

## Potent Immunomodulatory Effects of the Trifunctional Antibody Catumaxomab

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### Abstract

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 revealed a tumoricidal effect associated with reduced VEGF levels, CD69-expressing T cells, and the release of T-helper cell (T<sub>H</sub>)-1 cytokines. We comprehensively dissected the immunomodulatory effects of the CatmAb on the major subsets of malignant ascites-infiltrating leukocytes and the molecular fingerprint of tumor cell death. Herein 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] and stimulated inflammatory CD4<sup>+</sup> T<sub>H</sub>1 and CD8<sup>+</sup> T<sub>H</sub>1 to release IFN- $\gamma$  but failed to trigger T<sub>H</sub>17 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. *Cancer Res*; 73(15); 4663–73. ©2013 AACR.

### Introduction

The trifunctional bispecific monoclonal antibody catumaxomab (CatmAb) has two binding specificities directed at epithelial cell adhesion molecule [EpCAM (through a mouse immunoglobulin G (IgG)-2a) and CD3 (through a rat IgG2b)]. With its Fc portion, the antibody has the potential to engage accessory cells (through Fc $\gamma$ RI, Fc $\gamma$ RIIa, Fc $\gamma$ RIII) such as neutrophils, natural killer (NK) cells, macrophages, monocytes, and dendritic cells (DC; ref. 1). This therapeutic strategy is expected to promote an MHC-unrestricted pattern of killing, targeting EpCAM-positive tumor cells (most tumors of gastrointestinal origin and in some carcinomas of the genitourinary tract, i.e., about 90% of ascites causing carcinoma; refs. 2, 3), sparing EpCAM-negative mesothelial cells of the peritoneal cavity. *In vitro* studies showed that

cytokine release, perforin-dependent killing, antibody-dependent cytotoxicity (ADCC), and activation of accessory cells all acted in concert to eliminate tumor cells (4–6). EpCAM<sup>+</sup> tumors have a worse prognosis than their negative counterparts, specifically in triple-negative and node-negative breast cancers (7, 8). In tumor spheroids, CatmAb (alone or together with cisplatin) exerted regressions of tumor volumes associated with massive immune infiltration in an ADCC-dependent manner (9).

Pilot and phase I/II studies showed that intraperitoneal administration of CatmAb reduced tumor cell accumulation in ascitic fluids in a sustained manner (10, 11). 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 (the primary endpoint of the trial) was significantly higher in the CatmAb compared with the control group (median, 46 vs. 11 days, HR: 0.25; ref. 12). Overall survival in the pooled population showed a positive trend toward the CatmAb group with significant results in the subgroup of gastric cancers (12). Treatment with CatmAb delayed the deterioration of quality of life in patients with malignant ascites (13). Moreover, ascites concentrations of VEGF and CD133<sup>+</sup>EpCAM<sup>+</sup> cancer stem cells significantly dropped following CatmAb treatment, whereas 20% to 30% of CD4<sup>+</sup> and CD8<sup>+</sup> T cells acquired CD69 expression (14).

Therefore, we undertook a 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

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pattern toward an inflammatory T-helper cell (T<sub>H</sub>)-1 profile, turns on NK cells to express TRAIL, and causes inflammatory monocytes to harbor costimulatory molecules. In addition, when combined with oxaliplatin, CatmAb imprinted an immunogenic cell death pathway to tumor cells.

**Materials and Methods**

**PBMC or ascites-CatmAb coculture systems**

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

Analyses of T-cell activation and measurement of cytokine release in cell culture supernatants were conducted. Subsequent analyses included investigation of cell death of EpCAM<sup>+</sup> tumor cells and evaluation of immunologic cell death (ICD) markers (CRT, HMGB1, ATP).

**Ascites samples**

Ascites cells taken at screening (immediately before the study) were harvested by standard sample centrifugation or Ficoll density centrifugation if erythrocytes were present to eliminate the latter. The supernatant was 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), 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 (10<sup>6</sup> cells per well) in complete RPMI medium (RPMI-1640 containing 10% FBS, 2 mmol/L L-glutamine, 20 mmol/L HEPES, 1 mmol/L sodium pyruvate, 100 U/mL penicillin, and 100 µg/mL streptomycin), and CatmAb was added (or not) at 10 ng/mL. The plates were incubated in a 5% saturated CO<sub>2</sub> atmosphere at 37°C for 18 hours.

**Phenotype of peritoneal cells and activation markers**

FACS studies of plasma membrane markers were conducted with fluorescein isothiocyanate (FITC)-, phycoerythrin-, phycoerythrin cyanin 7 (PE-Cy7), Peridinin Chlorophyll Protein (PerCP), Peridinin Chlorophyll Protein Cyanin 5.5 (PerCP-Cy5.5), allophycocyanin Alexa750 (APC A750), allophycocyanin-conjugated antibodies. The following antibodies were used for FACS: anti-human CD45 (Beckman Coulter), CD3 (Miltenyi Biotec), 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

**Table 1. Patients' characteristics**

Patients	Pt 1	Pt 2	Pt 3	Pt 4	Pt 5	Pt 6	Pt 7	Pt 8	Pt 9	Pt 10	Pt 11	Pt 12
EpCAM Status (Pos/Neg)	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Neg	Neg
% EpCAM <sup>+/-</sup> ascites cells	8	0.5	1.6	0.12	0.07	1.8	0.06	24.4	0.55	11.1	—	—
Age, y	64	64	39	69	58	71	65	49	51	61	62	62
Gender (M/F)	F	F	F	F	F	F	F	M	F	F	M	M
Primary tumor	Ovary	Ovary	Ovary	Ovary	Ovary	Ovary	Ovary	Colon	Breast	Ovary	Colon	Colon
Stage	IIIb	IIIb	IIIb	IIIb	IIIb	IIIb	IIIb	IV	IIIb	IIIb	IV	IV
Ascites presentation (synchronous/metachronous)	Synch	Synch	Synch	Synch	Synch	Synch	Synch	Synch	Metach	Synch	Metach	Synch
Chemotherapy naive (yes/no)	Yes	No	Yes	No	Yes	Yes	Yes	Yes	No	Yes	No	No
Type of systemic chemotherapy	—	Carboplatin, acitaxel	—	Carboplatin, paclitaxel	—	—	—	—	Caelyx (Janssen) Zometa (Novartis)	—	Oxaliplatin, 5-FU, bevacizumab	Oxaliplatin 5-FU
Objective response to chemotherapy (yes/no)	—	Yes	—	Yes	—	—	—	—	No	—	No	No
Time to last chemo (d)	—	30	—	22	—	—	—	—	24	—	17	52
% CD45 <sup>+</sup> in ascites	17.6	10.3	76.9	55.7	37.3	95.4	84.1	64.9	2.25	97.4	14.7	73
CD45 <sup>+</sup> cells/EpCAM <sup>+</sup> cells	2	16	48	464	497	53	1402	3	4	974	—	—
% Dead cells in ascites	20.7	5	52.1	6	10.9	61	2.9	1	2.6	23.1	20.7	19

NOTE: Characteristics of the patients, the primary tumor, and the ascitic fluids harvested from 12 patients bearing EpCAM<sup>+/-</sup> cancers. Abbreviations: 5-FU, 5-fluorouracil; Neg, negative; Pos, positive.

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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), and Vivid Yellow (Molecular Probe).

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

**Intracellular staining.** After incubation for 18 hours, a protein transport inhibitor (BD Golgi-stop, Becton Dickinson) and CD107a-PE (Becton Dickinson) were added for 2 hours at 37°C.

Then membrane staining was conducted to identify T cells and NK cells (CD3, CD4, CD8, CD56), after which intracellular staining was conducted using BD CytoFIX CytoPerm Kit (Becton Dickinson) plus IFN- $\gamma$  -APC (Miltenyi) and -IL17A-FITC (Biolegend).

**Cytokines monitoring.** The cytokines, IFN- $\gamma$ , interleukin (IL)-2, IL-4, IL-13, TNF- $\alpha$  IL-6, IL-1 $\beta$ , IL-6, IL-17A, IL-12p40, and IL-5 were quantified in cell-free supernatants from cocultures 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 bead 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 round, a biotin-conjugated second antibody mixture was added. Finally, streptavidin-phycoerythrin was added. Acquisition was conducted by flow cytometry Cyan, and analysis of sample data was conducted with manufactured software (FlowCytomix; eBioscience).

#### Tumor cytotoxicity assays and ICD markers

Tumor cells coincubated with PBMC or CatmAb alone were used as controls. After incubation for 18 hours, 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 calreticuline (CRT) buffer (1 $\times$  PBS, 3% albumin bovin serum, pH 7.4) and then incubated with membrane markers for 20 minutes at 4°C. After two washes in CRT buffer, cells were incubated in rabbit anti-CRT antibody (1:100, Abcam) in FACS buffer at 4°C for 30 minutes. 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 minutes and then were washed twice with FACS buffer. Finally, cells were incubated for 15 minutes 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<sup>+/-</sup> cancers (Table 1) were freshly incubated with CatmAb for 18 hours. The percentages of CD3<sup>+</sup>CD4<sup>+</sup> and CD3<sup>+</sup>CD8<sup>+</sup> T cells among the CD45<sup>+</sup> fraction of ascites cells remained stable, 35.8  $\pm$  25 and 40.3  $\pm$  19 for CD4<sup>+</sup>T cells with or without CatmAb, respectively (Fig. 1A), and 20.5  $\pm$  11 and 19.9  $\pm$  11 for CD8<sup>+</sup> T cells with or without CATmAb, respectively (Fig. 2A) in EpCAM<sup>+</sup> malignant ascites. Both CD4<sup>+</sup> and CD8<sup>+</sup> T cells could degranulate (expressed CD107A) in the presence of CatmAb, only in EpCAM<sup>+</sup> ascites (Figs. 1B and 2B, left). Both CD4<sup>+</sup> and CD8<sup>+</sup> T cells secreted IFN- $\gamma$  in the presence of CatmAb, only in EpCAM<sup>+</sup> ascites (Figs. 1B and 2B, middle). CatmAb failed to significantly stimulate T cells to produce IL-17 (Figs. 1B and 2B, right). All T cells exhibited activation markers such as CD69, HLA-DR, and PD-1 upon recognition of EpCAM<sup>+</sup> tumors (Figs. 1C, 2C and D, right) but failed to express TRAIL (Fig. 2D, left). It is noteworthy that similar findings and also the production of IL-17 could be obtained by coculturing EpCAM<sup>+/-</sup> tumor cell lines with PBMCs from healthy volunteers in the presence of CatmAb (Supplementary Figs. S1 and S2). CatmAb-induced T-cell activation significantly increased the production of IFN- $\gamma$ , IL-17A, IL-6, and IL-1 $\beta$ , hence contributing to a modulation of the local microenvironment (Fig. 3A and B). CatmAb deviated the natural T<sub>H</sub>2 inflammation by decreasing the concentrations of IL-4 and IL-13 while augmenting the T<sub>H</sub>1 cytokines in most patients (Fig. 3C, left and right).

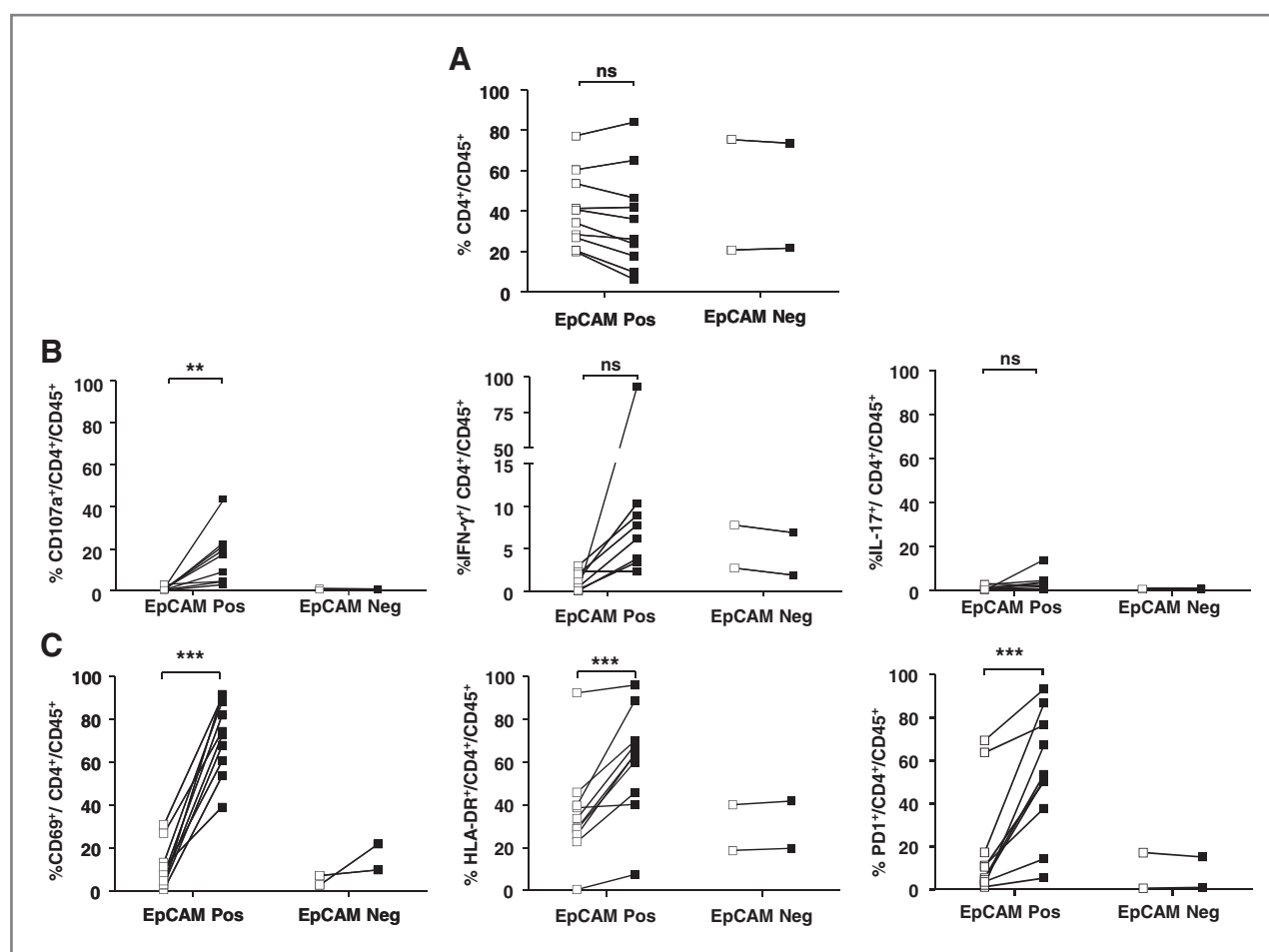
Malignant ascites contained up to 5% (8.25  $\pm$  5) of CD3<sup>+</sup>CD4<sup>+</sup>Foxp3<sup>+</sup> T cells among CD4<sup>+</sup>T cells (Fig. 4A) that could be analyzed according to CD45RA (15). Most of these regulatory T cells (Treg) became Foxp3<sup>high</sup> CD45RA<sup>-</sup>CD73<sup>+</sup> after exposure to CatmAb (Fig. 4B and C).

CatmAb activated local T cells and geared their polarization toward an inflammatory T<sub>H</sub>1 pattern despite the presence of ectoATPase-expressing Treg.

### Engagement of CD16-expressing cells in malignant ascites incubated with CatmAb

NK cells were not enriched following CatmAb treatment (Fig. 5A, left). Most NK cells from ascites TILs were CD16<sup>+</sup> at harvesting, but CatmAb did not significantly reduce CD16 expression on NK cells (Fig. 5A, right). However, NK cells were markedly activated by CatmAb, as shown by their upregulated levels of CD69 and TRAIL expression (Fig. 5B, left and middle, and, to a lesser extent NKp44 expression, right) only in EpCAM<sup>+</sup> ascites. Similar findings were achieved in cocultures of PBMC and allogeneic EpCAM<sup>+</sup> tumor cells modulated by CatmAb (Supplementary Fig. S3) in that NK cells became CD69<sup>+</sup> TRAIL<sup>+</sup> while expressing low levels of CD107A and IFN- $\gamma$  in the presence of the trifunctional Ab.

Most monocytes, albeit underrepresented in TILs (Fig. 5C, left), also expressed CD16 molecules (Fig. 5C, right), which upon engagement with CatmAb, decreased to some extent (Fig. 5C, right). CatmAb markedly increased CD80 and CD40



**Figure 1.** Activation of malignant ascites-derived CD4<sup>+</sup> T cells by CatmAb. Flow cytometric analyses of CD3<sup>+</sup>CD4<sup>+</sup> T cells gated in the CD45<sup>+</sup> fraction of TIL of various malignant ascites samples ( $n = 12$ ) incubated with [■] or without [□] CatmAb for 18 hours. A, percentages of CD4<sup>+</sup> T cells are indicated. B, intracellular staining for CD107A, IFN- $\gamma$ , and IL-17. C, membrane staining for CD69, HLA-DR, and PD-1. Each dot represents one patient's ascitic fluid. Two patients harboring an EpCAM-negative cancer were used as negative controls. Student  $t$  test to compare mean  $\pm$  SEM of two groups was used: \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ . ns, not statistically significant.

molecules on CD14<sup>+</sup>CD16<sup>low</sup> TIL monocytes (Fig. 5D, left and right) while maintaining HLA-DR expression (Fig. 5D, middle).

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

#### Tumor cell death fingerprint post-CatmAb

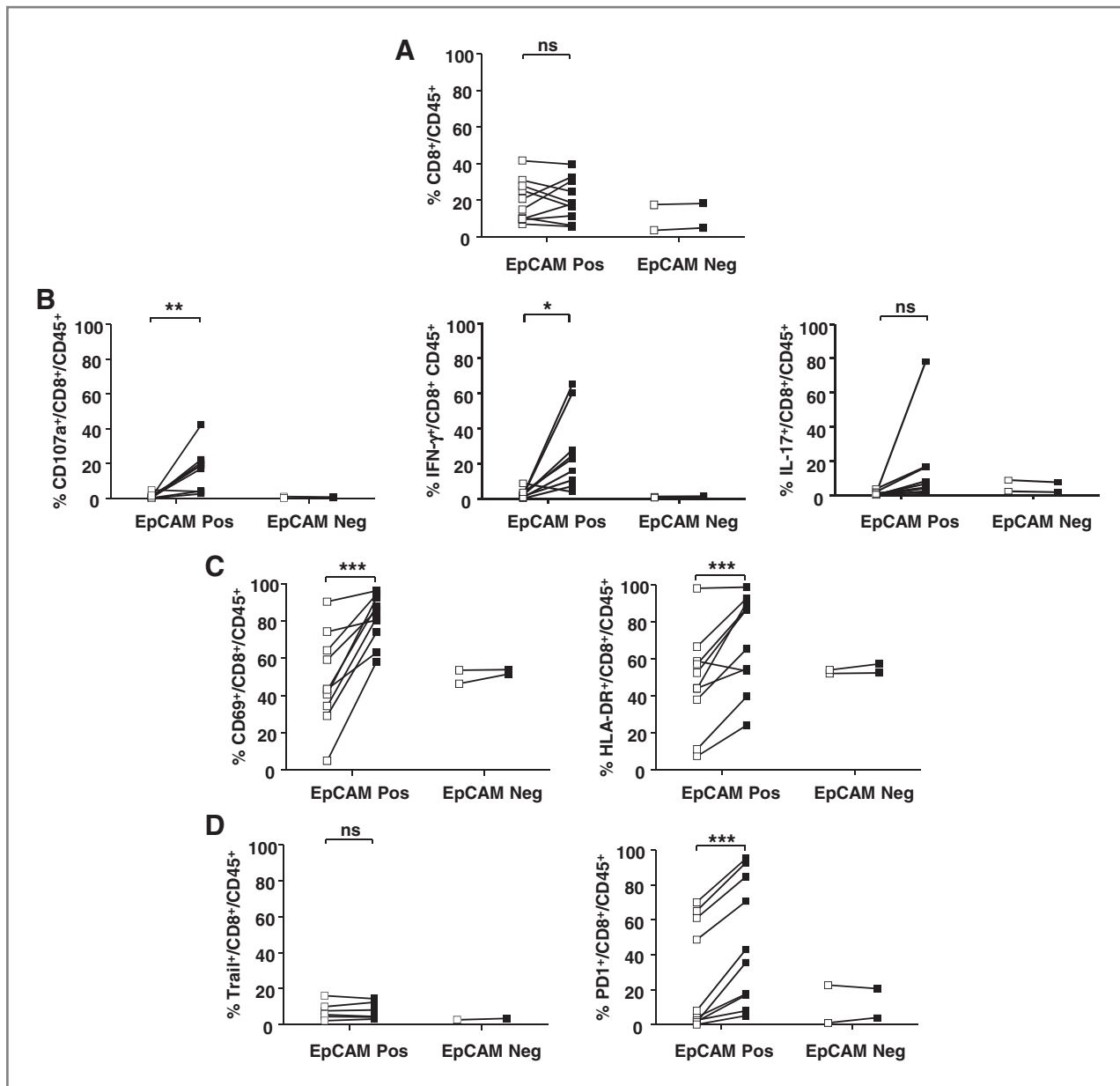
Immunogenic cell death can be defined as a modality of cell demise associated with T-cell-dependent tumor rejection and long-term survival, and correlated with the exposure or release of cell death-associated molecular (CDAM) patterns (16). Briefly, stress before death matters for an immunogenic cell death pathway to occur. First, an endoplasmic reticulum stress response culminating with 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 cell death could trigger an immunogenic cell death

program. We analyzed by flow cytometry the expression of Annexin V and 7AAD on EpCAM<sup>+</sup>CD45<sup>-</sup> 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) 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). In contrast, preincubation of tumor cells (SKBR3) with oxaliplatin did not increase cell death by CatmAb (Fig. 6C, left) but mediated stress and increased CRT exposure (Fig. 6C, right) and HMGB1 (Fig. 6D, left) and ATP release (Fig. 6D, right) 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 T<sub>H</sub>1 cells (capable of degranulating

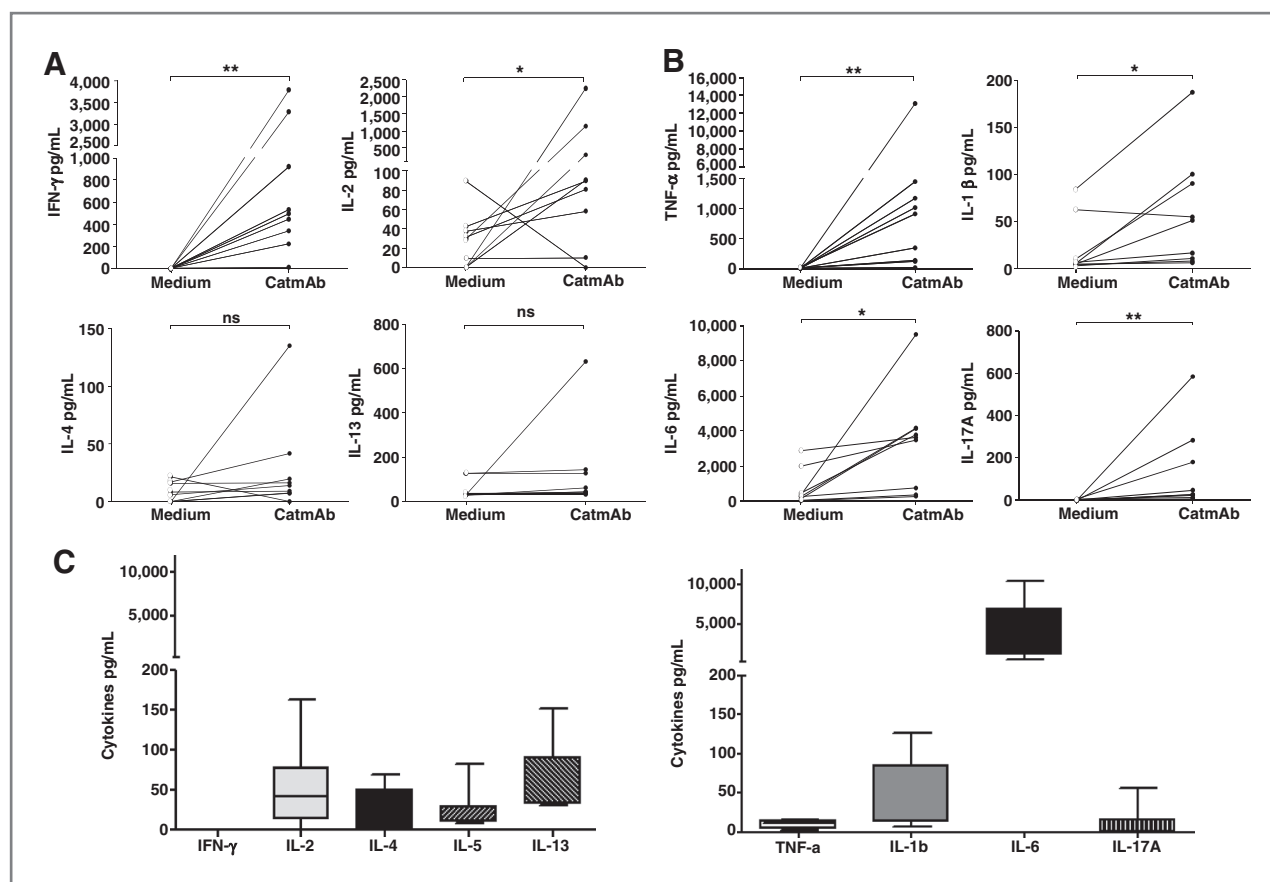


**Figure 2.** Activation of malignant ascites derived CD8<sup>+</sup> T cells by CatmAb. Flow cytometric analyses of CD3<sup>+</sup>CD8<sup>+</sup> T cells gated in the CD45<sup>+</sup> fraction of TIL of various malignant ascites samples ( $n = 12$ ) incubated with (■) or without (□) CatmAb for 18 hours. A, percentages of CD8<sup>+</sup> T cells are indicated. B, intracellular staining for CD107A, IFN- $\gamma$ , 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 harboring an EpCAM-negative cancer were used as negative controls. Student *t* test to compare mean  $\pm$  SEM of two groups was used: \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ . ns, not statistically significant.

and secreting IFN- $\gamma$ ), engages CD16<sup>+</sup> cells to express TRAIL (for NK cells) and costimulatory molecules (for monocytes). In conjunction with oxaliplatin, CatmAb triggered the 3 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, and (iii) stimulating antigen-presenting cells through the Fc $\gamma$ R. The increase rate of CD4<sup>+</sup> T cells showing surface mobilization of

CD107a was unsuspected because surface mobilization of CD107a by CD8<sup>+</sup> T cells means that they can release cytotoxic granules, a function not often associated with CD4<sup>+</sup> 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<sup>+</sup> T cells during viral infections. In such circumstances, CD4<sup>+</sup> T cells can acquire cytotoxic functions resulting in the surface expression of CD107a and the release of granzyme A and granzyme B (22–25).



**Figure 3.** Cytokine profiles of the ascites milieu are changed following exposure to CatmAb. A,  $T_H$  cytokines. Monitoring by Flow Cyto Mix (eBioscience) of 4 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 (eBioscience) of 4 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 (before incubation with CatmAb) of malignant ascitic fluids using commercial Flow Cyto Mix (eBioscience). Ten various malignant ascites samples were tested. Student  $t$  test to compare mean  $\pm$  SEM was used: \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

Although it is widely shown 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, 2 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 mesenteric nodular fibrosis and granulomatosis were observed postchemotherapy and surgery in adjuvant intraperitoneal CatmAb injections and seemed to be associated with a complete and long-term response in an advanced gastric adenocarcinoma (27). Ströhlein and colleagues showed tumor-reactive  $CD4^+/CD8^+$   $T_H1/Tc1$ -lymphocytes in PBMC after restimulation in 5 of 9 patients suffering from peritoneal carcinomatosis, indicating that a long-term specific antitumor 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 Fc $\gamma$ R-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 endoplasmic reticulum stress response culminating in the exposure, to the cell surface, of an endoplasmic reticulum-resident chaperone, CRT represents an "eat-me signal" for engulfment of tumor cells by inflammatory phagocytes (17, 34).

Although 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

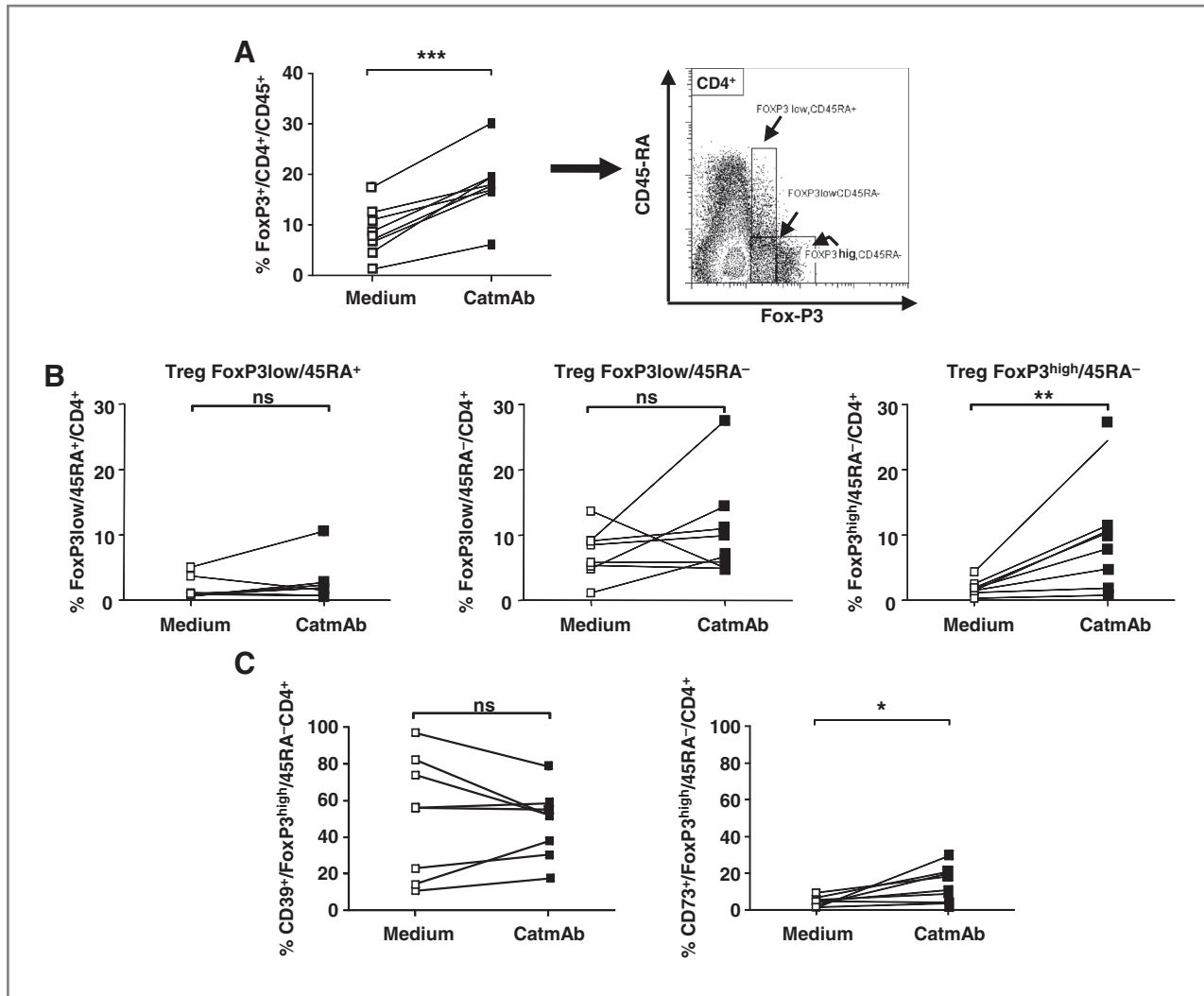
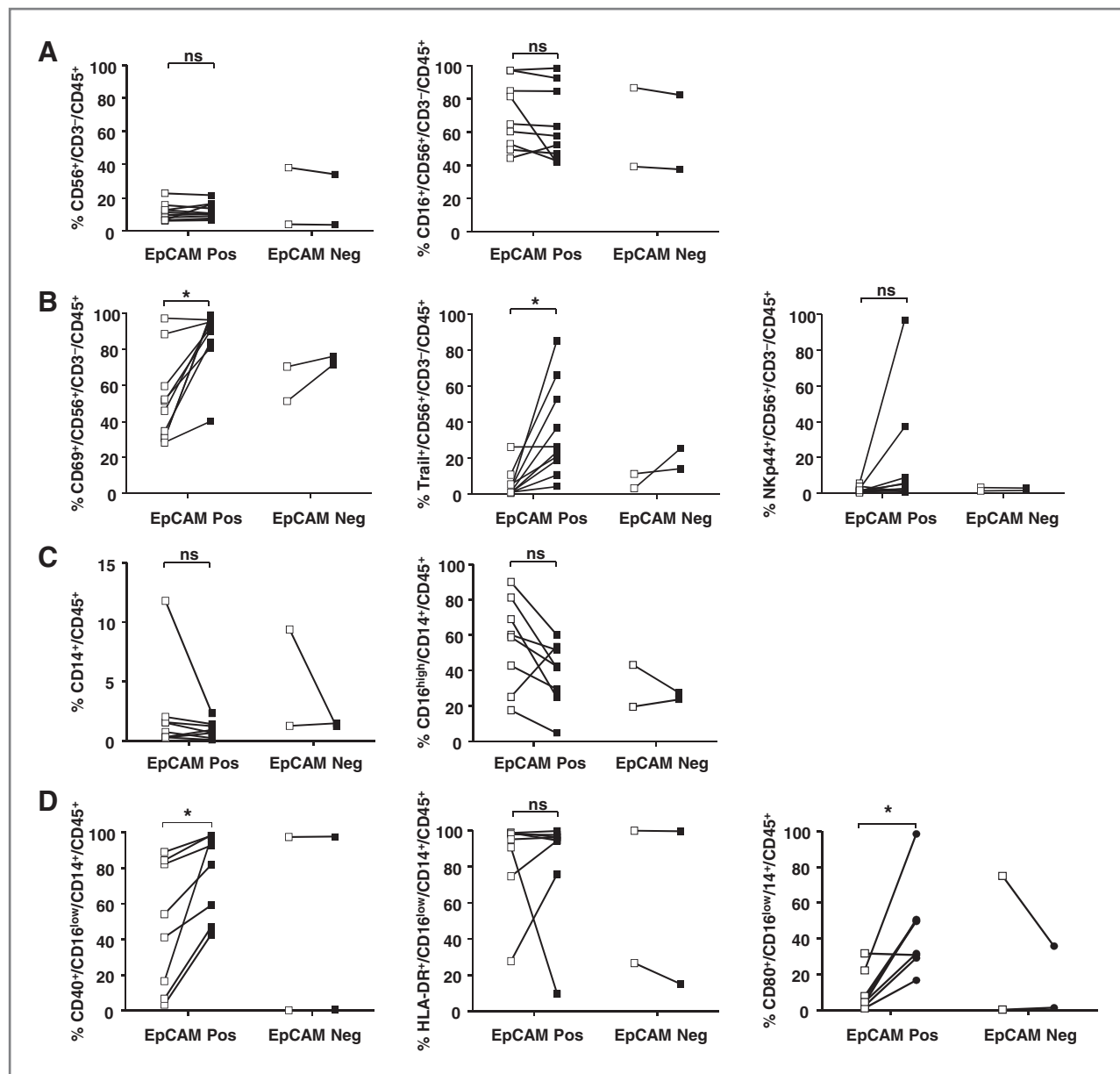


Figure 4. Treg expressed ecto-ATPases post-CatmAb. A and B, flow cytometric analyses of CD3<sup>+</sup>CD4<sup>+</sup> Foxp3<sup>+</sup> T cells gated in the CD45<sup>+</sup> fraction of TIL of various malignant ascites samples ( $n = 8$ ) incubated with (■) or without (□) CatmAb for 18 hours. Percentages of Foxp3<sup>+</sup>CD4<sup>+</sup> T cells are indicated in A as well as CD45RA<sup>+/−</sup>Foxp3<sup>+</sup> cells in B. C, membrane staining for CD39 and CD73 in the gate Foxp3<sup>high</sup>CD45RA<sup>−</sup>. Each dot represents one patient's ascitic fluid. Student *t* test to compare mean  $\pm$  SEM of two groups was used. ns, not statistically significant.

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, Hoves and colleagues showed that granzyme A/B-mediated lysis is involved in enhancing uptake and cross-presentation of dying bodies by CD8 $\alpha$ <sup>+</sup> DC in mouse models (36). They showed that blocking phosphatidyserine did not hamper this granzyme-dependent engulfment of dying tumor cells. It is conceivable that additional eat-me signals different from CRT be exposed postdegranulation by T cells that could be recognized by the human ortholog of mouse CD8 $\alpha$ <sup>+</sup> DC (37). However, very few BDCA3<sup>+</sup> DC could be recovered from ascitic fluids in our model system (data not shown). The lack of expression of CRT does not seem to be related to a low effector to tumor cell ratio. First, there was no significant correlation between the E:T ratio of CD45<sup>+</sup>/EpCAM<sup>+</sup> cells (ranging from 2–1,402; Table 1) and T-cell activation parameters. Second, in a randomized trial (13)

where the median proportion of EpCAM<sup>+</sup> cells in ascites cells was 0.6%, a clinical benefit associated with a decreased percentage of EpCAM<sup>+</sup> cells in ascites was reported.

CatmAb-mediated cell death could not promote the release of the nonhistone 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 knockdown 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



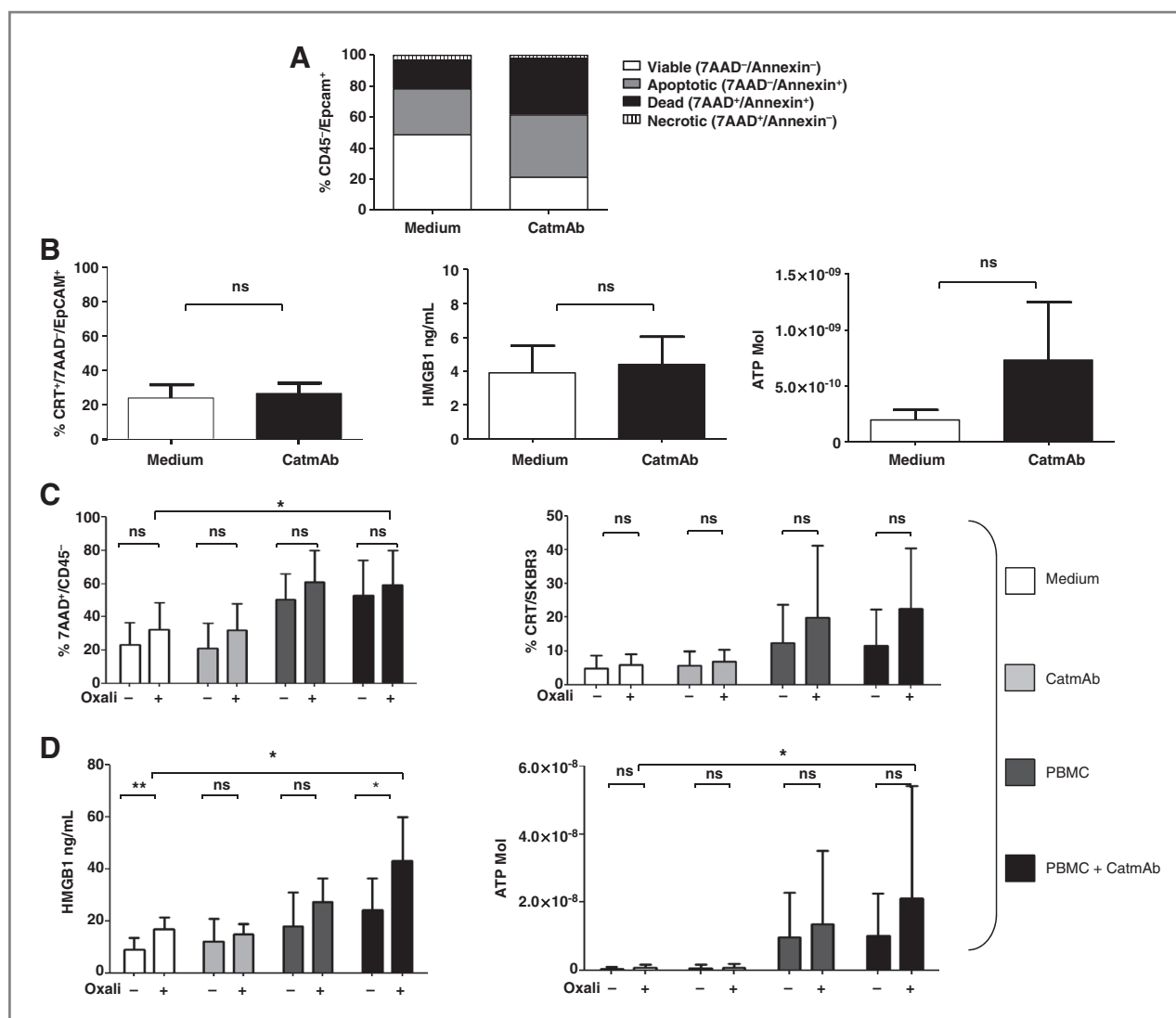
**Figure 5.** CatmAb-induced TRAIL and costimulatory molecule expression on CD16<sup>+</sup> cells. A and B, flow cytometry analyses of CD3<sup>-</sup> CD56<sup>+</sup> NK cells gated in the CD45<sup>+</sup> fraction of TIL of various malignant ascites samples ( $n = 12$ ) incubated with (■) or without (□) CatmAb for 18 hours. Percentages of NK cells and CD16<sup>+</sup> NK cells are indicated (A). Low levels of CD107A<sup>+</sup> or IFN- $\gamma$ <sup>+</sup> NK cells could be found after exposure with CatmAb (not shown). Membrane staining for CD69, TRAIL, and NKp44 (B). C and D, flow cytometric analyses of CD14<sup>+</sup> monocytes gated in the CD45<sup>+</sup> fraction of TIL of various malignant ascites incubated with (■) or without (□) CatmAb for 18 hours. Percentages of CD14<sup>+</sup> cells (left) and CD14<sup>+</sup>CD16<sup>high</sup> cells (right) are indicated (C). Expression levels of CD40, HLA-DR, and CD80 on CD14<sup>+</sup>CD16<sup>low</sup> cells (D). Each dot represents one patient's ascitic fluid. Two patients harboring an EpCAM-negative cancer were used as negative controls. Student  $t$  test to compare mean  $\pm$  SEM of two groups was used: \*,  $P < 0.05$ . ns, not statistically significant.

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 2 key events: first, the recruitment of CD11c<sup>+</sup> DC in tumor beds postchemotherapy or cell demise (26), second the engagement

of P2RX7 on DC, which activates the inflammasome NLRP3, culminating in IL-1 $\beta$  release and IL-1R1-dependent Tc1 polarization (39). Ecto-ATPases expressed on regulatory T<sub>H</sub>17 (40) and Tregs (41) might impair the recruitment of DC and/or the differentiation of IFN- $\gamma$  producing CTLs posttreatment. Indeed, CatmAb-treated malignant ascites contained increased levels of memory Treg expressing CD73 (Fig. 4). However, such Tregs did not seem to inhibit the strong polarization of TILs toward an inflammatory T<sub>H</sub>1 profile (TNF- $\alpha$ , IFN- $\gamma$ ,





**Figure 6.** Hallmark criteria of immunogenic cell death post-CatmAb. **A**, flow cytometric determination of living, apoptotic (7AAD<sup>-</sup>/Annexin<sup>V+</sup>), dead, or necrotic (Annexin V<sup>+</sup>/7AAD<sup>+</sup>) cells in the gate CD45<sup>+</sup> EpCAM<sup>+</sup>, featuring tumor cells of the ascitic 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** and **D**, allogeneic mixed PBMC/tumor *ex vivo* cocultures incubated with or without CatmAb for 18 hours (left) and after 2 hours of presensitization of oxaliplatin chemotherapy before cocultures (right). Flow cytometric analyses of 7AAD<sup>+</sup> cells (**C**), CRT exposure (**D**, left), ELISA assessment of HMGB1 secretion (**D**, middle), ATP release monitored by luciferase assays (**D**, right). Mean ± SEM for 4 to 6 independent experiments are depicted. ANOVA and Fisher exact method were used to compare mean ± SEM among four groups. Significant *P* values are indicated: \*, *P* < 0.05; \*\*, *P* < 0.01. ns, not statistically significant.

IL-2; Figs. 1B, 2B, and Fig. 3), starting from ascites containing T<sub>H</sub>2 cytokines (Fig. 3C) and poorly activated T cells (Figs. 1 and 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 postoperative 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

toward an inflammatory T<sub>H</sub>1 pattern, and (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.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** D. Goéré, C. Flament, D. Elias, N. Chaput, L. Zitvogel  
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**Study supervision:** D. Goéré, D. Elias, N. Chaput, L. Zitvogel

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