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Expression of CD39 is associated with T cell exhaustion in ovarian cancer and its blockade reverts T cell dysfunction

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ABSTRACT

Immune exhaustion is a hallmark of ovarian cancer. Using multiparametric flow cytometry, the study aimed to analyze protein expression of novel immunological targets on $CD3^+$ T cells isolated from the peripheral blood ($n = 20$), malignant ascites ($n = 16$), and tumor tissue ($n = 6$) of patients with ovarian cancer (OVCA). The study revealed an increased proportion of effector memory $CD8^+$ T cells in OVCA tissue and malignant ascites. An OVCA-characteristic PD-1^{high} $CD8^+$ T cell population was detected, which differed from PD-$1^{low}CD8^+$ T cells by increased co-expression of TIGIT, CD39, and HLA-DR. In addition, these OVCA-characteristic $CD8^+$ T cells showed reduced expression of the transcription factor TCF-1, which may also indicate reduced effector function and memory formation. On the contrary, the transcription factor TOX, which significantly regulates terminal T cell-exhaustion, was found more frequently in these cells. Further protein and gene analysis showed that CD39 and CD73 were also expressed on OVCA tumor cells isolated from solid tumors ($n = 14$) and malignant ascites ($n = 9$). In the latter compartment, CD39 and CD73 were also associated with the expression of the "don't eat me" molecule CD24 on tumor cells. Additionally, ascites-derived $CD24^+EpCAM^+$ tumor cells showed a higher frequency of $CD39^+$ or $CD73^+$ cells. Furthermore, CD39 expression was associated with unfavorable clinical parameters. Expression of CD39 on T cells was upregulated through CD3/CD28 stimulation and its blockade by a newly developed nanobody construct resulted in increased proliferation (eFluor), activation (CD25 and CD134), and production of cytotoxic cytokines (IFN-γ, TNF-α, and granzyme-B) of $CD8^+$ T cells.

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Introduction

The loss of T cell-mediated cytotoxicity with subsequent upregulation of inhibitory receptor co-expression including programmed cell death protein-1 (PD-1) and T-cell immunoglobulin and immunoreceptor tyrosine–based inhibitory motif (ITIM) domain (TIGIT) have been described as hallmarks during T cell exhaustion.[1,2] T cell exhaustion includes a transcriptional reprogram where exhausted T cells are regulated by specific transcription factors, making it difficult for the cells to be revived and activated by, for example, checkpoint blockade.[3]

Among tumor entities, ovarian cancer (OVCA) is considered an "immune-naïve" tumor.[4,5] In contrast to other tumor entities such as non-small cell lung cancer, melanoma or head and neck cancer, OVCA is characterized in the majority by a reduced infiltration of immune cells and a comparatively low response rate to immunotherapeutic strategies.[6] Interestingly, although the success of checkpoint blockade in OVCA has remained limited to date, clinical trials of antibody-based immunotherapeutic strategies have consistently observed OVCA patients in whom an anti-tumor immune response occurred.[7] Furthermore, in their multi-omics studies of OVCA, Sun et al. recently identified an immunologic subtype characterized by increased infiltration of activated $CD8^+$ T cells associated with a more favorable prognosis and higher response rates to chemotherapy.[8] These novel data suggest that lymphocyte subsets from OVCA having immunogenic properties do exist and that at least OVCA sub-entities are amenable to immunotherapeutic approaches.[9] Hence, the present study was performed to gain a comprehensive insight into characteristic immune profiles of OVCA associated T cells, thus providing a rational for further functional evaluation of lead candidates.

Based on the current data and our own preliminary immunophenotypic analyses, this study supports the importance of multiple markers associated with T cell exhaustion in

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OVCA.[10,11] TIGIT and the T-cell immunoglobulin and mucin-domain containing-3 (TIM-3) immunoreceptors are included in our analyses as they are known to be expressed by multiple tumor-associated CD8/CD4 T cells.[12,13] PD-1 was selected as reference immunoreceptor as it has been studied most extensively so far and is broadly expressed in OVCA.[14]

The ectonucleoside triphosphate diphosphohydrolase 1 (CD39) and the ecto-5′-nucleotidase (CD73) have recently been described in the context of T cell exhaustion. Purinergic signaling is involved in the regulation of tumor-associated lymphocyte activation and function. Adenosine triphosphate (ATP), which is released from tumor cells but also from immune cells, is sequentially dephosphorylated by CD39 and CD73 and converted to adenosine.[15,16] Via G protein-coupled receptors adenosine mediates immunosuppressive properties on different immune cells including T cells.[17,18] In addition, CD39 and CD73 itself have been shown to be upregulated on tumor cells and associated with a worse prognosis.[17,19]

To map signs of T cell activation, expression of Human Leukocyte Antigen–DR isotype (HLA-DR) a class II major histocompatibility complex (MHC) molecule expressed by antigen-experienced lymphocytes was assessed.[20,21] It is upregulated as a late-phase activation marker and associated with increased interferon gamma (IFN-γ) production.[22]

Regarding the transcriptional reprogram of T cell exhaustion, the study investigated two opposing transcription factors: the transcription factor thymocyte selection-associated high mobility group box (TOX), which regulates terminal T cell exhaustion[23–25] and the transcription factor T cell factor 1 (TCF-1) which is involved in the development of long-lived memory cells and self-renewal of T cell populations.[26,27]

The study aimed to improve our understanding of OVCA-characteristic immune profiles. Based on the phenotypic lead candidates, functional blockade of TIGIT and CD39 was studied.

Methods and material

Patient cohorts

For flow cytometry-based phenotypical and functional characterization of T cells, we analyzed lymphocytes from ovarian cancer patients treated in the gynecology department of the University Medical Center Hamburg-Eppendorf (Germany). Peripheral blood lymphocytes (PBL, $n = 20$), malignant ascites lymphocytes (MAL, $n = 16$), and tumor infiltrating lymphocytes (TIL, $n = 6$) were isolated and cryopreserved as previously described.[10] For the tumor cell analyses, specimen of solid tumor tissue ($n = 14$) and ascites ($n = 9$) were obtained from patients with OVCA during debulking surgery. Cell aggregates (ø 40–100 µm) were isolated and cryopreserved. Mononuclear cells from these specimens were phenotypically characterized by performing multiparametric flow cytometry. The protocol for the isolation of the 40–100 µm fractions of primary tumor cells or malignant ascites can be found in Supplementary Methods S1.

Patient characteristics are summarized in supplementary table S1.

Peripheral blood specimens from healthy age-matched female donors were used as controls (HD, $n = 14$). All healthy donors (HD) PBL were derived from buffy coats of anonymous HD kindly provided by the blood bank of the University Medical Center Hamburg-Eppendorf (Germany).

The UKE-RNAseq cohort includes material from 192 ovarian cancer patients who underwent primary debulking surgery according to current German guidelines. The study was conducted in accordance with the provisions of the Declaration of Helsinki and was approved by the local ethics board (#200814 and PV6012–4312-BO-ff). Additionally, mRNA expression data from serous ovarian adenocarcinomas were retrieved from The Cancer Genome Atlas (TCGA) Research Network. The patient characteristics of these cohorts have been previously described.[28]

Multiparameter flow cytometry, surface, and intracellular staining

Multiparameter Flow Cytometry (MFC) analysis was performed for the phenotypical characterization of PBL, MAL, TIL, and tumor cells of OVCA patients as well as for evaluation of T cell function in functional assays described below. In all MFC assays, samples were stained according to a similar protocol, which can be found in Supplementary Methods S2 and Supplementary Table S2. All samples were analyzed on a BD FACSymphony A3 with FACS Diva software version 8 (BD Biosciences).

RNA extraction and transcriptome data

RNA extraction from 192 ovarian cancer patients was performed from tumor tissue as recently described.[29] Tissue samples were collected intraoperatively and stored directly in liquid nitrogen. The histologic characteristics of each specimen were assessed by cryo- and hematoxylin-eosin-stained sections. When necessary, the tissue was further sectioned so it contained at least 70% tumor. For each patient, 20–30 cryosections (approximately 16 µm) were minced using the Precellys homogenizer (VWR International GmbH, Darmstadt, Germany) followed by RNA extraction (Qiagen GmbH, Hilden, Germany). RNA quantity and integrity were assessed using a Bioanalyzer instrument (Agilent, Santa Clara, CA, USA).

RNA sequencing was performed by BGI Genomics (Shenzhen, China) using the DNBseqTM technology platform. Sequence reads were processed using Trimmomatic (v0.38)[30] to remove sequences originating from sequencing adapters, as well as sequences of low quality (Phred quality score below 20) and short length (below 40 bases) from the 3' end of the sequence reads. Reads were then aligned to the human reference sequence (GRCh38.95) using STAR (v2.7.10a). Differential expression was assessed using DESeq2 (v1.34.0). Genes were classified as significantly differentially expressed if their absolute log2-transformed fold change (log2FC) was not less than 1 and the false discovery rate value did not exceed 0.1.

Chronic CD3/CD28 stimulation of naïve ($CCR7^+CD45RO^-$) healthy donor T cells

For functional evaluation of T cell properties, freshly isolated PBL of HD were used. The effect of T cell exhaustion on the expression of markers of interest (e.g., TIGIT, CD226, CD39,

CD73) was evaluated by MFC analyses of continuous CD3/CD28-stimulated T cells.

Based on fluorescence-activated cell sorting (FACS), HD PBL ($n = 3$) were sorted for naïve T cells defined as $CCR7^+CD45RO^-$ lymphocytes (Figure 5a) using a BD FACS Aria IIIu or a BD FACS AriaFusion.

$CCR7^+CD45RO^-$ lymphocytes (500 000–1x10^6 cells/mL) were cultured in RPMI 1640 (Gibco, Thermo Fisher Scientific, Paisley, Scotland, United Kingdom) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin-streptomycin (Sigma Aldrich, St Louis, MO, USA), and 30 U/mL interleukin 2 (IL-2) (PeproTech, Canbury, NJ, USA) in 48-well plate (Greiner bio-one, Frickenhausen, Germany) with or without Dynabeads™ Human T-Activator CD3/CD28 (Thermo Fisher Scientific, Vilnius, Lithuania) for up to 10 days. Dynabeads™ were used according to the manufacturer's instruction. Culture medium was changed every third day and cell count per well was adjusted up to 1.5x10^6 cells if necessary.

On days 0, 4, 7, and 10, unstimulated and stimulated cells were analyzed by MFC. Therefore, Dynabeads™ were removed, cells were transferred to FACS tubes, and samples were stained.

Information on further statistical evaluations can be found in the Supplementary Methods 3.

Proliferation, activation, and cytokine production of $CD8^+$ T cells of OVCA patients

Freshly isolated peripheral blood mononuclear cells (PBMC) of OVCA patients ($n = 6$) were obtained on the day of the debulking surgery. Notably, one OVCA patient (JW171) received neoadjuvant chemotherapy prior to debulking surgery.

PBMC were labeled with eBioscience™ Cell Proliferation Dye eFluor™ 670. For 4 days, labeled cells were stimulated with Dynabeads™ Human T-Activator CD3/CD28 and treated with 1 mM ATP together with different combinations of 102 µg/mL (5 µM) anti-CD39 nanobody (SB24 hFC-LALAPG), 102 µg/mL negative control nanobody (L10e hFC-LALAPG), 50 µg/mL anti-TIGIT antibody (A15153G; BioLegend), and 50 µg/mL negative control antibody (MOPC-173; BioLegend) to target CD39 and TIGIT alone or in combination. All conditions were plated in triplicates. Cells were incubated at 37°C and 5% CO_2 for 4 days. The nanobody constructs were kindly provided by the Koch-Nolte lab. Further details on the nanobody used can be found in the patent application with publication number WO2023021185A1. To estimate the respective concentration, titration of the nanobody constructs was performed (Supplementary Figure S7). After 4 days, MFC analyses of fluorescence intensity of proliferation dye and frequency of activation markers on $CD8^+$ T cells were performed. At day 1 and day 4 of stimulation, supernatants of cell cultures were collected and analyzed for inflammatory cytokines using bead-based multiplex analyses (LEGENDplex™ Human CD8/NK Panel (13-plex)).

Information on further statistical evaluations can be found in the Supplementary Methods 3.

Results

Ovarian cancer-derived T cells show a distinct phenotypic differentiation

The accumulation of intra-tumoral $CD8^+$ T cells in ovarian cancer (OVCA) delays disease progression and is associated with improved survival rates. In contrast, a high tumor infiltration by regulatory $CD4^+$ T cells is considered as prognostically unfavorable.[31]

To further investigate the local composition of regulatory and conventional $CD4^+$ and $CD8^+$ T cells in our OVCA patient cohort MFC analyses on T cell distribution were performed. Tumor-infiltrating $CD3^+$T lymphocytes (TIL $n = 6$), $CD3^+$ T lymphocytes from the ascites (MAL $n = 16$), and peripheral blood lymphocytes (PBL $n = 20$) were compared to age-matched HD PBLs.

The proportion of respective $CD8^+$, conventional $CD4^+$ ($CD4_{con}$), and regulatory $CD4^+$ ($CD4_{reg}$) was compared within the fraction of alive $CD19^-CD14^-EpCAM^-CD3^+$ T cells. The gating strategy is shown in the supplements (Supplementary Figure S1).

$CD8^+$ T cells in the MAL and TIL aspirates were more frequently found among the $CD3^+$ cells than in the PBL specimen of HDs ($p = 0.004$, $p = 0.05$). While $CD4_{con}$ T cells were reduced in the MAL and TILs compared to HD PBLs ($p = 0.003$; $p = 0.0006$), infiltration by $CD4_{reg}$ was increased in these two compartments ($p = 0.003$; $p = 0.0004$, Figure 1a).

To further study whether there are phenotypic variations that could lead to a functional difference, the differentiation status was raised: naïve (NA) = $CCR7^+CD45RO^-$, central memory (CM) = $CCR7^+CD45RO^+$, effector memory (EM) = $CCR7^-CD56RO^+$, and terminally differentiated effector memory (TEMRA) = $CCR7^-CD45RO^-$.

For $CD8^+$, $CD4_{reg}$ and $CD4_{con}$ MAL and TILs, a reduction of naïve cells in favor of an increased proportion of effector memory lymphocytes was observed compared to the distributions in HD-derived PBLs (Figure 1b–d).

In summary, we observed a preponderance of $CD8^+$ T cells in tissue compartments with a higher proportion of tumor cells. OVCA patient-derived $CD8^+$ as well as conventional and regulatory $CD4^+$ T cells showed an altered phenotypic differentiation with a shift in the proportion of naïve lymphocytes in favor of the memory fraction.

TIGIT and CD39 are more frequently co-expressed by PD-1^{high} than PD-1^{low} T cells in ovarian cancer

After the study found increased presence of EM $CD8^+$ T cells in tissue compartments containing OVCA tumor cells, we investigated whether these lymphocytes also exhibited differential expression of co-regulatory checkpoints involved in the dysfunction of T cells. As described at the outset, the co-inhibitory receptors such as PD-1, TIM-3, or TIGIT and the two ectoenzymes CD39 and CD73 have already been described in the context of immune exhaustion. By including HLA-DR in our analyses, we tried to obtain additional information about the activation status of the T cells. It has recently been shown that HLA-DR expression on CD8+ T cells reflects immune status as

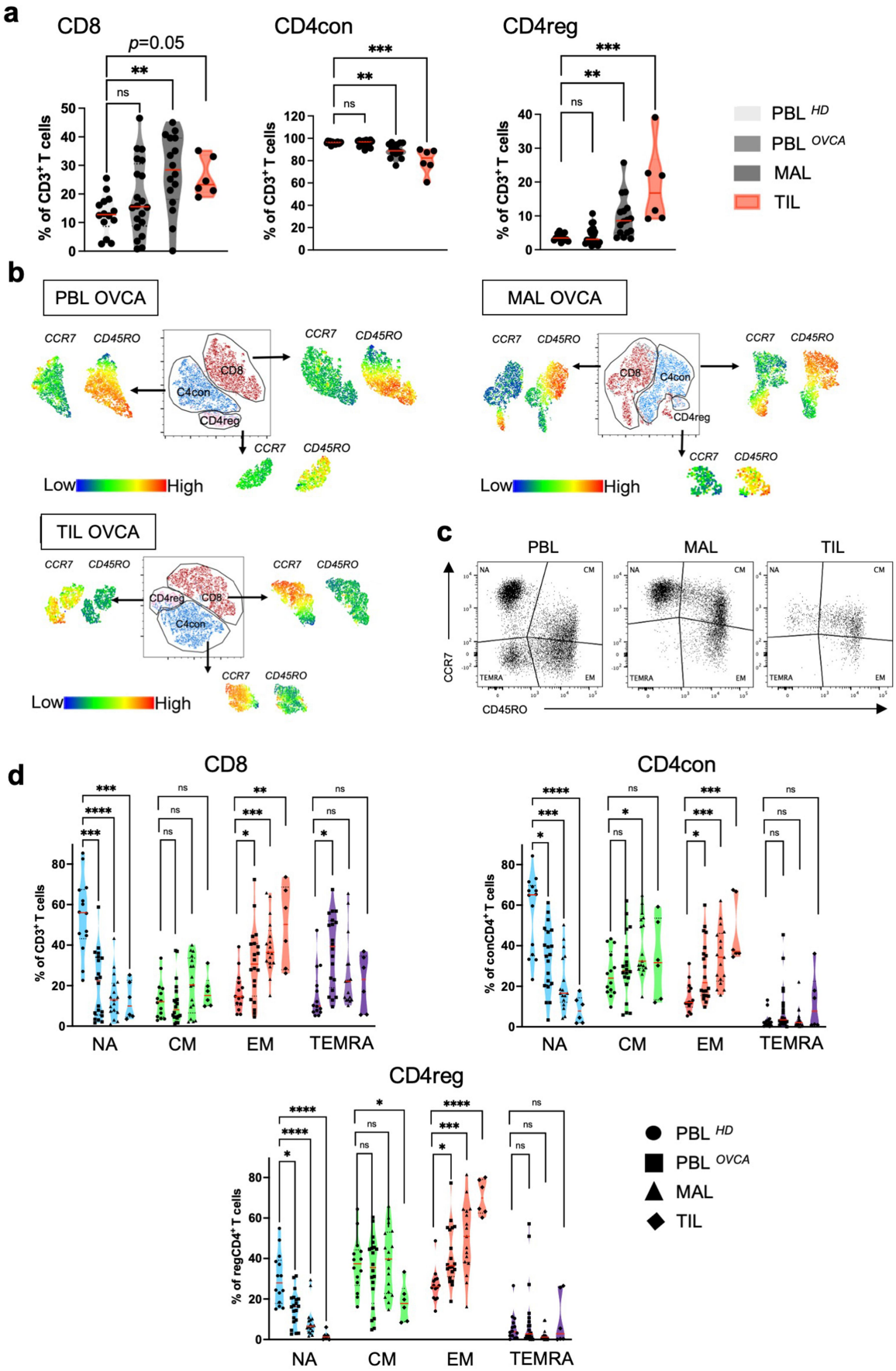

Figure 1. Infiltration and differentiation of $CD3^+$ T cells differ between tumor tissue, malignant ascites, and peripheral blood. Multiparametric flow cytometry (MFC) of the distribution of $CD8^+$, conventional $CD4^+$ (CD4con) and regulatory $CD4^+$ T (CD4reg) cells was performed on peripheral blood-derived lymphocytes (PBL) from healthy donors (HD, n = 14), patients with ovarian cancer (OVCA, n = 20), on aspirates of malignant ascites-derived lymphocytes (MAL) from OVCA patients (n = 16) and on specimen of tumor-infiltrating lymphocytes (TIL) from OVCA patients (n = 6). (a) Summary data show the proportion of $CD8^+$, CD4con, and CD4reg T cells within the

HLA-DR$^+$CD8$^+$ T cells showed high production of cytokines such as IFN-γ, TNF-α, and IL-2 upon stimulation.[32]

On the CD8$^+$ bulk, among the co-inhibitory receptors only PD-1 was more frequently expressed by OVCA-derived CD8$^+$ MALs and TILs (PBL HD vs. MAL OVCA $p = 0.007$; PBL HD vs. TIL OVCA $p = 0.023$; Supplementary Figure S2A, Figure 2a). Regarding the ectonucleotidases, CD39 was expressed by a significantly greater proportion of OVCA-patient-derived CD8$^+$ bulk, which further increased in aspirates of the ascites or tumor tissue (PBL HD vs. MAL OVCA $p = 0.0003$; PBL HD vs. TIL OVCA $p = 0.0009$; Supplementary Figure S2A, Figure 2a). Interestingly, these CD8$^+$ bulk displayed a reduced fraction of CD73$^+$ cells (PBL HD vs. MAL OVCA $p < 0.0001$; PBL HD vs. TIL OVCA $p = 0.0005$; Supplementary Figure S2A, Figure 2a).

The analyses of CD4$^+$ bulk cells revealed an increased frequency of TIGIT$^+$ and CD39$^+$CD4$_{con}$ MAL and TIL aspirates compared to PBL of HDs (Supplementary Figure S2B). In contrast, MAL and TIL-derived CD4$_{reg}$ showed only a higher proportion of TIGIT$^+$, but not CD39$^+$ cells, and the CD4$_{reg}$ TILs expressed more often PD-1 in comparison with HD PBLs (Supplementary Figure S2C).

HLA-DR was also more frequently expressed by CD8$^+$ T cells from the ovarian cancer patients (Supplementary S2A; Figure 2a).

Demonstrated by Simplified Presentation of Incredibly Complex Evaluations (SPICE) analyses, the frequency of PD-1, TIGIT, TIM-3, and CD39 co-expressing cells increased within the CD8$^+$ MAL and TILs (Figure 2b). Since the majority of CD8$^+$ T cells expressed PD-1, the PD-1$^+$CD8$^+$ population was analyzed in more detail. Interestingly, CD8$^+$ MAL and TIL harbored two PD1$^+$CD8$^+$ subpopulations: a PD-1^{high} and a PD-1^{low} population (Figure 2c). The subpopulation of PD-1^{high} expressing cells was significantly larger in MALs and TILs than in HD-derived PBLs ($p = 0.0007$, $p = 0.0009$; Supplementary Figure S3A and Figure 2c). Further comparison of the features of the PD-1^{high} and PD-1^{low} expressing CD8$^+$ T cells revealed a significant difference in co-expression patterns of additional molecules. PD-1^{high}CD8$^+$ T cells co-expressed TIGIT and CD39 more frequently. Our analyses revealed a consistently increased infiltration of TIGIT co-expressing PD-1^{high}CD8$^+$ T cells in all three compartments of ovarian cancer (PB, malignant ascites and tumor tissue; Figure 2d,e). The fraction of CD39$^+$ cells was also increased in the PD-1^{high}CD8$^+$ MAL and TILs (Figure 2d,e), and a remarkable co-expression of CD39 and TIGIT within the PD-1^{high}CD8$^+$ MALs and TILs was found (Figure 2f).

In addition, for a subgroup of donors (PBL HD $n = 4$, PBL OVCA $n = 5$, Mal $n = 5$, TIL $n = 3$), co-expression of HLA-DR was additionally analyzed. Interestingly, MAL and TIL-derived PD-1^{high}CD8$^+$ T cells exhibited a larger portion of HLA-DR$^+$ cells than the HD-derived controls ($p = 0.02$, $p = 0.01$; Supplementary Figure S3B). Moreover, PD-1^{high}CD8$^+$ T cells more frequently triple co-expressed HLA-DR, CD39, and TIGIT than their PD-1^{low}CD8$^+$ counterparts (Supplementary Figure S3C).

OVCA CD8$^+$ T cells showed reduced expression of the transcription factor TCF-1, which regulate several effector functions and memory formation (Figure 2g).[26,27] In contrast, the transcription factor TOX was more frequently expressed in CD39$^+$TIGIT$^+$PD-1^{high} than in CD39$^-$TIGIT$^-$PD-1^{high}CD8$^+$ T cells in OVCA (Figure 2h). TOX has been associated with regulation of terminal T cell exhaustion.[23–25]

In summary, a presumably exhausted subpopulation of OVCA-derived CD8$^+$ T cells could be identified, defined by their co-expression of co-inhibitory and stimulatory receptors as well as by their distinct transcriptional profile.

CD39 and CD73 are expressed by different tumor cell subpopulations

Since the expression of CD39 and CD73 has already been described for tumor cells, further investigation of their expression on OVCA tumor cells was conducted. To phenotype OVCA tumor cells in a more complex way, the expression analysis of the ectonucleotidases was also carried out on OVCA cell subgroups. Besides EpCAM, CD24, and CD90 were used as tumor markers. The Epithelial Cell Adhesion Molecule (EpCAM) is a type I transmembrane glycoprotein that is overexpressed in ovarian cancer. EpCAM expression correlates with tumor cell proliferation, differentiation and cellular signaling.[33] Thy-1 (CD90) and the protein Cluster of Differentiation 24 (CD24) have been shown in recent studies to be potential markers for various types of cancer.[34] CD90 is a regulator in cell-cell and cell-matrix interactions as well as in signal transduction and cytokine synthesis.[34] When the membrane receptor CD24 is expressed on the surface of tumor cells, it interacts with Siglec-10 expressed by immune cells, forming a so-called “don’t eat me” signal that allows the tumor to escape.[35]

Cell aggregates were isolated from solid tumor tissue $n = 14$ and ascites $n = 9$ during debulking surgery (Figure 3a). Tumor cells of both compartments were phenotypically characterized to evaluate the expression of ectoenzymes on EpCAM$^+$ cells. In addition, relevant co-expression patterns of the ectonucleotidases with two novel tumor lineage markers CD90 and CD24 were investigated.

The majority of the EpCAM$^+$ tumor cells co-expressed CD24 (median frequency of tumor-derived cellular aggregates 55.1% and of ascites-derived cellular aggregates 72.9%; Figure 3b), whereas a relevant expression of CD90 was only detected on EpCAM$^+$ tumor cells of tumor-derived cellular aggregates. Interestingly, these CD90$^+$ tumor cells also co-expressed the lineage marker CD24 (Figure 3b).

Regarding the ectoenzymes, CD39 was expressed more frequently on EpCAM$^+$ tumor-derived than on ascites-derived cellular aggregates (Figure 3c). The frequency of CD73$^+$EpCAM$^+$ cells was lower compared to CD39, but no

alive CD19$^-$CD14$^-$EpCAM$^-$CD3$^+$ T cells. (b) t-SNE analyses display the expression pattern of the differentiation markers CCR7 and CD45RO on different lymphocyte subsets. (c) Exemplary FACS plots showing the gating strategy for naïve (NA = CCR7$^+$CD45RO$^-$), central memory (CM = CCR7$^+$CD45RO$^+$), effector memory (EM= CCR7$^-$CD45RO$^+$), and terminal effector memory cells (TEMRA = CCR7$^-$CD45RO$^-$). (d) Summary data of the distribution of NA, CM, EM and TEMRA cells are depicted. p values were obtained by the ANOVA and Kruskal-Wallis test. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns not significant.

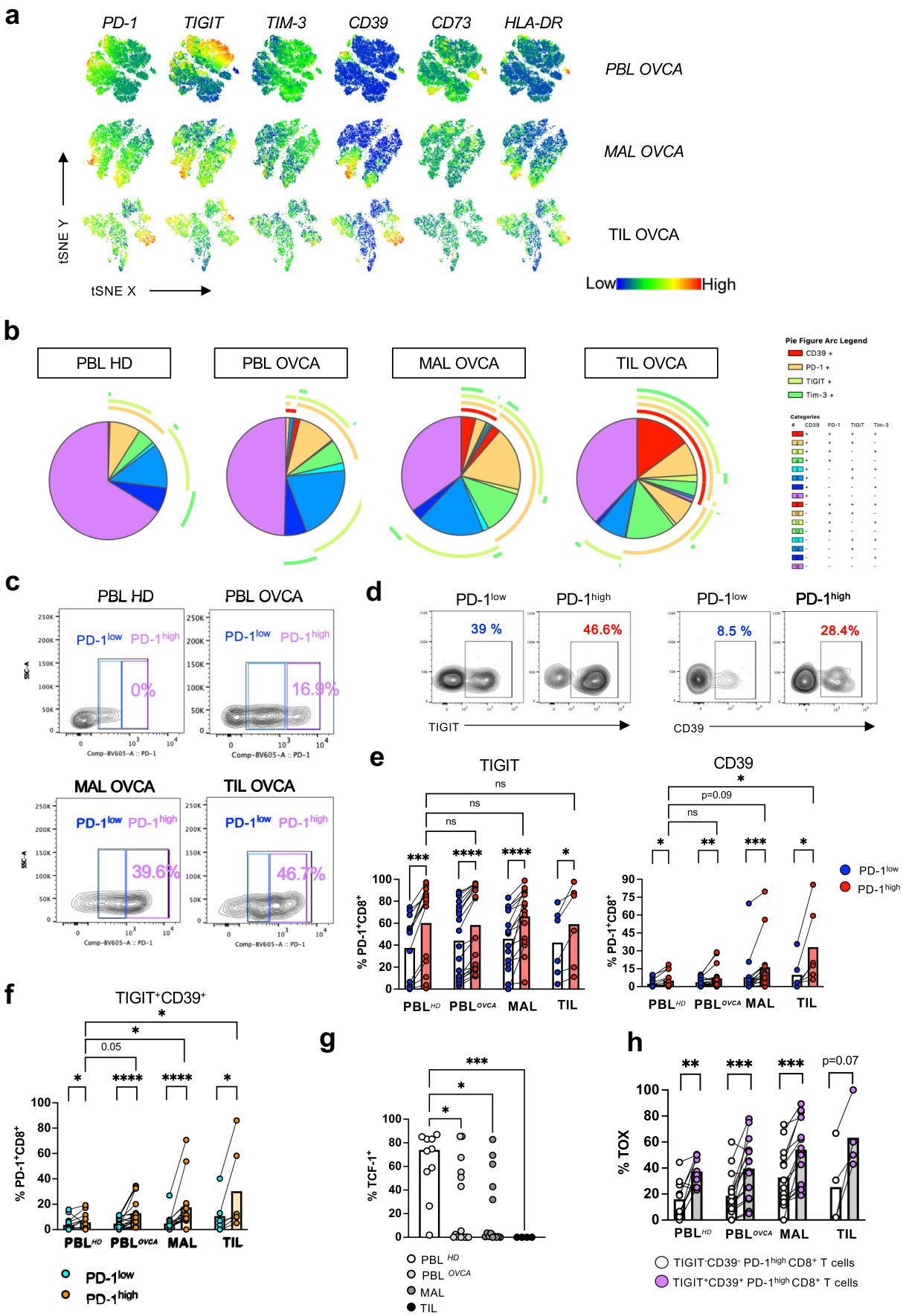

Figure 2. OVCA-associated CD8$^+$ T cells harbor a characteristic subpopulation defined by HLA-DR$^+$TOX$^+$TIGIT$^+$CD39$^+$PD-1^{high} cells. Expression of the co-regulatory receptors PD-1, TIGIT, TIM-3, CD39, and CD73 was assessed on peripheral blood-derived lymphocytes (PBL) of healthy donors (HD, n = 14), on PBL of patients with ovarian cancer (OVCA, n = 20), on malignant ascites-derived lymphocytes (MAL) aspirates from OVCA patients (n = 16) and on tumor-infiltrating lymphocytes (TIL) specimen from OVCA patients (n = 6) by multiparametric flow cytometry (MFC). HLA-DR was analyzed for a subgroup of the cohort (PBL HD n = 4, PBL OVCA n = 5, MAL

difference could be detected between the two compartments (tumor and ascites; Figure 3c).

As shown by t-distributed stochastic neighbor embedding (tSNE) analysis, no relevant co-expression of CD39 and CD73 was detected on the tumor cells in both compartments (Figure 3d).

Although the two ectonucleotidases were more frequently expressed on tumor-derived cellular aggregates, interestingly, for the ascites-derived fraction, we could detect a positive association between the expression of the ectonucleotidases and the tumor marker CD24 (Figure 3e). Furthermore, in both compartments, the ectonucleotidases were more frequently expressed by $CD24^+$ than by $CD24^-EpCAM^+$ tumor cells (Figure 3f). For solid tumors, our analyses for CD73 revealed at least an increased MFI on these cells (Figure 3f).

To sum up, CD39 and CD73 are also expressed on ovarian cancer tumor cells. Interestingly, mainly $CD24^+EpCAM^+$ tumor cells showed expression of the two ectoenzymes.

High expression of CD39 confers a negative prognosis in ovarian cancer patients

Next, we aimed to relate the immunoprofiling results to molecular gene expression data. We therefore analyzed the correlation between gene expression of the lead candidates CD39 and TIGIT. Therefore, analyses of tumor tissue samples from OVCA patients ($n = 192$) were conducted using available RNAseq data from the gynecology department at the UKE and the TCGA cohort. Details of the cohort and clinical parameters have been previously described.[28] The study included only gene expression data from tissue samples of ovarian cancer patients that contained at least 70% malignant tumor cells.

CD39 expression levels were correlated with available clinical-histopathological parameters. When the cohort was stratified into CD39 high vs. low expressors, a positive correlation with advanced FIGO state ($p = 0.047$; Figure 4a), stage ($p = 0.004$; Figure 4a), nodal involvement ($p = 0{,}038$; Figure 4a) and lymphovascular invasion ($p = 0.004$, $p = 0.002$; Figure 4a) was observed.

CD39 gene expression levels positively correlated with those of TIGIT ($r = 0.678$, $p < 0.001$; Figure 4b). The results are in line with our phenotypic data from protein analysis of MAL and TIL. Furthermore, a high TIGIT expression in patients was associated with the presence of high-grade OVCA ($p = 0.017$; Figure 4c).

In our cohort, both markers showed no significant impact on patient survival without further stratification. Stratification regarding the nodal status and CD39 expression level revealed an association of high CD39 expression with a poorer overall survival in patients with a negative nodal status (Figure 4d), which was also seen for patients harboring a high CD39 in combination with a high TIGIT expression (Figure 4e).

Taken together, our data indicate that CD39 and TIGIT are associated with unfavorable clinical parameters, indicating a prognostic relevance of both markers in ovarian cancer.

Chronic stimulation of naïve ($CCR7^+CD45RO^-$) healthy donor T cells increase frequencies of $CD39^+$ and $TIGIT^+$ $CD8^+$ T cells

$CD8^+$ T cells derived from MAL and TIL of OVCA patients showed increased expression and co-expression of CD39 and TIGIT. In addition, these cells displayed further signs of chronic stimulation, namely, increased co-expression of inhibitory receptors and increased expression of TOX. Next, we investigated whether the expression of CD39 and TIGIT is upregulated because of sustained TCR stimulation. Naïve T cells were isolated from HD ($n = 3$) by FACS (detecting the $CCR7^+CD45RO^-$ lymphocytes, Figure 5a). *In vitro* stimulation was performed as described in the Methods and Material section. The study compared the expression of crucial markers of the purinergic pathway and the TIGIT axis by MFC analysis over time.

The naïve $CD8^+$ T cells initially expressed only low levels of TIGIT, CD226, and CD39, whereas > 90% of respective T cells expressed CD73. With continuous stimulation, the amount of CD39, TIGIT, and CD226 expressing $CD8^+$ T cells increased while the amount of CD73 expressing $CD8^+$ T cells decreased (Figure 5b). Unstimulated $CD8^+$ T cells showed lower frequencies of $CD39^+$, $TIGIT^+$, and $CD226^+$ cells and higher proportions of $CD73^+$ cells during the 10 days of culturing (Figure 5b). In line with our previous data, the majority of TIGIT expressing $CD8^+$ T cells co-expressed CD39 after 10 days of T cell stimulation (Figure 5c; Supplementary Figure S4A).

Fluorescence intensity histograms of TIGIT, CD226, CD39, and CD73 gated on $CD8^+$ T cells illustrate the dynamic of the markers (Figure 5d; Supplementary Figure S4B). Furthermore, expression of the transcription factor TOX increases in continuously stimulated $CD8^+$ T cells compared to unstimulated ones (Figure 5d).

In summary, the data confirmed that sustained TCR stimulation leads to increased (co-) expression of TIGIT and CD39 on $CD8^+$ T cells.

Blockade of CD39 increases proliferation, activation, and cytotoxicity of OVCA patient-derived $CD8^+$ T cells in vitro

Co-expression of CD39 and TIGIT on OVCA-derived $CD8^+$ T cells might have a particularly potent effect on T cell

n = 5, TIL n = 3). (a) t-distributed stochastic neighbor embedding (tSNE) heat maps illustrate the expression of co-regulatory receptors on $CD3^+$ T cells from four PBL samples (upper row), four MAL samples (middle row), and two TIL samples (lower row) of OVCA patients. (b) Spice analyses show the co-expression of PD-1, TIGIT, TIM-3, and CD39 on PBL, MAL, and TIL. (c) Exemplary FACS plots demonstrating the distribution of PD-1^{low} and PD-$1^{high}CD8^+$ T cell subpopulations. (d) FACS plots illustrate the co-expression of TIGIT and CD39 within PD-1^{low} and PD-$1^{high}CD8^+$ T subpopulations. (e) Summary data compare the expression of TIGIT and CD39 on the PD-1^{low} vs. PD-$1^{high}CD8^+$ T cells. (f) Summary data show the frequency of $TIGIT^+CD39^+$ cells within the PD-1^{low} and the PD-$1^{high}CD8^+$ T cells. (g) The transcription factors T cell factor 1 (TCF-1) and thymocyte selection-associated HMG box transcription factor (TOX) were analyzed for a subgroup of the patient cohort (PBL HD n = 10, PBL OVCA n = 16, MAL n = 14, TIL n = 4) by MFC. (g) Summary data show the expression of TCF-1. (h) TOX expression is compared between $TIGIT^+CD39^+$PD-1^{low} and PD-$1^{high}CD8^+$ T cells. *p* values were obtained by the ANOVA and Kruskal-Wallis test and by the Wilcoxon matched-pairs signed rank test. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns not significant.

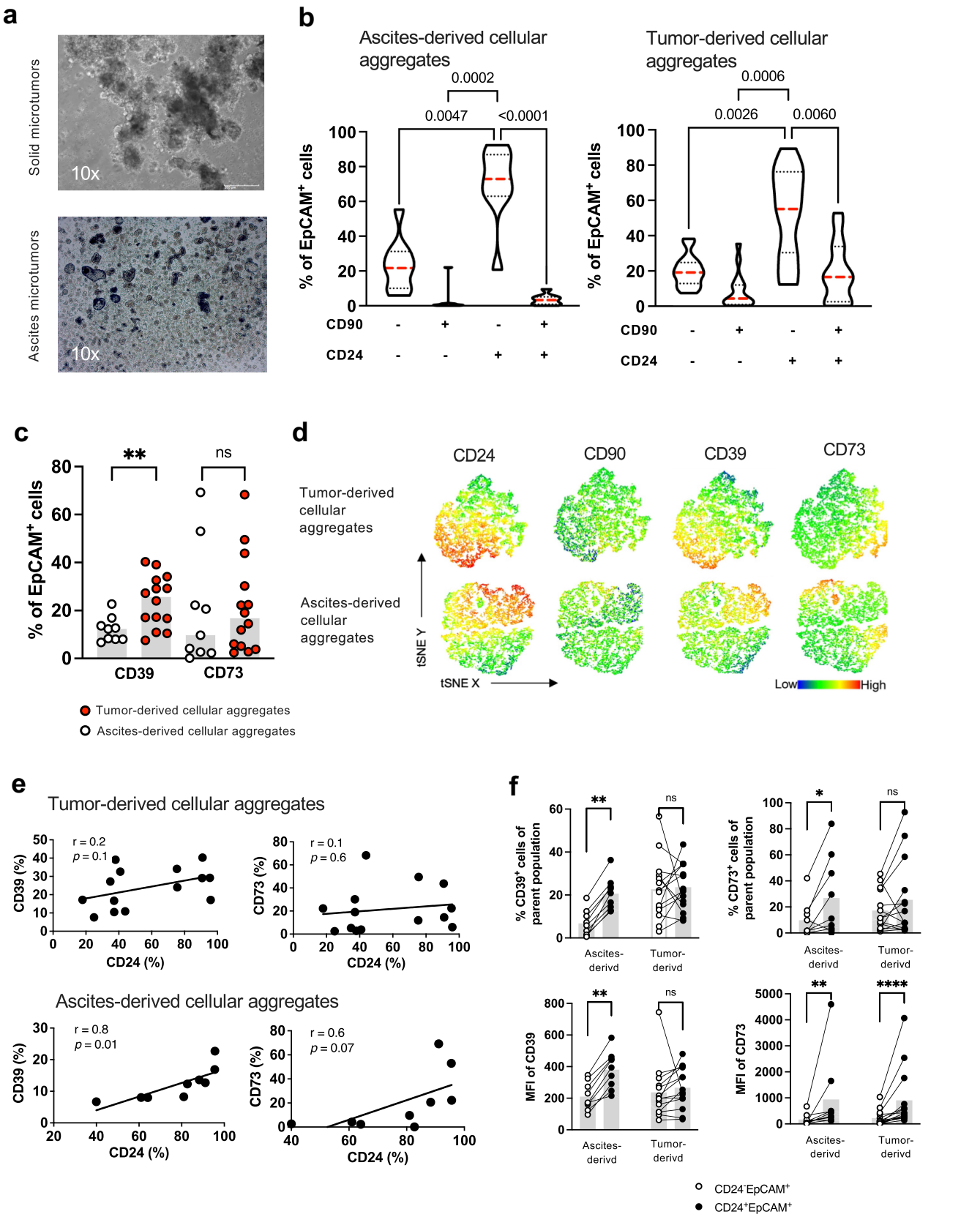

Figure 3. CD39 and CD73 are also expressed by ovarian cancer tumor cells.. Expression of CD39 and CD73 was assessed on ovarian cancer (OVCA)-derived solid tumors (n = 14) and malignant ascites (n = 9) and correlated to CD24 and CD90 using multiparametric flow cytometry (MFC). (a) Tumor or ascites-derived cellular aggregates were analyzed by microscopy. (b) Summary data show the distribution of CD24 and CD90 on EpCAM$^+$ tumor cells from tumor or ascites-derived cellular aggregates. (c) Expression of CD39 and CD73 was assessed on different tumor cell populations. (d) T-distributed stochastic neighbor embedding (tSNE) heat maps illustrate the expression of CD24, CD90, CD39 and CD73 on solid tumor cells of n = 3 OVCA patients (upper row) and malignant ascites tumor cells of n = 3 OVCA patients (lower row). (e) The expression of CD39 and CD73 was correlated with the expression of CD24 on EpCAM$^+$ Tumor cells. (F) The frequency of CD39$^+$ and CD73$^+$ tumor cells is compared between different tumor cell subpopulations. *p* values were obtained by the ANOVA and Kruskal-Wallis test and by the Wilcoxon matched-pairs signed rank test. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns not significant.

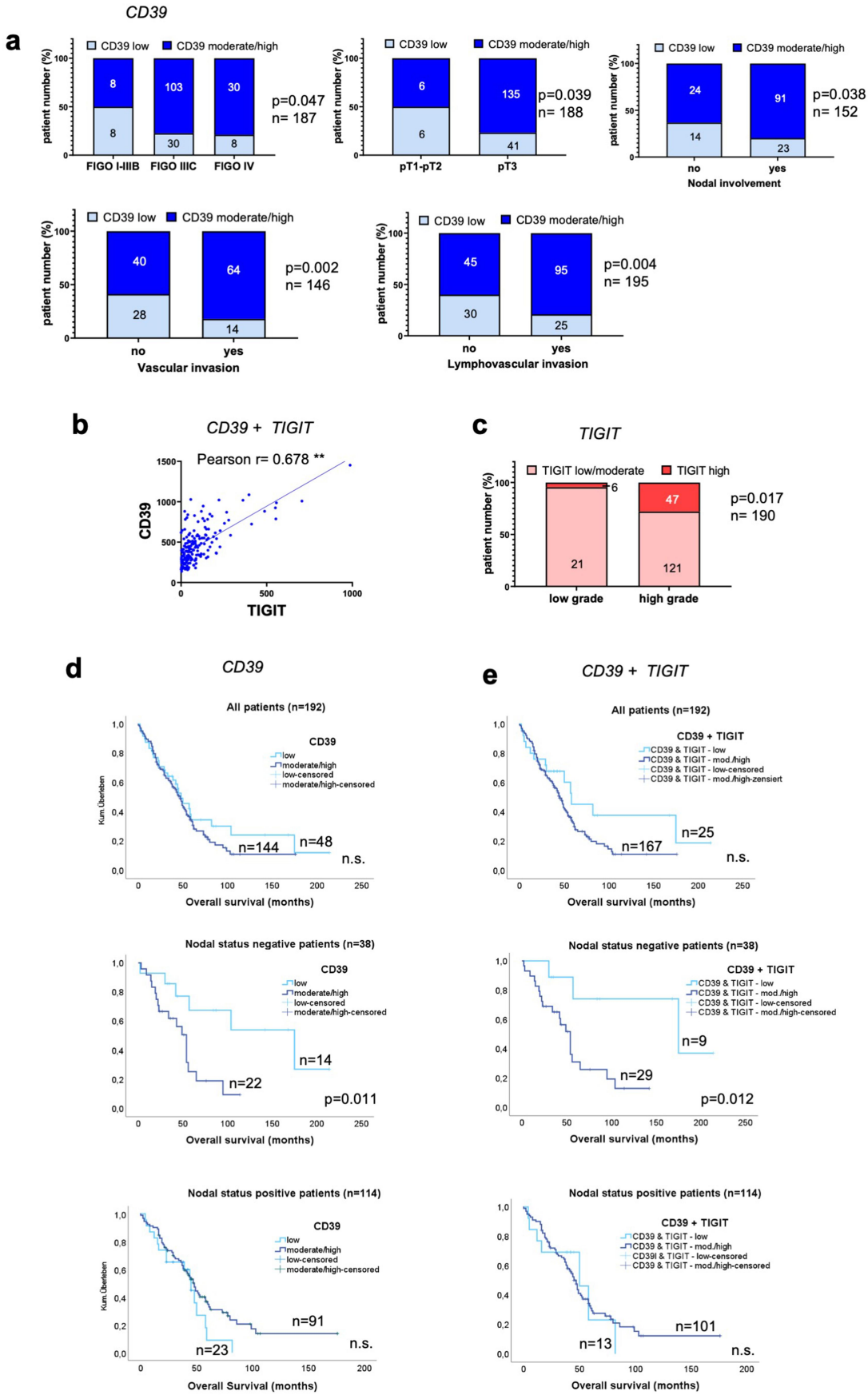

Figure 4. CD39 expression is associated with unfavorable clinical parameters. Gene expression of CD39 and TIGIT was assessed in a cohort of 192 patients diagnosed with ovarian cancer (OVCA) at the UKE and in the TCGA cohort. (a) Correlations between CD39 gene expression and clinical parameters including FIGO, stage, nodal involvement (UKE cohort) as well as vascular or lymphovascular invasion status (TCGA cohort). (b) Correlation analyses between CD39 and TIGIT mRNA expression in the UKE RNAseq cohort. (c) Association of TIGIT level and grading. (d) Kaplan–Meier analyses showing overall survival (OS) of patients with high (gene expression > the median) or low (gene expression < the median) CD39 gene expression for the entire UKE patient cohort as well as stratified for nodal status. (e) Kaplan–Meier analyses

dysfunction through the combination of the immunosuppressive metabolic property of adenosine with the inhibitory checkpoint receptor TIGIT. To investigate the potential of combined blockade of these two different targets, this study examined both single and combined blockade in proliferation and cytotoxicity assays. Therefore, enzymatic activity of CD39 was blocked alone or in combination with a blockade of TIGIT using OVCA patients-derived PBMC ($n = 6$) under the influence of exogenous ATP and CD3/CD28-stimulation. Proliferation, activation, and cytokine production of $CD8^+$ T cells were analyzed by MFC after 4 days. Although Figure 5 illustrates the peak expression of CD39 and TIGIT on naïve lymphocytes derived from HDs after 10 days of stimulation, our blocking assay involved a 4 day stimulation period. This was done as we utilized the entire PBMC fraction from OVCA patients, including naïve, central memory, effector memory, and terminally differentiated effector memory T cells. Building upon our observation in Figure 2, showing higher CD39 expression on T cells derived from OVCA patients compared to HD-derived T cells, we assumed an elevated CD39 expression from d0 in this assay (Supplementary Figure S6). Consequently, we choose to reduce the stimulation duration to 4 days.

Regarding proliferation, we analyzed the median fluorescence intensity (MFI) of eBioscience™ Cell Proliferation Dye eFluor™ 670. Blockade of CD39 resulted in increased proliferation of $CD8^+$ T cells as the MFI of eFluor670^{TM} was significantly lower in conditions with the anti-CD39 nanobody construct compared to conditions without the anti-CD39 nanobody construct (IgG2a + Ctr NB vs. IgG2a + anti-CD39 $p = 0.0312$; Figure 6a). The blockade of TIGIT did not affect the proliferation of $CD8^+$ T cells in our study (IgG2a + Ctr NB vs. anti-TIGIT + Ctr NB $p = 0.8438$). Also, combined targeting of CD39 and TIGIT did not increase the proliferation compared to single blockade of CD39 (IgG2a + anti-CD39 vs. anti-TIGIT + anti-CD39 $p = 0.6875$; Figure 6a).

In addition, the expression of the activation markers $CD25^+$ (IL2RA), $CD134^+$ (OX40), $CD137^+$ (4-1BB) and $CD154^+$ (CD40L) on $CD8^+$ T cells was evaluated (Figure 6b-e). In line with the findings on proliferation, blockade of CD39 resulted in an increased frequency of $CD8^+$ T cells expressing activation markers (IgG2a + Ctr NB vs. IgG2a + anti-CD39 for CD25 $p = 0.0312$, for CD134 $p = 0.0312$, for CD137 $p = 0.0625$, for CD154 $p = 0.0625$; Figure 6b-e). Again, single blockade of TIGIT as well as the combined blockade of TIGIT and CD39 did not affect the activation profile of $CD8^+$ T cells (Figure 6b–e).

To analyze cytokine production during the 4 days of T cell activation, the supernatants were collected at day 1 and day 4. At day 4, production of proinflammatory and cytotoxic cytokines, e.g. IL-2, tumor necrosis factor alpha (TNF-α), granzyme A and B and perforin was upregulated by the blockade of enzymatic activity of CD39 (Figure 6f ; Supplementary Figure S8). Blockade of TIGIT did not further increase the production for most of the cytokines. Notably, at day 1, combined targeting of CD39 and TIGIT resulted in increased TNF-α levels compared to the single blockade of CD39 (d1 IgG2a + anti-CD39 vs. d1 anti-TIGIT + anti-CD39 for TNF-α $p = 0.0312$; Figure 6f).

Taken together, blockade of CD39 with a novel developed nanobody construct was able to overcome immunosuppressive properties of ATP degradation. The blockade of enzymatic activity of CD39 resulted in an increased $CD8^+$ T cell proliferation, activation, and production of cytotoxic cytokines, whereas the additional blockade of TIGIT could only increase the production of TNF-α *in vitro*.

Discussion

OVCA-associated $CD8^+$, $CD4_{con}$ and $CD4_{reg}$ T cells had a decreased fraction of naïve cells, whereas the fraction of effector memory cells was increased compared to that of HDs. Analyses of checkpoint molecules identified a PD-1^{high}-$CD8^+$ subpopulation in OVCA, which was distinct from PD-1^{low} expressing $CD8^+$ T cells by an increased co-expression pattern of TIGIT and CD39. Interestingly, the stimulatory receptor HLA-DR was also more frequently expressed by this population. Regarding transcription factors, OVCA-associated $CD8^+$ T cells showed a decreased expression of TCF-1, whereas the transcription factor TOX was more frequently expressed in $TIGIT^+CD39^+PD\text{-}1^{high}CD8^+$ T cells.

Further protein and gene expression analysis revealed that CD39 and CD73 are also expressed in tumor tissue and associated with high TIGIT expression and unfavorable clinical parameters. Moreover, CD39 expression resulted in decreased overall survival in patients with negative nodal status.

Since the functional role of PD-1 in OVCA has already been investigated,[14,36] the study focused on the two novel therapeutic targets TIGIT and CD39. *In vitro*, blockade of TIGIT showed no significant effects on T cell proliferation and activation. However, blocking the enzymatic activity of CD39 achieved enhanced proliferation and activation of $CD8^+$ T cells and increased their production of IL-2, TNF-α, granzyme A and B.

Host immunity significantly affects cancer prognosis and can be assessed by the tumor infiltrating immune cells.[37] Different studies showed that occurrence of tumor infiltrating lymphocytes in the ovarian tumor microenvironment correlates with patient prognosis.[38] Sun et al. detected an immunological subtype of ovarian cancer characterized by increased infiltration of activated $CD8^+$ T cells and decreased $CD4_{reg}$ T cells that was associated with a favorable prognosis and improved therapeutic efficacy. The activated $CD8^+$ T cells contained a relevant fraction of effector memory populations specifically associated with favorable prognosis.[8] Our study revealed a higher proportion of $CD8^+$ but also CD4reg T cells in the tumor tissue and malignant ascites too. In subsequent phenotypic analyses, we also confirmed a loss of naïve cells and an increased fraction of effector memory cells.

showing overall survival (OS) of patients under consideration of the combined CD39 and TIGIT gene expression in the entire patient cohort as well as stratified for nodal status. *p* values were obtained by Chi-square and log-rank tests. *p < 0.05, **p < 0.01, ***p < 0.001.

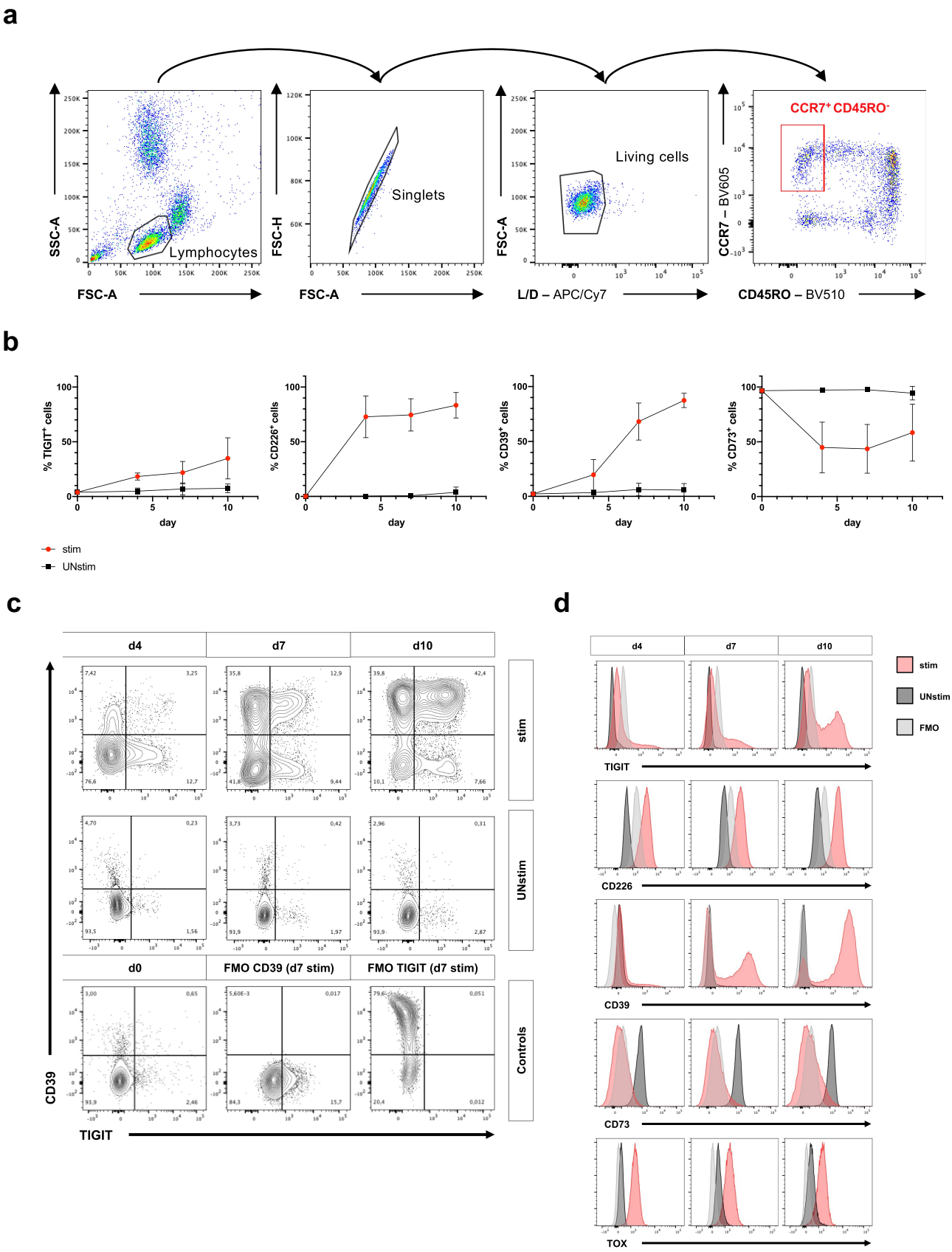

Figure 5. TIGIT and CD39 are upregulated upon TCR stimulation *in vitro*. Based on Fluorescence Activated Cell Sorting (FACS), peripheral blood mononuclear cells (PBMC) of $n = 3$ healthy donors (HD) were sorted for naïve lymphocytes defined as $CCR7^{+}CD45RO^{-}$ subpopulation. Naïve lymphocytes were stimulated with Dynabeads™ Human T-Activator CD3/CD28 or were cultured without stimulation for up to 10 days. Stimulated or unstimulated cells were analyzed by flow cytometry regarding markers of interest (e.g. TIGIT, CD226, CD39, CD73, TOX). The phenotyping was performed at day 0, 4, 7, and 10. (a) Exemplary gating strategy for FACS of naïve lymphocytes. (b) Summary data of all three runs show the frequency of $TIGIT^{+}$, $CD226^{+}$, $CD39^{+}$, or $CD73^{+}$ cells gated on $CD8^{+}$ T cells over the time of stimulation. (c) Exemplary flow cytometry data illustrate co-expression of TIGIT and CD39 on $CD8^{+}$ T cells over time of stimulation. (d) Exemplary histograms display expression of TIGIT, CD226, CD39, CD73 and TOX on $CD8^{+}$ T cells over time of stimulation.

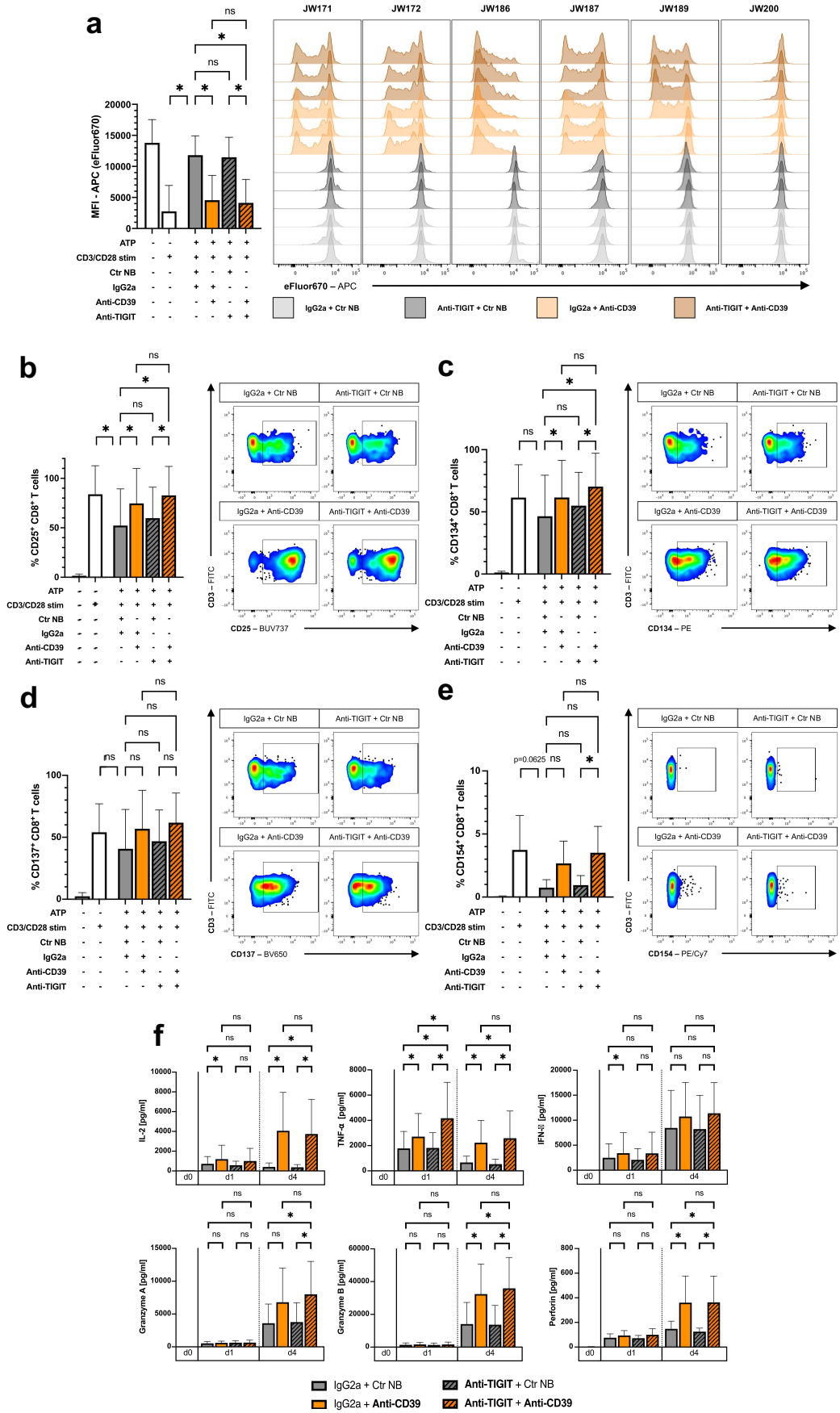

Figure 6. Blockade of CD39-enhanced CD8$^+$ T cell proliferation, activation, and cytotoxicity *in vitro*. The effects of blocking CD39 and TIGIT alone or in combination under the influence of exogenous adenosine triphosphate (ATP) on proliferation, activation, and cytokine production of CD3/CD28-stimulated CD8$^+$ T cells of OVCA patients (n = 6) were analyzed in an *in vitro* assay. (a) On the left, summary data show the proliferative activity of CD8$^+$ T cells illustrated by the median fluorescence intensity (MFI) of the proliferation dye gated on CD8$^+$ T cells. On the right, histograms showing the individual fluorescence intensity of the proliferation dye for every patient included in the assay. (b–e) Bar charts and exemplary MFC plots demonstrate the activation status of CD8$^+$ T cells by expression of CD25$^+$ (b), CD134$^+$ (c), CD137$^+$ (D) and CD154$^+$

Co-expression of several co-regulatory molecules has been considered as one of the most important characteristics of exhausted or dysfunctional T cells.[39] Using a multiparametric flow cytometry-based protein marker profiling, our study detected a characteristic PD-1^{high}CD8$^+$ T cell subset which has recently been reported for different cancer entities.[40,41] In the present study, OVCA-associated PD-1^{high}CD8$^+$ T cells co-expressed TIGIT and CD39. Using TCR detection, these three markers were recently shown to serve as a highly efficient and reliable source for CD8$^+$ TILs.[42] However, a characteristic co-expression pattern of TIGIT and CD39 has already been defined as a feature of T cell exhaustion for several tumor entities[43] TIGIT$^+$CD39$^+$PD-1^{high}CD8$^+$ T cells co-expressed HLA-DR more frequently. Several studies describe the subpopulation of PD1$^{+/high}$CD39$^+$CD8$^+$ TIL as characteristic tumor reactive TILs expressing activation markers like HLA-DR and CD25, whilst also showing clear signs of exhaustion.[44–46] Moreover, we could detect a similar immune profile not only on TIL but also on CD8$^+$ T cells derived from peripheral blood and malignant ascites of OVCA patients.

The transcription factors TOX and TCF-1 are among the major drivers of exhaustion and stem cell programs in CD8$^+$ T cells.[47,48] In the present study, OVCA-associated TIGIT$^+$CD39$^+$PD-1^{high}CD8$^+$ T cells exhibited an increased TOX expression, whereas TCF-1 was less frequently expressed by these cells. In line with our study, Kim et al. found reduced proportions of TCF-1 within PD-1^{high}CD8$^+$ T cells.[40] Khan et al. confirmed that TOX is dispensable for the formation of effector and memory T cells but plays a critical role in regulating T cell exhaustion by demonstrating that exhausted T cells do not form in the absence of TOX.[25] In line with our study, it was demonstrated that TOX is associated with checkpoint expression.[49] Moreover, robust TOX expression leads to the implementation of a specific transcriptional and epigenetic developmental program that ultimately prevents the reactivation of T cells by checkpoint blockade.[47]

Our study confirmed expression of CD39 and CD73 on different tumor cell populations at protein and gene level. Expression of CD39 and CD73 by cancer cells positive for the stem cell marker CD24 is interesting, as CD24 is involved in the development, invasion, and metastasis of cancer cells.[50] An impact of CD39 expression on overall survival was only observed in a subset of patients, namely, those with negative nodal status. Nodal status is not prognostic in ovarian cancer. In our experience, nodal involvement in ovarian cancer is rather related to retroperitoneal tumor dissemination, which represents an alternative metastatic route to the intraperitoneal tumor spread. We have recently described that these two metastatic pathways are characterized by the specific expression of certain biomarkers and may represent two distinct biological subtypes.[51–53] Although the detailed molecular mechanisms underlying these two subtypes are not yet known, they could potentially be responsible for the different response to therapy. Interestingly, in a previous study, we were able to show that CEACAM1, a known immune checkpoint, also has prognostic significance only in the subgroup of node-negative ovarian cancer patients.[54] This could indicate that patients with exclusively intraperitoneal metastasis, i.e. without lymph node involvement, could represent an interesting target group for immuno-oncological therapy. However, this result may also be due to the small cohort size. Recent studies including larger patient cohorts showed an association of CD39 and CD73 with shorter disease-free survival and overall survival in patients with OVCA.[19,55] Additionally, chemoprotective effects of CD39 have been demonstrated, supporting the further immunotherapeutic evaluation of these targets in patients with OVCA.[55]

Persistent CD3/CD28 stimulation of naïve HD T cells resulted in increased (co-) expression of TIGIT, CD39, and CD226 while expression of CD73 decreased on CD8$^+$ T cells. Raczkowski et al. described a similar dynamic for CD39 and CD73 on CD8$^+$ T cells when stimulating unsorted HD PBMC with anti-CD3 mAb.[56]

In line with our findings, upregulation of TIGIT mRNA and protein expression upon CD3/CD28 stimulation has been described for purified CD8$^+$ T cells.[57]

We and others showed that CD73 seems to be abundantly expressed on naïve CD8$^+$ T cells.[56,58] Furthermore, downregulation of CD73 and the absence of CD73 has been linked to T cell exhaustion, co-expression with TIGIT and impaired T cell function of CD8$^+$ T cells.[58]

With these findings in mind, we aimed to test the functional relevance of blockade of enzymatic activity of CD39. We hypothesized that combinatorial targeting of CD39 and TIGIT would increase activation and cytotoxicity of CD8$^+$ T cells.

Promising results of studies targeting CD39 with monoclonal antibodies revealed that the blockade of CD39 can counteract the inhibitory and anti-proliferative properties of ATP degradation on T and natural killer (NK) cells in context of cancer immunotherapy.[59,60] We recently showed that targeting CD39 together with TIGIT is able to enhance NK cell-mediated tumor cell lysis *in vitro*.[61]

In our current study, we could show that the novel anti-CD39 nanobody construct counteracted the ATP-mediated inhibition of proliferation, activation, and cytokine production of primary CD8$^+$ T cells of OVCA patients. Comparable trends in proliferation and activation can be observed when blocking CD39 on MAL-derived CD8$^+$ T cells of OVCA patients (Supplementary Figure S9). In previous studies, we showed that the anti-TIGIT antibody (A15153G) leads to enhanced *T*- and NK-cell-mediated lysis of acute myeloid leukemia (AML) or breast cancer cells.[61–63] Surprisingly, blockade of CD39 together with TIGIT did not result in an additional or synergistic effect in the current study. As our previous findings regarding additive effects of combined targeting of TIGIT and CD39

(e) on CD8$^+$ T cells. (f) Summary data showing concentrations of selected cytokines (IL-2, TNF-α, IFN-γ, granzyme A, granzyme B, perforin) in supernatant of stimulated cells at days 1 and 4. Concentration for further cytokines can be found in Supplementary Figure S8. All bar charts showing mean + SD. All *p* values were obtained by the Wilcoxon-matched pairs signed rank test. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns not significant.

were observed with an NK cell line, it is possible that the interaction of the TIGIT-axis and the purinergic pathway is different in primary $CD8^+$ T cells.

In conclusion, this study identified a subpopulation of $TIGIT^+CD39^+PD\text{-}1^{high}CD8^+$ T cells primarily present in OVCA ascites and tumor tissue. Beside its lymphatic expression, CD39 was also expressed by tumor cells themselves and was associated with unfavorable clinical parameters. Blockade of CD39 enzymatic activity achieved re-invigoration of proliferative capacity, elevated activation, and increased production of cytotoxic cytokines *in vitro*. Our findings further provide the rationale for testing CD39 blockade *in vivo* in OVCA.

Abbreviations

PD-1: programmed cell death protein-1; **ITIM**: immunoreceptor tyrosine – based inhibitory motif; **TIGIT**: T-cell immunoglobulin and immunoreceptor tyrosine – based inhibitory motif (ITIM) domain; **OVCA**: ovarian cancer; **TIM-3**: T-cell immunoglobulin and mucin-domain containing-3; **CD39**: ectonucleoside triphosphate diphosphohydrolase 1; **CD73**: ecto-5′-nucleotidase; **ATP**: adenosine triphosphate; **HLA-DR**: human leukocyte antigens – DR isotype; **MHC**: major histocompatibility complex; **IFN-γ**: interferon gamma; **TOX**: thymocyte selection-associated high mobility group box; **TCF-1**: transcription factor T cell factor 1; **PBL**: Peripheral blood lymphocytes; **MAL**: malignant ascites lymphocytes; **TIL**: tumor infiltrating lymphocytes; **HD**: healthy donor; **PBS**: phosphate-buffered saline; **MFC**: multiparameter flow cytometry; **FACS**: fluorescence activated cell sorting; **FcR**: Fc receptor; **FBS**: fetal bovine serum; **PBMC**: peripheral blood mononuclear cells; **IL-2**: interleukin 2; **$CD4_{con}$**: conventional $CD4^+$; **$CD4_{reg}$**: regulatory $CD4^+$ **NA**: naïve; **CM**: central memory; **EM**: effector memory; **TEMRA**: terminally differentiated effector memory; **SPICE**: Simplified Presentation of Incredibly Complex Evaluations; **EpCAM**: epithelial cell adhesion molecule; **tSNE**: t-distributed stochastic neighbor embedding; **MFI**: median fluorescence intensity; **TNF-α**: tumor necrosis factor alpha; **NK**: natural killer

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Competing interests

BC: Personal fees from AOK Germany, med update, Roche Pharma, Astra Zeneca, Bayer Healthcare, BioNTech, Bristol Myers Squipp, Jansen Cilag, Merck Serono, Oncology Drug Consult CRO, Sanofi Aventis; Invited speaker by AOK Germany, med update, Roche Pharma; Advisory board by Astra Zeneca, Bayer Healthcare, BioNTech, Bristol Myers Squipp, Jansen Cilag, Merck Serono, Oncology Drug Consult CRO, Sanofi Aventis. **SB**: Consulting and advisory board for Astra Zeneca, Roche, MSD, EISAI, GSK, Olympus; honoraria for presentations from Astra Zeneca, Roche, GSK, MSD, Clovis; travel grants Astra Zeneca, Roche, GSK; support for research from Roche, MSD, GSK, Astra Zeneca. **WJ**: patent for Amgen issued. **FW**: personal fees and non-financial support from AbbVie; grants, personal fees, and non-financial support from Amgen and Pfizer; and personal fees from Jazz Pharmaceuticals, Celgene, Morphosys, Ariad/Incyte, stem line therapeutics Daiichi Sankyo, and Servier outside the submitted work; in addition, FW has a patent for Amgen issued; and support for medical writing: Amgen, Pfizer, AbbVie. **BF**: Travel grants Daiichi Sankyo, Servier, Novartis; advisory board by Jazz. GmbH, Daiichi Sankyo. The remaining authors declare that they have no conflict of interest.

Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding authors on reasonable request (f.brauneck@uke.de).

Contributions

BF, OFL, FW, and WJ designed the research study, WM, SE, WP, DY, HL and BF performed the experiments and analyzed the data. OFL analyzed the RNAseq data, WM and FB wrote the manuscript. KNF and MS provided the nanobodies used in the project. SB, BC, FW and WJ monitored the data analyses and interpretation and reviewed the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the local ethics board of the Ärztekammer Hamburg (#200814 and PV6012–4312-BO-ff). Informed written consent was obtained from all patients.

Disclosure statement

The authors have no conflicts of interest to disclose.

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