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CHAPTER 3

A novel bispecific antibody for EpCAM-directed inhibition of the CD73/adenosine immune checkpoint in ovarian cancer

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SIMPLE SUMMARY

Blockade of the immunosuppressive CD73/ADO immune checkpoint has been suggested as a promising alternate immunotherapeutic approach for refractory ovarian cancer (OC). Despite promising preclinical results, midterm clinical trial reports indicate that the efficacy of the CD73-blocking antibody oleclumab is modest. The limited efficacy of oleclumab may be related to the fact that it indiscriminately binds and blocks to CD73 that is present on a massive surplus of normal cells. The aim of our study was to achieve a tumor-directed inhibition of the CD73 immune checkpoint on OC cells. To this end, we constructed a novel bispecific antibody, bsAb CD73xEpCAM, that blocks the CD73 immune checkpoint in an EpCAM-directed manner. Moreover, treatment of OC cells with bsAb CD73xEpCAM inhibited various pro-oncogenic features. Taken together, bsAb CD73xEpCAM may be useful as an alternate and more tumor-directed immunotherapeutic approach to overcome CD73-mediated immunosuppression in OC patients.

ABSTRACT

PD-1/PD-L1-inhibiting antibodies have shown disappointing efficacy in patients with refractory ovarian cancer (OC). Apparently, OC cells exploit nonoverlapping immunosuppressive mechanisms to evade the immune system. In this respect, the CD73-adenosine inhibitory immune checkpoint is of particular interest as it rapidly converts pro-inflammatory ATP released from cancer cells to immunosuppressive adenosine (ADO). Moreover, cancer cell-produced ADO is known to form a highly immunosuppressive extra-tumoral ‘halo’ that chronically inhibits the anticancer activity of various immune effector cells. Thus far, conventional CD73-blocking antibodies like oleclumab show limited clinical efficacy, probably due to the fact that it indiscriminately binds and blocks to CD73 on a massive surplus of normal cells. To address this issue, we constructed a novel bispecific antibody (bsAb) CD73xEpCAM that inhibits OC cell surface-expressed CD73 in an EpCAM-directed manner. Importantly, bsAb CD73xEpCAM showed potent capacity to inhibit CD73 enzyme activity in an EpCAM-directed manner and restored the cytotoxic activity of ADO-suppressed anticancer T cells. Additionally, treatment with bsAb CD73xEpCAM potently inhibited the proliferative capacity of OC cancer cells and enhanced their sensitivity to cisplatin, doxorubicin, 5FU, and ionizing radiation. BsAb CD73xEpCAM may be useful in the development of tumor-directed immunotherapeutic approaches to overcome CD73-mediated immunosuppression in patients with refractory OC.

INTRODUCTION

Typically, tumors in patients with ovarian cancer (OC) are often diagnosed at an advanced stage[1]. Currently, cytoreductive therapy with platinum-based chemotherapy is the mainstay of disease management, to which the majority of patients with OC initially respond. Unfortunately, 70% of these patients eventually develop refractory recurrences[2]. Therefore, novel therapeutic approaches in advanced OC are urgently needed. In this respect, an immunotherapeutic anticancer approach based on immune checkpoint inhibitors appears promising because OC tumors are frequently populated with tumor-infiltrating lymphocytes (TILs). Moreover, the degree of TIL infiltration in OC tumors independently correlates with patient survival[3]. However, recent trials have reported only modest activity for various PD-1/PD-L1-inhibiting antibodies in relapsed OC, with disappointing response rates of only 4% to 15%[4–6].

Apparently, OC exploits non-overlapping immunosuppressive mechanisms to achieve immune evasion. In this respect, the CD73-adenosine inhibitory immune checkpoint is of particular interest[7]. Many cancer types, including OC, appear to exploit CD73 overexpression to affect the immunosuppressive capacity of the tumor environment (TME). CD73 is an GPI-anchored ecto-5′-nucleotidase CD73 that is involved in the rapid conversion of pro-inflammatory extracellular ATP (eATP), released from cancer cells due to metabolic or therapy-induced stress, to adenosine (ADO), one of the most potent immunosuppressive regulatory molecules. In this process, eATP is rapidly metabolized by ecto-enzyme CD39 to adenosine monophosphate (AMP), which is a substrate for rate-limiting hydrolysis to ADO by CD73[8]. The produced ADO molecules form an immunosuppressive ‘halo’ in and beyond the TME that potently inhibits anticancer activity of immune effector cells. Notably, high level CD73 expression in the TME of OC cells was reported to abrogate the favorable prognosis associated with the presence of TILs[9].

Inhibition of the enzyme activity of CD73 is currently being explored as an alternative or complementary form of cancer immunotherapy[10]. Various CD73 antagonistic antibodies, the most notable being oleclumab (MEDI9447)[11], are currently undergoing clinical evaluation. Unfortunately, midterm reports on the treatment of relapsed OC patients with oleclumab alone or in combination with antagonistic PD-L1 antibody durvalumab indicate limited efficacy[12]. This lack of efficacy may be related to the fact that CD73 is broadly expressed on a wide variety of normal cells and tissues, potentially forming an antigen sink that precludes sufficient accretion of oleclumab at tumor lesions[13]. Moreover, generalized inhibition of CD73 enzyme activity by oleclumab may potentially lead to autoimmune-related adverse events analogous to those observed for other clinically used immune checkpoint inhibitors.

Therefore, novel approaches are urgently needed to inhibit OC cell-expressed CD73 in a more tumor-directed manner. In this respect, bispecific antibodies (bsAbs) may be useful to improve the efficacy and safety of cancer immunotherapy, as they can be engineered to more selectively direct immune checkpoint blockade to cancer cells[14]. Therefore, we engineered a novel tetravalent bsAb, designated bsAb CD73xEpCAM, that selectively binds to the pancarcinoma-associated cell surface antigen ‘Epithelial Cell Adhesion Molecule’ (EpCAM) and concurrently blocks the CD73 immune checkpoint. We selected EpCAM for this purpose as it is overexpressed by 55% to 75% of OC patients. Moreover, EpCAM overexpression in these patients correlated with enhanced cancer cell proliferation, oncogenic signaling, chemo-radiotherapy resistance[15,16], and decreased overall survival[16–18].

Here, we demonstrate that bsAb CD73xEpCAM has potent capacity to inhibit the CD73/adenosine immune checkpoint exposed on ovarian cancer cells in an EpCAM-directed manner. In particular, bsAb CD73xEpCAM restored the anticancer activity of T cells when incapacitated by OC cell-produced ADO. Additionally, *in vitro* treatment of OC cells with bsAb CD73xEpCAM potently inhibited their proliferative capacity and sensitized them to the cytotoxic activity of cisplatin, doxorubicin, 5FU, and ionizing radiation.

MATERIALS AND METHODS

Antibodies and reagents

Antibodies: Goat-anti-human-IgG APC (SouthernBiotech), mAbMM07 FITC (Sino Biological), and EpCAM APC (Abcam).

Reagents/proteins/kits: fluorescent caspase 3/8-488 probe (Biotium), crystal violet staining (Abcam), APCP (Sigma), recombinant soluble human CD73 (sCD73) (Abcam), recombinant soluble human EpCAM (sEpCAM) (Abcam), CFSE CellTrace Far Red cell proliferation kit (Thermofisher), colorimetric malachite green-based Pi assay kit (ab65622, Abcam), T cell activation/expansion beads (Miltenyi Biotec), and IFNγ ELISA kit (eBioscience)

Cell lines and transfectants

Cell lines OvCAR3, ES2, SKOV3, L37, SK-N-SH, A172, U87GM and CHO-K1 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). L37.EpCAM is a rat (Wag/Rij) squamous-cell lung carcinoma cell line stably transfected with human EpCAM[19]. Cells were cultured in RPMI-1640 or DMEM (Lonza) as indicated, supplemented with 10% fetal calf serum (FCS, Thermofisher) at 37°C in a humidified 5% CO_2 atmosphere (unless specified otherwise). CHO-K1 cells were cultured in GMEM (First Link), supplemented with 5% dialyzed FBS (Sigma Aldrich).

CHO.CD73 cells stably expressing human CD73 were generated by lipofection (Fugene-HD, Promega) using a eukaryotic plasmid containing cDNA encoding human CD73 (Origene). Likewise, CHO.EpCAM and L3745R.EpCAM cells were generated by lipofection using plasmids containing cDNA encoding human EpCAM (Sino Biological).

OvCAR3 CD73-Knockout (KO) cells were generated using CRISPR-Cas9 gene editing technology by transfection of pSpCas9 BB-2A-GFP (PX458) plasmid (Addgene plasmid #48138) containing the CD73-targeting sgRNA 5′-GCAGCACGTTGGGTTCGGCG-3′[20]. Likewise, OvCAR3 EpCAM-KO cells were generated by transfection of pSpCas9 BB-2A-GFP (PX458) plasmid containing the EpCAM-targeting sgRNA 5′-TAATGTTATCACTATTGATC-3′[21].

Construction of bsAb CD73xEpCAM-IgG2silent

DNA fragments encoding scFvCD73 and scFvEpCAM were generated by commercial gene synthesis service (Genscript) based on published VH and VL sequence data. For construction and production of bsAb CD73xEpCAM-IgG2silent, we used eukaryotic expression plasmid pbsAb, which contains 3 consecutive multiple cloning sites (MCS). MCS#1 and MCS#2 are interspersed by a 22 amino acid flexible linker[22]. MCS#1, MCS#2 were used for directional and in-frame insertion of DNA fragments encoding scFvCD73, scFvEpCAM, and MCS#3 for insertion of DNA fragments encoding human Fc IgG2s[23]. Analogously, pbsAb-CD73xMock-IgG2s encoding bsAb CD73xMock-IgG2s was constructed by replacing scFvEpCAM in pbsAb-CD73xEpCAM-IgG2s by scFvMCSP directed against CSPG4[24,25]. Likewise, pbsAb-MockxEpCAM-IgG2s encoding bsAb MockxEpCAM-IgG2s was constructed by replacing scFvCD73 in pbsAb-CD73xEpCAM-IgG2s by scFv4-4-20 directed against fluorescein[25–27].

Production of recombinant bsAbs

The Expi293 expression system (ThermoFisher) was used to produce bsAbs CD73xEpCAM-IgG2s, CD73xMock-IgG2s, and MockxEpCAM-IgG2s. Briefly, Expi293 cells were transfected with plasmid encoding the bsAb of choice and cultured on a shaker platform (125 rpm) at 37°C, 8% CO2 for 7 d. Next, conditioned culture supernatant was harvested and cleared by centrifugation (4000 x g for 30 min), after which bsAbs were purified using an HiTrap Mab-select column connected to an ÄKTA Start chromatography system (GE Healthcare Life Sciences).

Assessment dual binding activity of bsAb CD73xEpCAM

CHO, CHO.CD73 or CHO.EpCAM cells were incubated with increasing concentrations (0.01 - 10 µg/ml) of bsAbs CD73xEpCAM or bsAb CD73xMock at 4°C for 45 min. Unbound bsAb was removed by washing 3 times with cold PBS, after which cells were re-incubated with an APC-labeled secondary goat-anti-human-Ig-antibody at 4°C for 45 min. Binding data of the respective bsAbs were acquired using a Guava EasyCyte 6/2L flow cytometer (Merck Millipore) and analyzed using GuavaSoft 3.2 software.

The binding of bsAb CD73xEpCAM (1 µg/ml) to OvCAR3 cells was accessed in the competing presence of sCD73, sEpCAM, or a combination thereof (each 10 µg) at 4°C for 20 min. Binding of bsAb CD73xEpCAM to cancer cells was evaluated by flow cytometry using an APC-labeled goat-anti-human-Ig-antibody essentially as described above.

Binding of bsAb CD73xEpCAM (0.01 - 10 µg/ml) to L37 and L37.EpCAM rat cancer cells[19] was evaluated by flow cytometry using an APC-labeled goat-anti-human-Ig-antibody essentially as described above. Similar, binding of bsAb CD73xEpCAM (1 µg/ml) (or appropriate controls) to OvCAR3 parental, CD73-KO, and EpCAM-KO variants thereof was evaluated by flow cytometry using an APC-labeled goat-anti-human-Ig-antibody essentially as described above.

Binding of bsAb CD73xEpCAM to OvCAR3 cells was assessed over time. In short, OvCAR3 cells were incubated for 30 s with bsAb CD73xEpCAM (1 µg/ml) (or appropriate controls) and washed with cold PBS. Next, cells were cultured at 37 °C and, at indicated time points, washed again with cold PBS. Binding of bsAb CD73xEpCAM to OvCAR3 cells was evaluated by flow cytometry using an APC-labeled anti-human-Ig-antibody essentially as described above.

Assessment of internalization of bsAb CD73xEpCAM/antigen complexes

The capacity of bsAb CD73xEpCAM to internalize cancer cell surface-exposed CD73 upon concurrent binding to EpCAM was assessed using OvCAR3 cells by flow cytometry. In short, cancer cells were incubated with bsAb CD73xEpCAM (1 µg/ml) (or controls) at 37°C for 5 h, after which residual cancer cell surface presence of CD73 was assessed using anti-CD73 mAbMM07. Of note, mAbMM07 binds to a non-overlapping epitope on CD73 and as such does not interfere with the CD73-binding capacity of bsAb CD73xEpCAM.

Assessment capacity of bsAb CD73xEpCAM to inhibit the enzyme activity of CD73

Inorganic phosphate (Pi) formed during CD73-mediated hydrolysis of AMP to ADO was measured using a colorimetric malachite green-based Pi assay kit. In short, OvCAR3 cells were treated with bsAb CD73xEpCAM (1 µg/ml) (or appropriate controls) at 37°C. At indicated time points, cells were washed with cold PBS and incubated at 37°C for 24 h. Subsequently, cells were washed (20 mM HEPES, 120 mM NaCl, 5 mM KCl, 2 mM

MgCl2, 10 mM Glucose, pH 7.4) to remove residual Pi-containing medium and were then incubated with AMP (100 µM, Sigma-Aldrich) at 37°C for 40 min. The supernatant was mixed with phosphate reagent and color development was evaluated by measuring the absorbance at 650 nm using a microplate reader (VERSA max, Molecular Devices) and corrected by subtracting background levels.

The % of enzyme inhibition was calculated according to the following formula:

$$\% \text{ of CD73 enzyme inhibition} = 100 - ((\frac{\text{X}}{\text{OD650max}}) * 100)$$

X = is the OD value measured in a given experiment minus the background (OD650exp- OD650background)
OD650max = the amount of Pi present in the conditioned supernatant in the absence of bsAb

The EpCAM-directed capacity of bsAb CD73xEpCAM to inhibit enzyme activity of cancer cell surface-exposed CD73 was assessed using a panel of CD73^{pos}/EpCAM^{pos} (OvCAR3, ES2, and SKOV3) and CD73^{pos}/EpCAM^{neg} (SK-N-SH, A172, and U87GM) cell lines. In short, cancer cells were treated with bsAb CD73xEpCAM (1 µg/ml) (or appropriate controls) for 15 min, washed with cold PBS, and then incubated at 37°C for 24 h. Similarly, OvCAR3 WT and EpCAM-KO cells were treated with increasing concentrations (0.01 – 10 µg/ml) bsAb CD73xEpCAM for 15 min, washed with cold PBS, and then incubated at 37°C for 24 h. The CD73 enzyme activity was assessed essentially as described above.

The capacity of bsAb CD73xEpCAM to block enzyme activity CD73 by OvCAR3 cells EpCAM-directed manner was also assessed in the competing presence of recombinant sEpCAM (10 µg) at 4°C for 20 min. Subsequently, OvCAR3 cells were treated with bsAb CD73xEpCAM (1 µg/ml) for 15 min, washed with cold PBS and incubated at 37°C for 24 h, and then assessed for inhibition of the enzyme activity of CD73 essentially as described above.

Assessment capacity of bsAb CD73xEpCAM to restore proliferation capacity of ADO-suppressed T cells

Peripheral Blood Mononuclear Cells (PBMCs) obtained from healthy donors were labeled with cell permeable fluorescent dye CFSE-FarRed (Thermofisher) according to manufacturer protocol and re-suspended in RPMI-1640. Next, CFSE-labeled PBMCs were treated (or not) with bsAb CD73xEpCAM (1 µg/ml) (or appropriate controls) for 15 min, washed, and activated by addition of T cell activation/expansion beads (Miltenyi Biotec) in a bead-to-cell ratio of 1-1 in medium supplemented (or not) with AMP (100 µM). Live cell imaging technology (IncuCyte) was used to evaluate the size of T cell clusters by taking pictures at 4 x magnification every 6 h for 7 d. The area (µm^2/image) of activated T cell clusters was quantified using IncuCyte software 2019B.

Assessment capacity of bsAb CD73xEpCAM to restore anticancer activity of ADO-suppressed T cells

OvCAR3 cancer cells were treated (or not) with bsAb CD73xEpCAM (1 µg/ml) (or appropriate controls) for 15 min, washed and incubated in medium supplemented (or not) with AMP (100µM) for at 37°C for 24 h. Next, freshly isolated PBMCs were added at different effector (E) to target (T) cell ratios to OvCAR3 cancer cells, and then co-cultured at 37°C for 24 h. Subsequently, T cells present in the PBMC population were stimulated and re-directed to kill OvCAR3 cancer cells using EpCAM-directed/CD3-agonistic bispecific antibody BIS-1[28] at 37°C for 24 h. Of note, at the concentration range used, binding of BIS-1 to OvCAR3 cancer cells is not impaired by bsAb CD73xEpCAM. Apoptotic cancer cell death was assessed by flow cytometry and shown as percentage Annexin-V^{pos}/PI^{pos} events.

Assessment inhibitory effect of bsAb CD73xEpCAM on proliferation of cancer cells

OvCAR3 or ovarian carcinoma patient-derived cancer cells (8 × 10^3/well) were treated with bsAb CD73xEpCAM (1 µg/ml) (or appropriate controls) for 15 min, washed, and then seeded onto an E-plate 16 (ACEA Biosciences). Cell proliferation was monitored using the xCELLigence RTCA instrument (ACEA Biosciences) at 37°C for 60 h or 144 h, respectively.

OvCAR3 cancer cells (200/well) were treated with increasing concentrations (0.1 - 2 µg/ml) bsAb CD73xEpCAM (or appropriate controls) for 15 min, washed, and then cultured in a 6-well culture plate at 37°C for 14 d. Next, cancer cell colonies were washed with PBS and stained with crystal violet. Cancer cell colony number and size were analyzed using ImageJ software.

Assessment sensitization of cancer cells by bsAb CD73xEpCAM for chemotherapeutic agents and ionizing radiation

OvCAR3 cancer cells (3 × 10^3/well) were treated with bsAb CD73xEpCAM (1 µg/ml) (or appropriate controls) for 15 min, washed, seeded into a 96-well culture plate, and then incubated in the continuous presence (or absence) of 5FU (15 µg/ml), cisplatin (1 µg/ml), or doxorubicin (200 nM) at 37°C for 4 d. Live cell imaging technology (IncuCyte) was used to assess proliferation of cancer cells by taking pictures at 4 x magnification every 4 h for 5 d. Confluence (%) was measured using IncuCyte software 2019B.

OvCAR3 cells (200/well) were treated with (or without) bsAb CD73xEpCAM (1 µg/ml) (or appropriate controls) for 15 min, washed, and then incubated in the continuous presence (or absence) of 5FU (15 µg/ml), cisplatin (1 µg/ml), or doxorubicin (200 nM) in a 6-well culture at 37°C for 14 d. Next, cancer cell colonies were washed with PBS and stained with crystal violet. Cancer cell colony number and size were analyzed using ImageJ software.

OvCAR3 (200/well) were seeded in a 6-well culture plate, treated with (or without) bsAb CD73xEpCAM (1 µg/ml) (or appropriate controls) for 15 min, washed, irradiated with a 2 Gy dose of radiation, and then cultured at 37°C for 14 d. Subsequently, cancer cell colonies were washed with PBS and stained with crystal violet. Colony number and size were analyzed using ImageJ software. Irradiation (0.59 Gy/min) was performed using a ^{137}Ce source (IBL 637 Cesium-137 γ-ray machine).

Statistical analysis

Statistical analysis was done by (multiple) T-test or two-way ANOVA followed by Tukey post-hoc test, as indicated using Prism software. P<0.05 was defined as a statistically significant difference. Where indicated ns = $P>0.05$; * = $P<0.05$; ** = $P<0.01$; *** = $P<0.001$; **** = $P<0.0001$.

RESULTS

bsAb CD73xEpCAM has dual binding specificity for OC-exposed CD73 and EpCAM

BsAb CD73xEpCAM was constructed in a so-called tetravalent bispecific taFv-Fc format (supplementary Figure 1). Its dual binding activity was confirmed using Chinese Hamster Ovary (CHO) cells that were transfected with either CD73 or EpCAM (supplementary Figure 2). Binding of bsAb CD73xEpCAM to OC cells was only slightly reduced in the presence of soluble CD73 (s.CD73), whereas soluble EpCAM (s.EpCAM) strongly inhibited the binding. Binding of bsAb CD73xEpCAM was fully abrogated in the combined competing presence of s.CD73 and s.EpCAM (Figure 1A), indicating that bsAb CD73xEpCAM selectively and simultaneously binds to CD73 and EpCAM. Moreover, bsAb CD73xEpCAM dose-dependently bound to rat cells transfected with human EpCAM (L37.EpCAM) and not to parental L37 cells (Figure 1B). Additionally, low binding of bsAb CD73xEpCAM was detected to CD73-KO - and EpCAM-KO OC cells (Figure 1C). Binding analysis indicated that ~78% of bsAb CD73xEpCAM was still bound to OvCAR3 cells after 5 h, markedly outperforming the binding by oleclumab (~40% binding) (Figure 1D). Importantly, this difference in antibody binding is not due to displacement of cell surface-exposed CD73 caused by internalization of antibody/antigen complexes (Figure 1E).

bsAb CD73xEpCAM treatment potently inhibits CD73 enzyme activity in an EpCAM-directed manner

Treatment of OC cells with bsAb CD73xEpCAM rapidly inhibited the enzyme activity of cancer cell surface-exposed CD73, reaching its full inhibitory activity after 15 min (Figure 2A). In contrast, oleclumab reached its maximum CD73-inhibitory capacity only after 24 h (supplementary Figure 3). Accordingly, bsAb CD73xEpCAM significantly outperformed the CD73-inhibiting activity of both oleclumab and the small-molecule CD73-inhibitor APCP in 3 out of 3 $CD73^{pos}/EpCAM^{pos}$ OC cell lines (Figure 2B), but not in $CD73^{pos}/EpCAM^{neg}$ OC cell lines (Figure 2C). Importantly, bsAb CD73xEpCAM potently inhibited the CD73 enzyme activity in 3 out of 3 $CD73^{pos}/EpCAM^{pos}$ primary patient-derived OC cells (Figure 2D). Additionally, the capacity of bsAb CD73xEpCAM to inhibit CD73-mediated hydrolysis of AMP was largely abrogated in the presence of excess amounts of s.EpCAM (Figure 2E) and when treating EpCAM-KO OC cells (Figure 2F).

bsAb CD73xEpCAM treatment overcomes ADO-mediated suppression of T cell proliferation

When CFSE-labeled T cells were subjected to AMP, which is enzymatically converted to ADO by CD73, and subsequently stimulated to proliferate using T cell activation beads, their proliferative capacity was significantly repressed, as can be appreciated from the decrease in size of T cell clusters. Importantly, treatment of PBMCs with bsAb CD73xEpCAM fully overcomes the ADO-mediated inhibition of T cell proliferation, whereas oleclumab failed to do so (Figure 3A). These results corroborate the concurrent increase in clusters of activated T cells over time (Figure 3B).

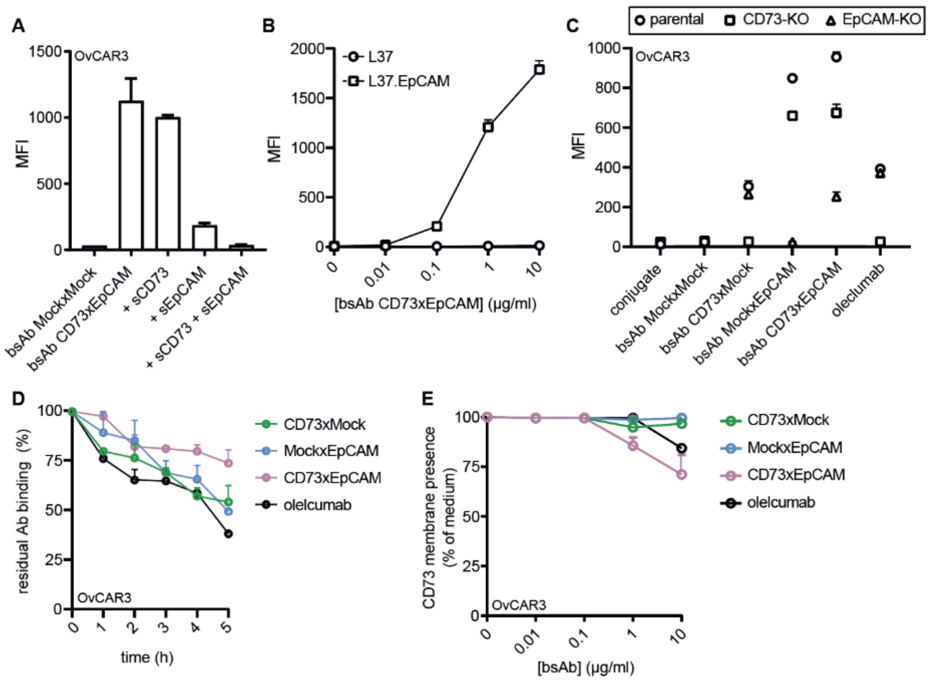


Figure 1: bsAb CD73xEpCAM has dual binding specificity for CD73 and EpCAM. *(A) Competitive binding assay in which bsAb CD73xEpCAM (1 µg/ml) was pretreated with excess amounts of soluble CD73 (sCD73), soluble EpCAM (sEpCAM), or a combination thereof (10 µg) and then evaluated for binding to OvCAR3 cancer cells. (B) Dose-dependent binding of bsAb CD73xEpCAM to L37 and L37.EpCAM. (C) Binding of bsAb CD73xEpCAM (1 µg/ml) (or controls) to OvCAR3 parental and corresponding CD73-KO and EpCAM-KO variants thereof. (D) Residual binding of bsAb CD73xEpCAM (1µg/ml) (or controls) to OvCAR3 cancer cells at indicated time points after 30 s of incubation and subsequent washing with PBS. (E) Residual CD73 membrane presence on OvCAR3 cells after treatment with bsAb CD73xEpCAM (0.01 – 10 µg/ml) (or controls) for 5 h. All experiments were analyzed by flow cytometry. All graphs represent mean ± SD.*

bsAb CD73xEpCAM treatment restores anticancer activity of ADO-suppressed cytotoxic T cells

When cytotoxic T cells were subjected to AMP and subsequently redirected to kill EpCAM-expressing OC cells using EpCAM-directed/CD3-antagonistic bispecific antibody BIS-1, induction of cancer cell death, evident from high caspase-3/8 activation levels in target cells, significantly dropped. Importantly, treatment of OC cells with bsAb CD73xEpCAM fully restored the cancer cell killing capacity of ADO-suppressed cytotoxic T cells (Figure 3C). These results corroborated ELISA data quantifying the restoration of capacity of ADO-suppressed cytotoxic T cells to secrete IFN-γ (Figure 3D).

bsAb CD73xEpCAM treatment inhibits the proliferative and colony-forming capacity of OC cells

Both CD73- and EpCAM-overexpression were reported to be implicated in enhancement of cancer cell proliferation[29,30] and in resistance towards cytotoxic agents[15,16,31,32]. In this respect, it is encouraging that treatment of OC cancer cells (Figure 4A) and primary OC patient-derived carcinoma cells (Figure 4B) with bsAb

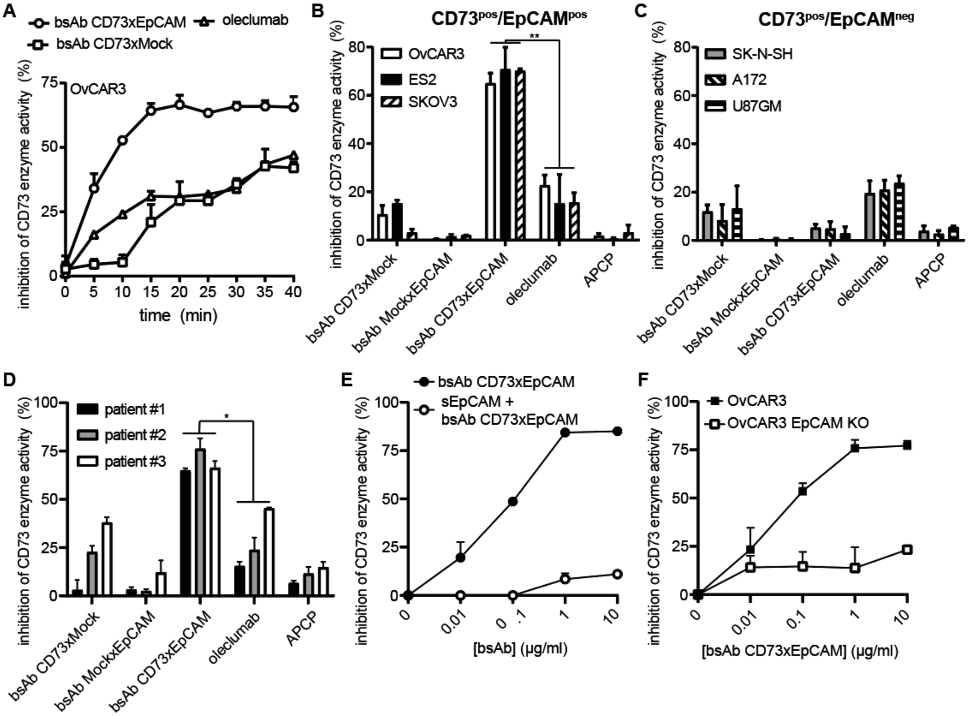
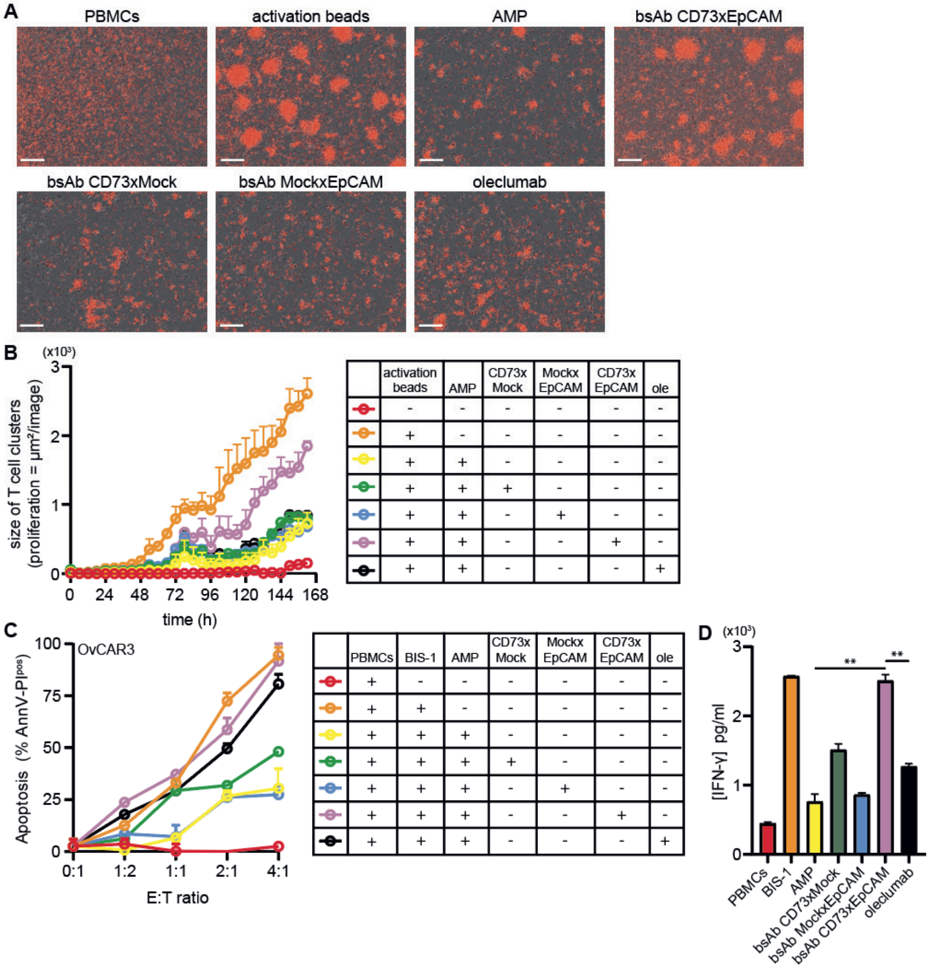


Figure 2: bsAb CD73xEpCAM potently inhibits the enzyme activity of CD73 in an EpCAM-directed manner. *(A) Kinetics of inhibition of OvCAR3-exposed CD73 enzyme activity by bsAb CD73xEpCAM (1 μg/ml) (or controls). Inhibition of CD73 enzyme activity by treatment (15 min) with bsAb CD73xEpCAM (1 μg/ml) (or controls) of (B) CD73^{pos}/EpCAM^{pos} cancer cell lines, (C) CD73^{pos}/EpCAM^{neg} cancer cell lines , and (D) primary OC patient-derived carcinoma cells, respectively. (E) Competitive CD73 enzyme inhibition assay on OvCAR3 cells after treatment (15 min) with bsAb CD73xEpCAM in the presence of excess amounts of soluble EpCAM (sEpCAM). (F) Dose-dependent Inhibition of CD73 enzyme activity by bsAb CD73xEpCAM (0.01 – 10 μg/ml) exposed on parental OvCAR3 cells versus OvCAR3 EpCAM-KO cells. CD73-mediated hydrolysis of AMP to ADO was evaluated using a colorimetric malachite green-based Pi assay. All graphs represent mean ± SD. Statistical analysis in B and D (group-mean) was performed using unpaired T-test (**$p < .01$).*

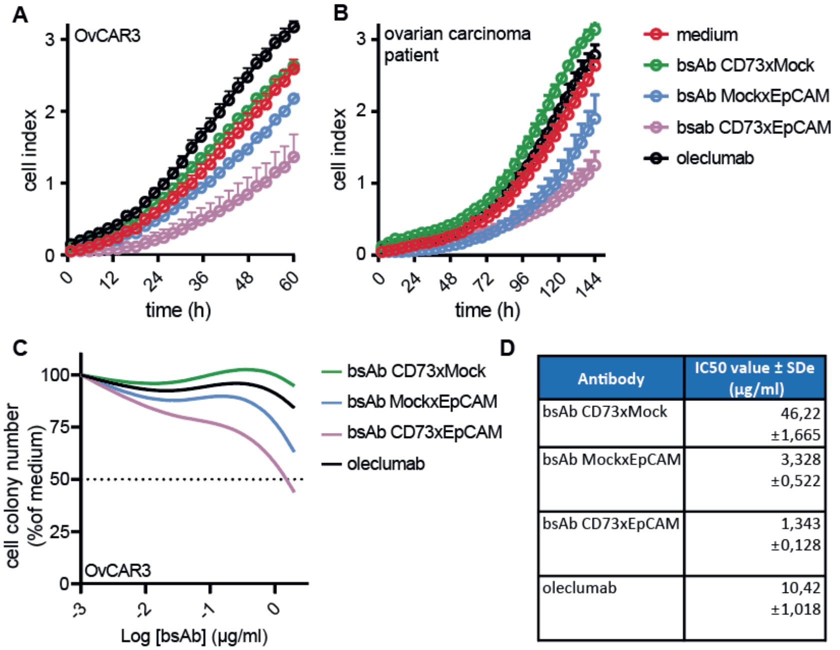
CD73xEpCAM significant decreased their proliferative- and colony-forming capacity (Figure 4C and Supplementary Figure 4). In particular, the corresponding colony-forming IC$_{50}$ value calculated for bsAb CD73xEpCAM (1.343 μg/ml) was superior compared to that of oleclumab (10.42 μg/ml) (Figure 4D).



	activation beads	AMP	CD73x Mock	Mockx EpCAM	CD73x EpCAM	ole
●	-	-	-	-	-	-
●	+	-	-	-	-	-
●	+	+	-	-	-	-
●	+	+	+	-	-	-
●	+	+	-	+	-	-
●	+	+	-	-	+	-
●	+	+	-	-	-	+

	PBMCs	BIS-1	AMP	CD73x Mock	Mockx EpCAM	CD73x EpCAM	ole
●	+	-	-	-	-	-	-
●	+	+	-	-	-	-	-
●	+	+	+	-	-	-	-
●	+	+	+	+	-	-	-
●	+	+	+	-	+	-	-
●	+	+	+	-	-	+	-
●	+	+	+	-	-	-	+

Figure 3: bsAb CD73xEpCAM restores the anticancer activities of ADO-suppressed T cells. *(A) Representative images of activated CFSE-labeled PBMCs, treated (15 min) (or not) with bsAb CD73xEpCAM (1 µg/ml) (or controls), washed, and then cultured in medium supplemented with AMP at 37°C for 7 d. Scale bar = 400 µm. (B) Cluster size (µm2/image) of activated proliferating T cell quantified using live cell imaging by taking pictures at 4 x magnification at 37°C every 6 h. (C) Percentage of AnnexinV-PI^{pos} (apoptotic) OvCAR3 cancer cells, treated (15 min) with bsAb CD73xEpCAM (1 µg/ml) (or controls), washed, and then cultured in medium supplemented with AMP at 37°C for 24 h. Subsequently, PBMCs were redirected to kill OvCAR3 cancer cells at increasing effector (E) to target (T) cell ratios. (D) IFN-γ levels excreted in culture supernatant of (C) were measured by ELISA. All graphs represent mean ± SD. Ole = oleclumab in graphs B and C. Statistical analysis in D was performed using un-paired T test (**p < .01).*



Antibody	IC50 value ± SDe (µg/ml)
bsAb CD73xMock	46,22 ±1,665
bsAb MockxEpCAM	3,328 ±0,522
bsAb CD73xEpCAM	1,343 ±0,128
oleclumab	10,42 ±1,018

Figure 4: bsAb CD73xEpCAM inhibits the proliferative capacity of EpCAM-expressing cancer cells. *Proliferation of (A) OvCAR3 and (B) primary OC patient-derived carcinoma cells after treatment with bsAb CD73xEpCAM (or controls) (1 µg/ml) using the RTCA xCELLigence instrument. The readout is indicated as cell-index, an arbitrary unit for attachment of adherent cells and cell proliferation measured at 37 °C every 15 min. (C) Inhibition of colony formation by OvCAR3 after treatment (15 min) with bsAb CD73xEpCAM (0.1 – 2 µg/ml) (or controls) and cultured at 37 ºC for 14 d. (D) Table with IC$_{50}$ values (µg/ml) calculated for graph (C). All graphs represent mean ± SD.*

bsAb CD73xEpCAM treatment sensitizes OC cells towards chemotherapeutic agents and ionizing radiation

In vitro treatment of OC cells with bsAb CD73xEpCAM sensitized these cells towards the cytotoxic activity of cisplatin, doxorubicin, and 5FU. In particular, single treatment of OC cells with bsAb CD73xEpCAM decreased cell confluence by ~30% (Figure 5A), which decreased further when combined with cisplatin (~66%, Figure 5B), doxorubicin (~66%, Figure 5C), or 5FU (~53%, Figure 5D), respectively. Additionally, co-treatment of bsAb CD73xEGFR with cisplatin, doxorubicin, or 5FU significantly reduced the colony-forming capacity of OC cells (Figure 5E). Similarly, co-treatment with bsAb CD73xEGFR enhanced sensitivity of OC cells towards cytotoxicity induced by ionizing radiation up to ~45% (Figure 5F).

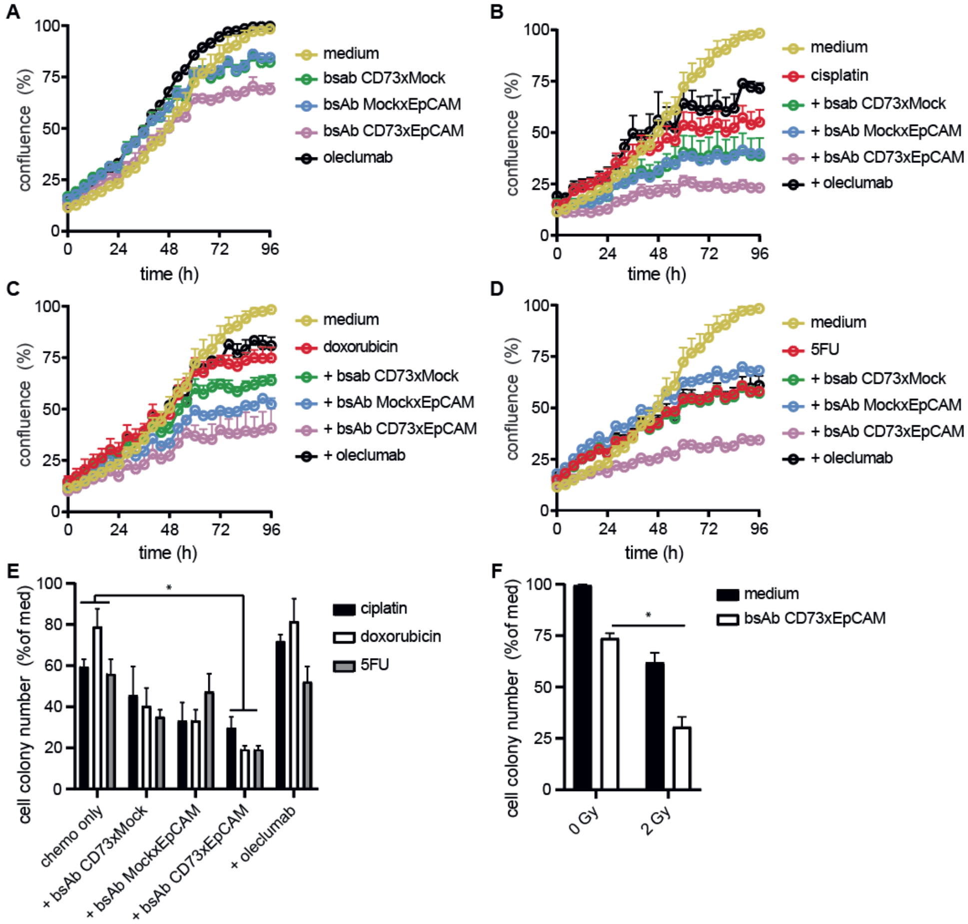


Figure 5: bsAb CD73xEpCAM sensitizes OvCAR3 cancer cells towards chemo- and radio-therapy. *OvCAR3 cell confluence after pretreatment (15 min) with (A) bsAb CD73xEpCAM (1 µg/ml) (or controls), and subsequent treatment with (B) cisplatin (1 µg/ml), (C) doxorubicin (200 nM), or (D) 5FU (15 µg/ml), respectively. Cell confluence was evaluated using live cell imaging technology by taking pictures at 4 x magnification every 4 h for 4 d. (E) Percentage of OvCAR3 cell colonies after pretreatment (15 min) with bsAb CD73xEpCAM (1 µg/ml) (or controls), and subsequent treatment with 5FU (15 µg/ml), cisplatin (1 µg/ml) or doxorubicin (200 nM) at 37°C for 14 d. (F) Percentage of OvCAR3 cell colonies after pretreatment (15 min) with bsAb CD73xEpCAM (1 µg/ml), irradiated (or not) with 2 Gy and subsequent cultured at 37°C for 14 d, respectively. All graphs represent mean ± SD. Statistical analysis in E (group means) and F was performed using un-paired T test (*$p < .05$).*

DISCUSSION

Antibodies that inhibit the PD-1/PD-L1 immune checkpoint have shown limited clinical benefit in patients with advanced OC[4–6]. This reduced efficacy may be attributable (in part) to the highly immunosuppressive TME and low PD-L1 expression in OC tumors. Additionally, OC cells may exploit alternate and/or additional inhibitory immune checkpoints to achieve immune evasion and subsequent malignant progression. In this respect, immunosuppressive cell surface nucleotidase CD73 is of particular interest, as it is implicated in tumor progression and metastasis in OC. High levels of CD73 have been shown to be negatively correlated with disease-free survival - and overall survival rates in patients with high-grade serous (HGS) OC. Furthermore, high CD73 expression levels in OC tumors abrogated the favorable prognosis associated with increased levels of intraepithelial CD8^{pos} cytotoxic T cells. Moreover, *in vitro* studies have indicated that both elevated CD73 expression and enhanced extracellular ADO levels promote OC tumor growth and induce the expression of anti-apoptotic BCL-2 family members in OC cells[9].

Several preclinical studies suggested that CD73 is a promising target for cancer treatment. Currently, several multicenter trials are ongoing to evaluate the clinical potential of antagonistic CD73 antibody oleclumab[11] in patients with advanced solid malignancies, including OC. However, recent midterm reports indicate that as a single treatment modality, the efficacy of oleclumab remains modest at best[19,33–35]. Clearly, novel strategies are urgently needed to enhance the clinical impact of CD73-inhibiting antibodies in OC patients.

Bispecific antibodies (bsAbs) are a promising class of immunotherapeutics with the potential to improve the clinical efficacy and safety of immune checkpoint-inhibiting approaches. An increasing number of bsAb-based immunotherapeutics are currently being evaluated in preclinical - and clinical studies. To achieve OC-directed inhibition of the CD73-ADO immune checkpoint, we produced bsAb CD73xEpCAM, which was constructed in a tetravalent bispecific taFv-Fc format[36]. We selected the clinically relevant pan-carcinoma target antigen EpCAM for this purpose as is selectively overexpressed in 55%-75% of OC patients.

In our study, bsAb CD73xEpCAM outperformed oleclumab with respect to its binding capacity to CD73 exposed on EpCAM^{pos} OC cells. After 5 h, ~78% of the initial cell-bound fraction of bsAb CD73xEpCAM remained bound to OC cells in the presence of serum at 37 °C. In contrast, under identical conditions, only ~40% of oleclumab was still bound to the cancer cell surface. Previously, Hey et al.[11] demonstrated that oleclumab can displace CD73 from the cell surface by driving the internalization of antibody/antigen complexes. Therefore, we investigated whether or not cell surface displacement of CD73 induced by oleclumab would drastically affect the results of our binding analyses. This investigation indicated that after treatment with oleclumab for 5h cell surface presence of CD73 was only moderately reduced by ~18% .

Treatment of OC cells with bsAb CD73xEpCAM resulted in potent inhibition of cell surface-exposed CD73 to convert extracellular AMP to ADO. Maximum inhibition of OC cell-exposed CD73 enzyme activity by bsAb CD73xEpCAM was reached after only 15 min, whereas oleclumab and bsAb CD73xMock required 24 h to do so. Moreover, bsAb CD73xEpCAM outperformed oleclumab in inhibiting the CD73 enzyme activity in 3 out of 3 primary patient-derived OC cells. This remarkable activity of tetravalent bsAb CD73xEpCAM is most likely attributable to its enhanced avidity for binding to OC cell surface-exposed EpCAM and concurrent blocking of CD73 activity. Previously, we reported similar attributes for analogous tetravalent bsAbs designed to inhibit immune checkpoints PD-L1[24,26], CD47[37,38], and CD73 (bsAb CD73xEGFR, manuscript under revision, Ploeg *et al.*), respectively in a tumor-directed manner.

It is well established that EpCAM overexpression is associated with increased cancer cell proliferation[39]. In this respect, it is noteworthy that *in vitro* treatment with bsAb CD73xEpCAM potently inhibited the proliferative capacity of both OvCAR3 cells and CD73^{pos}/EpCAM^{pos} primary patient-derived OC cells by ~49% and 56%, respectively. Moreover, bsAb CD73xEpCAM showed potent capacity (IC50 1.3 μg/ml) to inhibit colony-forming capacity of OC cells, which was superior compared to that of oleclumab (IC50 10.4 μg/ml), bsAb CD73xMock (IC50 46.2 μg/ml), and bsAb MockxEpCAM (IC50 3.3 μg/ml).

Both CD73 - and EpCAM overexpression were reported to be associated with enhanced resistance to chemotherapeutic agents and ionizing radiation[15,16,31,32]. Remarkably, platinum-based chemotherapeutic agents appear to enhance CD73 expression levels in OC cells[31]. Additionally, platinum-based chemotherapeutic agents preferentially eliminate EpCAM-negative OC cells, suggesting that the remaining EpCAM-positive cells may contribute to tumor recurrence after chemotherapy[40]. In this respect, it is encouraging that *in vitro* treatment with bsAb CD73xEpCAM sensitized CD73^{pos}/EpCAM^{pos} OC cells to cytotoxic activity of cisplatin, doxorubicin, and 5FU by ~66%, ~66%, and ~53%, respectively. Similarly, *in vitro* treatment of OC cells with bsAb CD73xEpCAM enhanced their sensitivity towards radiation-induced cytotoxicity by ~25%. These results appear in line with data that indicate that siRNA-mediated silencing of EpCAM and CD73 enhances sensitivity to chemotherapeutic agents and ionizing radiation in breast, pancreatic, and prostate cancer cells[31,41–43].

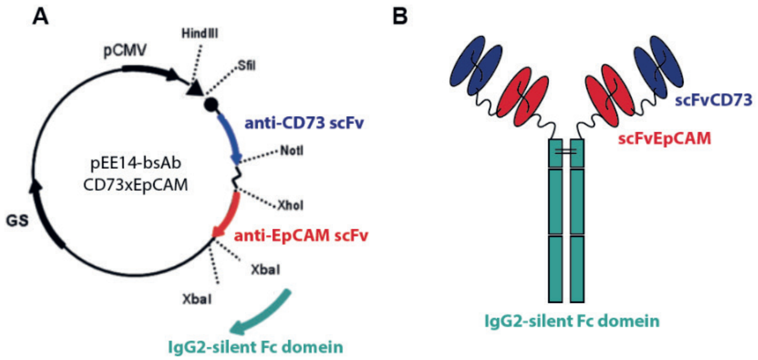
In conclusion, bsAb CD73xEpCAM harbors multiple and possibly mutually reinforcing anticancer activities, including: 1. enhanced binding avidity for CD73^{pos}/EpCAM^{pos} OC cells; 2. potent and EpCAM-dependent inhibition of CD73 enzyme activity of (primary patient-derived) OC cells; 3. restoration of ADO-mediated suppression of T cell proliferation; 4. restoration of the anticancer activity of ADO-suppressed cytotoxic T cells; 5. inhibition of the proliferative capacity of (primary patient-derived) OC cells; and 6. sensitization of OC cells to chemotherapeutic agents and ionizing radiation. Taken together, bsAb CD73xEpCAM may be useful to devise an alternate and more tumor-selective immunotherapeutic approach to overcome CD73-mediated immunosuppression in patients with EpCAM-overexpressing refractory OC.

REFERENCES

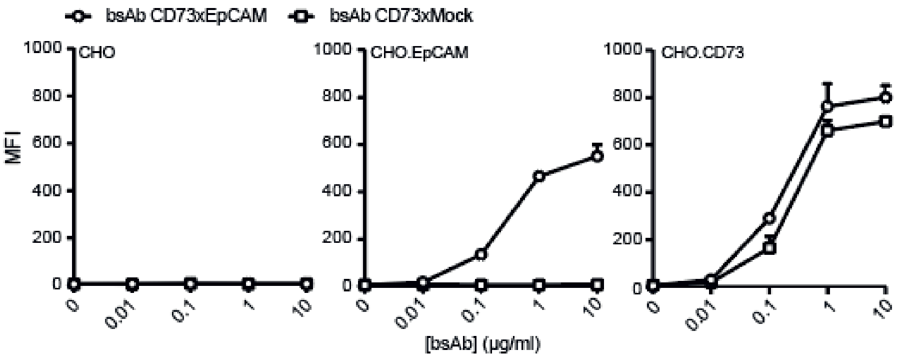
1. Coleman, R. L., Monk, B. J., Sood, A. K. & Herzog, T. J. Latest research and treatment of advanced-stage epithelial ovarian cancer. Nat Rev Clin Oncol 10, 211–24 (2013).
2. Herzog, T. J. & Pothuri, B. Ovarian cancer: a focus on management of recurrent disease. Nat Clin Pract Oncol 3, 604–11 (2006).
3. Zhang, L. et al. Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer. N Engl J Med 348, 203–13 (2003).
4. Brahmer, J. R. et al. Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. N Engl J Med 366, 2455–65 (2012).
5. Hamanishi, J. et al. Safety and Antitumor Activity of Anti-PD-1 Antibody, Nivolumab, in Patients With Platinum-Resistant Ovarian Cancer. J Clin Oncol 33, 4015–22 (2015).
6. Zamarin, D. et al. Randomized Phase II Trial of Nivolumab Versus Nivolumab and Ipilimumab for Recurrent or Persistent Ovarian Cancer: An NRG Oncology Study. J Clin Oncol 38, 1814–1823 (2020).
7. Minor, M., Alcedo, K. P., Battaglia, R. A. & Snider, N. T. Cell type- and tissue-specific functions of ecto-5′-nucleotidase (CD73). Am J Physiol Cell Physiol 317, C1079–C1092 (2019).
8. Alcedo, K. P., Bowser, J. L. & Snider, N. T. The elegant complexity of mammalian ecto-5′-nucleotidase (CD73). Trends Cell Biol 31, 829–842 (2021).
9. Turcotte, M. et al. CD73 is associated with poor prognosis in high-grade serous ovarian cancer. Cancer Res 75, 4494–503 (2015).
10. Allard, D., Chrobak, P., Allard, B., Messaoudi, N. & Stagg, J. Targeting the CD73-adenosine axis in immuno-oncology. Immunol Lett 205, 31–39 (2019).
11. Hay, C. M. et al. Targeting CD73 in the tumor microenvironment with MEDI9447. Oncoimmunology 5, e1208875 (2016).
12. Mirza, M. et al. 1195 Results of NSGO-OV-UMB1/ENGOT-OV30 study: a phase II study of durvalumab and oleclumab in patients with relapsed ovarian cancer (OC). International Journal of Gynecologic Cancer 31, A376 (2021).
13. Keizer, R. J., Huitema, A. D. R., Schellens, J. H. M. & Beijnen, J. H. Clinical pharmacokinetics of therapeutic monoclonal antibodies. Clin Pharmacokinet 49, 493–507 (2010).
14. Mazor, Y. et al. Insights into the molecular basis of a bispecific antibody's target selectivity. MAbs 7, 461–9 (2015).

15. Richter, C. E. et al. High-grade, chemotherapy-resistant ovarian carcinomas overexpress epithelial cell adhesion molecule (EpCAM) and are highly sensitive to immunotherapy with MT201, a fully human monoclonal anti-EpCAM antibody. Am J Obstet Gynecol 203, 582.e1–7 (2010).
16. Bellone, S. et al. Overexpression of epithelial cell adhesion molecule in primary, metastatic, and recurrent/chemotherapy-resistant epithelial ovarian cancer: implications for epithelial cell adhesion molecule-specific immunotherapy. Int J Gynecol Cancer 19, 860–6 (2009).
17. Spizzo, G. et al. Overexpression of epithelial cell adhesion molecule (Ep-CAM) is an independent prognostic marker for reduced survival of patients with epithelial ovarian cancer. Gynecol Oncol 103, 483–8 (2006).
18. Zheng, J., Zhao, L., Wang, Y., Zhao, S. & Cui, M. Clinicopathology of EpCAM and EGFR in Human Epithelial Ovarian Carcinoma. Open Med (Wars) 12, 39–44 (2017).
19. Kroesen, B. J. et al. Reduction of EGP-2-positive pulmonary metastases by bispecific-antibody-redirected T cells in an immunocompetent rat model. Int J Cancer 61, 812–8 (1995).
20. Zhang, J.-P. et al. Different Effects of sgRNA Length on CRISPR-mediated Gene Knockout Efficiency. Sci Rep 6, 28566 (2016).
21. Yang, J. et al. EpCAM associates with integrin and regulates cell adhesion in cancer cells. Biochem Biophys Res Commun 522, 903–909 (2020).
22. Helfrich, W. et al. A rapid and versatile method for harnessing scFv antibody fragments with various biological effector functions. J Immunol Methods 237, 131–45 (2000).
23. Vafa, O. et al. An engineered Fc variant of an IgG eliminates all immune effector functions via structural perturbations. Methods 65, 114–26 (2014).
24. Koopmans, I. et al. Bispecific Antibody Approach for Improved Melanoma-Selective PD-L1 Immune Checkpoint Blockade. J Invest Dermatol 139, 2343-2351.e3 (2019).
25. Ploeg, E. M. et al. Bispecific antibody CD73xEpCAM selectively inhibits the adenosine-mediated immunosuppressive activity of carcinoma-derived extracellular vesicles. Cancer Lett 521, 109–118 (2021).
26. Koopmans, I. et al. A novel bispecific antibody for EGFR-directed blockade of the PD-1/PD-L1 immune checkpoint. Oncoimmunology 7, e1466016 (2018).
27. Helbert, H. et al. A proof-of-concept study on the use of a fluorescein-based 18F-tracer for pretargeted PET. EJNMMI Radiopharm Chem 7, 3 (2022).
28. Parisi, A. et al. BIS-1: a novel bispecific monoclonal antibody for CEA-expressing carcinoma radioimmunoscintigraphy and radioimmunotherapy. Year Immunol 7, 96–105 (1993).
29. Gao, Z.-W. et al. CD73 promotes proliferation and migration of human cervical cancer cells independent of its enzyme activity. BMC Cancer 17, 135 (2017).
30. Lund, E., Güttinger, S., Calado, A., Dahlberg, J. E. & Kutay, U. Nuclear export of microRNA precursors. Science 303, 95–8 (2004).
31. Nevedomskaya, E. et al. A Systems Oncology Approach Identifies NT5E as a Key Metabolic Regulator in Tumor Cells and Modulator of Platinum Sensitivity. J Proteome Res 15, 280–90 (2016).
32. Chowanadisai, W. et al. Cisplatin Resistant Spheroids Model Clinically Relevant Survival Mechanisms in Ovarian Tumors. PLoS One 11, e0151089 (2016).
33. Kondo, S. et al. Safety, tolerability, pharmacokinetics, and antitumour activity of oleclumab in Japanese patients with advanced solid malignancies: a phase I, open-label study. Int J Clin Oncol 27, 1795–1804 (2022).
34. Somaiah, N. et al. A phase II multi-arm study to test the efficacy of oleclumab and durvalumab in specific sarcoma subtypes. Journal of Clinical Oncology 40, TPS11594–TPS11594 (2022).
35. Overman, M. J. et al. Safety, efficacy and pharmacodynamics (PD) of MEDI9447 (oleclumab) alone or in combination with durvalumab in advanced colorectal cancer (CRC) or pancreatic cancer (panc). Journal of Clinical Oncology 36, 4123 (2018).
36. Brinkmann, U. & Kontermann, R. E. The making of bispecific antibodies. MAbs 9, 182–212 (2017).
37. Hendriks, M. A. J. M. et al. Bispecific antibody approach for EGFR-directed blockade of the CD47-SIRPα ‘don’t eat me’ immune checkpoint promotes neutrophil-mediated trogoptosis and enhances antigen cross-presentation. Oncoimmunology 9, 1824323 (2020).
38. van Bommel, P. E. et al. CD20-selective inhibition of CD47-SIRPα ‘don’t eat me’ signaling with a bispecific antibody-derivative enhances the anticancer activity of daratumumab, alemtuzumab and obinutuzumab. Oncoimmunology 7, e1386361 (2018).
39. Winter, M. J. et al. Expression of Ep-CAM shifts the state of cadherin-mediated adhesions from strong to weak. Exp Cell Res 285, 50–8 (2003).
40. Tayama, S. et al. The impact of EpCAM expression on response to chemotherapy and clinical outcomes in patients with epithelial ovarian cancer. Oncotarget 8, 44312–44325 (2017).
41. Ni, J. et al. Epithelial cell adhesion molecule (EpCAM) is associated with prostate cancer metastasis and chemo/radioresistance via the PI3K/Akt/mTOR signaling pathway. Int J Biochem Cell Biol 45, 2736–48 (2013).
42. Nguyen, A. M., Zhou, J., Sicairos, B., Sonney, S. & Du, Y. Upregulation of CD73 Confers Acquired Radioresistance and is Required for Maintaining Irradiation-selected Pancreatic Cancer Cells in a Mesenchymal State. Mol Cell Proteomics 19, 375–389 (2020).
43. Gao, J., Yan, Q., Liu, S. & Yang, X. Knockdown of EpCAM enhances the chemosensitivity of breast cancer cells to 5-fluorouracil by downregulating the antiapoptotic factor Bcl-2. PLoS One 9, e102590 (2014).

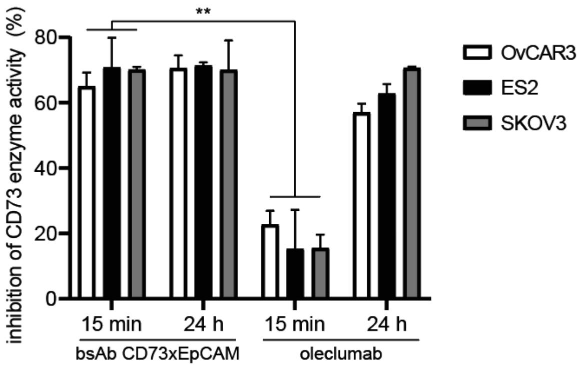
SUPPLEMENTARY DOCUMENTATION



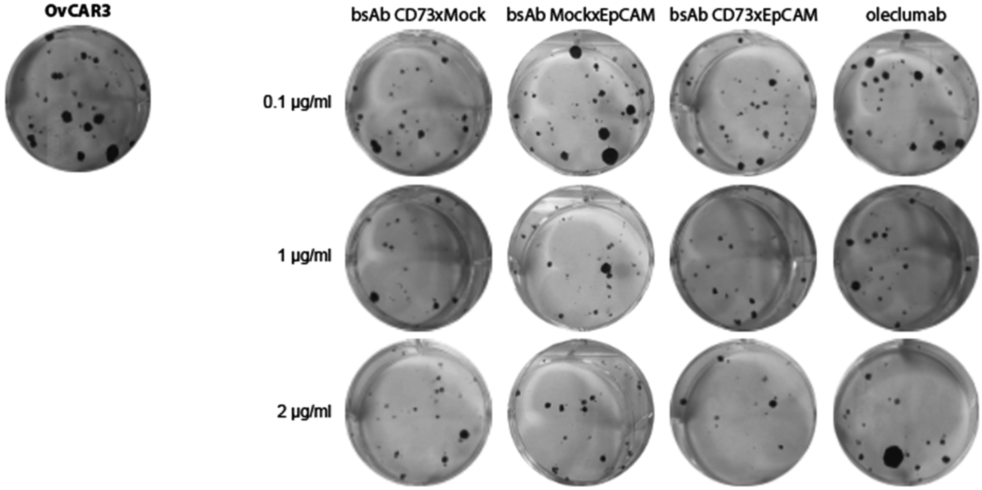
Supplementary figure 1: bsAb CD73xEpCAM. *(A) Topology of expression plasmid pbsAb encoding bsAb CD73xEpCAM-IgG2s and (B) schematic depiction of bsAb CD73xEpCAM-IgG2s protein.*



Supplementary figure 2: bsAb CD73xEGFR has dual binding specificity for CD73 and EGFR. *Dose-dependent binding of bsAb CD73xEGFR and bsAb CD73xMock to CHO, CHO.hEGFR, and CHO.hCD73, respectively. Experiments were analyzed by flow cytometry. All graphs represent mean ± SD.*



Supplementary figure 3: bsAb CD73xEpCAM rapidly inhibits the enzyme activity of CD73, outperforming oleclumab. *Percentage inhibition of CD73 enzyme activity on a panel of CD73*^{pos}*/EpCAM*^{pos} *OC cell lines after treatment (15 min or 24 h) with bsAb CD73xEpCAM or oleclumab (both 1 µg/ml). Graph represent mean ± SD. Statistical analysis (group-mean) was performed using unpaired T-test (**p < .01).*



Supplementary figure 4: bsAb CD73xEpCAM inhibits the colony-forming activity of OvCAR3 cancer cells. *Representative pictures of OvCAR3 cell colonies after pretreatment (15 min) with bsAb CD73xEpCAM (0.1 – 2 µg/ml) (or controls) and subsequent culturing at 37 ºC for 14 d. Cell colonies were stained with crystal violet.*