Bispecific antibody CD73xEGFR inhibits the CD73/adenosine immune checkpoint in a tumor-directed manner and concurrently counteracts pro-oncogenic activities of CD73 and EGFR

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**ABSTRACT**

**Background:** CD73 is an ecto-enzyme that is involved in the conversion of proinflammatory extracellular ATP (eATP) excreted by cancer cells under stress to anti-inflammatory adenosine (ADO). A broad variety of solid cancer types was shown to exploit CD73 overexpression as a suppressive immune checkpoint. Consequently, CD73-antagonistic antibodies, most notably oleclumab, are currently evaluated in several multicenter trials for clinical applicability. However, the efficacy of conventional monospecific CD73-inhibiting antibodies may be limited due to on-target/off-tumor binding to CD73 on normal cells. Therefore, a novel approach that more selectively directs CD73 immune checkpoint inhibition towards cancer cells is warranted.

**Methods:** To address this issue, we constructed a novel tetravalent bispecific antibody (bsAb), designated bsAb CD73xEGFR. Subsequently, the anticancer activities of bsAb CD73xEGFR were evaluated using in vitro and in vivo tumor models.

**Results:** *In vitro* treatment of various carcinoma cell types with bsAb CD73xEGFR potently inhibited the enzyme activity of CD73 (~71%) in an EGFR-directed manner. In this process, bsAb CD73xEGFR induced rapid internalization of antigen/antibody complexes, which resulted in a prolonged concurrent displacement of both CD73 and EGFR from the cancer cell surface. In addition, bsAb CD73xEGFR sensitized cancer to the cytotoxic activity of various chemotherapeutic agents and potently inhibited the proliferative/migratory capacity (~40%) of cancer cells. Unexpectedly, we uncovered that treatment of carcinoma cells with oleclumab appeared to enhance several pro-oncogenic features, including upregulation and phosphorylation of EGFR, tumor cell proliferation (~20%), and resistance towards cytotoxic agents and ionizing radiation (~39%). Importantly, in a tumor model using immunocompetent BALB/c mice inoculated with syngeneic CD73pos/EGFRpos CT26 cancer cells, treatment with bsAb CD73xEGFR outperformed oleclumab (65% vs. 31% tumor volume reduction). Compared to oleclumab, treatment with bsAb CD73xEGFR enhanced the intratumoral presence of CD8pos T-cells and M1 macrophages.

**Conclusions:** BsAb CD73xEGFR outperforms oleclumab as it inhibits the CD73/ADO immune checkpoint in an EGFR-directed manner and concurrently counteracts several oncogenic activities of EGFR and CD73. Therefore, bsAb CD73xEGFR may be of significant clinical potential for various forms of difficult-to-treat solid cancer types.

**KEY MESSAGE**

**What is already known on this topic:** Inhibition of the CD73 immune checkpoint has been hailed as a promising alternate or complementary approach in cancer immunotherapy. However, recent midterm clinical trial reports indicate that, as a single treatment modality, the efficacy of CD73 antagonistic antibody oleclumab remains modest at best.

**What this study adds:** A novel tetravalent bsAb CD73xEGFR is presented that allows to inhibit the CD73/adenosine immune checkpoint on cancer cells in an EGFR-directed manner. Its mode-of-action involves the rapid co-internalization and prolonged concurrent displacement of CD73 and EGFR from the cancer cell surface. BsAb
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CD73xEGFR showed potent capacity to reinvigorate the anticancer activities of ADO-suppressed cytotoxic T-cells and concurrently counteracted cancer cell-surface CD73- and EGFR-mediated pro-oncogenic activities. Of note, we uncovered that identical treatment of carcinoma cells with CD73-antagonistic antibody oleclumab appeared to enhance several pro-oncogenic features, including the upregulation and phosphorylation of EGFR, enhancement of tumor cell proliferation, and promotion of resistance towards chemotherapeutic agents and ionizing radiation.

How this study might affect research, practice, or policy: The uncovered negative attributes of CD73 antagonistic antibody oleclumab can have major ramifications for its future application in cancer treatment. Therefore, alternate approaches that inhibit the CD73 immune checkpoint in a more tumor-selective manner while counteracting the pro-oncogenic activity of EGFR and CD73 appear warranted. In this respect, the multiple and more tumor-selective anticancer activities of bsAb CD73xEGFR may be of particularly promise.

INTRODUCTION

Immunotherapy has significantly contributed to the therapeutic armamentarium currently available to cancer patients with advanced disease. In particular, antagonistic antibodies towards immune checkpoints PD-1/PD-L1 and CTLA-4 have strongly improved therapeutic outcome, albeit only for a relatively small subgroup of these patients. Apparently, the majority of cancer patient harbors malignant cells that exploit alternate and/or additional immunosuppressive checkpoints to achieve immune evasion. Consequently, there is an unmet need for novel inhibitors that can selectively block such alternate immune checkpoints. In this respect, antagonistic antibodies that can selectively inhibit the CD73 immune checkpoint, such as oleclumab, appear to be of clinical promise.

CD73 is a cell surface-expressed enzyme that is key in maintaining immune system homeostasis by the stepwise hydrolysis of the autocrine and paracrine danger signals conveyed by extracellular ATP (eATP) into anti-inflammatory adenosine (ADO). Infection, tissue injury, ischemia, and metabolic stress are known to result in a sharp elevation of eATP release at the site of such lesion(s), where it serves to initiate pro-inflammatory immune responses by attracting and activating various types of immune cells. These pro-inflammatory responses are appropriately locally counterbalanced by the concerted action of cell surface-anchored ectonucleotides CD39 and CD73, which sequentially convert eATP via AMP to ADO. In this process, the enzyme activity of CD73 is the rate-limiting step in catalyzing the conversion AMP to ADO. This catalysis results in a rapid local increase of ADO levels, which engages the immunosuppressive actions of ADO receptors on various, locally present immune cells, thereby providing a self-limiting mechanism for a timely and localized resolution of immune responses.

Due to intrinsic high metabolic stress, numerous cancer cell types excrete remarkably high levels of pro-inflammatory eATP, which they rapidly convert into anti-inflammatory ADO by concurrently overexpressing CD73. Subsequently, diffusion of tumor-produced ADO molecules results in the formation of a potent immunosuppressive ‘halo’ that acts not only locally in the tumor microenvironment, but also outside of the tumor site. Consequently, a halo of ADO molecules chronically

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suppresses the anticancer immune response, which promotes the induction of immune
tolerance, immune escape, and subsequent cancer progression 3.

Preclinical studies have demonstrated that CD73-inhibiting antibodies may be
used to overcome this form of tumor-induced immune suppression in a broad variety
of cancer types, including various carcinomas. In particular, oleclumab (MEDI9447), a
recombinant fully human antibody, selectively binds to CD73 and subsequently inhibits
its enzyme activity 4. Currently, several multicenter trials are ongoing in patients with
advanced solid malignancies to evaluate the clinical potential of oleclumab. However,
the efficacy of a conventional monospecific CD73-inhibiting antibody like oleclumab is
anticipated to be hampered by the ‘on-target/off-tumor’ binding to a vast surplus of
CD73 molecules present on normal cells and tissues 5, potentially precluding its
sufficient accumulation at the tumor site(s). Moreover, CD73-inhibiting antibodies may
induce a generalized (re-)activation of T-cells, potentially leading to autoimmune-
related adverse events, analogous to what is observed for other immune checkpoint
inhibitors.

Intriguingly, we uncovered that in vitro treatment of carcinoma cells with
oleclumab induced upregulation and phosphorylation of oncogenic proteins EGFR and
HER2, which coincided with a marked enhancement of cancer cell proliferation and
resistance towards cytotoxic regimes.

To address these issues, we constructed a novel tetravalent bispecific antibody
(bsAb), designated ‘bsAb CD73xEGFR’, that was engineered to inhibit cancer cell
surface-expressed CD73 in an EGFR-directed manner. Our results demonstrate that
treatment with bsAb CD73xEGFR reduced the outgrowth of inoculated syngeneic
tumors in immunocompetent mice, which coincided with a marked increase in tumor-
infiltration by CD8 pos effector T-cells and activated macrophages. Moreover, bsAb
CD73xEGFR sensitized cancer to the cytotoxic activity of various chemotherapeutic
agents and that of ionizing radiation in vitro and potently inhibited the proliferative and
migratory capacity of cancer cells in vivo.

METHODS
Antibodies and reagents
Antibodies: FITC-labeled-anti-CD73 (clone-MM07, Sino Biological), APC-labeled-anti-
EGFR (clone-528, Santa Cruz), FITC-labeled-Annexin-V (Immunotools), goat-anti-
human-IgG-APC (Southern Biotech), sheep-anti-human-CD73 (R&D), rabbit-anti-
human-EGFR (Abcam), rabbit-anti-human-HER2 (R&D), rabbit-anti-human/mouse-β-
Actin (Abcam), rabbit-anti-sheep-IgG (Thermofisher), goat-anti-rabbit-IgG (Dako),
anti-CD4 (ab183685, Abcam), anti-CD8 (98941, Cell Signalling Technology), anti-
FoxP3 (ab99964; Abcam), anti-F4/80 (ab240946, Abcam), anti-CD206 (ab64693, Abcam),
anti-CD11c (ab52632; Abcam).

Reagents/proteins: VivoGlo (Promega), CFSE, CFSE-FarRed (Thermofisher),
Vybrant DiD, DiO, propidium iodide (PI), fluorescent caspase 3/8-488 probe (Biotium),
Adenosine 5’-(α,β-methylene)diphosphate (APCP, Sigma), anti-humanFc saporin-6
toxin-labeled Fab (Fab-ZAP, Advanced Targeting Systems), CypHer5E (Fisher
Scientific), AMP (Sigma-Aldrich), T-cell activation/expansion beads (Milenyi Biotec),
recombinant soluble human-CD73 (s.hCD73) (Abcam), IFNy-ELISA (eBioscience),
Colorimetric malachite green-based Pi (ab65622, Abcam).
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**Cell lines and transfectants**

Cell lines 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 the American Type Culture Collection (ATCC, Manassas, VA). A549, A549.EGFR-KO 6, H1650, and H1650 EGFR-KO were kindly provided by Prof. Dr. H.J. Haisma. H292-luc cancer cells were purchased from Cellomix Technology (SC-1087).

Cells were cultured in RPMI-1640 or DMEM (Lonza), supplemented with 10% fetal calf serum (Thermofisher) at 37°C in a humidified 5% CO2 atmosphere. CHO-K1 cells were cultured in GMEM (FirstLink), supplemented with 5% dialyzed FBS (Sigma Aldrich).

CHO.CD73 cells stably expressing human CD73 were generated by lipofection (Fugene-HD, Promega) of a plasmid containing cDNA encoding human CD73 (Origene). Likewise, CHO.EGFR cells were generated by lipofection of a plasmid containing cDNA encoding human EGFR (Sino Biological).

CD73-knockout (KO) cells were generated using CRISPR-Cas9 by transfection of pSpCas9 BB-2A-GFP (PX458) plasmid (Addgene plasmid #48138) containing CD73-targeting sgRNA 5’-GCAGCACGTTGGGTTCGGCG-3’ 7. Likewise, EGFR-KO cells were generated by transfection of pSpCas9 BB-2A-GFP (PX458) plasmid containing EGFR-targeting sgRNA 5’-GAGTAACAAGCTCAGGCAGT-3’ (Genscript).

**Construction of bsAb CD73xEGFR-IgG2silent**

DNA fragments encoding scFvCD73 and VHH-EGFR were generated by commercial gene synthesis service (Genscript) based on published VH and VL sequence data from oleclumab 4 and EGFR-directed camelid single-domain antibody fragment NRC-sdAb028 8, respectively. For construction of bsAb CD73xEGFR-IgG2silent and controls, we refer to the supplementary documentation.

**Production of recombinant bsAbs**

BsAbs were produced using the Expi293 expression system (ThermoFisher) and purified using áKTA-Start chromatography system as described previously by our group 9.

**Assessment dual binding activity of bsAb CD73xEGFR**

Cancer cells were incubated at given concentration with bsAbs CD73xEGFR (or controls) at 4°C for 45 min. Subsequently, cells were re-incubated with APC-labeled secondary anti-human-Ig-antibody at 4°C for 45 min. Binding data of the respective bsAbs were acquired using Guava EasyCyte 6/2L flowcytometer (Merck Millipore) and analyzed by GuavaSoft 3.2 software.

Similar, competitive binding of bsAb CD73xEGFR (1 µg/ml) was assessed in the presence of either recombinant soluble human CD73 (s.CD73), EGFR-competing bsAb MockxEGFR, or a combination thereof (each 10 µg) at 4°C for 20 min and evaluated by flowcytometry essentially as described above.

**Assessment of internalization of bsAb CD73xEGFR/antigen complexes**

Cancer were incubated with increasing concentrations (0.01 - 10 µg/ml) bsAb CD73xEGFR (or appropriate controls) at 37°C for 24 h, after which residual CD73- and
EGFR-cell-surface exposure was assessed using anti-CD73 mAbMM07 and anti-EGFR mAb528, which bind to non-overlapping epitopes on CD73 and EGFR, respectively.

Cancer cells were incubated with bsAb CD73xEGFR-pHAb (CypHer5E) (1 µg/ml) (or controls) in the presence (or absence) of EGFR-competing bsAb MockxEGFR (10 µg/ml) at 37 °C for 0, 10-, 60-, 240-, or 360-min. Internalization of pHAb-labeled bsAbs by cancer cells was evaluated by flow-cytometry.

Cancer cells were incubated with bsAb CD73xEGFR (1 µg/ml) (or controls) in the presence of anti-human Fc saporin-6 toxin-labeled Fab (Fab-ZAP, Advanced Targeting Systems). Apoptotic cancer cell death was evaluated after 24 h by flow-cytometry using Annexin-V/PI staining.

**Assessment capacity of bsAb CD73xEGFR 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 as described previously by our group 9.

**Assessment capacity of bsAb CD73xEGFR to restore proliferation capacity of ADO-suppressed T-cells**

CFSE-labeled Peripheral Blood Mononuclear Cells (PBMCs) were activated by addition of T cell activation/expansion beads in a bead-to-cell ratio of 1-1. Subsequently, PBMCs were cultured in medium supplemented (or not) with AMP (100 µM) and incubated with bsAb CD73xEGFR (1 µg/ml) (or controls) for 5 d. T cell proliferation was analyzed by CFSE dilution using flow-cytometry.

Similar, proliferation of CFSE-FarRed-labeled PBMCs evaluated using live cell imaging was performed as described previously by our group 9.

**Assessment capacity of bsAb CD73xEGFR to restore anticancer activity of ADO-suppressed T-cells in vitro**

PBMCs were cultured in medium supplemented with AMP (100 µM) and incubated with increasing concentrations (0.01 - 10 µg/ml) bsAb CD73xEGFR (or controls) for 24 h. Next, the T-cells present were stimulated and re-directed to kill EpCAM-expressing PC3M prostate cancer cells and evaluated using live cell imaging as described previously by our group 9.

**Assessment capacity of bsAb CD73xEGFR to restore anticancer activity of ADO-suppressed T-cells in vivo**

Syngeneic tumors were established by subcutaneous injection of 5×10⁵ CT26 cells suspended in 0.1 ml of PBS into the right flanks of 4-to-6-week-old female BALB/c mice (Janvier, France) in accordance with Dutch Act on Animal Experimentation. Animals were randomized using RandoMice into 5 groups of 9 mice each based on body weight (Total = 45 animals. Sample size was determined using G*Power Tool). bsAb CD73xEGFR and controls were administered by I.P. injection at 7.5 mg/kg on day 4, 7, 10, and 14. Tumor volume was assessed by caliper measurements. Mice were euthanized using cardiac puncture, followed by cervical dislocation after 21 d. Tumor and organs and processed for multiplexed immunofluorescence or histology. Of note, E.M.P. was aware of group allocation at the different stages of the experiment (during
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allocation, conduct of the experiment, outcome assessment, and data analysis). Additionally, we did not adjust for cofounding since randomization was successful.

**Multiplexed immunofluorescence to assess tumor-infiltrating leukocytes**
Multiplexed immunofluorescence to evaluate T cell and macrophage populations in tumors resected from immunocompetent mice was performed as described previously 10.

**Assessment inhibitory effect of bsAb CD73xEGFR on proliferation of cancer cells in vitro**
Cancer cells were seeded into an E-plate 16 (ACEA Biosciences) and incubated with bsAb CD73xEGFR (1 µg/ml) (or controls) at 37°C for 60 h. Cell proliferation was monitored using the xCELLigence RTCA instrument (ACEA Biosciences).

Cancer cells were seeded into a 96-well culture plate and incubated with bsAb CD73xEGFR (1 µg/ml) (or controls) at 37°C. Live cell imaging technology (IncuCyte) was used to follow proliferation of cancer cells by taking pictures at 4 x magnification every 4 h for 3d. Confluence (%) was measured using IncuCyte software 2019B.

**Assessment inhibitory effect of bsAb CD73xEGFR on proliferative capacity of cancer cells in vivo**
Tumors were established by subcutaneous injection of $1\times10^6$ H292-Luc cells suspended in 0.1 ml of PBS into the right flanks of a 4-to-6-week-old female Athymic Nude Mouse (Crl:NU(NCr)-Foxn1™, Charles River, Germany). Animals were randomized using RandoMice into 5 groups of 9 mice each based on body weight (Total = 45 animals. Sample size was determined using G*Power Tool). bsAb CD73xEGFR (or controls) was administered via I.P. injection at 7.5 mg/kg on day 7, 10 and 17. Bioluminescent imaging (IVIS spectrum, 75 mg/kg VivoGlo; Promega) and caliper measurements were performed to evaluate tumor growth. Mice were euthanized using cardiac puncture, followed by cervical dislocation after 28 d. All procedures using animals were in accordance with Dutch Act on Animal Experimentation and approved by the institutional Animal Welfare Committee at the Rijksuniversiteit Groningen/University Medical Center Groningen. Of note, E.M.P. was aware of group allocation at the different stages of the experiment (during allocation, conduct of the experiment, outcome assessment, and data analysis). Additionally, we did not adjust for cofounding since randomization was successful.

**Assessment inhibitory effect of bsAb CD73xEGFR on migratory capacity of cancer cells**
Cancer cells were seeded in 24-well plates, equipped with a stopper, in the presence of bsAb CD73xEGFR (1 µg/ml) (or controls) at 37°C for 24 h. Subsequently, the stopper was removed and Live cell imaging technology (IncuCyte) was used to assess the treatment effect on the migratory capacity of cancer cells by taking pictures at 4 x magnification every 0.5 h for 3 d. Closure of the standardized cell-free zone (%) was measured using IncuCyte software 2019B.
Assessment capacity of bsAb CD73xEGFR to sensitize cancer cells towards chemotherapeutic agents and radiation

Cancer cells were seeded into a 96-well culture plate and incubated with bsAb CD73xEGFR (1 µg/ml) (or controls) with (or without) 5FU (50 µg/ml), taxol (50 nM), cisplatin (1 µg/ml), or doxorubicin (50 nM) at 37°C. Live cell imaging technology (IncuCyte) was used to assess treatment effect on proliferative capacity on cancer cells by taking pictures at 4 x magnification every 6 h for 6 d. Confluence (%) was measured using IncuCyte software 2019B.

Cancer cells were seeded into a 6-well culture plate and incubated with increasing concentrations (0.01 - 10 µg/ml) of bsAb CD73xEGFR (or controls) at 37°C for 14d. Subsequently, cell colonies were washed with PBS and then stained with crystal violet. Colony number and size were analyzed using ImageJ software.

Cancer cells were seeded into a 6-well culture plate, irradiated with an increasing dose of radiation (0.5–2 Gy), and then cultured at 37°C for 14 d. Subsequently, cell colonies were washed, stained, and analyzed as described above. Irradiation (0.59 Gy/min) was performed using a 137Ce source (IBL637 Cesium-137 γ-ray machine).

Statistical analysis

Statistical analysis was done by (multiple) T-test, one-way ANOVA followed by Tukey post-hoc test, or (non-)linear regression, 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 CD73xEGFR has dual binding specificity for CD73 and EGFR

BsAb CD73xEGFR was constructed in the bispecific taFv-Fc format equipped with 2 identical CD73-directed scFv antibody fragments derived from oleclumab and 2 identical EGFR-directed cameld single-domain antibody fragments (supplementary Figure 1). Dual binding activity of bsAb CD73xEGFR was confirmed using Chinese Hamster Ovary (CHO) cells transfected with either human CD73 or human EGFR. BsAb CD73xEGFR bound-dose-dependently to both CHO.CD73 and CHO.EGFR cells, and not to parental CHO cells (Figure 1A). Binding of bsAb CD73xEGFR towards SK-BR-3 breast cancer cells was partly reduced in the presence of soluble human CD73 (s.CD73), whereas antagonistic EGFR bsAb MockxEGFR strongly inhibited the binding. Of note, binding of bsAb CD73xEGFR towards SK-BR-3 breast cancer cells was partly reduced in the presence of soluble human CD73 (s.CD73), whereas antagonistic EGFR bsAb MockxEGFR strongly inhibited the binding. Of note, binding of bsAb CD73xEGFR towards CD73 and EGFR. Importantly, binding levels of bsAb CD73xEGFR towards a panel of 4 CD73pos/EGFRpos cell lines closely correlated with their respective expression levels for EGFR (Figure 1C, supplementary Figure 2). Moreover, bsAb CD73xEGFR showed reduced binding activity towards EGFR-KO/CD73pos cell lines (Figure 1C). Additionally, bsAb CD73xEGFR showed capacity to simultaneously bind to CD73 present on one cell type and EGFR present on another cell type when both cell types are in close proximity. In particular, bsAb CD73xEGFR cellularly bridged FaDu cancer cells (DiO-labeled) and CHO.CD73 cells (DiD-labeled), as was evident from a marked increase in DIOpos/DIDpos cell clusters detected by flow cytometry (supplementary Figure 3).
bsAb CD73xEGFR induces rapid co-internalization and subsequent prolonged displacement of both CD73 and EGFR from the cancer cell surface

Treatment of CD73\textsuperscript{pos}/EGFR\textsuperscript{pos} H292 cancer cells with bsAb CD73xEGFR resulted in a concurrent and dose-dependent cancer cell surface displacement of CD73 and EGFR. At a concentration of 1 \( \mu \text{g/ml} \), treatment with bsAb CD73xEGFR reduced cell surface exposure of CD73 and EGFR by 81\% and 73\%, respectively (Figure 2A and 2B, supplementary Figure 4), which persisted for up to 96 h (Figure 2C and 2D). Comparable prolonged displacement of CD73 and EGFR was detected when primary patient-derived ovarian- and colon cancer cells were treated with bsAb CD73xEGFR (Figure 2E). Internalization of bsAb CD73xEGFR/antigen complexes occurred after 10 – 60 min, which was abolished in presence of saturating amounts of EGFR-competing bsAb MockxEGFR (Figure 2F). Likewise, ‘piggybacking’ of Fab-ZAP toxin on internalizing bsAb CD73xEGFR resulted in saporin-mediated apoptotic cell death (Figure 2G). Analogously, treatment of CD73\textsuperscript{pos}/EGFR\textsuperscript{pos} cancer cells with bsAb CD73xEGFR significantly reduced CD73 expression levels and attenuated EGFR tyrosine phosphorylation (supplementary Figure 5).

Figure 1: bsAb CD73xEGFR has dual binding specificity for CD73 and EGFR. (A) Dose-dependent binding of bsAb CD73xEGFR and bsAb CD73xMock to CHO, CHO.hEGFR and CHO.hCD73, respectively. (B) Competitive binding assay in which bsAb CD73xEGFR (1 \( \mu \text{g/ml} \)) was pre-incubated with excess amounts of soluble human CD73 (s.hCD73), EGFR-competing bsAb MockxEGFR or a combination thereof.
and then evaluated for binding to SK-BR-3 cancer cells. (C) Binding of bsAb CD73xEGFR and bsAb CD73xMock (both 1 µg/ml) to parental cancer cells (FaDu, H292, A549, H1650) and corresponding EGFR-KO variants thereof. All experiments were analyzed by flow-cytometry. All graphs represent mean ± SD. Statistical analysis in B was performed using one-way ANOVA followed up by Tukey post-hoc test (***p < .01, ****p < .001).

**bsAb CD73xEGFR internalizes cancer cell surface-exposed CD73 in an EGFR-directed manner**

BsAb CD73xEGFR showed a marked capacity to displace CD73 from CD73pos/EGFRpos cancer cells, whereas only minimal CD73 displacement was observed when treating CD73pos/EGFRneg cancer cells (supplementary Figure 6A). Analogously, bsAb CD73xEGFR essentially failed to internalize CD73 when treating A549 EGFR-KO and H1650 EGFR-KO cells (supplementary Figure 6B). Importantly, treatment of a series of 10 individual CD73pos/EGFRpos cancer cell line types with bsAb CD73xEGFR demonstrated a positive linear dependence (R² = 0.89) between residual CD73 and EGFR cell surface-expression levels, whereas identical treatment with oleclumab failed to do so (R² = 0.0003) (Figure 2H). Again, this indicated that upon treatment with bsAb CD73xEGFR, cancer cell surface-expressed CD73 and EGFR are subject to antibody-mediated co-internalization.

**bsAb CD73xEGFR potently inhibits the CD73 enzyme activity in an EGFR-directed manner**

Treatment of various carcinomas with bsAb CD73xEGFR dose-dependently inhibited the CD73 enzyme activity (Figure 3A, supplementary Figure 7A) and in this respect significantly outperformed oleclumab in 3 out of 3 cancer cell lines (group average; 71% vs. 52%, respectively) (Figure 3B) and in 8 out of 9 primary patient-derived carcinoma cells (group average; 55% vs. 40%, respectively) (Figure 3C). The respective IC₅₀ values of bsAb CD73xEGFR calculated for 6 cell lines ranged from 0.001 – 0.038 µg/ml, whereas those of oleclumab ranged from 0.005 – 0.563 µg/ml (supplementary Figure 7B). Additionally, inhibition of CD73 enzyme activity of H929 cancer cells by bsAb CD73xEGFR was dose-dependently decreased in presence of the EGFR-competing bsAb MockxEGFR (Figure 3D). The CD73 enzyme activity of A549 EGFR-KO cancer cells was only marginally reduced upon treatment with bsAb CD73xEGFR (Figure 3E).

**bsAb CD73xEGFR overcomes ADO-mediated suppression of T cell proliferation**

When activated CFSE-labeled T-cells were subjected to AMP, which is locally enzymatically converted to ADO by CD73, their proliferation capacity was significantly repressed. Importantly, treatment with bsAb CD73xEGFR or oleclumab fully abrogated the ADO-mediated inhibition of T cell proliferation, as can be appreciated from the successive dilution of the CFSE-dye (Figure 4A). These results corroborated the increase in activated T cell cluster size (Figure 4B).
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Figure 2: bsAb CD73xEGFR induces rapid co-internalization and subsequent prolonged displacement of both CD73 and EGFR from the cancer cell surface. Residual (A) CD73 and (B) EGFR membrane presence on H292 cells after treatment with bsAb CD73xEGFR (0.01 – 10 μg/ml) (or controls) for 24 h. Residual (C) CD73 and (D) EGFR membrane presence on H292 cells after treatment with bsAb CD73xEGFR (1 μg/ml) (or controls) for 1, 4, 24, 48, 72 and 96 h. (E) Residual CD73 and EGFR membrane presence after treatment of primary patient-derived ovarian and colon cancer cells with bsAb CD73xEGFR (1 μg/ml) (or controls) for 24 h. (F) Mean fluorescent intensity (MFI) of H292 cells after incubation with bsAb CD73xEGFR (1 μg/ml) (or controls) labeled with pH-sensitive dye Cypher5E for 10, 60, 240 and 360 min. (G) Percentage of Annexin-V pos/PIpos H292 cancer cells after incubation for 24 h with bsAb CD73xEGFR (1 μg/ml) (or controls) in the presence of an anti-human IgG-Fab fragment labeled with a saporin-based toxin. (H) Residual cell surface EGFR-expression levels plotted against residual cell surface CD73-expression levels of a panel of 10 cancer cell lines after treatment with bsAb CD73xEGFR or oleclumab (both 1 μg/ml) for 24 h. All experiments were analyzed by flow-cytometry. All graphs represent mean ± SD. Statistical analysis in C (based on group-means) and E was performed using unpaired T-test (*p < .05, **p < .01).
Figure 3: bsAb CD73xEGFR potently inhibits the enzyme activity of CD73 in an EGFR-directed manner. (A) Percentage of inhibition CD73 enzyme activity on H292 cells after treatment with bsAb CD73xEGFR (0.01 - 10 μg/ml) (or controls) for 24 h. (B) Percentage of inhibition CD73 enzyme activity on H292, OvCAR3, and H322 cancer cell lines or on primary patient-derived cancer cells after treatment with bsAb CD73xEGFR (1 μg/ml) (or controls) for 24 h. (D) Competitive CD73 enzyme inhibition assay in which H292 cells were pretreated with excess amounts EGFR-competing bsAb MockxEGFR prior to incubation with bsAb CD73xEGFR (0.01 - 2 μg/ml). (E) Percentage of inhibition CD73 enzyme activity on A549 parental and EGFR-KO cells after treatment with bsAb CD73xEGFR or bsAb CD73xMock (both 1 μg/ml). CD73-mediated hydrolysis of AMP into ADO was evaluated using a colorimetric malachite green-based Pi assay. All graphs represent mean ± SD. Statistical analysis in B and C was performed using unpaired T-test (based on group-means) (***p < .01).
**BsAb CD73xEGFR inhibits the CD73 immune checkpoint in a tumor-directed manner**

**BsAb CD73xEGFR restores anticancer activity of ADO-suppressed cytotoxic T-cells**

When cytotoxic T-cells were subjected to AMP and subsequently redirected to kill EpCAM-expressing cancer cells using EpCAM-directed/CD3-agonistic bispecific antibody BIS-1, induction of cancer cell death, evident from high caspase-3/8 activation levels in target cells, significantly dropped (Figure 4C and 4D, supplementary Figure 8). Importantly, treatment with bsAb CD73xEGFR dose-dependently restored the capacity of these ADO-suppressed T-cells to eliminate cancer cells (Figure 4E). These results corroborate ELISA data quantifying the restored capacity of cytotoxic T-cells to secrete IFN-γ (Figure 4F).

**BsAb CD73xEGFR increases the tumor-infiltrating capacity of leukocytes in immunocompetent mice**

Subsequently, the apparent effect of in vitro treatment with bsAb CD73xEGFR on the anticancer activities of cytotoxic T-cells was evaluated in vivo using BALB/c mice inoculated with CT26 cancer cells (in vitro data CT26; supplementary Figure 9). At 4, 7, 10, and 14 days after inoculation, animals were treated I.P. with 7.5 mg/kg bsAb CD73xEGFR, oleclumab, or controls (Figure 5A). In vivo treatment with bsAb CD73xEGFR significantly reduced tumor volume (65%) and tumor wet-weight (77%), thereby markedly outperforming oleclumab (31% reduction in tumor volume) (Figure 5B and 5C, supplementary Figure 10). Importantly, tumors procured from animals treated with bsAb CD73xEGFR showed increased infiltration of CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes and a decreased infiltration of FoxP3<sup>+</sup> lymphocytes (Figure 5D – 5G, supplementary Figure 11). Moreover, in these tumors an increased infiltration of F4/80<sup>+</sup> (total macrophage) and CD11c<sup>+</sup> (M1 macrophage) cells, and a decreased infiltration of CD206<sup>+</sup> (M2 macrophage) cells was observed (Figure 5H – 5K, supplementary Figure 12). In contrast, tumors procured from animals treated with oleclumab only demonstrated an increased infiltration with CD8<sup>+</sup> lymphocytes and a decreased infiltration of total and M2 macrophages. Importantly, animals treated with bsAb CD73xEGFR appeared to show no signs of systemic toxicity in vital organs (supplementary Figure 13).

**BsAb CD73xEGFR inhibits proliferative and migratory capacity of cancer cells in vitro**

Both enzymatic and non-enzymatic attributes of CD73 overexpression were reported to be implicated in enhancement of cancer cell proliferative/migratory capacity and resistance to chemo/radio-therapy<sup>12</sup>. In this respect, it is noteworthy that treatment with bsAb CD73xEGFR decreased the proliferative capacity of carcinoma cell line types (4 out of 4) by ~40% (Figure 6A and 6B, supplementary Figure 14). Additionally, treatment with bsAb CD73xEGFR significantly reduced the colony forming capacity of cancer cells both in number and size (Figure 6C – 6E). In this respect, IC<sub>50</sub> values calculated for bsAb CD73xEGFR ranged from 0.16 – 0.65 μg/ml, which was superior compared to oleclumab (IC<sub>50</sub> 9.38 – 24.33 μg/ml) in 3 out of 3 cell lines tested (supplementary Figure 15). Representative light-microscopic pictures shown in Figure 6F indicate that treatment of cancer cells with bsAb CD73xEGFR almost fully abrogated their migratory capacity. These results corroborate the observed delayed closure of the cell-free zone over time upon treatment with bsAb CD73xEGFR (Figure 6G).
Figure 4: bsAb CD73xEGFR restores anticancer activities of ADO-suppressed T-cells. (A) Cell count of activated CFSE-labeled PBMCs, cultured in medium supplemented with AMP, in the presence (or absence) of bsAb CD73xEGFR (1 µg/ml) (or controls) at 37 °C for 5 d. Subsequently, CFSE-dye dilution was evaluated by flow-cytometry. Both unstimulated (purple) and ADO-inhibited T-cells are indicated by bright single peaks on the right side of the histogram. The discrete successive peaks (green) in the histograms represent successive generations of live T-cells due to dilution of cytoplasmic CFSE-dye. (B) Similar, the size of proliferating T-cells clusters (µm²/image) was quantified using live cell imagine by taking pictures at 10 x magnification at 37°C every 1.5 h for 2 d. (C) Caspase-3/8 activation (apoptotic cancer cell death) in PC3M cancer cells, killed by redirected PBMCs using BIS-1 (bsAb CD3xEpCAM) in an effector (E) to target (T) cell ratio of 4:1. Importantly, PBMCs were incubated with (or without) AMP, in the presence, or absence, of bsAb CD73xEGFR, oleclumab or (D) controls (all 1 µg/ml). Caspase-3/8 activation (count per image) was evaluated using live cell imagine technology by taking pictures at 10 x magnification at 37°C every 1.5 h for 2.5 d. (E) Similar, PBMCs were incubated with increasing amounts of bsAb CD73xEGFR (0.01 – 10 µg/ml), subjected to AMP, redirected in order to kill PC3M cancer cells and analyzed as indicated above. (F) IFN-γ levels in culture supernatant of E were measured by ELISA. All graphs represent mean ± SD. Ole = oleclumab in graph B. Statistical analysis in D was performed using unpaired T-test. Statistical analysis in F was performed using one-way ANOVA followed up by Tukey post-hoc test (*p < .05, **p < .01).
Figure 5: bsAb CD73xEGFR markedly enhanced the intratumoral presence of CD8<sup>+</sup> T-cells and M1 macrophages in immunocompetent mice. (A) Immunocompetent BALB/c mice were inoculated with murine CT26 colon carcinoma cells S.C. into the flank and injected I.P. with bsAb CD73xEGFR (7.5 mg/kg) or controls on day 4, 7, 10, and 14. Tumor and organs were harvested for analysis 21 days post tumor injection. (B) Mean tumor volume and (C) wet tumor weight of each 9 animals per group. (D) Representative images of multiplexed immunofluorescence staining and quantification for (E) CD4, (F) CD8, (G) and FoxP3 in tumor sections derived from isotype, bsAb CD73xEGFR or oleclumab treated mice. (H) Representative images of multiplexed immunofluorescence staining and quantification for (I) F4/80, (J) CD11c and (K) CD206 in tumor sections derived from isotype, bsAb CD73xEGFR or oleclumab treated mice. All graphs represent mean ± SD. Scale bar in D and H = 200 µm. Statistical analysis in B and E-K was performed using unpaired T-test. Statistical analysis in C was performed using one-way ANOVA followed up by Tukey post-hoc test (*p < .05, **p < .01, ***p < .001).
Unexpectedly, \textit{in vitro} treatment of various carcinoma cell line types with oleclumab (or small-molecule CD73-inhibitor APCP) appeared to rather promote than inhibit cancer cell growth (Figure 6B). Moreover, treatment with oleclumab enhanced the migration capacity of cancer cells by \( \sim 20\% \) (Figure 6G). These results may be explained by the fact that \textit{in vitro} treatment with oleclumab enhanced (both total and phosphorylated) EGFR and HER2 levels in these cancer cells (supplementary Figure 5). Of note, oleclumab-induced upregulation of cell-surface expressed EGFR is also apparent in Figure 2H in 8 out of 10 cell lines evaluated.

**bsAb CD73xEGFR inhibits the proliferative capacity of xenografted cancer cells in immunodeficient mice**

Subsequently, the apparent opposing effect of \textit{in vitro} treatment with bsAb CD73xEGFR versus oleclumab treatment on the proliferative capacity of cancer cells was evaluated \textit{in vivo} using athymic nude mice inoculated with H292-Luc2 cells. At 7, 10, and 17 days after inoculation, animals were treated I.P. with 7.5 mg/ml bsAb CD73xEGFR, oleclumab, or controls (Figure 6H). Compared to treatment with isotype control, \textit{in vivo} treatment with bsAb CD73xEGFR inhibited tumor growth by 56\% (Figure 6I and 6J, supplementary Figure 16). In comparison, the volume of tumors that developed in animals treated with oleclumab or bsAb CD73xMock increased by 24\% and 40\%, respectively.

**bsAb CD73xEGFR sensitizes cancer cells towards treatment with chemotherapeutic agents and ionizing radiation**

\textit{In vitro} co-treatment with bsAb CD73xEGFR sensitized cancer cells towards the cytotoxic activity of cisplatin, doxorubicin, taxol, and 5FU by \( \sim 1\text{-}, 0.45\text{-}, 0.74\text{-}, \) and 0.4-fold, respectively (Figure 7A and 7B, supplementary Figure 17). In contrast, treatment with oleclumab enhanced resistance of cancer cells towards cisplatin, doxorubicin, and 5FU by \( \sim 2\text{-}, 1.8\text{-}, \) and 1.44-fold, respectively. Similarly, co-treatment with bsAb CD73xEGFR enhanced sensitivity of cancer cells towards cytotoxicity induced by ionizing radiation (Figure 7C). In this respect, IC\(_{50}\) values calculated for bsAb CD73xEGFR treatment decreased from 1.35 Gy to 0.536 Gy (Figure 7E). In contrast, identical treatment with oleclumab enhanced resistance towards ionizing radiation up to 39\% at 2 Gy (Figure 7C), whereas calculated IC\(_{50}\) values increased from 1.35 Gy to 3.77 Gy (Figure 7E). Importantly, treatment of CD73-KO cancer cells with oleclumab did not change their resistance towards ionizing radiation (Figure 7D).
BsAb CD73xEGFR inhibits the CD73 immune checkpoint in a tumor-directed manner

**Figure 6:** bsAb CD73xEGFR inhibits the proliferative and migratory capacity of cancer cells. (A) Proliferation assay in which H292 and corresponding CD73-KO cancer cells were incubated with bsAb CD73xEGFR (1 μg/ml) (or controls) and then evaluated using the RTCA xCELLigence instrument. Readout is indicated as cell-index, which is an arbitrary unit for attachment of adherent cells and cell proliferation measured at 37°C every 15 min. (B) Proliferation assay in which a panel of cell lines (H292, H322, FaDu, H2, etc.) were treated with different concentrations of bsAb CD73xEGFR. (C) Colony formation assay showing the effect of bsAb CD73xEGFR on colony formation in H292 and CD73-KO cancer cells. (D) Dose-response curves for bsAb CD73xEGFR on colony formation. (E) Colony area versus log [bsAb] (μg/ml). (F) Morphological changes in H292 cells treated with bsAb CD73xEGFR. (G) Closure of cell-free area (%) in H292 and CD73-KO cancer cells treated with bsAb CD73xEGFR. (H) Time course of tumor growth in athymic nude mice (C57BL/6) following injection of H292 luciferase-expressing cells and treatment with bsAb CD73xEGFR. (I) Bioluminescence imaging of tumor growth in response to bsAb CD73xEGFR treatment. (J) Tumor volume over time following injection of H292 luciferase-expressing cells and treatment with bsAb CD73xEGFR.
and OvCAR3) was incubated with bsAb CD73xEGFR (1 µg/ml) (or controls). The percentage of cell confluence was evaluated after 72 h. (C) Representative images of H292 cell colonies after treatment with bsAb CD73xEGFR (1 µg/ml) or indicated controls at 37 °C for 14 d. (D) Percentage of OvCAR3 cell colonies after treatment with bsAb CD73xEGFR (0.01 – 10 µg/ml) (or controls) at 37 °C for 14 d. Subsequently, (D) number and (E) area of OvCAR3 cell colonies were calculated using ImageJ software. (F) Representative images of H292 cells, seeded in a culture plate equipped with a stopper, which enabled the formation of a cell-free detection zone, and incubated with bsAb CD73xEGFR (1 µg/ml) (or controls) at 37 °C for 72 h. (G) Similar, closure of cell-free detection zone (migration) evaluated over time using live cell imaging technology by taking pictures at 4 x magnification at 37°C every 0.5 h for 3 d. (H) Athymic nude mice (Crl:NU(NCr)-Foxn<sup>nu</sup>) were inoculated S.C. in the flank with human H292-luc2 non-small lung cancer cells and injected I.P. with bsAb CD73xEGFR (7.5 mg/kg) (or controls) on day 7, 10 and 17. (I) Representative bioluminescent images of bsAb CD73xEGFR or control treated mice on day 28 post tumor injection. (J) Mean tumor volume (mm<sup>3</sup>) of each 9 animals per group determined by caliper measurements. All graphs represent mean ± SD. Statistical analysis in B was performed using one-way ANOVA followed up by Tukey post-hoc test. Statistical analysis in J was performed using unpaired T-test (**p < .01). (*p < .05, **p < .01).

**Figure 7:** bsAb CD73xEGFR sensitizes cancer cells towards chemotherapeutic agents and radiation. Cell confluence of H292 cancer cells treated (or not) with bsAb CD73xEGFR (1 µg/ml) or controls in the presence (or absence) of (A/B) doxorubicin (50 nM), (B) cisplatin (1 µg/ml), taxol (50 nM) or 5FU (50 µg/ml) and then evaluated using live cell imaging by taking pictures at 4x magnification every 6 h at 37 °C for 6 d. (C) Percentage of H292 or (D) corresponding CD73-KO cell colonies after treatment with bsAb CD73xEGFR (1 µg/ml) or controls, irradiated (0.5, 1, 1.5 and 2 Gy) and then incubated at 37 °C for 14 d. Cell colonies were calculated using ImageJ software. (E) IC<sub>50</sub> values (Gy) calculated for graph C. All graphs represent mean ± SD. Statistical analysis in B was performed using un-paired T-test (**p < .05, **p < .01).
DISCUSSION
Antagonistic CD73 antibodies like oleclumab appear useful to overcome the inhibitory activity of the CD73/ADO immune checkpoint in a broad variety of cancer types. Currently, several multicenter trials are ongoing to evaluate the clinical potential of oleclumab as an immune checkpoint inhibitor in patients with advanced solid malignancies, including various carcinomas. Unfortunately, recent midterm clinical trial reports indicate that, as a single treatment modality, the efficacy of oleclumab remains modest at best. Possibly, monospecific antagonistic CD73 antibodies like oleclumab suffer from ‘on-target/off-tumor’ binding to CD73 molecules present on normal cells, which limits CD73-inhibitory activity at the tumor site(s).

Here, we report on the construction and preclinical evaluation of bsAb CD73xEGFR that was engineered to inhibit cancer cell surface-expressed CD73 in an EGFR-directed manner. We selected EGFR for this purpose as it is a well-established tumor-associated cell surface target antigen that is frequently mutated and/or overexpressed by various difficult-to-treat solid malignancies. Moreover, many malignancies were shown to selectively co-overexpress CD73 and EGFR. BsAb CD73xEGFR is equipped with two identical scFv antibody fragments derived from oleclumab and two identical EGFR-directed nanobodies. Of note, these antibody domains bind to their respective human - and mouse orthologues with similar affinities. This strategy allows for preclinical evaluation of bsAb CD73xEGFR in both human and murine tumor model systems. To exclude ADCC-mediated antitumor effects during such evaluation, we equipped bsAb CD73xEGFR with an IgG2-silent Fc domain with nullified effector function.

Importantly, compared to oleclumab, bsAb CD73xEGFR showed superior antagonistic activity towards cancer cells that co-overexpress CD73 and EGFR. This superiority is most likely attributable to the enhanced avidity associated with tetravalent format of bsAb CD73xEGFR. Previously, we reported on similar attributes for tetravalent bsAbs that were designed to block immune checkpoints PD-L1 and CD47 in a tumor-directed manner. More recently, we reported on tetravalent bsAb CD73xEpCAM, which allowed to potently inhibit CD73 exposed on carcinoma-derived exosomes, whereas oleclumab showed no or very limited capacity to do so. Intriguingly, in vitro treatment of CD73pos/EGFRpos cancer cells, both various cell lines and primary patient-derived cancer cell types, with bsAb CD73xEGFR induced the rapid internalization of bsAb/antigen complexes, resulting in the prolonged and concurrent displacement of CD73 and EGFR from the cancer cell surface for up to 96 hours. The bsAb CD73xEGFR-induced internalization of cancer cell surface-exposed CD73 resulted in a similarly prolonged incapacity of these cancer cells to convert extracellular AMP to ADO. Importantly, in vitro treatment of ADO-suppressed cytotoxic T-cells with bsAb CD73xEGFR potently reinvigorated their anticancer activities. Moreover, compared to controls, treatment with bsAb CD73xEGFR of immunocompetent BALB/c mice (7.5 mg/kg) inoculated with syngeneic CT26 colorectal carcinoma cells, resulted in an average reduction of ~65% in tumor size development, whereas identical treatment with oleclumab reduced tumor size by only ~31%. Importantly, the in vivo application of bsAb CD73xEGFR increased the presence of tumor-infiltrating CD4pos T-cells, - CD8pos T-cells, and - macrophages by 38%, 52%, and 82%, respectively. These results appear favorable compared to those reported by Hay et al. using essentially the same mouse tumor model in which treatment with
oleclumb (10 mg/kg) resulted in a reduction in tumor size of ~60% and an increase in tumor-infiltrating CD4\textsuperscript{pos} T-cells, - CD8\textsuperscript{pos} T-cells, and - macrophages by only 15%, 25%, and 20%, respectively.

Several reports indicated that overexpression of CD73 is implicated in the enhancement of various oncogenic attributes of cancer cells, including increased cell proliferation and migration capacity \textsuperscript{12}. To evaluate whether bsAb CD73xEGFR has capacity to counteract the oncogenic attributes of CD73 overexpression, we treated immunodeficient mice inoculated with EGFR-overexpressing H292-luc tumor cells with bsAb CD73xEGFR and assessed its treatment effect on tumor outgrowth. Indeed, treatment with bsAb CD73xEGFR potently reduced the size of xenografted tumors. In contrast, and rather unexpectedly, identical treatment with oleclumab (or bsAb CD73xMock) appeared to enhance the growth capacity of xenografted H292-luc carcinoma cells. Intriguingly, \textit{in vitro} treatment of H292-luc cells with oleclumab also resulted in enhancement of tumor growth, which coincided with enhanced expression - and phosphorylation levels of oncogenic proteins EGFR and HER2 in these cancer cells. In this respect, our observations appear to contradict with those mentioned in some previous reports \textsuperscript{12,27,28}. Apparently, apart from its ADO-producing activity, cancer cell-exposed CD73 appears to serve as a signaling transduction molecule that can deliver opposing intracellular signals. Because CD73 is linked to the cell surface by a GPI anchor, the transduction of such signals is probably indirectly mediated by a lateral interaction with juxtaposed transmembrane molecules \textsuperscript{29}. Depending on the particular membrane context, CD73 signaling may thus be coupled to either their tyrosine kinase or phosphatase activities (or possibly both). Similarly, it has been previously reported that CD73 present on CD8\textsuperscript{pos} T-cells can act as co-stimulatory signal by laterally interacting with receptor-linked protein tyrosine phosphatase CD45RC \textsuperscript{30}. We speculate that cancer cell-expressed CD73 may have analogous capacity to modulate oncogenic activities of EGFR and HER2 in a direct or indirect manner. The underlying mechanism by which oleclumab treatment promoted tumor growth and enhanced expression and phosphorylation levels of EGFR and HER2 in our model systems remains to be elucidated by follow-up studies.

Previously, it was reported that cancer patients treated with chemotherapeutic agents, such as carboplatin, gemcitabine, and paclitaxel, show enhanced CD73 expression levels on cancer cells \textsuperscript{31}, which correlate with adopting a multidrug-resistance phenotype \textsuperscript{32,33}. To evaluate whether bsAb CD73xEGFR has capacity to counteract multidrug-resistance, we treated cancer cells \textit{in vitro} with bsAb CD73xEGFR and assessed its treatment effect on the sensitivity of these cells towards cytotoxicity induced by various chemotherapeutic agents. In this respect, it is encouraging that bsAb CD73xEGFR sensitized cancer cells towards the cytotoxicity of cisplatin, doxorubicin, taxol and 5FU, respectively. Surprisingly, combined treatment of cancer cells with bsAb CD73xMock and bsAb MockxEGFR or oleclumab and cetuximab did not have similar effects as treatment with bsAb CD73xEGFR alone. This suggests that bsAb CD73xEGFR sensitizes cancer cells towards the cytotoxicity of chemotherapeutic agents through the concurrent removal of (juxtaposed) CD73 and EGFR molecules from the cell surface.

Analogously, \textit{in vitro} treatment with bsAb CD73xEGFR enhanced the sensitivity of H292 non-small-lung cancer cells towards radiation-induced cytotoxicity up to ~40%. In contrast, identical treatment with oleclumab reduced sensitivity of these
cancer cells towards ionizing radiation and increased the IC₅₀ value from 1.35 Gy to 3.77 Gy. The latter observation appears to corroborate with those of Dietrich et al. who reported that inhibition of the enzyme activity of CD73 by small-molecule CD73-inhibitor APCP promoted the proliferative capacity of irradiated cancer cells, thereby enhancing their radiation-resistance, due to the absence of (locally) accumulated ADO. This suggests that CD73-produced ADO potentiates radiation-induced cell death in certain cancer types. Of note, in our study, treatment of H292 cancer cells with bsAb CD73xMock did not increase their resistance to radiation. This may be due to the fact that, compared to oleclumab, bsAb CD73xMock has a somewhat lower capacity to inhibit the enzyme activity of CD73. Whether the observed oleclumab-induced enhancement to radiation also applies to other cancer cell lines/types remains to be evaluated in follow-up studies.

In conclusion, bsAb CD73xEGFR appears to be a promising approach to overcome the inhibitory activity of the CD73/ADO immune checkpoint in broad variety of cancer types. In particular, it allows to inhibit the CD73 enzyme activity in a more tumor-directed manner, while simultaneously counteracting pro-oncogenic activities of CD73 and EGFR. Therefore, bsAb CD73xEGFR may be of significant clinical potential for various forms of difficult-to-treat solid cancer types.

REFERENCES
SUPPLEMENTARY DOCUMENTATION

METHODS USED IN SUPPLEMENTARY DOCUMENTATION

Construction of bsAb CD73xEGFR-IgG2silent

DNA fragments encoding scFvCD73 and VHH EGFR were generated by commercial gene synthesis service (Genscript) based on published VH and VL sequence data from oleclumab\(^4\) and EGFR-directed cameld single-domain antibody fragment NRC-sdAb028\(^8\), respectively. For construction of bsAb CD73xEGFR-IgG2silent and controls eukaryotic expression plasmid pbsAb was used, which contains 3 consecutive multiple cloning sites (MCS). MCS#1 and MCS#2 are interspersed by a 22 amino acid flexible linker and used for directional and in-frame insertion of DNA fragments encoding scFvCD73, VHH EGFR. MCS#3 contain DNA fragments encoding human Fc IgG2-silent, which mitigates immune effector functions. Analogously, pbsAb-CD73xMock-IgG2s encoding bsAb CD73xMock-IgG2s was constructed by replacing VHH EGFR in pbsAb-CD73xEGFR-IgG2s by scFvMCSP directed against CSPG4. Likewise, pbsAb-MockxEGR-1gG2s encoding bsAb MockxEGR-1gG2s was constructed by replacing scFvCD73 in pbsAb-CD73xEGFR-IgG2s by scFv4-4-20 directed against fluorescein. Plasmid pbsAb-MockxMock-IgG2s encoding bsAb MockxMock-IgG2s was constructed by replacing scFvCD73 in pbsAb-CD73xMock-IgG2s by scFv4-4-20.

Immunoblot analysis

Protein content of lysed cell samples was determined using the Bradford method after which samples with equal protein content (20 μg) were subjected to SDS/PAGE. In short, samples were mixed with 4x Laemmli sample buffer (containing 355 mM 2-mercaptoethanol) and heated at 95°C for 5 min. Subsequently, the separated proteins were transferred to a 0.45 μm nitrocellulose membrane. Separate lanes of the membrane were incubated with indicated primary antibodies at 4°C for 16 h and subsequently incubated with an appropriate horseradish peroxidase-conjugated secondary antibody at room temperature for 2 h. Immunoreactive bands were visualized with SuperSignal West Pico Chemiluminescent Substrate (Thermofisher) and analyzed using Bio-Rad ImageLab.
**Assessment dual binding activity of bsAb CD73xEGFR**

The capacity of bsAb CD73xEGFR to simultaneously bind to CD73 on one cell type and EGFR on a proximal other cell type was assessed by flow cytometry. In short, CD73^{pos}/EGFR^{pos} FaDu cancer cells (DiO-labeled) were co-cultured with CD73^{pos}/EGFR^{neg} CHO.CD73 (DiD-labeled) or CD73^{neg}/EGFR^{neg} CHO.Mock (DiD-labeled) and incubated with bsAb CD73xEGFR (1 µg/ml) at 4°C for 45 min. The percentage of DiO^{pos}/DiD^{pos} cell clusters was evaluated by flow cytometry.

**Histological assessment of signs of toxicity**

Various organs and tissues were fixed in 10% formalin, embedded in paraffin, and stained with hematoxylin and eosin (H&E). Histological images were evaluated for signs of toxicity by two independent pathologists.

**EGFR phosphorylation antibody array**

Cancer cells were incubated with bsAb CD73xEGFR, bsAb MockxEGFR or oleclumab (all 1 µg/ml) for 24 h and washed twice with cold PBS. Protein content of lysed cell samples was determined using the Bradford method. The Human EGFR Phosphorylation Antibody Array (Abcam, ab134005) was used according to protocol for detection of 17 phosphorylated human EGF receptors; EGFR (Tyr845), EGFR (Tyr992), EGFR (Tyr1045), EGFR (Tyr1068), EGFR (Tyr1086), EGFR (Tyr1148), EGFR (Tyr1173), EGFR (Ser1046/1047), EGFR (Ser1070), ErbB2 (Tyr877), ErbB2 (Tyr1112), ErbB2 (Tyr1212), ErbB2 (Tyr1248), ErbB2 (Thr686), ErbB2 (Ser1113), ErbB3 (Tyr1289), and ErbB4 (Tyr1284). Pictures were taken with Bio-Rad Chemidoc and analyzed using Bio-Rad ImageLab.
BsAb CD73xEGFR inhibits the CD73 immune checkpoint in a tumor-directed manner

Supplementary figure 1: bsAb CD73xEGFR. (A) Topology of expression plasmid pbsAb encoding bsAb CD73xEGFR-IgG2s and (B) schematic depiction of bsAb CD73xEGFR-IgG2s protein.

Supplementary figure 2: Relative CD73 and EGFR expression levels in 16 cell lines. Relative CD73 and EGFR expression levels on a panel of 16 cell lines used in this study. - = MFI < 5, + = MFI 5 – 20, ++ = MFI 20 – 200, +++ = MFI 200 – 500, ++++ = MFI > 500. Expression levels were analyzed by flow cytometry.
Supplementary figure 3: bsAb CD73xEGFR simultaneously binds to CD73 and EGFR. Binding of bsAb CD73xEGFR (0.01 – 10 μg/ml) cellurally bridged FaDu cancer cells (DiO-labeled) and CHO.CD73 (black) or CHO.Mock cells (white) (DiD-labeled). Binding experiments were analyzed by flow cytometry. Data shown in the graph represent mean ± SD.

Supplementary figure 4: bsAb CD73xEGFR induces co-internalization of both CD73 and EGFR from the cancer cell surface. Residual (A) CD73 and (B) EGFR membrane presence on H292 cells after treatment with bsAb CD73xEGFR or controls (1 μg/ml) for 24 h. Data shown in the graph represent mean ± SD.
Supplementary figure 5: Oleclumab treatment increases EGFR and HER2 expression levels in cancer cells. Representative images of (A) immunoblot analysis and subsequent (B) quantification of CD73, EGFR, phosphorylated-HER2 (Y1248) and β-actin expression by H292 cancer cells after treatment with bsAb CD73xEGFR or controls (1 µg/ml) at 37°C for 24 h. Equal protein concentration (20 µg) of each sample was loaded. Representative pictures of (C) EGFR growth factor array and subsequent (D) quantification of (phosphorylated) EGFR and (phosphorylated) HER2 expressed by H292 cancer cells after treatment with bsAb CD73xEGFR or controls (1 µg/ml) at 37°C for 24 h. Pictures were analyzed using Bio-Rad ImageLab software. Statistical analysis in D was performed using one-way ANOVA followed by Tukey post-hoc test (*p < .05, **p < .01, ****p < .0001).
Supplementary figure 6: bsAb CD73xEGFR co-internalize CD73 and EGFR in an EGFR-directed manner. (A) Residual CD73 cell surface expression levels of CD73pos/EGFRneg (A375m, A2058, and SK-MEL-28) and CD73pos/EGFRpos (OvCAR3, H292, H322, and SK-BR-3) cell lines after treatment with bsAb CD73xEGFR or bsAb CD73xMock (both 1 μg/ml) for 24 h. (B) Residual CD73 and EGFR cell surface expression levels of parental cancer cells (A549 and H1650) and corresponding EGFR-KO variants thereof after treatment with bsAb CD73xEGFR or bsAb CD73xMock (both 1 μg/ml) for 24 h. All experiments were analyzed by flow cytometry. All graphs represent mean ± SD.

Supplementary figure 7: bsAb CD73xEGFR potently inhibits the enzyme activity of CD73. (A) Percentage of inhibition CD73 enzyme activity on H292 cells after treatment with bsAb CD73xEGFR or controls (0.01 – 10 μg/ml) for 24 h. (B) IC50 values (μg/ml) calculated for a panel of 6 carcinoma cell lines that were treated with bsAb CD73xEGFR or controls (0.01 - 10 μg/ml) for 24 h and then evaluated for their capacity to inhibit CD73 enzyme activity. Graph A represents mean ± SD.
Supplementary figure 8: bsAb CD73xEGFR restores anticancer activities of ADO-suppressed T-cells. Caspase-3/8 activation (apoptotic cancer cell death) in PC3M cancer cells subjected to bsAb CD3xEpCAM-redirected PBMCs at an effector (E) to target (T) cell ratio of 4:1. PBMCs were incubated with (or without) AMP, in the presence or absence of bsAb CD73xEGFR, oleclumab, or controls (all 1 µg/ml). Caspase-3/8 activation was evaluated using live-cell imaging technology. Graph represents mean ± SD.

Supplementary figure 9: In vitro assessment bsAb CD73xEGFR using CT26 murine cancer cells. (A) Immunoblot analysis of murine CD73 (mCD73), murine EGFR (mEGFR), and murine β-actin (mβ-actin) expression levels in CT26 murine cancer cells (cell lysate protein 40 µg/lane). (B) Flow cytometric analysis of binding of bsAb CD73xEGFR or controls (1 µg/ml) to CT26 cancer cells. (C) Immunoblot analysis of mCD73, mEGFR, and mβ-actin expression by CT26 cells after treatment with bsAb CD73xEGFR or controls (1 µg/ml) at 37°C for 24 h. Equal amounts of protein (40 µg) of each sample were loaded per lane. (D) Percentage inhibition of mCD73 enzyme activity on CT26 cells after treatment with bsAb CD73xEGFR or controls (1 µg/ml) at 37°C for 24 h. All graph represent mean ± SD.
Supplementary figure 10: bsAb CD73xEGFR reduces syngeneic tumor size in immunocompetent mice. Representative picture of CT26 syngeneic tumors, harvested from BALB/c mice 21 d post tumor injection. Mice were treated I.P. with bsAb CD73xEGFR or controls (7.5 mg/kg) on day 4, 7, 10 and 14. 5 tumors per treatment-group are shown. Scale bar = 1 cm.
Supplementary figure 11: bsAb CD73xEGFR increases the tumor-infiltrating capacity of lymphocytes in immunocompetent tumor-bearing mice. Representative images of multiplexed immunofluorescence staining for CD4, CD8, and FoxP3 in CT26-tumor sections, harvested 21 d post tumor inoculation. Mice were treated I.P. with bsAb CD73xEGFR or controls (7.5 mg/kg) on day 4, 7, 10 and 14. Scale bar = 200 µm.
Supplementary figure 12: bsAb CD73xEGFR increases the tumor-infiltrating capacity of leukocytes in immunocompetent tumor-bearing mice. Representative images of multiplexed immunofluorescence staining for CD206, CD11c, and F4/80 in CT26-tumor sections, harvested 21 d post tumor inoculation. Mice were treated I.P. with bsAb CD73xEGFR or controls (7.5 mg/kg) on day 4, 7, 10 and 14. Scale bar = 200 µm.
Supplementary figure 13: bsAb CD73xEGFR-treatment induces no or minimal systemic toxicity in immunocompetent mice. Representative histological images (H&E staining) of various organs and tissues harvested 21 d post tumor inoculation from mice treated with bsAb CD73xEGFR, oleclumab, or controls. Mice were treated I.P. with bsAb CD73xEGFR or controls (7.5 mg/kg) on day 4, 7, 10 and 14. Pictures were taken at 200 x magnification.
Supplementary figure 14: bsAb CD73xEGFR inhibits the proliferative capacity of cancer cells. Proliferation assay in which a panel of cell lines (H292, H322, FaDu, and OvCAR3) was incubated with bsAb CD73xEGFR or controls (1 μg/ml). The percentage of cell confluence was evaluated after culturing for 72 h. Graph represent mean ± SD.

Supplementary figure 15: bsAb CD73xEGFR reduces cell colony formation of carcinoma cells. IC50 values (μg/ml) calculated for OvCAR3, H322 and H292 cell lines that were treated with bsAb CD73xEGFR or controls (0.01 - 10 μg/ml) at 37 °C for 14 d and subsequently assessed for the number of cell colonies.

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<td>OvCAR3</td>
<td>IC50/SDe</td>
<td>9.193 ± 1,821</td>
<td>1.254 ± 0.258</td>
<td>0.653 ± 0.163</td>
<td>24.33 ± 7,667</td>
</tr>
<tr>
<td>H322</td>
<td>IC50/SDe</td>
<td>4.760 ± 0.834</td>
<td>1.257 ± 0.250</td>
<td>0.775 ± 0.106</td>
<td>9.381 ± 2,311</td>
</tr>
<tr>
<td>H292</td>
<td>IC50/SDe</td>
<td>1.228 ± 0.202</td>
<td>0.409 ± 0.065</td>
<td>0.165 ± 0.032</td>
<td>15.07 ± 3,118</td>
</tr>
</tbody>
</table>

IC50 and SD values (μg/ml) clonogenic assay
Supplementary figure 16: Treatment with bsAb CD73xEGFR inhibits the proliferative capacity of xenografted cancer cells in immunodeficient mice. (A) Representative bioluminescent images of athymic nude mice (Crl:NU(NCr)-Foxn nu) inoculated S.C. in the flank with human H292-luc2 non-small lung cancer cells on day 0, 7, 11, 17 and 28 post tumor inoculation. Mice were treated I.P. with bsAb CD73xEGFR or controls (7.5 mg/kg) on day 7, 10 and 17. (B) Representative pictures of 3 excised H292-luc2 tumors after indicated treatment, 28 d post tumor inoculation. Scale bar = 1 cm.
Supplementary figure 17: bsAb CD73xEGFR sensitizes cancer cells towards treatment with chemotherapeutic agents. Cell confluence of H292 (A) or FaDu (B) cancer cells treated (or not) with bsAb CD73xEGFR (1 μg/ml) or controls in the presence (or absence) of cisplatin (1 μg/ml) at 37 °C for 4 d and then evaluated using live cell imaging by taking pictures at 4x magnification every 6 h. All graphs represent mean ± SD. All graph represent mean ± SD.
BsAb CD73xEGFR inhibits the CD73 immune checkpoint in a tumor-directed manner