Society of Medical Education & Research

2024, Volume 4, Issue 2, Page No: 122-140
ISSN: 3108-4834

Archive of International Journal of Cancer and Allied Science

Selective Cancer Cell Targeting via a Bispecific CD73×EGFR Antibody Enhances Immune Checkpoint Inhibition and Suppresses Oncogenic Signaling

Emily Rachel Thompson[1] ★, Lauren Michelle Scott[1], Gianni Alberto Conti[2], Marco Stefano Vitale[2], Anna Lee Wilson[1]

[1]Department of Oncology, University of Melbourne, Melbourne, Australia.
[2]Department of Cancer Medicine, University of Bologna, Bologna, Italy.

***E-mail** ✉ e.thompson.unimelb@outlook.com

Abstract

CD73 functions as an ecto-enzyme that converts extracellular ATP (eATP), released under stress by tumor cells, into immunosuppressive adenosine (ADO). Many solid tumors exploit CD73 overexpression to evade immune responses, positioning it as an immune checkpoint target. Antibodies such as oleclumab are currently being tested in clinical trials. However, standard monospecific CD73 antibodies may affect normal tissues due to widespread CD73 expression, limiting tumor selectivity. Hence, strategies that focus checkpoint inhibition specifically on tumor cells are needed. To achieve targeted inhibition, we developed a tetravalent bispecific antibody, bsAb CD73xEGFR, and tested its anticancer effects in both in vitro carcinoma models and in vivo tumor-bearing mice. Treatment of carcinoma cells with bsAb CD73xEGFR reduced CD73 activity by approximately 71% in an EGFR-directed manner. The antibody promoted rapid internalization of CD73/EGFR complexes, causing prolonged concurrent removal of both antigens from the tumor cell surface. BsAb CD73xEGFR also enhanced the efficacy of cytotoxic chemotherapy and decreased tumor cell proliferation and migration by roughly 40%. Surprisingly, oleclumab treatment alone enhanced certain pro-tumor processes, including EGFR phosphorylation, increased proliferation (~20%), and heightened resistance to cytotoxic drugs and radiation (~39%). In immunocompetent BALB/c mice carrying syngeneic CD73^pos/EGFR^pos CT26 tumors, bsAb CD73xEGFR achieved superior tumor reduction compared with oleclumab (65% vs. 31%) and increased the presence of intratumoral CD8^pos T cells and M1 macrophages. BsAb CD73xEGFR demonstrates superior antitumor activity relative to oleclumab, selectively inhibiting CD73 in an EGFR-targeted fashion while simultaneously mitigating oncogenic signaling from EGFR and CD73. These findings highlight the potential of bsAb CD73xEGFR for the treatment of hard-to-treat solid tumors.

Keywords: Immunotherapy, Immune checkpoint inhibition, Tumor microenvironment, Adenosine

Introduction

Immunotherapy has become an integral component of current treatment strategies for patients with advanced malignancies. Specifically, antagonistic antibodies targeting immune checkpoints, including programmed cell death protein 1 (PD-1)/programmed cell death ligand 1 (PD-L1) and cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4), have achieved significant clinical benefit, albeit in a limited subset of patients. The majority of tumors appear to utilize additional or alternative immune checkpoints to evade immune surveillance, highlighting the unmet need for novel therapies capable of selectively inhibiting these pathways. In this context, antibodies that target the CD73 immune checkpoint, such as oleclumab, show substantial clinical potential [1, 2].

CD73 is a membrane-anchored enzyme that regulates immune homeostasis by catalyzing the conversion of extracellular ATP (eATP) into anti-inflammatory adenosine (ADO). Elevated eATP levels arise in

Access this article online https://smerpub.com/

Received: 24 May 2024; **Accepted:** 06 October 2024

How to cite this article: Thompson ER, Scott LM, Conti GA, Marco Stefano Vitale MS, Wilson AL. Selective Cancer Cell Targeting via a Bispecific CD73×EGFR Antibody Enhances Immune Checkpoint Inhibition and Suppresses Oncogenic Signaling. Arch Int J Cancer Allied Sci. 2024;4(2):122-40. https://doi.org/10.51847/aQD3nFv64H

response to infection, tissue injury, ischemia, or metabolic stress, acting as danger signals that recruit and activate immune cells to trigger pro-inflammatory responses. These responses are tightly controlled locally by ectonucleotidases CD39 and CD73, which sequentially convert eATP to AMP and then to ADO, with CD73 functioning as the rate-limiting enzyme in this pathway. The resulting accumulation of ADO engages adenosine receptors on surrounding immune cells, creating a negative feedback loop that limits inflammation in a spatially and temporally regulated manner [1].

Cancer cells, due to their high metabolic stress, release large amounts of eATP and simultaneously overexpress CD73, rapidly converting eATP to ADO. The diffusion of tumor-derived ADO establishes a potent immunosuppressive microenvironment both within and beyond the tumor niche. This “ADO halo” dampens anticancer immune responses, facilitates immune tolerance, and promotes tumor progression [3].

Preclinical studies have shown that CD73-blocking antibodies can mitigate this tumor-driven immunosuppression across diverse cancer types, including multiple carcinoma models. Oleclumab (MEDI9447), a fully human recombinant antibody, binds CD73 selectively and inhibits its enzymatic function [4]. Currently, several multicenter clinical trials are evaluating its efficacy in patients with advanced solid tumors. However, the therapeutic effectiveness of conventional monospecific CD73 antibodies may be limited due to “on-target/off-tumor” interactions with abundant CD73 expressed on normal tissues, potentially reducing tumor accumulation [5]. Additionally, CD73 blockade may trigger generalized T cell activation, potentially resulting in autoimmune-related adverse events similar to those observed with other checkpoint inhibitors.

Unexpectedly, in vitro exposure of carcinoma cells to oleclumab was associated with increased expression and phosphorylation of oncogenic receptors EGFR and HER2, coinciding with enhanced tumor cell proliferation and resistance to cytotoxic therapies.

To overcome these limitations, we developed a tetravalent bispecific antibody, bsAb CD73xEGFR, designed to selectively inhibit CD73 on cancer cells via EGFR-directed engagement. Treatment with bsAb CD73xEGFR effectively reduced tumor growth in syngeneic, immunocompetent mouse models, accompanied by increased infiltration of CD8^pos effector T cells and activated macrophages. Furthermore, bsAb CD73xEGFR enhanced the sensitivity of cancer cells to chemotherapeutic agents and ionizing radiation in vitro and markedly impaired tumor cell proliferation and migration in vivo.

Materials and Methods

Antibodies and reagents

Antibodies: The following antibodies were used: FITC-conjugated anti-CD73 (clone MM07, Sino Biological), APC-conjugated anti-EGFR (clone 528, Santa Cruz), FITC-labeled annexin V (ImmunoTools), goat anti-human IgG-APC (SouthernBiotech), sheep anti-human CD73 (R&D Systems), rabbit anti-human EGFR (Abcam), rabbit anti-human HER2 (R&D Systems), rabbit anti-human/mouse β-Actin (Abcam), rabbit anti-sheep IgG (Thermo Fisher), goat anti-rabbit IgG (Dako), anti-CD4 (ab183685, Abcam), anti-CD8 (98941, Cell Signaling Technology), anti-FoxP3 (ab99964, Abcam), anti-F4/80 (ab240946, Abcam), anti-CD206 (ab64693, Abcam), and anti-CD11c (ab52632, Abcam).

Other reagents: VivoGlo substrate (Promega), CFSE and CFSE-Far Red (Thermo Fisher), Vybrant DiO/DiD dyes, propidium iodide (PI), fluorescent caspase 3/8-488 probe (Biotium), adenosine 5’-(α,β-methylene)diphosphate (APCP, Sigma), Fab-ZAP (anti-human Fc saporin-6, Advanced Targeting Systems), CypHer5E (Fisher Scientific), AMP (Sigma-Aldrich), T-cell activation/expansion beads (Miltenyi Biotec), recombinant soluble human CD73 (Abcam), IFN-γ ELISA kit (eBioscience), and malachite green phosphate assay (ab65622, Abcam).

Cell lines and engineered models

CHO-K1, SK-BR-3, 4T1, FaDu, H292, OvCAR3, H322, PC3M, A375m, A2058, SK-MEL-28, MDA-MB-231, DLD1, CT26, and HEK293AD were obtained from ATCC (Manassas, VA, USA). A549, A549.EGFR-KO, H1650, and H1650 EGFR-KO were provided by Prof. H.J. Haisma. H292-luc cells were sourced from Cellomics Technology (SC-1087).

Cells were maintained in RPMI-1640 or DMEM supplemented with 10% fetal calf serum at 37°C under 5% CO2. CHO-K1 cells were cultured in GMEM with 5% dialyzed fetal bovine serum.

CHO.CD73 cells were generated via FuGENE-HD-mediated lipofection of a plasmid encoding human CD73 (OriGene). CHO.EGFR cells were similarly established

using a plasmid containing human EGFR (Sino Biological).

CRISPR-Cas9 mediated knockout of CD73 was performed using PX458 (Addgene #48138) plasmid containing CD73-targeting sgRNA 5'-GCAGCACGTTGGGTTCGGCG-3' [6]. EGFR-KO cells were produced using PX458 carrying EGFR-specific sgRNA 5'-GAGTAACAAGCTCAGGCAGT-3' (GenScript).

Design of bsAb CD73xEGFR-IgG2 silent

Sequences for scFvCD73 and VHH-EGFR were synthesized commercially (GenScript) based on previously reported sequences from oleclumab [4] and camelid single-domain antibody NRC-sdAb028 [7].

Production of recombinant bispecific antibodies

BsAbs were expressed in Expi293 cells (Thermo Fisher) and purified using ÄKTA-Start chromatography, following previously established protocols [8].

Dual-target binding evaluation

Cancer cells were incubated with defined concentrations of bsAb CD73xEGFR or control antibodies at 4°C for 45 minutes, followed by secondary staining with APC-labeled anti-human IgG for 45 minutes at 4°C. Flow cytometry analysis was performed using a Guava easyCyte 6/2L cytometer (Merck Millipore) and GuavaSoft v3.2.

For competitive binding, bsAb CD73xEGFR (1 µg/mL) was incubated with either soluble recombinant CD73 (s.CD73), EGFR-targeting control bsAb MockxEGFR, or both (10 µg each) at 4°C for 20 minutes. Binding was analyzed as described above.

Assessment of internalization of bsAb CD73xEGFR/antigen complexes

Cancer cells were exposed to escalating concentrations of bsAb CD73xEGFR (0.01–10 µg/mL) or relevant control antibodies at 37°C for 24 hours. Surface levels of CD73 and EGFR were subsequently quantified using anti-CD73 mAb MM07 and anti-EGFR mAb 528, which recognize distinct, non-overlapping epitopes on each antigen.

For dynamic internalization studies, cancer cells were incubated with bsAb CD73xEGFR labeled with the pH-sensitive CypHer5E dye (pHAb) at 1 µg/mL, either alone or combined with 10 µg/mL EGFR-competitive bsAb MockxEGFR, at 37°C for 0, 10, 60, 240, or 360 minutes. CypHer5E is a non-toxic dye that fluoresces under acidic conditions (endosomes/lysosomes) but remains non-fluorescent at neutral/basic pH (extracellular medium). Internalization of the labeled bsAbs was quantified by flow cytometry.

Cancer cells were also treated with bsAb CD73xEGFR or control antibodies (1 µg/mL) in the presence of Fab-ZAP (anti-human Fc saporin-6 conjugate; Advanced Targeting Systems), a monovalent goat anti-human IgG fragment linked to the ribosome-inactivating protein saporin. Apoptotic induction, which depends on internalization of Fab-ZAP, was assessed after 24 hours using annexin-V/PI staining and flow cytometry.

Evaluation of CD73 enzymatic inhibition by bsAb CD73xEGFR

Hydrolysis of AMP to adenosine (ADO) by CD73 was measured by quantifying inorganic phosphate (Pi) formation using a colorimetric malachite green assay, following previously described procedures [8].

Assessment of bsAb CD73xEGFR on ADO-suppressed T-cell proliferation

Peripheral blood mononuclear cells (PBMCs) were labeled with CFSE and activated using T-cell activation/expansion beads at a 1:1 bead-to-cell ratio. Cells were cultured for 5 days in the presence or absence of AMP (100 µM) and incubated with bsAb CD73xEGFR or control antibodies (1 µg/mL). Proliferation was assessed via CFSE dilution by flow cytometry.

In parallel, CFSE-Far Red-labeled PBMC proliferation was monitored using live-cell imaging as described previously [8].

Restoration of anticancer T-cell activity in vitro

PBMCs were pre-cultured with AMP (100 µM) and exposed to increasing concentrations of bsAb CD73xEGFR or controls (0.01–10 µg/mL) for 24 hours. T cells were then stimulated and redirected against EpCAM-expressing PC3M prostate carcinoma cells, with cytotoxic activity monitored by live-cell imaging according to established protocols [8].

Restoration of anticancer T-cell activity in vivo

Syngeneic tumors were established by subcutaneous injection of 5×10^5 CT26 cells in 0.1 mL PBS into the right flank of 4–6-week-old female BALB/c mice (Janvier, France). Animals were randomized using

RandoMice software into five groups (n=9 per group; total=45 mice) based on body weight, with sample size calculated using G*Power. BsAb CD73xEGFR or control antibodies were administered intraperitoneally at 7.5 mg/kg on days 4, 7, 10, and 14 post-inoculation. Tumor growth was measured using calipers. Mice were euthanized after 21 days via cardiac puncture followed by cervical dislocation. Tumors and organs were harvested for multiplex immunofluorescence and histological analysis.

Multiplexed immunofluorescence for analysis of tumor-infiltrating immune cells

Tumors harvested from immunocompetent mice were analyzed for T cell and macrophage populations using a multiplexed immunofluorescence approach, following the previously established protocol [9].

In vitro evaluation of bsAb CD73xEGFR effects on cancer cell proliferation

Cells were plated in E-Plate 16 wells (ACEA Biosciences) and incubated with bsAb CD73xEGFR or control antibodies at 1 µg/mL for 60 hours at 37°C. Proliferation was continuously monitored using the xCELLigence RTCA platform (ACEA Biosciences).

Additionally, cells were seeded into 96-well plates and treated with bsAb CD73xEGFR or controls (1 µg/mL) at 37°C. Growth dynamics were tracked using live-cell imaging (IncuCyte) with images captured at 4× magnification every 4 hours over three days. Percent confluence was quantified with IncuCyte software 2019B.

In vivo evaluation of bsAb CD73xEGFR on tumor proliferation

Tumors were generated by subcutaneous injection of 1×10^6 H292-Luc cells in 0.1 mL PBS into the right flanks of 4–6-week-old female athymic nude mice (Crl:NU(NCr)-Foxn1^nu, Charles River, Germany). Mice were randomized using RandoMice software into five groups (n=9 per group; total 45 animals) based on body weight. Sample size calculations were performed using G*Power. BsAb CD73xEGFR or control antibodies were administered intraperitoneally at 7.5 mg/kg on days 7, 10, and 17. Tumor progression was monitored by bioluminescence imaging (IVIS Spectrum, 75 mg/kg VivoGlo; Promega) and caliper measurements. Animals were euthanized after 28 days using cardiac puncture followed by cervical dislocation.

Assessment of bsAb CD73xEGFR effects on cancer cell migration

Cancer cells were plated in 24-well plates with a physical stopper in the presence of bsAb CD73xEGFR or control antibodies (1 µg/mL) at 37°C for 24 hours. Following stopper removal, migration into the cell-free region was monitored with live-cell imaging (IncuCyte) at 4× magnification every 30 minutes over 3 days. The percentage closure of the gap was quantified using IncuCyte software 2019B.

Evaluation of bsAb CD73xEGFR on chemosensitivity and radiosensitivity

Cells were seeded in 96-well plates and treated with bsAb CD73xEGFR or controls (1 µg/mL) alongside chemotherapeutics: 5FU (50 µg/mL), taxol (50 nM), cisplatin (1 µg/mL), or doxorubicin (50 nM). Cell proliferation was monitored using live-cell imaging (IncuCyte) at 4× magnification every 6 hours for 6 days, and confluence was calculated using IncuCyte software 2019B.

For colony formation assays, cells were cultured in 6-well plates with bsAb CD73xEGFR or controls (0.01–10 µg/mL) for 14 days, washed with PBS, stained with crystal violet, and analyzed for colony number and size via ImageJ.

For radiation experiments, cells in 6-well plates were treated with bsAb CD73xEGFR or controls (1 µg/mL) and irradiated at doses of 0.5–2 Gy using a ^137Ce γ-ray source (IBL637, 0.59 Gy/min). Colonies were incubated for 14 days, then stained and quantified as described above.

Animal experimentation compliance

All animal procedures adhered to the Dutch Act on Animal Experimentation and were approved by the Institutional Animal Welfare Committee of the University Medical Center Groningen (AVD1050020209544). EMP was aware of group assignments during allocation, study execution, outcome assessment, and data analysis. No adjustments were made for confounders, as randomization was successfully applied.

Statistical evaluation

Data analysis was performed using t-tests, one-way ANOVA with Tukey's post hoc test, or (non-)linear regression, as appropriate, in Prism software. Pooled

datasets (n-number and technical replicates noted in figure legends) were analyzed. Statistical significance was set at P<0.05. Notation: ns = P>0.05; * = P<0.05; ** = P<0.01; *** = P<0.001; **** = P<0.0001.

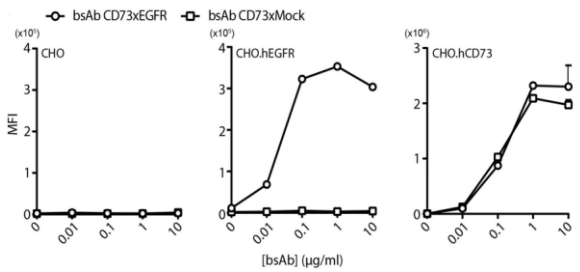
Results and Discussion

BsAb CD73xEGFR demonstrates simultaneous recognition of CD73 and EGFR

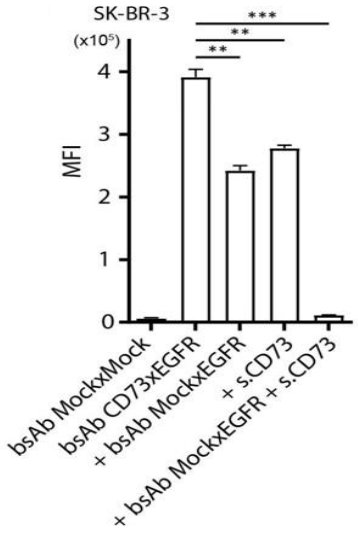
The tetravalent bispecific antibody, bsAb CD73xEGFR, was designed in a taFv-Fc configuration [10], combining two identical scFv fragments targeting CD73 from oleclumab with two identical camelid-derived single-domain antibodies specific for EGFR [9]. Its ability to bind both antigens was examined using CHO cells engineered to express either human CD73 or EGFR. Dose-dependent binding of bsAb CD73xEGFR was observed for CHO.CD73 and CHO.EGFR cells, whereas unmodified CHO cells showed no detectable interaction **(Figure 1a)**.

In SK-BR-3 breast cancer cells, the presence of soluble human CD73 (s.CD73) partially interfered with bsAb CD73xEGFR binding, while EGFR-blocking bsAb MockxEGFR largely reduced antibody attachment. Strikingly, simultaneous exposure to s.CD73 and bsAb MockxEGFR completely eliminated bsAb CD73xEGFR binding **(Figure 1b)**, demonstrating selective dual engagement of both CD73 and EGFR.

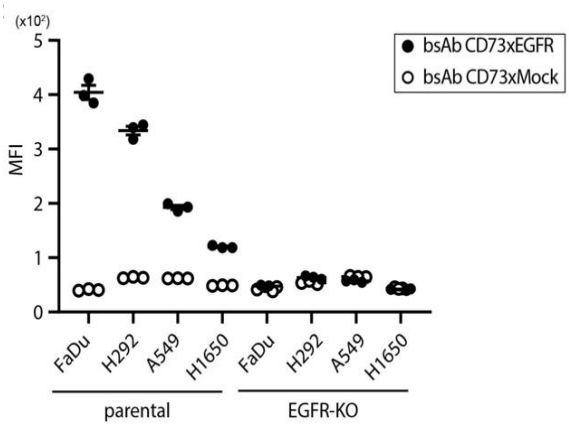
Analysis of four CD73^pos/EGFR^pos tumor cell lines revealed that bsAb CD73xEGFR binding intensity corresponded closely with their EGFR surface expression levels **(Figure 1c)**. Reduced binding was noted in EGFR-KO/CD73^pos variants **(Figure 1c).** Moreover, bsAb CD73xEGFR was capable of bridging separate cell populations: DiO-labeled FaDu cancer cells and DiD-labeled CHO.CD73 cells formed DiO^pos/DiD^pos clusters, reflecting simultaneous engagement of CD73 on one cell and EGFR on a neighboring cell as detected by flow cytometry.



a)



b)



c)

Figure 1. BsAb CD73xEGFR exhibits simultaneous recognition of CD73 and EGFR.

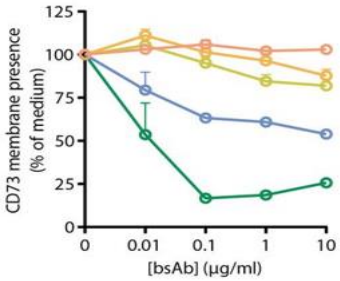
(a) Binding of bsAb CD73xEGFR and bsAb CD73xMock to CHO, CHO.hEGFR, and CHO.hCD73 cells was assessed in a concentration-dependent manner. (b) In a competitive binding setup, bsAb CD73xEGFR (1 µg/mL) was pre-treated with either excess soluble human CD73 (s.hCD73), EGFR-targeting bsAb MockxEGFR, or both, and its binding to SK-BR-3 cancer cells was measured. (c) Interaction of bsAb CD73xEGFR and bsAb CD73xMock (both 1 µg/mL) with parental tumor cells (FaDu, H292, A549, H1650) and their EGFR-KO variants was evaluated. Flow cytometry was employed for all assessments. Graphs A–B show representative data: n=3 (two technical repeats); graph C: n=3 (three technical repeats). Data are represented as mean±SD. One-way ANOVA followed by Tukey post hoc test was

used for statistical analysis in B (**p<0.01, ***p<0.001). Abbreviations: bsAb, bispecific antibody; CHO, Chinese hamster ovary; KO, knockout; MFI, mean fluorescence intensity.

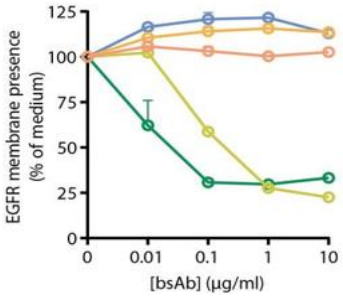
BsAb CD73xEGFR induces fast co-internalization and prolonged removal of CD73 and EGFR from cancer cells

Treatment of CD73^pos/EGFR^pos H292 cells with bsAb CD73xEGFR caused simultaneous, dose-dependent reduction of surface CD73 and EGFR. At 1 µg/mL, bsAb CD73xEGFR lowered CD73 and EGFR surface levels by 81% and 73%, respectively **(Figures 2a and 2b** and these effects persisted for up to 96 hours **(Figures 2c and 2d)**. Extended surface removal was also observed in primary ovarian and colon cancer cells **(Figure 2e)**.

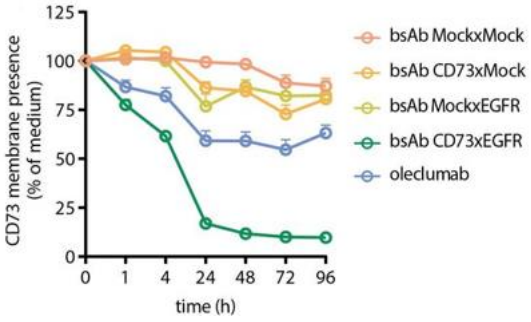
Internalization of the bsAb CD73xEGFR/antigen complexes was observed between 10–60 minutes and was blocked in the presence of saturating EGFR-competing bsAb MockxEGFR **(Figure 2f)**. Moreover, "piggybacking" of Fab-ZAP toxin on internalized bsAb CD73xEGFR triggered apoptosis via saporin **(Figure 2g)**. Additionally, treatment with bsAb CD73xEGFR decreased CD73 surface expression and reduced EGFR tyrosine phosphorylation in CD73^pos/EGFR^pos tumor cells.



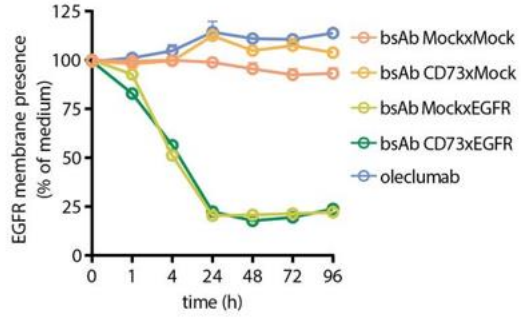
a)



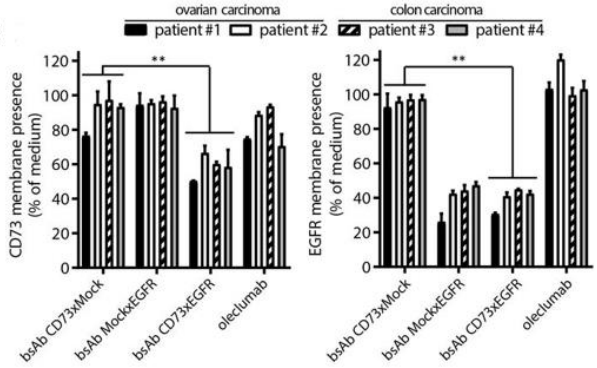
b)



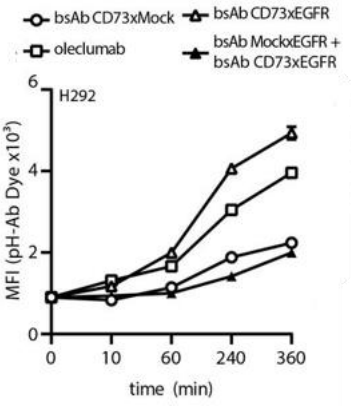
c)



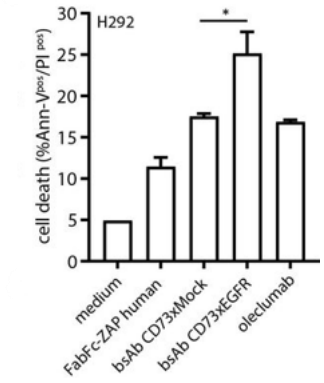
d)



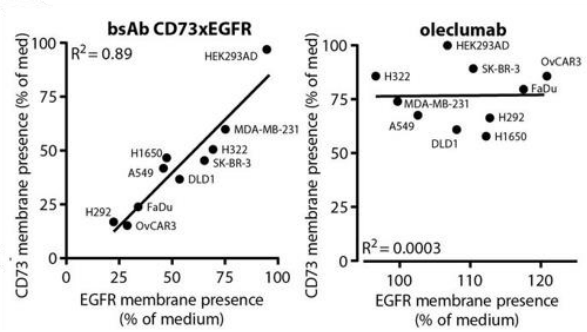
e)



f)



g)



h)

Figure 2. The bispecific antibody CD73xEGFR promotes fast co-internalization and persistent removal of CD73 and EGFR from cancer cell membranes. Remaining (A) CD73 and (B) EGFR levels on H292 cells after 24-hour exposure to bsAb CD73xEGFR or control treatments at concentrations of 0.01–10 µg/mL. Remaining (C) CD73 and (D) EGFR on H292 cells following treatment with bsAb CD73xEGFR or controls (1 µg/mL) across 1, 4, 24, 48, 72, and 96 hours. (E) Surface CD73 and EGFR levels in primary patient-derived ovarian and colon cancer cells after 24-hour incubation with bsAb CD73xEGFR or controls (1 µg/mL). (F) Mean fluorescence intensity (MFI) of H292 cells labeled with the pH-sensitive dye CypHer5E and incubated with bsAb CD73xEGFR or controls (1 µg/mL) for 10, 60, 240, and 360 minutes. (G) Fraction of annexin-Vpos/PIpos H292 cells after 24-hour treatment with bsAb CD73xEGFR or controls (1 µg/mL) combined with anti-human IgG-Fab conjugated to saporin. (H) Correlation of residual EGFR and CD73 surface expression in 10 cancer cell lines treated for 24 hours with bsAb CD73xEGFR or oleclumab (both 1 µg/mL). All data were obtained using flow cytometry. Graphs A–D: n=4 (two technical replicates), graph E: n=1 (four technical replicates), graph F (representative): n=3 (two technical replicates), graph G: n=2 (three technical replicates), graph H: n=3 (two technical replicates). Values are expressed as mean±SD. Statistical testing in E (group mean comparison) and G used the unpaired t-test (*p<0.05, **p<0.01). Linear regression was applied in graph H to calculate the regression coefficient. bsAb, bispecific antibody.

EGFR-targeted CD73 internalization by bsAb CD73xEGFR

bsAb CD73xEGFR efficiently removes CD73 from cells expressing both CD73 and EGFR, whereas cells lacking EGFR show negligible CD73 internalization. Likewise, A549 EGFR-KO and H1650 EGFR-KO cells exhibited minimal CD73 uptake after treatment. Analysis across 10 CD73pos/EGFRpos cancer cell lines revealed a strong linear association (R^2=0.89) between residual surface CD73 and EGFR levels, whereas oleclumab treatment produced no correlation (R^2=0.0003) **(Figure 2h)**. These findings indicate that bsAb CD73xEGFR induces co-internalization of CD73 and EGFR on cancer cell surfaces.

Inhibition of CD73 enzymatic activity via EGFR-targeted bsAb CD73xEGFR

Exposure to bsAb CD73xEGFR caused a dose-dependent decrease in CD73 enzyme activity across several carcinoma cell lines **(Figure 3a)**, outperforming oleclumab in all three tested lines (mean inhibition: 71% vs 52%) **(Figure 3b)** and in eight of nine primary patient-derived carcinoma samples (mean: 55% vs 40%) **(Figure 3c).** IC50 values of bsAb CD73xEGFR ranged from 0.001 to 0.038 µg/mL in six cell lines, compared with 0.005–0.563 µg/mL for oleclumab. In H929 cells, the presence of EGFR-blocking bsAb MockxEGFR reduced bsAb CD73xEGFR-mediated CD73 inhibition in a dose-dependent manner **(Figure 3d)**. In contrast, A549 EGFR-KO cells showed only a minor decrease in CD73 activity when treated with bsAb CD73xEGFR **(Figure 3e)**.

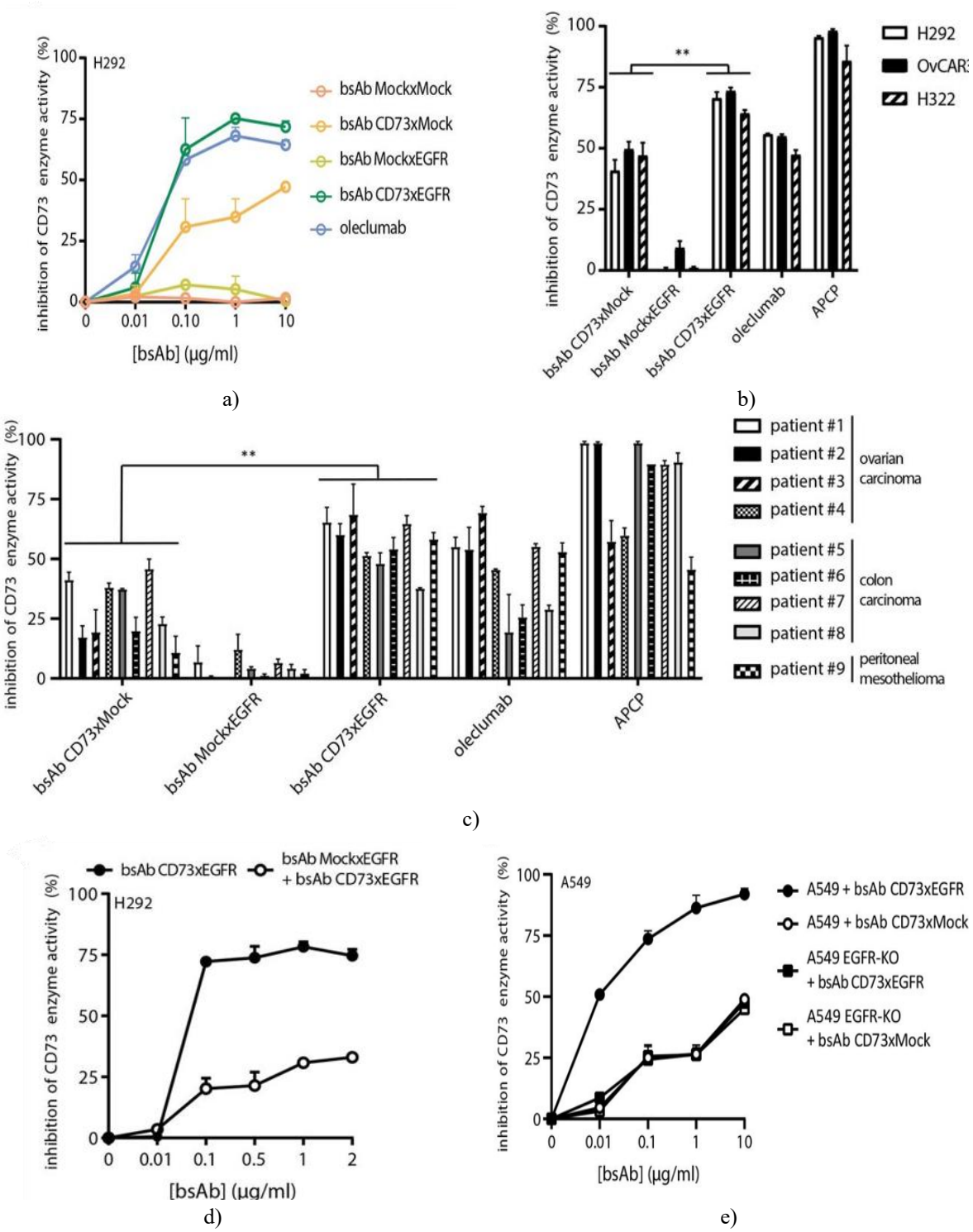
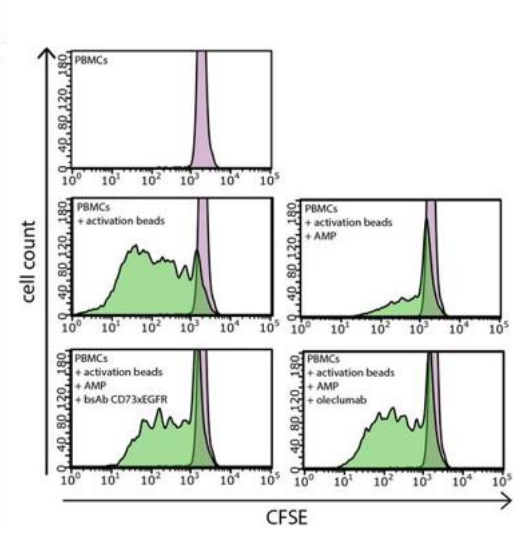


Figure 3. bsAb CD73xEGFR strongly blocks the enzymatic function of CD73 in an EGFR-specific manner. (a) H292 cells were exposed for 24 hours to bsAb CD73xEGFR or control compounds at concentrations ranging from 0.01 to 10 µg/mL, and CD73 inhibition was quantified. (b) Suppression of CD73 activity was measured in H292, OvCAR3, and H322 cancer cell lines, and (c) in primary patient-derived tumor cells, after 24 hours of treatment with bsAb CD73xEGFR or controls (1 µg/mL). (d) For competitive inhibition, H292 cells were first incubated with an excess of EGFR-targeting bsAb MockxEGFR before adding bsAb CD73xEGFR (0.01–

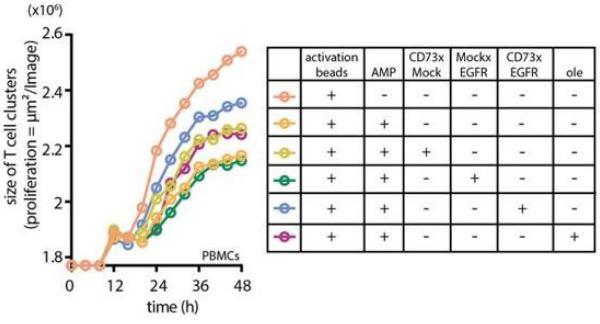
2 µg/mL). (e) CD73 activity was compared in A549 parental versus EGFR-KO cells following treatment with bsAb CD73xEGFR or bsAb CD73xMock (both 1 µg/mL). AMP-to-adenosine conversion was assessed using a malachite green-based colorimetric assay detecting inorganic phosphate. Sample sizes: Graphs A–B: n=4 (two technical replicates), Graph C: n=1 (four technical replicates), Graphs D–E: n=3 (two technical replicates). Data are shown as mean±SD. Statistical tests for B and C used unpaired t-tests on group means (**p<0.01). bsAb, bispecific antibody; KO, knockout.

bsAb CD73xEGFR counteracts adenosine-mediated T-cell proliferation inhibition

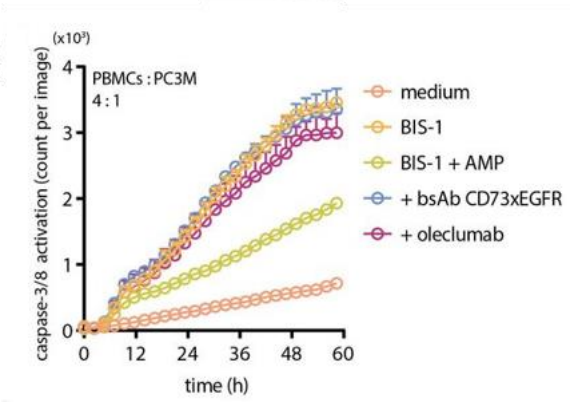
Activated CFSE-labeled T cells experienced a strong reduction in proliferation when AMP was present, due to its enzymatic conversion to adenosine by CD73. Application of bsAb CD73xEGFR or oleclumab fully restored T-cell proliferation, as evidenced by progressive CFSE dye dilution **(Figure 4a),** which was accompanied by enlargement of activated T-cell clusters **(Figure 4b)**.



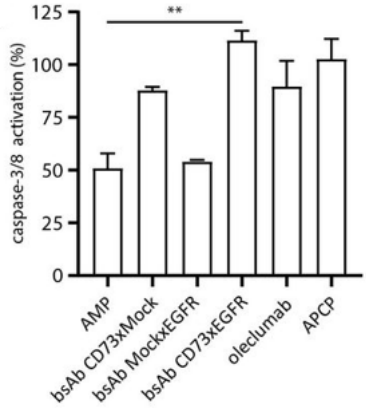
a)



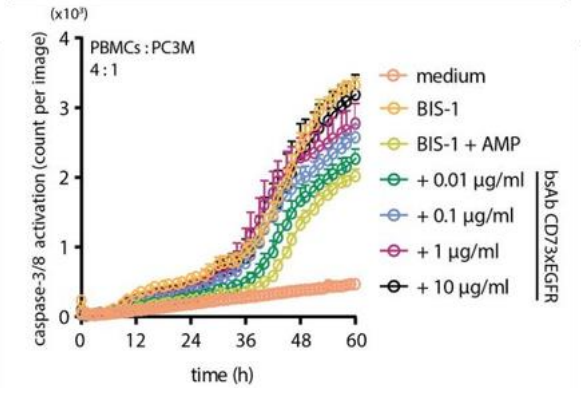
b)



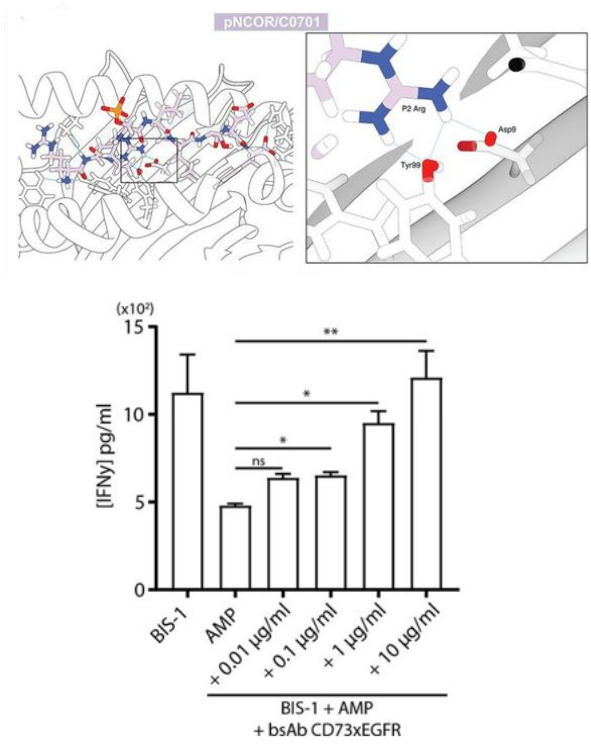
c)



d)



e)



f)

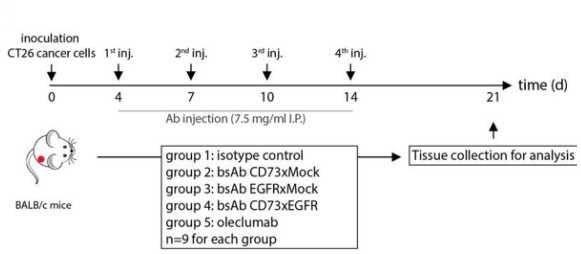
Figure 4. bsAb CD73xEGFR restores T-cell antitumor function that is suppressed by adenosine (ADO). (A) Activated PBMCs labeled with CFSE were cultured for 5 days at 37°C in medium containing AMP, with or without bsAb CD73xEGFR or control antibodies (1 µg/mL). Proliferation was assessed by tracking CFSE dye dilution using flow cytometry. Unstimulated T cells (purple) and ADO-suppressed T cells are visible as single peaks on the right of the histogram, while successive generations of dividing T cells appear as sequential green peaks. (b) The area of proliferating T-cell clusters (µm²/image) was determined via live-cell imaging at 10× magnification, with images captured every 1.5 hours for 2 days at 37°C. (C) Apoptosis in PC3M cancer cells was measured through caspase-3/8 activation after redirection with BIS-1 (bsAb CD3xEpCAM) at an effector-to-target ratio of 4:1, with PBMCs incubated in the presence or absence of AMP and bsAb CD73xEGFR, oleclumab, or control antibodies (1 µg/mL). (D) Caspase-3/8 signals (count per image) were recorded by live-cell imaging every 1.5 hours over 2.5 days at 37°C and 10× magnification. (E) PBMCs exposed to increasing concentrations of bsAb CD73xEGFR (0.01–10 µg/mL) were treated with AMP, redirected to kill PC3M cells, and analyzed as above. (F) IFN-γ levels in supernatants from E were quantified via ELISA. Sample sizes: Graph A (representative): n=3 (two technical replicates), Graph B (representative): n=3 (four technical replicates), Graph C (representative): n=3 (four technical replicates), Graph D: n=3 (four technical replicates), Graph E (representative): n=3 (four technical replicates), Graph F: n=3 (four technical replicates). Data are shown as mean±SD. Statistical analysis in D and F used one-way ANOVA with Tukey's post hoc test (*$p<0.05$, **$p<0.01$). ADO, adenosine; bsAb, bispecific antibody; IFN, interferon; PBMCs, peripheral blood mononuclear cells; EpCAM, epithelial cell adhesion molecule; CFSE, carboxyfluorescein succinimidyl ester.

bsAb CD73xEGFR reverses ADO-mediated suppression of cytotoxic T cells

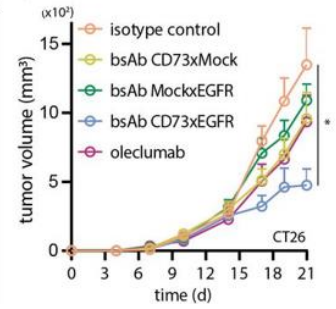
Exposure of cytotoxic T cells to AMP markedly impaired their ability to kill EpCAM-expressing target cells via BIS-1 (bsAb CD3xEpCAM), as indicated by decreased caspase-3/8 activity in PC3M cells **(Figures 4c and 4d)**. Treatment with bsAb CD73xEGFR restored T-cell-mediated killing in a dose-dependent manner **(Figure 4e),** which was supported by elevated IFN-γ secretion measured by ELISA **(Figure 4f).**

bsAb CD73xEGFR enhances immune cell infiltration and antitumor activity in vivo

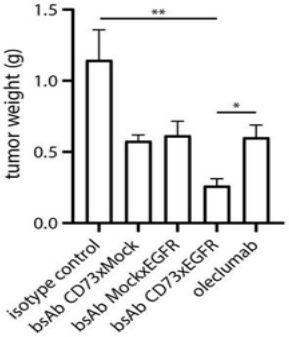
The in vivo efficacy of bsAb CD73xEGFR was tested in BALB/c mice bearing CT26 tumors (in vitro reference: CT26). Animals were injected intraperitoneally with 7.5 mg/kg of bsAb CD73xEGFR, oleclumab, or control agents on days 4, 7, 10, and 14 post-tumor inoculation **(Figure 5a)**. Treatment with bsAb CD73xEGFR reduced tumor volume by 65% and tumor weight by 77%, outperforming oleclumab (31% volume reduction) **(Figures 5b and 5c)**. Tumors from bsAb CD73xEGFR-treated mice had increased $CD4^+$ and $CD8^+$ T-cell infiltration and decreased $FoxP3^+$ regulatory T cells **(Figures 5d–5g)**. Furthermore, enhanced infiltration of $F4/80^+$ total macrophages and $CD11c^+$ M1 macrophages, along with reduced $CD206^+$ M2 macrophages, was observed **(Figures 5h–5k)**. In contrast, oleclumab-treated tumors primarily showed increased $CD8^+$ T cells and reduced total and M2 macrophages. No signs of systemic toxicity were detected in vital organs of bsAb CD73xEGFR-treated animals.


inoculation
CT26 cancer cells
1st inj.
2nd inj.
3rd inj.
4th inj.
0
4
7
10
14
21
time (d)
Ab injection (7.5 mg/ml I.P.)
BALB/c mice
group 1: isotype control
group 2: bsAb CD73xMock
group 3: bsAb EGFRxMock
group 4: bsAb CD73xEGFR
group 5: oleclumab
n=9 for each group
Tissue collection for analysis

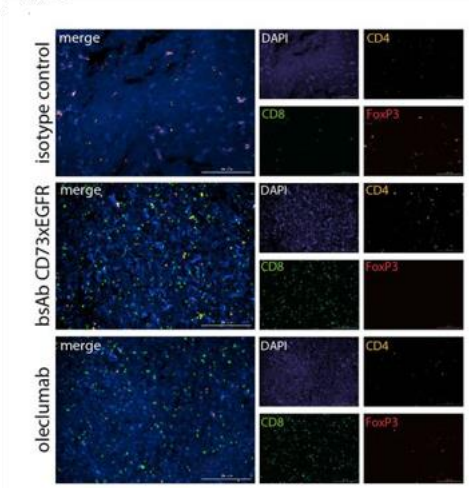
a)


(x10²)
isotype control
bsAb CD73xMock
bsAb MockxEGFR
bsAb CD73xEGFR
oleclumab
tumor volume (mm³)
CT26
time (d)

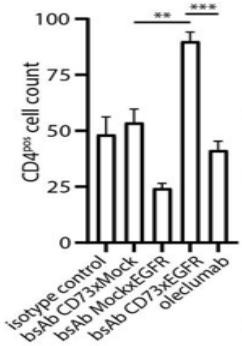
b)


tumor weight (g)
isotype control
bsAb CD73xMock
bsAb MockxEGFR
bsAb CD73xEGFR
oleclumab

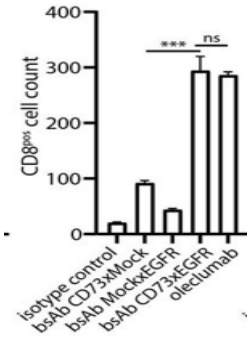
c)


isotype control
bsAb CD73xEGFR
oleclumab
merge
DAPI
CD4
CD8
FoxP3

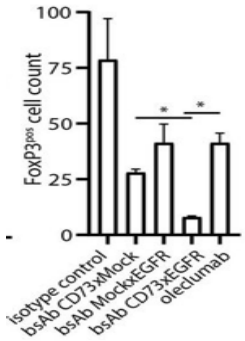
d)


CD4pos cell count
isotype control
bsAb CD73xMock
bsAb MockxEGFR
bsAb CD73xEGFR
oleclumab

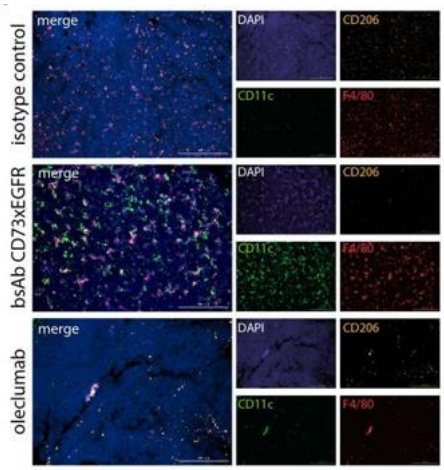
e)


CD8pos cell count
isotype control
bsAb CD73xMock
bsAb MockxEGFR
bsAb CD73xEGFR
oleclumab

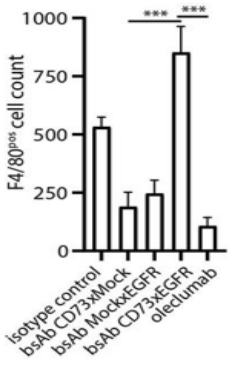
f)


FoxP3pos cell count
isotype control
bsAb CD73xMock
bsAb MockxEGFR
bsAb CD73xEGFR
oleclumab

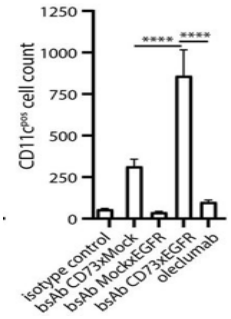
g)


isotype control
bsAb CD73xEGFR
oleclumab
merge
DAPI
CD206
CD11c
F4/80

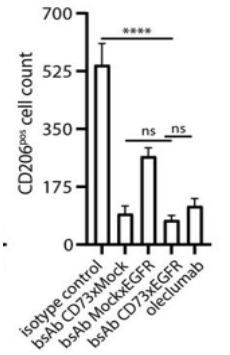
h)



i)



j)

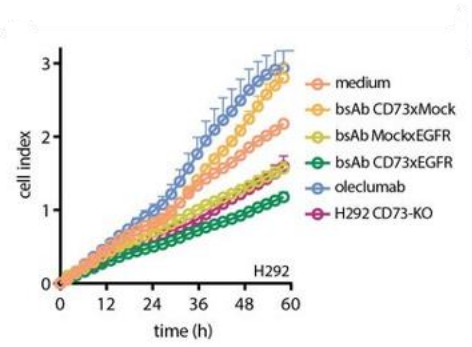


k)

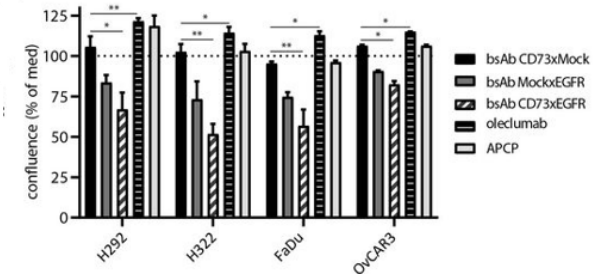
Figure 5. bsAb CD73xEGFR substantially increased the infiltration of CD8⁺ T cells and M1 macrophages within tumors in immunocompetent mice. (a) BALB/c mice received subcutaneous injections of murine CT26 colon carcinoma cells in the flank. Treatments with bsAb CD73xEGFR (7.5 mg/kg) or control antibodies were administered intraperitoneally on days 4, 7, 10, and 14. Tumor tissues and organs were collected 21 days after tumor implantation for downstream analyses. (b) Mean tumor volumes and (c) wet weights for each group of nine animals. (d) Representative images of multiplexed immunofluorescence staining and corresponding quantifications of (e) CD4⁺, (F) CD8⁺, and (g) FoxP3⁺ cells in tumor sections from isotype control, bsAb CD73xEGFR, or oleclumab-treated mice. (h) Representative images and quantifications of (i) F4/80⁺, (j) CD11c⁺, and (k) CD206⁺ macrophages in the same treatment groups. Data are shown as mean±SD. Scale bar in D and H = 200 µm. Statistical comparisons for B (day 21), C, E–G, and I–K were conducted using one-way ANOVA followed by Tukey's post hoc test (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns = not significant). bsAb, bispecific antibody; I.P., intraperitoneal.

bsAb CD73xEGFR limits cancer cell proliferation and migration in vitro

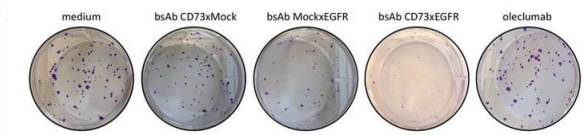
Overexpression of CD73 has been linked to enhanced proliferation, migration, and therapy resistance via both enzymatic and non-enzymatic mechanisms [11]. In vitro, bsAb CD73xEGFR treatment reduced proliferation across four carcinoma cell lines by ~40% **(Figures 6a and 6b)**. Colony-forming assays demonstrated a pronounced reduction in both the number and size of colonies following treatment **(Figures 6c–6e).** Calculated IC50 values for bsAb CD73xEGFR ranged from 0.16 to 0.65 µg/mL, showing superior potency compared to oleclumab (IC50 9.38–24.33 µg/mL) in all three cell lines tested. Light microscopy images (**Figures 6f)** illustrate that bsAb CD73xEGFR nearly abolished migratory behavior. This was further confirmed by the delayed closure of the wound area in migration assays over time **(Figure 6g)**.



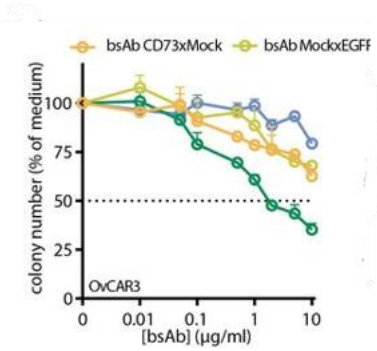
a)



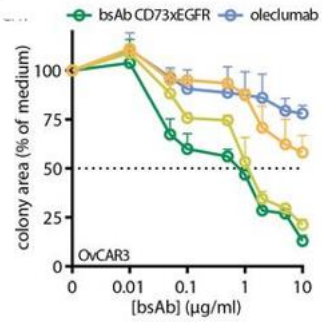
b)



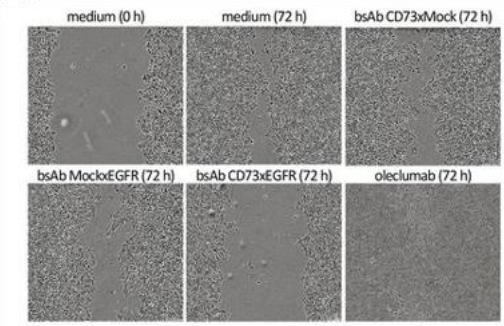
c)



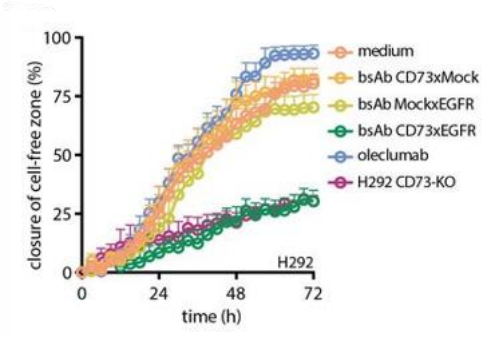
d)



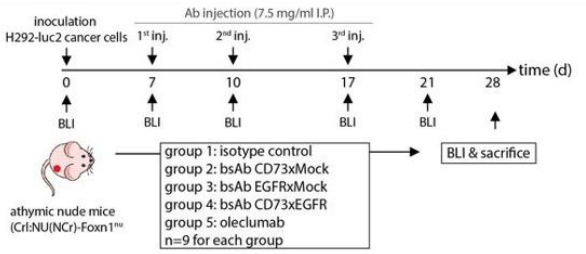
e)



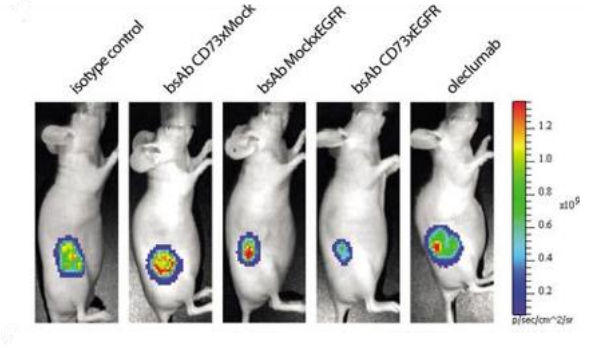
f)



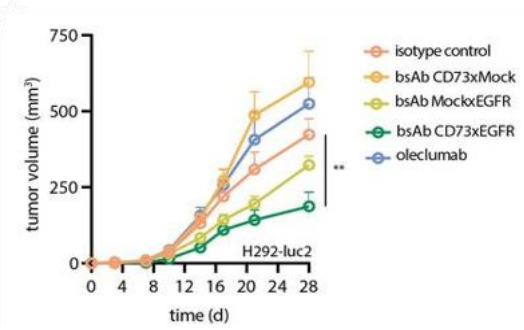
g)



h)



i)



j)

Figure 6. bsAb CD73xEGFR limits tumor cell growth and motility.

(a) Cell growth was assessed by treating H292 cells and matched CD73-deficient counterparts with bsAb CD73xEGFR or control agents (1 µg/mL), followed by continuous monitoring using the RTCA xCELLigence platform. Data are presented as cell index, an arbitrary

parameter reflecting adhesion and proliferation, recorded every 15 min at 37 °C.
(b) A multi–cell line proliferation assay (H292, H322, FaDu, OvCAR3) was performed after incubation with bsAb CD73xEGFR or control antibodies (1 µg/mL). Cell confluence (%) was determined at 72 h.
(c) Representative colony formation images of H292 cells treated with bsAb CD73xEGFR (1 µg/mL) or corresponding controls for 14 days at 37 °C.
(d, e) Quantification of OvCAR3 colony formation (%) following exposure to bsAb CD73xEGFR or controls (0.01–10 µg/mL) for 14 days at 37 °C. Colony count and surface area were analyzed using ImageJ.
(f) Representative images of H292 cells cultured in stopper-containing plates to generate a defined cell-free area and treated with bsAb CD73xEGFR or controls (1 µg/mL) for 72 h at 37 °C.
(g) Cell migration was quantified as progressive closure of the detection gap using live-cell imaging, captured every 0.5 h for 3 days at 37 °C with 4× magnification.
(h) Athymic nude mice (Crl:NU(NCr)-Foxnnu) received subcutaneous flank injections of H292-luc2 human non–small cell lung cancer cells and were administered intraperitoneal injections of bsAb CD73xEGFR or control treatments (7.5 mg/kg) on days 7, 10, and 17.
(i) Representative bioluminescence images of mice treated with bsAb CD73xEGFR or controls obtained on day 28 after tumor implantation.
(j) Average tumor volumes (mm^3) from nine mice per group measured by calipers. Graphs A–B: n = 3 (two technical replicates); D–E: n = 3 (two technical replicates); G: n = 3 (two technical replicates). Values are shown as mean ± SD. Statistical comparisons in B and J (day 28) were conducted using one-way ANOVA followed by Tukey's post-hoc test (*p < 0.05, **p < 0.01). APCP, adenosine 5′-(α,β-methylene)diphosphate; bsAb, bispecific antibody; I.P., intraperitoneal; KO, knockout.

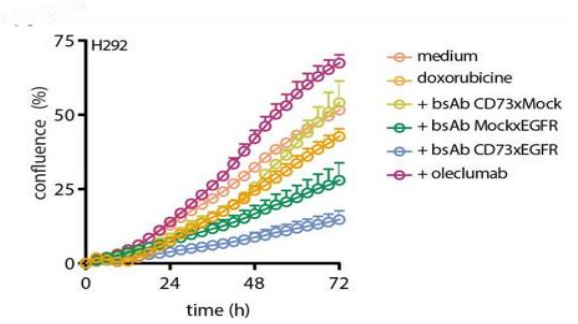
Contrary to expectations, in vitro exposure of diverse carcinoma cell lines to oleclumab (or the CD73 inhibitor APCP) resulted in enhanced, rather than reduced, cellular proliferation **(Figure 6b).** Furthermore, oleclumab increased migratory behavior by approximately 20% **(Figure 6g).** This phenomenon may be linked to oleclumab-induced elevation of EGFR and HER2 expression, including both total and phosphorylated forms, in tumor cells. Consistently, increased surface EGFR expression following oleclumab treatment was observed in 8 of 10 tested cell lines **(Figure 2h).**

bsAb CD73xEGFR suppresses tumor expansion in xenograft models

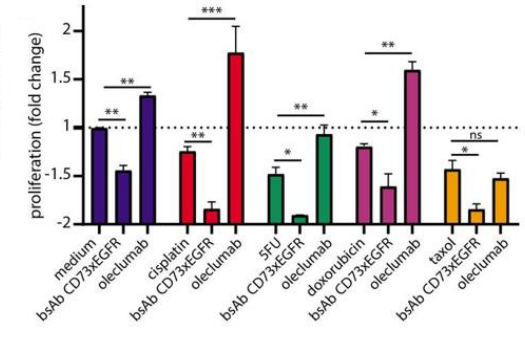
The divergent in vitro effects of bsAb CD73xEGFR and oleclumab on tumor cell proliferation were next examined in vivo using athymic nude mice bearing H292-Luc2 xenografts. Mice were treated intraperitoneally with bsAb CD73xEGFR (7.5 mg/mL), oleclumab, or control antibodies on days 7, 10, and 17 after tumor implantation **(Figure 6h)**. Compared with isotype control treatment, bsAb CD73xEGFR reduced tumor growth by 56% **(Figure 6i and 6j)**. In contrast, tumor volumes increased by 24% and 40% in oleclumab- and bsAb CD73xMock-treated animals, respectively.

bsAb CD73xEGFR increases responsiveness to chemotherapy and irradiation

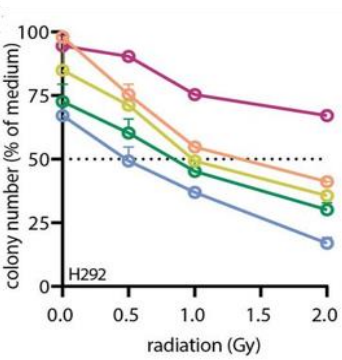
Combined in vitro treatment with bsAb CD73xEGFR enhanced tumor cell sensitivity to cisplatin, doxorubicin, taxol, and 5-fluorouracil by approximately 1-fold, 0.45-fold, 0.74-fold, and 0.4-fold, respectively **(Figures7a and 7b)**. In comparison, oleclumab exposure promoted resistance to cisplatin, doxorubicin, and 5-fluorouracil by ~2-fold, 1.8-fold, and 1.44-fold, respectively.



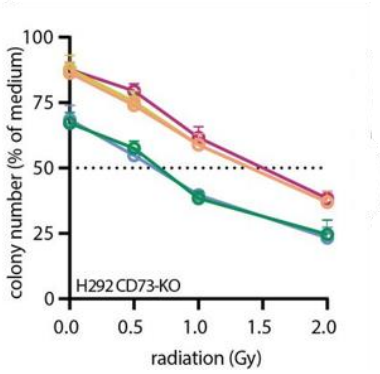
a)



b)



c)



d)

antibody	IC50/SDe
medium	1.351 ± 0.088
CD73xMock	1.086 ± 0.205
MockxEGFR	0.811 ± 0.255
CD73xEGFR	0.536 ± 0.182
oleclumab	3.775 ± 0.405

e)

Figure 7. bsAb CD73xEGFR increases tumor cell responsiveness to chemotherapy and ionizing radiation.

H292 cell confluence was monitored following treatment, or no treatment, with bsAb CD73xEGFR (1 µg/mL) or control reagents, in the presence or absence of (a) doxorubicin (50 nM), (b) cisplatin (1 µg/mL), taxol (50 nM), or 5-FU (50 µg/mL). Cell growth was tracked by live-cell imaging at 37 °C, capturing images at 4× magnification every 6 h for a total of 6 days.

(c) Colony formation (%) of H292 cells or (d) matched CD73-KO cells after exposure to bsAb CD73xEGFR (1 µg/mL) or controls, followed by irradiation at 0.5, 1, 1.5, or 2 Gy and incubation at 37 °C for 14 days. Colony numbers were quantified using ImageJ.

(e) IC_{50} values (Gy) derived from the data shown in panel c. All panels: n = 3 (two technical replicates). Data are presented as mean ± SD. Statistical testing in b used an unpaired t-test (*p < 0.05, **p < 0.01, ***p < 0.001). bsAb, bispecific antibody; KO, knockout.

In line with these findings, combined treatment with bsAb CD73xEGFR also potentiated cancer cell susceptibility to damage induced by ionizing radiation **(Figure 7c).** Accordingly, IC_{50} values for bsAb CD73xEGFR–treated cells were reduced from 1.35 Gy to 0.536 Gy **(Figure 7e)**. By contrast, exposure to oleclumab increased resistance to radiation by up to 39% at 2 Gy **(Figure 7c)**, with IC_{50} values rising from 1.35 Gy to 3.77 Gy **(Figure 7e).** Notably, oleclumab treatment did not alter radiation sensitivity in CD73-KO tumor cells **(Figure 7d).**

Blocking antibodies against CD73, such as oleclumab [4], have been proposed as a strategy to counteract the immunosuppressive CD73/adenosine checkpoint across multiple cancer entities. At present, several multicenter clinical studies are investigating oleclumab as an immune checkpoint inhibitor in patients with advanced solid tumors, including diverse carcinoma types. However, interim analyses from recent trials suggest that oleclumab, when used as monotherapy, shows only limited clinical benefit [12–15]. One possible explanation is that monospecific CD73 antibodies may exhibit "on-target/off-tumor" binding to CD73 expressed on healthy tissues [5], thereby reducing effective CD73 blockade within tumor sites.

In this study, we describe the design and preclinical characterization of bsAb CD73xEGFR, engineered to selectively inhibit CD73 on tumor cells via EGFR targeting. EGFR was chosen because it represents a well-validated tumor-associated surface antigen that is frequently overexpressed and/or mutated in multiple hard-to-treat solid cancers [16, 17]. In addition, many tumor types display concurrent overexpression of CD73 and EGFR [18–20]. BsAb CD73xEGFR consists of two identical scFv fragments derived from oleclumab and two identical EGFR-specific nanobodies. Importantly, these binding domains recognize both human and murine orthologs with comparable affinity, enabling evaluation of bsAb CD73xEGFR in human as well as mouse tumor models. To avoid antibody-dependent cellular

cytotoxicity during these studies, the molecule was generated with an IgG2-silent Fc region lacking effector function [21].

Crucially, bsAb CD73xEGFR demonstrated markedly stronger antagonistic effects than oleclumab in tumor cells co-expressing CD73 and EGFR. This enhanced activity is most likely explained by the increased avidity conferred by the tetravalent architecture of the bsAb. Similar advantages of tetravalent bispecific antibodies have previously been reported for tumor-selective blockade of immune checkpoints such as PD-L1 [22, 23] and CD47 [24, 25]. More recently, a tetravalent bsAb targeting CD73 and EpCAM was shown to efficiently inhibit CD73 present on carcinoma-derived exosomes, an effect that oleclumab failed to achieve or achieved only marginally [8].

Intriguingly, exposure of $CD73^+/EGFR^+$ tumor cells—including established cell lines and primary patient-derived samples—to bsAb CD73xEGFR in vitro triggered rapid uptake of bsAb–antigen complexes. This process caused sustained and simultaneous removal of CD73 and EGFR from the tumor cell surface for as long as 96 h. Notably, internalization of oleclumab (CypHer5E-labeled) exceeded that observed for bsAb CD73xMock. This difference is likely attributable to more efficient CypHer5E NHS-ester labeling of a conventional monoclonal antibody such as oleclumab compared with the bispecific taFv-Fc format of the bsAbs, potentially yielding a higher dye-to-antibody ratio for oleclumab. Although not directly examined, this technical aspect may explain the observed discrepancy.

Internalization of surface CD73 induced by bsAb CD73xEGFR led to a similarly prolonged inability of tumor cells to convert extracellular AMP into ADO. Importantly, in vitro treatment of ADO-inhibited cytotoxic T cells with bsAb CD73xEGFR robustly restored their antitumor function. Furthermore, in immunocompetent BALB/c mice bearing syngeneic CT26 colorectal tumors, administration of bsAb CD73xEGFR (7.5 mg/kg) resulted in an average 65% reduction in tumor growth, whereas the same dosing regimen with oleclumab achieved only a 31% decrease. In vivo treatment with bsAb CD73xEGFR also increased infiltration of $CD4^+$ T cells, $CD8^+$ T cells, and macrophages by 38%, 52%, and 82%, respectively. These outcomes compare favorably with findings reported by Hay *et al*. [4], where oleclumab treatment (10 mg/kg) reduced tumor size by 57% at 16 days and increased tumor-infiltrating $CD4^+$ T cells, $CD8^+$ T cells, and macrophages by only 15%, 25%, and 20%, respectively. Notably, our in vitro data confirm that bsAb CD73xEGFR efficiently inhibits the enzymatic activity of murine CD73 on CT26 cells. However, unlike human tumor cells, bsAb CD73xEGFR did not induce co-internalization of murine CD73 and murine EGFR in CT26 cells. The basis for this human–mouse difference remains unresolved.

Multiple studies have shown that CD73 overexpression contributes to enhanced oncogenic properties, including increased tumor cell proliferation and migration [11]. To determine whether bsAb CD73xEGFR can counter these CD73-driven malignant features, immunodeficient mice implanted with EGFR-overexpressing H292-luc tumors were treated with bsAb CD73xEGFR and tumor growth was monitored. Consistently, bsAb CD73xEGFR markedly suppressed xenograft expansion. In contrast, and unexpectedly, treatment with oleclumab or bsAb CD73xMock promoted growth of H292-luc tumors. Similarly, in vitro exposure of H292-luc cells to oleclumab enhanced tumor cell proliferation, accompanied by increased expression and phosphorylation of the oncogenic receptors EGFR and HER2. These observations diverge from some earlier reports [12, 26, 27]. Collectively, the data suggest that, beyond its role in ADO production, tumor-associated CD73 may function as a signaling mediator capable of transmitting divergent intracellular cues. As CD73 is anchored to the membrane via a glycosylphosphatidylinositol (GPI) moiety, such signaling is likely indirect and mediated through lateral interactions with neighboring transmembrane proteins [28]. Depending on the membrane context, CD73-associated signaling may engage tyrosine kinase or phosphatase pathways, or both. In line with this concept, CD73 on $CD8^+$ T cells has been reported to deliver co-stimulatory signals through lateral association with the receptor-linked protein tyrosine phosphatase CD45RC [29]. We hypothesize that CD73 expressed on tumor cells may similarly regulate EGFR and HER2 oncogenic signaling, either directly or indirectly. The precise mechanisms by which oleclumab enhances tumor growth and elevates EGFR and HER2 expression and phosphorylation in our models require further investigation.

Earlier studies showed that cancer patients receiving chemotherapeutic drugs such as carboplatin, gemcitabine, or paclitaxel display elevated CD73 expression on tumor cells [30], a feature associated with

the acquisition of multidrug resistance [31, 32]. To determine whether bsAb CD73xEGFR can mitigate this resistance, tumor cells were exposed in vitro to bsAb CD73xEGFR and their susceptibility to cytotoxic effects of multiple chemotherapeutics was examined. Notably, bsAb CD73xEGFR increased cancer cell sensitivity to cisplatin, doxorubicin, taxol, and 5-FU. Unexpectedly, the combined application of bsAb CD73xMock with bsAb MockxEGFR, or oleclumab with cetuximab, failed to reproduce the effects observed with bsAb CD73xEGFR alone. These findings indicate that bsAb CD73xEGFR enhances chemosensitivity through simultaneous elimination of adjacent CD73 and EGFR molecules from the tumor cell surface.

In a similar manner, in vitro exposure to bsAb CD73xEGFR augmented the responsiveness of H292 non–small cell lung cancer cells to radiation-induced cytotoxicity by up to ~40%. By contrast, treatment with oleclumab diminished radiation sensitivity and increased the IC_{50} from 1.35 Gy to 3.77 Gy. This result is consistent with observations by Dietrich *et al*. [33], who demonstrated that blocking CD73 enzymatic function with the small-molecule inhibitor APCP enhanced the proliferation of irradiated cancer cells and thereby promoted radioresistance, likely due to reduced local accumulation of ADO. These data suggest that CD73-generated ADO can amplify radiation-triggered cell death in certain tumor contexts. Notably, bsAb CD73xMock did not increase radiation resistance in H292 cells, possibly because its capacity to inhibit CD73 enzymatic activity is lower than that of oleclumab. Whether oleclumab-induced radioresistance extends to additional cancer cell types remains to be clarified in future investigations.

Conclusion

In summary, bsAb CD73xEGFR represents a promising strategy to counteract the suppressive CD73/ADO immune checkpoint across a wide spectrum of malignancies. Importantly, this approach enables more tumor-selective inhibition of CD73 enzymatic activity while concurrently antagonizing the pro-tumorigenic functions of both CD73 and EGFR. Consequently, bsAb CD73xEGFR may hold substantial clinical promise for the treatment of diverse, therapy-resistant solid tumors.

Acknowledgments: None

Conflict of Interest: None

Financial Support: This work was supported by the Dutch Cancer Society (project numbers 11464 and 6986, to WH).

Ethics Statement: None

References

1. Allard D, Allard B, Gaudreau PO, et al. Cd73-adenosine: a next-generation target in Immuno-oncology. Immunotherapy 2016;8:145–63. 10.2217/imt.15.106
2. Allard B, Allard D, Buisseret L, et al. The adenosine pathway in Immuno-oncology. Nat Rev Clin Oncol 2020;17:611–29. 10.1038/s41571-020-0382-2
3. Antonioli L, Blandizzi C, Malavasi F, et al. Anti-Cd73 Immunotherapy: A viable way to Reprogram the tumor Microenvironment. Oncoimmunology 2016;5:e1216292. 10.1080/2162402X.2016.1216292
4. Hay CM, Sult E, Huang Q, et al. Targeting Cd73 in the tumor Microenvironment with Medi9447. OncoImmunology 2016;5:e1208875. 10.1080/2162402X.2016.1208875
5. Keizer RJ, Huitema ADR, Schellens JHM, et al. Clinical pharmacokinetics of therapeutic Monoclonal antibodies. Clin Pharmacokinet 2010;49:493–507. 10.2165/11531280-000000000-00000
6. Zhang J-P, Li X-L, Neises A, et al. Different effects of sgRNA length on CRISPR-mediated gene knockout efficiency. Sci Rep 2016;6:28566. 10.1038/srep28566
7. Rossotti MA, Henry KA, van Faassen H, et al. Camelid single-domain antibodies raised by DNA immunization are potent inhibitors of EGFR signaling. Biochem J 2019;476:39–50. 10.1042/BCJ20180795
8. Ploeg EM, Ke X, Britsch I, et al. Bispecific antibody Cd73Xepcam selectively inhibits the adenosine-mediated immunosuppressive activity of carcinoma-derived extracellular Vesicles. Cancer Lett 2021;521:109–18. 9.1016/j.canlet.2021.08.037
9. Xiong X, Ke X, Wang L, et al. Neoantigen-based cancer vaccination using Chimeric RNA-loaded Dendritic cell-derived extracellular Vesicles. J

Extracell Vesicles 2022;11:e12243. 10.1002/jev2.12243
10. Brinkmann U, Kontermann RE. The making of Bispecific antibodies. MAbs 2017;9:182–212. 10.1080/19420862.2016.1268307
11. wei GZ, Dong K, zhong ZH. The roles of Cd73 in cancer. Biomed Res Int 2014;2014:460654.
12. 12.Kondo S, Iwasa S, Koyama T, 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 2022;27:1795–804. 10.1007/s10147-022-02242-5
13. Mirza M, Henriksen JR, Maenpaa J, 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). ESGO SoA 2021 Conference Abstracts; October 2021. 10.1136/ijgc-2021-ESGO.668
14. Somaiah N, Livingston JAA, Ravi V, et al. A phase II multi-arm study to test the efficacy of Oleclumab and Durvalumab in specific sarcoma subtypes. JCO 2022;40:TPS11594. 10.1200/JCO.2022.40.16_suppl.TPS11594 Available: 10.1200/JCO.2022.40.16_suppl.TPS11594
15. Overman MJ, LoRusso P, Strickler JH, 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). JCO 2018;36:4123. 10.1200/JCO.2018.36.15_suppl.4123 Available: 10.1200/JCO.2018.36.15_suppl.4123
16. Selvaggi G, Novello S, Torri V, et al. Epidermal growth factor receptor overexpression correlates with a poor prognosis in completely Resected non-small-cell lung cancer. Ann Oncol 2004;15:28–32. 10.1093/annonc/mdh011
17. Yoshida T, Okamoto I, Okabe T, et al. n.d. Matuzumab and Cetuximab activate the Epidermal growth factor receptor but fail to trigger downstream signaling by AKT or Erk. Int J Cancer;122:1530–8. 10.1002/ijc.23253
18. Zhi X, Wang Y, Yu J, et al. Potential Prognostic biomarker Cd73 regulates Epidermal growth factor receptor expression in human breast cancer. IUBMB Life 2012;64:911–20. 10.1002/iub.1086
19. Griesing S, Liao BC, Yang JCH. Cd73 is regulated by the EGFR-ERK signaling pathway in non-small cell lung cancer. Anticancer Res 2021;41:1231–42. 10.21873/anticanres.14880
20. Tu E, McGlinchey K, Wang J, et al. Anti–PD-L1 and anti-Cd73 combination therapy promotes T cell response to EGFR-Mutated NSCLC. JCI Insight 2022;7:e142843. 10.1172/jci.insight.142843
21. Vafa O, Gilliland GL, Brezski RJ, et al. An engineered FC variant of an IgG eliminates all immune Effector functions via structural perturbations. Methods 2014;65:114–26. 10.1016/j.ymeth.2013.06.035
22. Koopmans I, Hendriks D, Samplonius DF, et al. A novel Bispecific antibody for EGFR-directed blockade of the PD-1/PD-L1 immune Checkpoint. Oncoimmunology 2018;7:e1466016. 10.1080/2162402X.2018.1466016
23. Koopmans I, Hendriks M, van Ginkel RJ, et al. Bispecific antibody approach for improved Melanoma-selective PD-L1 immune Checkpoint blockade. J Invest Dermatol 2019;139:2343–51. 10.1016/j.jid.2019.01.038
24. Hendriks MAJM, Ploeg EM, Koopmans I, 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 2020;9:1824323. 10.1080/2162402X.2020.1824323
25. van Bommel PE, He Y, Schepel I, 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 2018;7:e1386361. 10.1080/2162402X.2017.1386361
26. Gao Z, Wang H, Lin F, et al. Cd73 promotes proliferation and migration of human Cervical cancer cells independent of its enzyme activity. BMC Cancer 2017;17. 10.1186/s12885-017-3128-5
27. Biscardi JS, Maa MC, Tice DA, et al. C-SRC-mediated Phosphorylation of the Epidermal growth factor receptor on Tyr845 and Tyr1101 is associated with modulation of receptor function. J Biol Chem 1999;274:8335–43. 10.1074/jbc.274.12.8335
28. Dianzani U, Redoglia V, Bragardo M, et al. Co-stimulatory signal delivered by Cd73 molecule to human Cd45Rahicd45Rolo (naive) Cd8+ T lymphocytes. J Immunol 1993;151:3961–70.

29. Airas L, Niemelä J, Salmi M, et al. Differential regulation and function of Cd73, a Glycosyl-Phosphatidylinositol-linked 70-kD adhesion molecule, on lymphocytes and endothelial cells. J Cell Biol 1997;136:421–31. 10.1083/jcb.136.2.421
30. Samanta D, Park Y, Ni X, et al. Chemotherapy induces enrichment of Cd47+/Cd73+/Pdl1+ immune evasive triple-negative breast cancer cells. Proc Natl Acad Sci U S A 2018;115:E1239–48. 10.1073/pnas.1718197115
31. Ujházy P, Klobusická M, Babusíková O, et al. Ecto-5'-Nucleotidase (Cd73) in multidrug-resistant cell lines generated by doxorubicin. Int J Cancer 1994;59:83–93. 10.1002/ijc.2910590117
32. Loi S, Pommey S, Haibe-Kains B, et al. Cd73 promotes anthracycline resistance and poor prognosis in triple negative breast cancer. Proc Natl Acad Sci U S A 2013;110:11091–6. 10.1073/pnas.1222251110
33. Dietrich F, Figueiró F, Filippi-Chiela EC, et al. Ecto-5'-Nucleotidase/Cd73 contributes to the Radiosensitivity of T24 human bladder cancer cell line. J Cancer Res Clin Oncol 2018;144:469–82. 10.1007/s00432-017-2567-3