Anti-LYPD1/CD3 T-Cell-Dependent Bispecific Antibody for the Treatment of Ovarian Cancer

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ABSTRACT

Ovarian cancer is a diverse class of tumors with very few effective treatment options and suboptimal response rates in early clinical studies using immunotherapies. Here we describe L6/PLAUR domain containing 1 (LYPD1) as a novel target for therapeutic antibodies for the treatment of ovarian cancer. LYPD1 is broadly expressed in both primary and metastatic ovarian cancer with ~70% prevalence in the serous cancer subset. Bispecific antibodies targeting CD3 on T cells and a tumor antigen on cancer cells have demonstrated significant clinical activity in hematologic cancers. We have developed an anti-LYPD1/CD3 T-cell-dependent bispecific antibody (TDB) to redirect T-cell responses to LYPD1 expressing ovarian cancer. Here we characterize the nonclinical pharmacology of anti-LYPD1/CD3 TDB and show induction of a robust polyclonal T-cell activation and target dependent killing of LYPD1 expressing ovarian cancer cells resulting in efficient in vivo antitumor responses in PBMC reconstituted immune-deficient mice and human CD3 transgenic mouse models. Anti-LYPD1/CD3 TDB is generally well tolerated at high-dose levels in mice, a pharmacologically relevant species, and showed no evidence of toxicity or damage to LYPD1 expressing tissues.

Introduction

Ovarian cancer is the fifth-leading cause of cancer-related death in women and comprises a histologically and genetically broad range of tumors (1–3). The standard treatment for ovarian cancer is surgical debulking followed by platinum-based chemotherapy (4) with additional treatments including targeted and hormonal therapies (4). Early clinical studies using single agent inhibitors of programmed cell death protein 1 (PD-1)/programmed death-ligand 1 (PD-L1) checkpoint pathway have yielded only modest results in ovarian cancer treatment with median response rates of 10% to 15% (5). Therefore, despite recent therapeutic advances, ovarian cancer remains a disease with a less than 50% 5-year survival rate, in need of more effective therapies.

CD3 bispecific antibodies are a novel class of therapeutics that can over-ride endogenous T-cell receptor (TCR)-mediated antigen specificity by binding to both a T cell and an antigen on tumor cells. Previously, we have shown (6) that bispecific antibodies trigger a polyclonal, MHC-independent T-cell response to tumors resulting in T-cell proliferation and chemokine-mediated T-cell recruitment (7). Because of their unique mechanism of action, CD3 bispecific antibodies may be an efficacious immunotherapy in indications with lower mutation load, such as ovarian cancer (8), where checkpoint inhibitors have yielded limited benefit.

LY6/PLAUR domain containing 1 (LYPD1) is a highly conserved glycosylphosphatidylinositol (GPI)-anchored protein (9). LYPD1 contains one cysteine rich extracellular leukocyte antigen-6 (Ly6)/uPAR (PLAUR) domain followed by signal sequence that triggers posttranslational GPI-anchor addition incorporating mature, LYPD1 into the plasma membrane. Although the exact function of LYPD1 is not fully understood, the neural expression pattern and structural features suggest a role in acetylcholine signal modulation (9) with known nicotinic acetylcholine receptor (nAChR) association and in vitro modulation (10). LYPD1 knockout mice are viable and fertile without obvious anatomical abnormality, gross motor behavior changes, or sensory processing effects (10).

We have identified LYPD1 as a tumor antigen overexpressed in human ovarian cancers and characterized its expression in tumors and normal tissues. In addition, we describe the activity of a novel ovarian cancer immunotherapy, anti-LYPD1/CD3 T-cell-dependent bispecific antibody (TDB), that redirects T cells to kill LYPD1 expressing ovarian cancer cells. Our data show that anti-LYPD1/CD3 TDB demonstrates robust in vivo antitumor activity and is well tolerated at high doses in mice. Overall, we demonstrate that LYPD1 is a promising novel therapeutic target in ovarian cancer, supporting development of anti-LYPD1/CD3 TDB for immunotherapy for these patients.

Materials and Methods

Therapeutic anti-LYPD1 antibodies

The extracellular domain (ECD) of human LYPD1 (NP_653187.3, amino acid sequence 1 to 115) was fused with C-terminal polyhistidine tag and expressed in Hi-5 insect cell using baculovirus. Phage display library (10^10 clones) was panned against recombinant LYPD1 ECD protein (11). Fab fragments were grafted onto appropriate IgG constant regions (11), affinity matured by hard randomization approach (11) to obtain H2 and H6 (K DS = 0.2–0.7 nmol/L; Scatchard on OVCAR3, HCT-116-gD-LYPD1, respectively). H6 was used in subsequent bispecific antibodies.

Bispecific antibodies

Bispecific antibodies were produced as described previously (12, 13). All TDBs include N297G Fc substitution and the mouse CD3 reactive...
LYPD1-TDB (muIgG2a) consisted of murine anti-LYPD1 annealed with chimeric anti-murine CD3 2C11 (14). Anti-HER2 arms were based on trastuzumab (humanized 4D5; ref. 15).

Antibodies for IHC
LYPD1-knockout mice were immunized with purified mouse IgG2a Fc-tagged recombinant human LYPD1. Isolated spleen cells were fused with myeloma cells for hybridoma screening by ELISA.

Antibodies for flow cytometry and Western blot analysis
Antibodies were purchased from BD Biosciences unless otherwise stated. Anti-LYPD1 antibody 22E3 (Genentech) was used in Western blot analysis with anti-β-actin (Sigma, A2228) and anti-GAPDH (Novus, NB100-56875) loading controls.

Cancer cell lines
Cell lines were verified using short tandem repeat (STR) profiling (Promega PowerPlex 16 System) and high-throughput SNP profiling (Fluidigm multiplexed assays).

Gene expression analyses
Data for a panel of normal tissues were obtained from Gene Logic (Affymetrix HG-U133 platform) and normalized using standard rma normalization. RNA-sequencing data for GTex are based on release phs000424.v6.p1. RNA-sequencing data for TCGA were obtained from the National Cancer Institute Genomic Data Commons (https://gdc.cancer.gov). Data sets were processed using HTSeqGenie (Pau G, Reeder J. HTSeqGenie: a NGS analysis pipeline. R package version 3.14.0. 2012) aligned to the reference genome GRCh38 using GSNAP (16). Only uniquely mapping reads were used to obtain reads per kilobase of exon model per million mapped reads normalized by size factor (nRPKM). A two-sided t test was used to compare ovarian cancer and normal tissue expression.

qPCR analysis
qPCR for hULYPD1 and huβ-actin was performed with gene expression assays ABI Hs00375992_ml and Hs01060665_g1 and Taqman Universal Master Mix (ABI; catalog no. 4304437). Expression was assessed in Origene Tissue Scan cdNA arrays for Human Normal (catalog no. HMR104) and Ovarian Cancer (catalog no. HORT101e-104). Cell line RNA was extracted using Qiagen RNeasy Mini Kit (catalog no. 74104) and gene expression assessed using TaqMan RNA-to-CT(ABI; catalog no. 4392653). Normal human tissues in the Human Normal cdNA Array included adipose, bone marrow, cerebellum, whole brain, fetal brain, colon, heart, liver, fetal liver, lung, mammary gland, pancreas, prostate, salivary gland, skeletal muscle, skin, small intestine, smooth muscle, spinal cord, spleen, stomach, testis, thymus, trachea, uterus, fetal eye and retina (presented in their respective order, Fig. 2B).

Generation of LYPD1 expressing CT26, ID8, and KPL4 cell lines
Human LYPD1 (NM_144586) was cloned into lentiviral vector (pLVX_CMY_Puro; Takara 632164 to transfect CT-26 or pLVX_EFla_Puro; Takara 63125 to transfect ID-8 and KPL4). Cell lines were infected with human LYPD1 lentivirus and selected in 2 μg/mL Puromycin (Sigma, P8833).

LYPD1 mutations
cBioPortal (17, 18) was used to assess mutations in ovarian tumors, sourcing the pan-cancer TCGA data from the Broad Firehose (19).

Bispecific Antibody for the Treatment of Ovarian Cancer

IHC
Chromogenic LYPD1 IHC was performed on 4-μm formalin-fixed, paraffin-embedded tissue sections using the Ventana Benchmark XT Autostainer (Ventana Medical Systems Inc.). Slides were pretreated with CC1, for 56 minutes, followed by anti-LYPD1 antibody (Clone 3.21A2HC.RbtIgG.A80C. Genentech) at 10 μg/mL and Ventana Optview DAB KIT detection (Ventana Medical Systems Inc.). Sections were counter stained with hematoxylin, dehydrated, and cover-slipped.

LYPD1 expression analysis by flow cytometry
Cells were lifted using nonenzyme dissociation buffer (Sigma), incubated with 3 μg/mL anti-LYPD1 (Genentech; clone H6) on ice for 15 minutes and stained with goat anti-human-IgG-Fc (Jackson ImmunoResearch) or Alexa Fluor-647 Goat Anti-Human IgG, Fcγ Fragment (Jackson ImmunoResearch).

Isolation of human CD8+ T cells
CD8+ T cells were isolated from peripheral blood mononuclear cells (PBMC) as described previously (12).

In vitro cytotoxicity assay by flow cytometry and Cell Titer Glo viability assay
Analysis of cell viability was performed as described previously (12, 20).

T-cell activation in vitro
Target cells and purified CD8+ T cells were mixed ±TDB, incubated, and stained with CD8-FITC, CD69-PE, and CD107a-Alexa Fluor-647 (eBioscience) and analyzed by flow cytometry.

Flow cytometry analysis for T-cell activation in mouse blood cells
Mouse peripheral blood was collected in EDTA tubes (BD Biosciences) and lysed twice using ACK lysis buffer (Thermo Fisher Scientific). Remaining cells were stained with anti-CD8-Pacific Blue, anti-CD69-PE, anti-CD25-APC followed by flow cytometry analysis.

Detection of soluble granzymes and perforin
Soluble perforin (Cell Sciences) and granzyme B (eBioscience) were detected from growth media by ELISA according to manufacturer’s protocols.

TDB binding to human CD8+ T cells
Human CD8+ T cells were incubated with TDB on ice for 15 minutes, washed with FACS buffer and stained with goat anti-human-IgG-APC (Jackson ImmunoResearch) and anti-CD8-FITC on ice for 15 minutes followed by flow cytometry analysis.

Pharmacokinetics in mice
Male or female BALB/C mice (12 per group; Charles River Laboratories, 251) received a single intravenous dose of anti-LYPD1/CD3 or anti-gD/CD3 at 1 or 10 mg/kg. Total antibody concentrations in serum were determined by GRIP ELISA and used for pooled PK evaluations. A time point at day 21 from group 2 (10 mg/kg anti-LYPD1/CD3 female group) was excluded due to potential ADA impact. NCA parameters with standard error values were estimated using Phoenix WinNonlin 64 with sparse sampling and intravenous bolus input and SE values were provided.
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Antibody radiolabeling
Radiosynthesis of 111In labeled antibodies (~9 µCi/µg) was achieved through incubation of 111InCl3 and 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic (DOTA-NHS, macrocyclics)-conjugated (randomly through lysines) mAb in 0.3 M ammonium acetate pH 7 at 37°C for 1 hour. Radioimmunoconjugate purification was performed using NAP5 columns (GE Healthcare) and confirmed by radio-size-exclusion chromatography.

Mouse tissue distribution studies
Female human CD3ε transgenic mice (huCD3 TG, 6–8 weeks, 20–25 g; ref. 21) were inoculated on left flanks with CT26-LYPD1 syngeneic tumors and right flanks with LYPD1-nonexpressing CT26 syngeneic tumors (22). Mice with 400 to 500 mm³ mean tumor volumes were randomly assigned treatment (n = 3 per group) and administered a single tail vein bolus of 111In-labeled antibodies (5 µCi) together with the respective unmodified antibody (5 mg/kg total dose). Tissues were PBS rinsed, blotted dry, and weighed and blood was processed for plasma. Tissue and blood was then counted for radioactivity using a 1480 WIZARD Gamma Counter in the energy window for the 245 keV photon peak of 111In (decay t1/2 = 2.8 days).

Longitudinal SPECT-CT imaging
Noninvasive in vivo distribution was obtained by single-photon emission computed tomography/X-ray computed tomography (SPECT-CT) imaging of huCD3 TG mice bearing CT26-LYPD1 positive and negative tumors with 111In-labeled TDBs as described previously (22). Tumors were generated as in the invasive biodistribution studies and mice received an average dose of 1 mCi of 111In-labeled TDBs (5 µg/kg) and imaging performed at 2, 24, 48, and 72 hours. SPECT was performed on the photopeaks of 111In using a 5-pinhole collimator with 5.5 cm radius of rotation immediately after CT acquisition, tissues were collected for gamma counting as in the nonimaging study arm and SPECT images were visualized (Amira software, TGS).

In vivo efficacy in mice
Ten million ID8-LYPD1 cells/mouse suspended in HBSS+Matrigel were inoculated into the thoracic mammary fat pad (0.2 mL) of female C57BL-6 mice (Charles River) and a 0.2 mL inoculum was injected into the thoracic mammary fat pad of female C57BL-6 mice (Charles River-Hollister). Mice with 200 to 300 mm³ tumor volume were treatment randomized and dosing was initiated (day 0). Treatments were administered qw3 by tail vein injection (0.1 mL). Three million XPL4-LYPD1 cells suspended in HBSS+Matrigel were inoculated in the right second/third mammary fat pad of female NOD/SCID mice NOD.CB17-Prkdcscid/J; The Jackson Laboratory West). One day after inoculation, mice were injected intraperitoneally with 10 million human PBMcs cultured overnight in non-activating conditions. Control mice received no PBMcs. Mice with 100 to 250 mm³ tumors were treatment randomized and dosed via tail vein injection (0.1 mL) on day 0. TDBs were diluted in vehicle (20 mmol/L Histidine acetate, 240 mmol/L sucrose, 0.02%Tween-20, pH 5.5 buffer).

Analysis of peripheral cytokines in mice
Mouse blood was collected in serum separator tubes (BD Biosciences), centrifuged at 200 × g for 10 minutes, and sera were stored at −80°C for cytokine analysis using the mouse cytokine Luminex assay (Bio-Rad) according to manufacturer’s instructions.

Assessment of antibody brain concentration in mice
Forty huCD3E tg.B6N female mice were subcutaneously inoculated with 4 million ID8-LYPD1 cells resuspended in 200 µL of HBSS + Matrigel. When tumors reached ~100 to 120 mm³, mice received single intravenous administration of 10 mg/kg anti-gD or 10 mg/kg LYPD1-TDB (n = 20). One or four days after administration (n = 10 per time point), mice were anesthetized and terminally perfused with PBS. Half of the brain hemisphere (~0.1 g) was lysed in 1 mL 1% NP40 buffer (Life Technologies) plus protease inhibitor (Roche Diagnostics). Brain tissues were homogenized using a TissueLyrser II (Qiagen) and ~250 µL aliquots were applied to GRIP ELISA to measure antibody concentration.

Statistical analysis
Welch t test was used to test for differences between groups. P values were assessed by unpaired, two-tailed, Student t test, unless otherwise indicated.

Study approval
All in vivo mouse experimental procedures conformed to the guiding principles of the American Physiological Society and were approved by Genentech’s Institutional Animal Care and Use Committee.

Results
LYPD1 is a novel therapeutic target for ovarian cancer
LYPD1 was identified as a significantly overexpressed transcript in ovarian cancer compared with normal tissues (P < 2.2e−16; two-sided t test; Fig. 1A). LYPD1 RNA expression was also elevated in brain tumors (glioblastoma multiforme, GBM; low-grade glioma, LGG). Further analysis of the LYPD1 RNA expression profile in the Affymetrix GeneLogic microarray normal-tissue dataset indicated that LYPD1 RNA is expressed in normal ovaries, brain, nerve, and pancreas (Supplementary Fig. S1A). Analysis of NIH GTEx data demonstrated that LYPD1 RNA is broadly expressed in different regions of human brain (Supplementary Fig. S1B), confirmed with expression in brain and spinal cord by qRT-PCR (Fig. 1B). LYPD1 expression was further analyzed in sets of ovarian cancer and matched normal ovary from 23 patients by qRT-PCR. The majority of tumors expressed higher relative levels of LYPD1 compared with noncancerous samples (Fig. 1C). Only three of 398 ovarian tumors harbored a mutation in LYPD1 and amplification and deletion events were rare (Supplementary Fig. S1C). In summary, RNA analysis identified overexpression of LYPD1 at high prevalence in ovarian cancer.

We developed a LYPD1 IHC assay to confirm LYPD1 mRNA levels reflected protein expression. Development and optimization were performed using cell lines expressing endogenous or transfected LYPD1 (Supplementary Fig. S2A). Human ovarian cancers from a tissue microarray (TMA; n = 208) were then stained (Fig. 1D) and scored as 0, 1, 2, or 3 for negative, low, moderate, or high IHC signal (n = 125; Fig. 1D). LYPD1 was detected in both primary and metastatic samples with 50% (104/208) of samples positive for LYPD1. When present, expression was diffuse with 2.4% of samples scored as 3, 16.8% as 2, and 50.4% as 1. Ovarian serous carcinoma prevalence was high (70%; 87/125) and no correlation between LYPD1 expression and tumor grade, tumor stage, or patient age (11–81 years) was detected. Normal human tissue LYPD1 IHC expression was also evaluated (TMA; n = 23; Fig. 1E). Brain demonstrated positive staining in the cytoplasm of rare single cells or neuropil from cerebrum (n = 5). Cerebellum was negative (n = 5). Staining was also detected in human heart and appeared to align with intercalated discs.

Next, we extended the analysis to full sections of normal tissues suspected to be positive for LYPD1 staining. Staining was negative in...
Figure 1.
LYPD1 expression in cancers and normal human tissues. A, Differential expression in tumor (red) and normal (blue) samples from The Cancer Genome Atlas using RNASeq. The boxplots show gene expression levels by indication. The box represents the middle 50% of the data, with median indicated. The box edges (hinges) represent 25th and 75th percentiles. The whiskers mark the largest or smallest observation that falls within a distance of 1.5 times the box size from the nearest hinge; any observations farther away, are considered outliers. ACC, adrenocortical carcinoma; PRAD, prostate adenocarcinoma; SARC, sarcoma; DLBC, lymphoid neoplasm diffuse large B cell lymphoma; THCA, thyroid carcinoma; LUSC, lung squamous cell carcinoma; KIRC, kidney renal clear cell carcinoma; BRCA, breast invasive carcinoma; KICH, kidney chromophobe; LIHC, liver hepatocellular carcinoma; BLCA, bladder urothelial carcinoma; CESC, cervical squamous cell carcinoma and endocervical adenocarcinoma; HNSC, head and neck squamous cell carcinoma; KIRP, kidney renal papillary cell carcinoma; SKCM, skin cutaneous melanoma; COAD, colon adenocarcinoma; READ, rectum adenocarcinoma; PAAD, pancreatic adenocarcinoma; UCS, uterine carcinosarcoma; LUAD, lung adenocarcinoma; UCEC, uterine corpus endometrial carcinoma; LGG, brain lower grade glioma; GBM, glioblastoma multiforme; OV, ovarian serous cystadenocarcinoma. B, qRT-PCR analysis of LYPD1 RNA expression in normal tissue samples (blue) and positive control cell line (OVXF 1023; black). Identity of normal tissues is indicated in materials and methods (1 = brain, cerebellum; 2 = brain, whole; 3 = brain, fetal; 4 = spinal cord). C, Expression of LYPD1 in ovarian cancer (red) and adjacent normal ovarian tissue (blue) was measured with qPCR. D, Representative IHC images of ovarian cancer and (E) normal human tissues stained with LYPD1.
peripheral nerve ($n = 8$). Epithelial cells in ovary stained positive (2/4), but ovarian stroma was consistently LYPD1 negative ($n = 14$; Fig. 1E). A variety of anatomical heart regions also demonstrated staining that appeared to align with intercalated discs. Western blot analysis of lysates from various heart regions confirmed human heart expression (Supplementary Fig. S2B). Dual immunofluorescent staining demonstrated colocalization of LYPD1 and N-cadherin, a known zonula adherens junctional complex, within intercalated disc (Supplementary Fig. S2C).

**In vitro pharmacology of anti-LYPD1/CD3**

To generate anti-LYPD1 antibodies, a phage display library was screened against the ECD (Fig. 2A) of LYPD1 to discover LYPD1 specific antibodies that were further affinity matured to low nmol/L $K_D$ binding affinity. The amino acid sequence of the LYPD1 ECD is identical between human, mouse and cynomolgus monkey; therefore, therapeutic antibodies are expected to bind to the target in the aforementioned species. Affinity matured anti-LYPD1 was reformed as a “knob” (23, 24) and was coupled with an anti-CD3 “hole” to generate anti-LYPD1/CD3 TDB (Fig. 2B, Supplementary Fig. S3). FC mediated effector functions were attenuated by N297G substitution. Anti-LYPD1/CD3 TDB stimulated target dependent T-cell activation (Fig. 2C, Supplementary Fig. S4A) and dose-dependent degranulation of granzyme B and perforin (Fig. 2D), which resulted in robust killing of multiple LYPD1 expressing ovarian cancer cell lines (Fig. 2E) with no effect on target negative cells (OVCAR8 and SKBR3). TDB induced killing of all LYPD1 expressing cell lines, even those with low expression (Fig. 2G). However, cells with high target expression were substantially more sensitive (Fig. 2F and G), indicating that killing activity correlates with tumor antigen expression level. The impact of CD3 affinity on the activity of the anti-LYPD1/CD3 TDB was tested using two anti-CD3 variants previously described in detail (22, 25). Expected binding to T cells was confirmed by flow cytometry (Fig. 2H) and the molecule with a lower CD3 affinity demonstrated ~50-fold reduced killing activity compared with the molecule with higher affinity (Fig. 2I). Therefore, the higher CD3 affinity molecule was selected for in vivo studies.

Figure 2.

**In vitro pharmacology of anti-LYPD1/CD3 TDB.** A, Schematic representation of the structural features of LYPD1 protein (SS, signal sequence; N, N-linked glycosylation; GPI, GPI link/cleavage; C:C, disulphide bridge). B, Schematic representation of the anti-LYPD1/CD3 TDB. C, TDB-mediated T-cell activation was measured using flow cytometry. D, Soluble granzyme B and perforin was assayed from the growth media using ELISA. OVCAR-3 was used as a target cell. E, TDB mediated killing of ovarian cancer cell lines measured by flow cytometry, EC50 are indicated. F, LYPD1 expression and TDB mediated killing of CT-26 cell lines. Color codes indicate the LYPD1 expression level in respective transfected cell line. G, TDB mediated killing of LYPD1 transfected cell lines. H, Flow cytometry analysis of TDB affinity variant binding to human PBMC. I, Killing activity mediated by TDB affinity variants was analyzed by flow cytometry.
LYPD1 expression in mice

LYPD1 mouse tissue IHC expression was detected in brain, ovary, fallopian tube, and heart (Supplementary Fig. S5), mirroring human expression. In mouse brain, heterogeneous signal was present within the neuropil and various areas had cells with cytoplasmic expression (Supplementary Fig. S5A), also consistent with what was detected in human brain. Analysis of regional expression by rough rostrocaudal anatomic level in mouse demonstrated septo-striatal, caudal diencephalon and mesencephalon, and caudal cerebellum (granular layer) expression (Supplementary Fig. S5A). Positive regions correlated well with published ISH data (26). Positive signal was also detected in ovary and fallopian tube (Supplementary Fig. S5B) as well as heart (Supplementary Fig. S5C) and aligned with intercalated discs.

Distribution and pharmacokinetics of anti-LYPD1/CD3 TDB in mice

Serum concentration–time profiles of the anti-LYPD1/CD3 were assessed with control antibody anti-gD/CD3 in male and female BALB/C mice at two different dose levels (Fig. 3A). The PK data of each group were characterized by noncompartmental analysis (NCA; Supplementary Table S1) and similar systemic exposures were observed in all mice at 10 mg/kg dose, suggesting that sex does not impact the PK of anti-LYPD1/CD3 in mice. In addition, dose proportional exposure was observed between 1 and 10 mg/kg, no difference was observed between anti-LYPD1/CD3 and anti-gD/CD3 groups and overall, anti-LYPD1/CD3 exposure is similar to a typical human IgG1 antibody.

As vascular features of syngeneic CT26 tumors have been previously characterized (22), this model was selected for distribution studies. huCD3-TG mice express both mouse and human CD3 on T cells (Supplementary Fig. S6; ref. 27). The huCD3ζ subunit can both structurally and functionally substitute for murine CD3ζ in the transgenic mouse. In addition, the chimeric T-cell receptor in this model supports normal T-cell development and selection of repertoires in vivo and can mediate activity of the huCD3ζ-specific HER2/CD3 bispecific antibody (27). huCD3-TG mice bearing both LYPD1...
positive and negative tumors received a single, 5 mg/kg i.v. dose of 111In-TDB and were subjected to tissue distribution analysis 72 hours later. Distribution of anti-LYPD1/CD3 TDB with anti-human CD3 was compared with TDB including the same target arm and anti-mouse CD3 arm (clone 2C11; ref. 14) that binds to mouse CD3 (~30-fold lower affinity compared with the anti-human CD3).

Whole blood and plasma concentrations showed no difference between the TDBs (Fig. 3B). Significantly (~2- 5-fold) more TDB distributed to LYPD1 expressing tumor compared with LYPD1 negative tumor (P = 0.05 and 0.0006 for the anti-mouse CD3 and anti-human CD3 TDB, respectively; Fig. 3B), demonstrating clear target dependent tumor distribution (20.4 ± 5.3%ID/g for anti-mouse CD3 TDB; 23.6 ± 2.6%ID/g for anti-human CD3 TDB; Fig. 3B).

Although the LYPD1 targeting arm cross reacts with mouse LYPD1 expressed in mouse brain, ovaries, and heart, only a minimal distribution was seen to these tissues (Fig. 3B). Although a nonbinding antibody control was not included, the values recorded from brain and heart are within expected background level uptake (22).111In signal detected in samples collected from brain or heart were not significantly higher compared with LYPD1 negative tissues suggesting limited target-dependent distribution to normal tissues (Fig. 3B).

Human CD3 reactive TDB with higher CD3 affinity was significantly more enriched in secondary lymphatic tissues compared with mouse CD3 reactive TDB. Lymphatic (10.5 ± 1.4%ID/g vs. 5.9 ± 1.2%ID/g, P = 0.01) and splenic (6.7 ± 1.2%ID/g vs. 3.4 ± 0.7%ID/g, P = 0.01) enrichment was roughly ~2× higher for anti-human CD3 TDB with higher CD3 affinity (Fig. 3B).

Longitudinal SPECT CT imaging of single mice dosed with 5 mg/kg of TDB confirmed all above findings without statistical power. Additional imaging at time points of 1, 24, and 48 hours suggest that there are no gross differences in distribution kinetics (Fig. 3C; Supplementary Fig. S7).

In vivo efficacy of anti-LYPD1/CD3 TDB

Syngeneic mouse ovarian tumor model ID8-LYPD1 (Supplementary Fig. S4B) was used to demonstrate in vivo efficacy in mice with intact immune system (Fig. 4A). Tumor bearing female C57BL-6 mice were treated with surrogate anti-LYPD1/CD3 mIgG2a TDB, which uses a mouse reactive CD3 arm (2C11; ref. 14). Weekly treatment with the TDB induced tumor regression at 0.5 to 5 mg/kg dose levels.

To further test in vivo activity of anti-LYPD1/CD3 TDB with a higher affinity anti-human CD3 arm, KPL4-LYPD1 (Supplementary Fig. S4C) tumor bearing female NOD/SCID mice were engrafted with human PBMC (Fig. 4B). TDB induced complete responses and prevented tumor growth at 0.05 mg/kg dose, with activity comparable to the previously described anti-HER2/CD3 TDB (12, 20, 25). In our experience, NSG mice inoculated with human PBMCs typically start displaying symptoms of GVHD ~20 to 30 days inoculation. In some instances, human immune cells also impact the growth of tumor xenografts (Fig. 4B), potentially due to allo-reactivity. Timing and severity of GVHD is highly variable between tumor models and PBMC donors, but also between individual mice (Fig. 4B), likely due to differences in PBMC engraftment.

In vivo pharmacology and tolerability of anti-LYPD1/CD3 TDB in mice

LYPD1 is expressed in mouse brain, female reproductive organs, and heart muscle. As the amino acid sequence is identical between human and mouse LYPD1, mouse is a pharmacologically relevant species, in which to investigate potential on target/off tumor effects. Pharmacologic effects and acute tolerability of anti-LYPD1/CD3 TDB in female ID8-LYPD1 tumor bearing hu.CD3ε.tg.B6N mice (Fig. 5A) were investigated with single 10 mg/kg doses of anti-LYPD1/CD3 TDB (higher CD3 affinity variant) or nonbinding control antibody (anti-gD).

Treatment with anti-LYPD1/CD3 TDB did not result to any observable effects on vital signs, behavior or neurologic signs, but did induce a robust activation of T cells and peripheral lymphocyte margination (Fig. 5B). Elevated levels of serum cytokines and chemokines were detected 2 to 6 hours after the TDB dose (Fig. 5C), but generally returned near baseline at 24 hours.

Concentration of TDB in the brain homogenate of mice was analyzed 1 or 4 days after high-dose anti-LYPD1/CD3 TDB treatment. Brain TDB concentration was ~300-fold lower than serum concentration, consistent with previously reported concentration ratios (0.1–0.3%; refs. 28, 29) and was similar to nontarget binding control anti-gD IgG1 antibody (Fig. 5D).

In a histologic analysis of mice treated with a single 10 mg/kg dose of anti-LYPD1/CD3 TDB for 1 or 4 days, brain, fallopian tube, ovary, kidney, spleen, heart, small intestine, and large intestine were without significant abnormality. Multiple necroinflammatory foci (Fig. 5E), variable perivascular and periportal chronic inflammatory infiltrate, and focal early necrosis of hepatocytes surrounding the central vein, portal tracks, and within lobules were noted in liver 1 day after administration of anti-LYPD1/CD3 TDB, consistent with acute hepatocellular toxicity expected from TDB treatment (25). Liver findings were absent 4 days after treatment, suggesting that the effects are transient.

In summary, anti-LYPD1/CD3 TDB induces expected pharmacodynamic response including transient cytokine release and transient liver toxicity, with no apparent on target/off tumor toxicity on LYPD1-expressing normal tissues. No evidence was found that would suggest blood–brain barrier impairment.

Discussion

LYPD1 is expressed in ovary, fallopian tube, and ovarian cancer, where its function remains unknown and in brain and GBM where its expression is logical due to its proposed functional role. As the blood–brain barrier interferes with antibody distribution to the brain, our efforts were focused on ovarian cancer. Our genetic analysis of human tumors suggests that amplification of LYPD1 is not frequent in ovarian cancer. Transcriptional and posttranslational regulation of LYPD1 in not well understood and mechanistic basis of dysregulation in cancer remains unclear.

GPI anchored molecules can be solubilized by proteolytic or lipolytic cleavage. Cleavage of the LYPD1 ECD may interfere with antibody binding to tumor cells. It could also serve as a potential diagnostic to identify target positive tumors and as an indirect marker for antitumor activity. Further studies are required to evaluate the effect of potential LYPD1 shedding on the function of anti-LYPD1/CD3 bispeciﬁc antibody.

We developed a novel anti-LYPD1/CD3 bispeciﬁc antibody for treatment of LYPD1 positive ovarian cancer. Consistent with previous studies, the pharmacologic activity of anti-LYPD1/CD3 correlated with expression level of the tumor antigen (12, 20, 30). TDB activity also correlated with high CD3 afﬁnity. This result is in contrast to our previous anti-HER2/CD3 TDB studies (25) where CD3 afﬁnity was dispensable for antitumor activity. Contrasting results support the hypothesis that potency of a CD3-bispeciﬁc molecule is a sum of interdependent factors (31). Examples of other parameters that impact biological activity include tumor antigen copy number (12, 20,
structural properties, and antibody binding tumor antigen epitope (6) as well as tumor antigen affinity and valency (20, 22, 25). Despite the advantages in understanding critical parameters, properties required to generate an optimal molecule are still difficult to predict accurately and substantial experimentation and optimization are required for each molecule.

Clinical and preclinical safety studies have demonstrated the risk of on target toxicities with T-cell re-targeting drugs in treatment of solid tumors (25, 32–35). Mouse reactive antibodies and target expression in respective mouse tissues enable anti-LYPD1/CD3 safety studies in mice, where we found no evidence of acute on-target toxicity. Previous reports have indicated less than 0.5% serum level CNS uptake of therapeutic antibodies due to the blood–brain barrier (28, 29, 36). We therefore hypothesized that TDB brain exposure would be insufficient to cause on-target toxicities and confirmed low brain exposure, suggesting functional blood–brain barrier despite induction of systemic cytokines in our model. We hypothesize that lack of damage to ovary and heart is due to insufficient LYPD1 expression or, in the case

Figure 4.
In vivo efficacy of anti-LYPD1/CD3 TDB. A, Individual tumor volume responses of ID8-LYPD1 tumors to anti-LYPD1/CD3 TDB with mouse reactive CD3 arm (2C11; IV; qwx3) in female C57BL-6 mice (n = 10). Animals that were removed from study prematurely displayed severe skin ulceration due to tumor growth. B, Individual tumor volume responses of KPL4-LYPD1 tumors to LYPD1-TDB treatment (higher CD3 affinity; intravenous; single dose) in female NOD/SCID mice engrafted with human PBMC as indicated (n = 8). Animals that were removed from study prematurely displayed signs of huPBMC induced GVHD. Trellis plots of individual and fitted tumor volumes are presented with study day on the x-axis and tumor volume on the y-axis. Each panel in the trellis depicts one dose group (panel headers indicate group numbers). Solid black lines indicate the fitted tumor volume for each dose group. Dashed blue lines indicate the fitted tumor volume for the control group (vehicle: Histidine buffer). Red lines indicate the tumor response over time in individual animals.
of heart, accessibility of antibody or T cells to tight junction localization of LYPD1. Similar reversible inflammation and lack of tissue damage, despite normal tissue target expression, was recently reported in preclinical studies using anti-MUC16/CD3 (37).

Anti-LYPD1-CD3 induced reversible inflammation in liver independent of LYPD1 expression in the organ. Differentiating between direct T-cell activity on tissue and secondary toxicity caused by inflammation and cytokines is challenging, and the hepatic findings may potentially be secondary to cytokine-induced hepatocellular damage and related immune cell infiltration. Target independent liver findings have been previously reported for similar solid tumor targets (25). We used the mouse model to demonstrate initial acute tolerability. However, the current studies have limitations and further studies addressing impact of multiple doses with broad analysis of observable physiologic effects are required in model species that recapitulates the expected systemic cytokine response before clinical use.

In summary, limited targeted therapies are available for ovarian cancer. CD3 bispecific antibodies are a novel class of immunotherapy substantially differentiated from checkpoint inhibitors and have shown transformational clinical activity in treatment of hematologic cancers. We identify LYPD1, a broadly expressed novel therapeutic target for ovarian cancer, demonstrate that anti-LYPD1/CD3 TDB immunotherapy has promising preclinical antitumor activity and present data that supports development of anti-LYPD1/CD3 TDB for treatment of ovarian cancer.

Authors’ Disclosures
No disclosures were reported.

Authors’ Contributions
A.A. Lo: Data curation, formal analysis, supervision, methodology, writing—original draft, writing—review and editing. J. Johnston: Formal analysis, validation, investigation, methodology. J. Li: Formal analysis, validation, investigation, methodology. D. Mandikian: Formal analysis, validation, investigation, methodology. M. Hristopoulos: Formal analysis, validation, investigation, methodology. R. Clark: Formal analysis, validation, investigation, methodology. D. Nickles: Data
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