAM : cell death-associated molecular pattern
ER : endoplasmic reticulum
Precis

This study reports a comprehensive dissection of the immunomodulatory effects of a bispecific mAb specific for a widely expressed tumor cell adhesion molecule and the T cell molecule CD3, which is one of the first bispecific mAbs to be explored in clinic.
Catumaxomab (CatmAb), a trifunctional bispecific antibody directed against the epithelial cell adhesion molecule (EpCAM) and the T cell antigen CD3, is approved as intraperitoneal therapy for the treatment of malignant ascites in patients with EpCAM positive carcinomas. The immunomonitoring results of a Phase II/III study using CatmAb reported tumoricidal effect, associated with reduced VEGF levels, CD69 expressing T cells and the release of Th1 cytokines (1). Here, we comprehensively dissected the immunomodulatory effects of the CatmAb on the major subsets of malignant ascites-infiltrating leukocytes (TILs) and the molecular fingerprint of tumor cell death. We show that in the presence of EpCAM-positive tumor targets CatmAb markedly enhanced T cell activation (CD69, CD107A (LAMP1), HLA-DR and PD-1(PDCD1) expression), stimulated inflammatory CD4+Th1 and CD8+ Th1 to release IFNγ but failed to trigger Th17 cells. Engagement of CD16-expressing cells caused upregulation of TRAIL (TNFSF10) and costimulatory CD40 and CD80 molecules. CatmAb promoted tumor cell death associated with ATP release and strongly synergized with oxaliplatin for the exposure of the three hallmarks of immunogenic cell death (calreticulin, HMGB1 and ATP). These findings warrant validation as potential biomarkers of efficacy of CatmAb.
Introduction

The trifunctional bispecific monoclonal antibody CatmAb has two binding specificities directed at EpCAM (through a mouse IgG2a) and CD3 (through a rat IgG2b). With its Fc portion, the antibody has the potential to engage accessory cells (through FcγRI, FcγRIIa, FcγRIII) such as neutrophils, NK cells, macrophages, monocytes and dendritic cells (2). This therapeutic strategy is expected to promote an MHC-unrestricted pattern of killing, targeting EpCAM positive tumor cells (most tumours of gastrointestinal origin and in some carcinomas of the genitourinary tract, i.e about 90% of ascites-causing carcinoma (3,4)) sparing EpCAM negative mesothelial cells of the peritoneal cavity. In vitro studies showed that cytokine release, perforin-dependent killing, antibody-dependent cytolyis (ADCC) and activation of accessory cells all acted in concert to eliminate tumor cells (5-7). EpCAM+ tumors have a worse prognosis than their negative counterparts, specifically in triple negative and node negative breast cancers (8,9). In tumor spheroids, CatmAb (alone or together with cisplatin) exerted regressions of tumor volumes associated with massive immune infiltration in an ADCC-dependent manner (10).

Pilot and Phase I/II studies showed that intraperitoneal administration of CatmAb reduced tumor cell accumulation in ascitic fluids in a sustained manner (11,12). Next, an open label, multicenter, randomized Phase II/III trial in patients with malignant ascites due to epithelial cancer compared the effects of CatmAb with control paracentesis. Puncture-free survival (primary endpoint of the trial) was significantly higher in the CatmAb compared with the control group (median 46 versus 11 days, HR: 0.25) (13). Overall survival in the
pooled population showed a positive trend towards the CatmAb group with significant results in the subgroup of gastric cancers (13). Treatment with CatmAb delayed the deterioration of quality of life in patients with malignant ascites (14). Moreover, ascites concentrations of VEGF and CD133+EpCAM+ cancer stem cells significantly dropped post-CatmAb while 20-30% of CD4+ and CD8+ T cells acquired CD69 expression (1).

Therefore, we undertook the comprehensive phenotypic analysis of the immune infiltrates and the tumor cell death fingerprint in ascitic fluids incubated ex vivo with CatmAb. Our findings indicate that CatmAb skews the T cell cytokine pattern towards an inflammatory Th1 profile, turns on NK cells to express TRAIL and inflammatory monocytes to harbour costimulatory molecules. In addition, when combined with oxaliplatin, CatmAb imprinted an immunogenic cell death pathway to tumor cells.

Material and Methods

**PBMC or Ascites-CatmAb coculture systems.** Either co-cultures of peripheral blood mononuclear cells (PBMC) from healthy volunteers (n=5) and EpCAM+ tumor cells (SKBR3) (allogeneic system) or ascites cells from patients with malignant ascites (autologous system) were incubated in the presence of CatmAb (CatmAb) at a concentration of 10 ng/mL for 18-48 hours. Ascitic fluids harvested from 12 patients bearing EpCAM+/-cancers were used (table 1).

Analyses of T cell activation and measurement of cytokine release in cell culture supernatants were performed. Subsequent analyses included investigation of cell death of EpCAM+ tumor cells and evaluation of immunologic cell death markers (ICD) (CRT, HMGB1, ATP).
Ascites samples. Ascites cells taken at screening (immediately prior to the study) were harvested by standard sample centrifugation or Ficoll density centrifugation if erythrocytes were present to eliminate the latter. The supernatant were frozen at -80°C to evaluate the assessment of cytokines. Cells harvested from samples at screening were stained for fluorescence-activated cell sorted (FACS) analysis with the antibody anti-CD45-A750 (Beckman Coulter) and anti-Epcam-PE (CD326, Becton Dickinson) and Vivid Yellow (Molecular Probe) to assess viability. Malignant ascitic fluids were defined as “positive” when EpCAM specific staining gating on viable CD45 negative cells was detectable in flow cytometry using the appropriate isotype control antibodies. No precise threshold of positivity was required.

Then, cells were seeded in 12-well plates (10⁶ cells per well) in complete RPMI medium (RPMI 1640 containing 10% FBS, 2 mM L-glutamine, 20 mM Hepes, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin) and CatmAb was added (or not) at 10ng/ml. The plates were incubated in a 5% saturated CO₂ atmosphere at 37°C, for 18h.

Phenotype of peritoneal cells and activation markers: Fluorescence-activated cell sorting (FACS) studies of plasma membrane markers. They were performed with the use of fluorescein isothiocyanate-(FITC)-, phycoerythrin (PE)-, phycoerythrin cyanin 7 (PE-Cya7)-, Peridinin Chlorophyll Protein (PerCP)-, Peridinin Chlorophyll Protein Cyanin 5.5 (PerCP-Cy5.5)-, allophycocyanin Alexa750 (APC A750)-, allophycocyanin -conjugated antibodies. The following antibodies used for FACS were: anti-human CD45 (Beckman Coulter), CD3 (Miltenyi Biotech), CD4 (Becton Dickinson), CD8 (Beckman Coulter), CD56 (Beckman Coulter), FoxP3 (eBioscience), CD45RA (eBioscience), CD14 (Biolegend), CD16 (Beckman Coulter), CD80 (Biolegend), CD39 (eBioscience), CD40(Becton Dickinson), CD19 (Becton
Dickinson), CD25 (Beckman Coulter), CD15 (Biolegend), CD73 (eBioscience), CD69 (Becton Dickinson), HLA-DR (Beckman Coulter), CD253 (TRAIL, eBioscience), CD279 (PD-1, eBioscience), CD336 (NKp44, Miltenyi Biotech), TCR VD2 (Beckman Coulter), TCR VG/D PAN (Beckman Coulter), CD123 (Becton Dickinson), Vivid Yellow (Molecular Probe).

After 30 minutes of incubation at 2°C to 8°C, samples were washed. Samples were analyzed in a FACS Cyan (Beckman Coulter). FACS data were analyzed with FlowJo software (TreeStar Inc, Ashland, OR).

**Intracellular staining.** After incubation for 18h, a protein transport inhibitor (BD Golgi-stop, Becton Dickinson) and CD107a-PE (Becton Dickinson) were added for 2h at 37°C. Then, membrane staining was performed to identify T- and NK cells (CD3, CD4, CD8, CD56). Then, intracellular staining was performed using BD CytoFIX CytoPerm Kit (Becton Dickinson) plus IFN-γ-APC (Miltenyi) and -IL17A-FITC (Biolegend).

**Cytokines monitoring.** The cytokines IFN-γ, IL-2, IL-4, IL-13, TNF-α, IL-6, IL-1β, IL-6, IL-17A, IL-12p40, IL-5, was quantified in cell-free supernatants from co-cultures after the end of the incubation period. The supernatants were stored at −80°C until analysis. Cytokines were quantified by cytometric bead array (FlowCytomix; eBioscience), according to the manufacturer’s instructions. Briefly, 13 beads populations with distinct spectral addresses and size precoated with cytokine-specific capture antibodies were mixed and incubated with recombinant cytokine standards or test samples. In a second time a biotin-conjugated second antibody mixture was added. Finely, Streptavidin-Phycoerythrin was added. Acquisition was performed by flow cytometry Cyan and analysis of sample data was performed with manufactured software (FlowCytomix; eBioscience).
**Tumor Cytotoxicity assays and ICD markers.** Tumour cells co-incubated with PBMC or CatmAb alone were used as controls. After incubation for 18h, cells were analyzed on a FACS after membrane staining with CD45-Alexa A750 (Beckman Coulter) and staining with 7AAD (Becton Dickinson) for determination of cell viability, and Annexin V-APC (Becton Dickinson) conjugated for analysis of apoptosis.

**Fluorescence detection of cell surface CRT.** Cells (in 12-well plates) were first washed with CRT buffer (1× PBS, 3% albumin bovin serum, PH 7.4) and then incubated with membrane markers for 20 min at 4°C. After 2 washes in CRT buffer, cells were incubated in rabbit anti-CRT antibody (1:100, Abcam) in FACS buffer at 4°C for 30 min. Cells reacted with secondary anti-rabbit IgG (H+L) Alexa fluor 488-conjugates (Becton Dickinson) (1:100) in CRT buffer at 4°C for 30 min and then were washed twice with FACS buffer. Finally, cells were incubated 15 min with 7AAD and Annexin V-APC in Annexin Buffer. Data analysis was conducted using a FACS Cyan (Beckman Coulter).

**HMGB1 and ATP dosages.** HMGB1 in cell-free supernatant was detected using an ELISA kit (IBL International GMBH) and ATP in cell-free supernatant was detected using a kit for ATP measurement (ENLITEN ATP Assay System Bioluminescence from PROMEGA).

**Results**

**CatmAb-mediated T cell triggering**

Ascitic fluids harvested from 12 patients bearing EpCAM⁺⁻ cancers (Table 1) were freshly incubated with CatmAb for 18 hours. The percentages of CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells
among the CD45+ fraction of ascites cells remained stable, 35.8 ± 25 and 40.3 ± 19 for CD4+T cells with or without CatmAb respectively (Fig. 1A), 20.5 ± 11 and 19.9 ± 11 for CD8+T cells with or without CATmAb respectively (Fig. 2A) in EpCAM+ malignant ascites. Both CD4+ and CD8+ T cells could degranulate (expressed CD107A) in the presence of CatmAb, only in EpCAM+ ascites (Fig. 1B, Fig. 2B, left panels). Both CD4+ and CD8+ T cells secreted IFNγ in the presence of CatmAb, only in EpCAM+ ascites (Fig. 1B, Fig. 2B, middle panels). CatmAb failed to significantly stimulate T cells to produce IL-17 (Fig. 1B, Fig. 2B, right panels). All T cells exhibited activation markers such as CD69, HLA-DR and PD-1 upon recognition of EpCAM+ tumors (Fig. 1C, 2C, 2D right panel) but failed to express TRAIL (Fig. 2D, left panel). It is noteworthy that similar findings and also the production of IL-17, could be obtained by coculturing EpCAM+- tumor cell lines with peripheral blood mononuclear (PBMC) cells from healthy volunteers in the presence of CatmAb (Supplemental Fig. 1 and Fig. 2). CatmAb-induced T cell activation significantly increased the production of IFNγ, IL-17A, IL-6 and IL-1β, hence contributing to a modulation of the local microenvironment (Fig. 3A, 3B). CatmAb deviated the natural Th2 inflammation by decreasing the concentrations of IL-4 and IL-13 while augmenting the Th1 cytokines in most patients (Fig. 3C, left and right panels).

Malignant ascites contained up to 5% (8.25 ± 5) of CD3+CD4+Foxp3+ T cells among CD4+T cells (Fig. 4A), that could be analyzed according to CD45RA (15). Most of these Treg became Foxp3high CD45RA−CD73+ after exposure to CatmAb (Fig. 4B, Fig. 4C). CatmAb activated local T cells and geared their polarization towards an inflammatory Th1 pattern despite the presence of ectoATPase-expressing Treg.
Engagement of CD16 expressing cells in malignant ascites incubated with CatmAb

NK cells were not enriched post-CatmAb (Fig. 5A, left panel). Most NK cells from ascites TILs were CD16+ at harvesting but CatmAb did not significantly reduce CD16 expression on NK cells (Fig. 5A, right panel). However, NK cells were markedly activated by CatmAb as shown by their upregulated levels of CD69 and TRAIL expression (Fig. 5B left and middle panels and, to a lesser extent NKp44 expression, right panel) only in EpCAM+ ascites. Similar findings were achieved in cocultures of PBMC and allogeneic EpCAM+ tumor cells modulated by CatmAb (Supplemental Fig. 3) in that NK cells became CD69+ TRAIL+ while expressing low levels of CD107A and IFNγ in the presence of the trifunctional Ab.

Most monocytes, albeit underrepresented in TILs (Fig. 5C, left panel), also expressed CD16 molecules (Fig. 5C, right panel) which, upon engagement with CatmAb, decreased to some extent (Fig. 5C, right panel). CatmAb markedly increased CD80 and CD40 molecules on CD14+CD16low TIL monocytes (Fig. 5D, left and right panels) while maintaining HLA-DR expression (Fig. 5D, middle panel).

Hence, CatmAb induced TRAIL expression on NK cells and costimulatory molecules on inflammatory monocytes.

Tumor cell death fingerprint post-CatmAb

Immunogenic cell death can be defined as a modality of cell demise associated with T cell-dependent tumor rejection and long term survival, and correlated with the exposure or release of cell death-associated molecular (CDAM) patterns (16). Briefly, stress before
death matters for an immunogenic cell death pathway to occur. First, an ER stress response culminating with calreticuline (CRT) exposure to the tumor cell surface (17), then late apoptosis associated with the exodus of the chromatin binding HMGB1 protein (18) and finally, activation of the autophagy machinery leading to ATP release (19) all contribute to the adequate phagocytosis, processing and antigen presentation of the dying tumor cells to T lymphocytes (20,21). Therefore, we addressed whether CatmAb-mediated tumor cells death could trigger an immunogenic cell death program. We analyzed by flow cytometry the expression of annexin V and 7AAD on EpCAM+CD45- tumor cells of the malignant ascites in the presence (or absence) of CatmAb at 18 hours. The bispecific mAb increased the fraction of dying and dead cells (Fig. 6A) and promoted the release of ATP in the milieu (Fig. 6B, right panel) but failed to induce the exposure of CRT or the exodus of HMGB1 in the extracellular environment of ascites (n=10)(Fig. 6B, left and middle panels). In contrast, pre-incubation of tumor cells (SKBR3) with oxaliplatin did not increase cell death by CatmAb (Fig. 6C left panel) but mediated stress and increased CRT exposure (Fig. 6C, right panel), HMGB1 (Fig. 6D, left panel) and ATP release (Fig. 6D, right panel) in the presence of CatmAb.

CatmAb-mediated immunomodulatory effects on malignant ascites synergized with oxaliplatin to induce CDAM on tumor cells.

Discussion

Here we show –using malignant ascites ex vivo experimental model systems- that CatmAb
switches the polarization of local T cells into inflammatory Th1 cells (capable of degranulating and secreting IFN\(\gamma\)), engages CD16+ cells to express TRAIL (for NK cells) and costimulatory molecules (for monocytes). In conjunction with oxaliplatin, CatmAb triggered the three hallmark criteria of immunogenic cell death in allogeneic tumor cell lines cocultured with PBMC. CatmAb has been described as a trifunctional antibody capable of i) engaging T cells in a MHC-independent manner, ii) targeting tumor cells in an EpCAM-dependent fashion, iii) stimulating antigen presenting cells through the Fc\(\gamma\)R. The increase rate of CD4+ T cells showing surface mobilization of CD107a was unsuspected because surface mobilization of CD107a by CD8+ T cells identifies that can release cytotoxic granules, a function not often associated with CD4+ T cells. Although mostly described for cytolytic CTLs recognizing target cells, membrane expression of CD107a, a lysosomal marker associated with degranulation in CTLs, could also be found on activated CD4+ T cells during viral infections. In such circumstances, CD4+ T cells can acquire cytotoxic functions resulting in the surface expression of CD107a and the release of granzyme A and granzyme B (22-25).

While it is widely demonstrated that CatmAb has local antitumor effects in malignant ascites (13) and mediates a strong tumoricidal activity in mixed tumor lymphocyte cocultures (5-7), whether CatmAb mediates distant effects on systemic immunity remains an open conundrum. Interestingly, two case reports suggested that a systemic immunity might take place post-CatmAb. An extraperitoneal skin metastasis of a platinum-refractory ovarian cancer regressed after an intraperitoneal treatment with CatmAb given for a massive ascites (26). Delayed inflammatory lesions of the peritoneum corresponding to mesenterial nodular fibrosis and granulomatosis were observed post-chemotherapy and
surgery in adjuvant intraperitoneal CatmAb injections and appeared to be associated with a complete and long term response in an advanced gastric adenocarcinoma (27). Stroehlein et al. showed tumor-reactive CD4+/CD8+ Th1/Tc1-lymphocytes in PBMC after restimulation in 5 of 9 patients suffering from peritoneal carcinomatosis, indicating that a long term specific anti-tumor immunity can be elicited with CatmAb (28). Finally, humoral responses to CatmAb (human anti-mouse antibodies) correlated with clinical outcome (puncture-free survival, time to next puncture and overall survival) in a Phase II/III clinical trial (29). Several mechanisms could be involved to account for this systemic immunity. The FcγR-mediated cross-presentation of dying cells is an efficient pathway for the priming of naïve T cells (30). Alternatively, reactivation of tumor-specific effector T cells into long lived cells integrating the memory T cell pool and recirculating to protect the host against relapse independently of a de novo priming in lymph nodes has been suggested or described (31-33). Finally, the nature of tumor cell stress preceding cell demise matters to condition long term immunity against dying cells. The ER stress response culminating in the exposure, to the cell surface, of an ER-resident chaperone, calreticulin (CRT) represents an « eat-me signal » for engulfment of tumor cells by inflammatory phagocytes (17,34).

While CatmAb alone failed to promote CRT exposure on most malignant ascites-derived tumor cells (and in vitro in mixed lymphocyte tumor cocultures), it enhanced the ability of oxaliplatin to induce it. On one hand, this negative result is surprising given the fact that TRAIL–mediated lysis can promote CRT exposure on tumor cells (35). It may be explained by the fact that TRAIL-expressing NK cells may not engage in privileged contacts with dying tumor cells in the context of the trifunctional bispecific antibody. On the other hand, Trapani and coll. showed that granzyme A/B-mediated lysis is involved in enhancing
uptake and cross-presentation of dying bodies by CD8α+ DC in mouse models (36). They showed that blocking phosphadidylserine did not hamper this granzyme-dependent engulfment of dying tumor cells. It is conceivable that additional eat-me signals different from CRT be exposed post-degranulation by T cells that could be recognized by the human ortholog of mouse CD8α+ DC (37). However, very few BDCA3+ DC could be recovered from ascitic fluids in our model system (data not shown). The lack of expression of CRT does not appear to be related to a low effector to tumor cell ratio. First, there was no significant correlation between the E:T ratio of CD45+/EpCAM+ cells (ranging from 2 to 1402, Table 1) and T cell activation parameters. Second, in a randomized trial (13) where the median proportion of EpCAM+ cells in ascites cells was 0.6%, a clinical benefit associated with a decreased percentage of EpCAM+ cells in ascites was reported.

CatmAb-mediated cell death could not promote the release of the non-histone chromatin-binding transcription factor, HMGB1, in tumor cells from malignant ascites treated with the bispecific antibody alone but could do so, in mixed lymphocyte tumor allogeneic cocultures after presensitization with oxaliplatin (Fig. 6D). This negative result could be explained by the frequent deficiency in nuclear HMGB1 observed in advanced cancers (our unpublished data). Indeed, HMGB1 is the ligand for TLR4 mediating efficient processing and presentation of the phagocytic cargo in DC to Tc1 cells (18). The specific knock down of HMGB1 (created by means of siRNA interfering with HMGB1 translation in tumor cells) suppressed the immunogenicity of cell death induced by oxaliplatin in vivo (18). Therefore, it is conceivable that CatmAb may not mediate its full potential in peritoneal carcinomatosis exhibiting HMGB1 deficiencies. Interestingly, when combined with oxaliplatin, CatmAb was more potent in promoting HMGB1 release, urging the use of
combinatorial regimen to exploit the immunogenicity of the cell death process.

Finally, ATP release, associated with the activation of the autophagy machinery in tumor cells (19,38), is critical for two key events: first, the recruitment of CD11c⁺ DC in tumor beds post-chemotherapy or cell demise (26), second the engagement of P2RX7 on DC which activates the inflammasome NLRP3, culminating in IL-1β release and IL-1R1-dependent Tc1 polarization (39). Ecto-ATPases expressed on regulatory Th17 (40) and Tregs (41) might impair the recruitment of DC and/or the differentiation of IFNγ producing CTLs post-treatment. Indeed, CatmAb-treated malignant ascites contained increased levels of memory Treg expressing CD73 (Fig. 4). However, such Tregs did not appear to inhibit the strong polarization of TILs towards an inflammatory Th1 profile (TNFα, IFNγ, IL-2, Fig. 1B, Fig. 2B, Fig. 3), starting from ascites containing Th2 cytokines (Fig. 3C) and poorly activated T cells (Fig. 1, Fig. 2). This might be explained by the significant release of ATP induced by CatmAb and amplified by presensitization of tumor cells with oxaliplatin (Fig. 6).

A multicenter, randomized, prospective Phase II study aimed at addressing the efficacy of CatmAb in adjuvant settings in post-operative gastric cancer carcinomatosis responding to neoadjuvant chemotherapy (platinum-based regimen) will be conducted in France. We will validate the capacity of CatmAb i) to induce immunogenic cell stress or death in peritoneal cancer cells, ii) to switch the polarization of TIL towards an inflammatory Th1 pattern, iii) to promote the expression of TRAIL and costimulatory molecules on NK cells and monocytes respectively, with the intent to correlate one of these immune biomarkers to the time to progression.
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References


Table 1 – Patients’ characteristics

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<td>464</td>
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<td>52.1</td>
<td>6</td>
<td>10.9</td>
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Table and Figure Legends

Table 1. Characteristics of the patients, the primary tumor and the ascitic fluids harvested from 12 patients bearing EpCAM+/− cancers

Figure 1. Activation of malignant ascites derived- CD4+ T cells by CatmAb.
Flow cytometry analyses of CD3+CD4+ T cells gated in the CD45+ fraction of TIL of various malignant ascites samples (n=12) incubated with [■] or without [□] CatmAb for 18 hours.

A. Percentages of CD4+ T cells are indicated. B. Intracellular staining for CD107A, IFNγ and IL-17. C. Membrane staining for CD69, HLA-DR and PD-1. Each dot represents one patient’s ascitic fluid. Two patients harbouring an EpCAM negative cancer were used as negative controls. Student t’ test to compare means ±SEM of two groups was used: *p<0.05, **p<0.01, ***p<0.001.

Figure 2. Activation of malignant ascites derived- CD8+ T cells by CatmAb.
Flow cytometry analyses of CD3+CD8+ T cells gated in the CD45+ fraction of TIL of various malignant ascites samples (n=12) incubated with [■] or without [□] CatmAb for 18 hours.

A. Percentages of CD8+ T cells are indicated. B. Intracellular staining for CD107A, IFNγ and IL-17. C. Membrane staining for CD69, HLA-DR. D. Membrane staining for TRAIL and PD-1.
Each dot represents one patient’s ascitic fluid. Two patients harbouring an EpCAM negative cancer were used as negative controls. Student t’ test to compare means ±SEM of two groups was used: *p<0.05, **p<0.01, ***p<0.001.
Figure 3. Cytokine profiles of the ascites milieu are changed following exposure to CatmAb.

A. T helper cytokines. Monitoring by Flow Cyto Mix (eBiosciences) of four cytokines preferentially secreted by polarized T cells in TIL of various malignant ascites samples (n=12) incubated with [●] or without [○] CatmAb for 18 hours. B. Inflammatory cytokines. Monitoring by Flow Cyto Mix (eBiosciences) of four cytokines preferentially secreted by the tumor microenvironment in various malignant ascites incubated with [●] or without [○] CatmAb for 18 hours. C. Cytokines of the ascitic fluids. Direct assessment of cytokine concentrations after fresh harvesting (prior to incubation with CatmAb) of malignant ascetic fluids using commercial Flow Cyto Mix (eBiosciences). 10 various malignant ascites samples were tested. Student t’ test to compare means ±SEM was used: *p<0.05, **p<0.01, ***p<0.001.

Figure 4. Regulatory T cells expressed ecto-ATPases post-CatmAb.

A-B. Flow cytometry analyses of CD3+CD4+ Foxp3+ T cells gated in the CD45+ fraction of TIL of various malignant ascites samples (n=8) incubated with [■] or without [○] CatmAb for 18 hours. Percentages of Foxp3+CD4+ T cells are indicated (A) as well as CD45RA+/Foxp3+ cells (B). C. Membrane staining for CD39 and CD73 in the gate Foxp3highCD45RA-. Each dot represents one patient’s ascitic fluid. Student t’test to compare means ±SEM of two groups was used.

Figure 5. CatmAb-induced TRAIL and costimulatory molecule expression on CD16+ cells.
**A-B.** Flow cytometry analyses of CD3-CD56+ NK cells gated in the CD45+ fraction of TIL of various malignant ascites samples (n=12) incubated with [■] or without [□] CatmAb for 18 hours. Percentages of NK cells and CD16+ NK cells are indicated (A). Low levels of CD107A+ or IFNγ+NK cells could be found after exposure with CatmAb (not shown). Membrane staining for CD69, TRAIL and NKp44 (B). **C-D.** Flow cytometry analyses of CD14+ monocytes gated in the CD45+ fraction of TIL of various malignant ascites incubated with [■] or without [□] CatmAb for 18 hours. Percentages of CD14+ cells (left panel) and CD14+CD16high cells (right panel) are indicated (C). Expression levels of CFD40, HLA-DR and CD80 on CD14+CD16low cells (D). Each dot represents one patient’s ascitic fluid. Two patients harbouring an EpCAM negative cancer were used as negative controls. Student t’ test to compare means +SEM of two groups was used: *p<0.05, **p<0.01, ***p<0.001.

**Figure 6. Hallmark criteria of immunogenic cell death post-CatmAb.**

**A.** Flow cytometry determination of living, apoptotic (7AAD-annexin V*), dead or necrotic (annexin V-7AAD+) cells in the gate CD45- EpCAM+ featuring tumor cells of the ascetic fluids (n=7). The percentages of live versus apoptotic versus dead cells are represented for all the patients. **B.** Monitoring of CRT exposure by direct flow cytometry gating on living tumor cells (left), of HMGB1 release by commercial ELISA (middle) and ATP release by luciferase assays (right) for 7 malignant ascites samples incubated with or without CatmAb (right). **C-D.** Allogeneic mixed PBMC/ tumor ex vivo cocultures incubated with or without CatmAb for 18h (left panels) and after 2h of presensitization of oxaliplatin chemotherapy before cocultures (right panels). Flow cytometry analyses of 7AAD+ cells (C), CRT exposure (D, left panels), ELISA assessment of HMGB1 secretion (D, middle panels), ATP release
monitored by luciferase assays (D, right panels). Means±SEM for 4-6 independent experiments are depicted. Anova and Fisher’s exact method were used to compare means ±SEM in between four groups. Significant $p$ values are indicated: *$p<0.05$, **$p<0.01$, ***$p<0.001$. 
Figure 1

A. 

B. 

C. 

ns

EpCAM Pos EpCAM Neg

EpCAM Pos EpCAM Neg

EpCAM Pos EpCAM Neg

EpCAM Pos EpCAM Neg

EpCAM Pos EpCAM Neg

EpCAM Pos EpCAM Neg

ns

ns

% CD4+/CD45+

% CD107a+/CD4+/CD45+

% IFN-\(\gamma\)+/CD4+/CD45+

% IL-17+/CD4+/CD45+

% CD69+/CD4+/CD45+

% HLA-DR+/CD4+/CD45+

% PD1+/CD4+/CD45+

% EpCAM Pos EpCAM Neg

% EpCAM Pos EpCAM Neg

% EpCAM Pos EpCAM Neg

% EpCAM Pos EpCAM Neg

% EpCAM Pos EpCAM Neg

% EpCAM Pos EpCAM Neg

% EpCAM Pos EpCAM Neg
Figure 3
Figure 4
Figure 5

A.

B.

C.

D.

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.
Figure 6

A. 

B. 

C. 

D.
Potent immunomodulatory effects of the trifunctional antibody catumaxomab

Diane Goere, Caroline Flament, Sylvie Rusakiewicz, et al.

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