On line supplemental file 1

Materials

The mouse monoclonal antibody against the 52-, 48-, and 34-kDa forms of human cath-D (#610801) was purchased from BD Transduction Laboratories (San Jose, CA). The anti-human, cath-D monoclonal antibody M2E8 recognizes only 52-kDa cath-D, and not 48- and 34 kDa cath-D[1, 2]. The anti-human cath-D rabbit polyclonal antibody (H-75; sc-10725) to detect 52-kDa human pro-cath-D was from Santa Cruz Biotechnology (Dallas, TX). The anti-mouse cath-D goat polyclonal antibody (clone AF1029) was from R&D Systems (Minneapolis, MN). The 11H4 hybridoma against the C-terminal part of the LRP1β chain was provided by P. van der Geer (San Diego State University, USA). The mouse monoclonal anti-human M6P/IGF2 receptor antibody (clone MEM-238) was from Origene. The mouse monoclonal anti-human cath-D antibody (clone E-7; sc-13148) to detect 52-kDa pro-cath-D, the mouse monoclonal anti-human cath-D antibody (clone C-5), and the anti- HSC70 antibody (clone B-6, sc-7298) were purchased from Santa Cruz Biotechnology (Dallas, TX). The rabbit monoclonal anti-androgen receptor (AR) antibody (clone D6F11, #5153) was from Cell Signaling Technology. The anti-β actin polyclonal antibody (#A2066) and the anti-human cath-D mouse monoclonal antibody (clone CTD19) were from Sigma-Aldrich (Saint-Louis, MO). The mouse monoclonal anti-tubulin antibody (clone 236-10501, #A11126) and Hoechst (#33342) were from Thermo Fisher Scientific (Waltham, MA). Human F1 IgG1, human F1M1 IgG1 and Fc-mutated F1M1 IgG1 (F1M1-Fc^+ with the mutations S239D, H268F, S324T, I332E and F1M1-Fc^- with the mutations L234A, L235A and P329G) were constructed from the scFv (F1, F1M1) sequences using gene synthesis, expressed in the Chinese hamster ovary (CHO) cell line, and purified on protein-A HiTrap columns (GE Healthcare) by Evitria AG (Schlieren, Switzerland). The PC5-conjugated anti-CD107a (clone H4A3) IgG1, and PE-conjugated anti-IFNγ (clone B27) IgG1 were from BD Biosciences. Unconjugated and APC-conjugated anti-hCD16a (clone 3G8) IgG1 were from Beckman Coulter. Enzalutamide was purchased from Euromedex and 3(4,5-dimethyl-thiazol-2-yl)2,5-diphenol tetrazolium bromide (MTT) from Sigma-Aldrich. The anti-human CD20 chimeric IgG1 antibody rituximab (Truxima®) was from Healthcare Celltrion. Paclitaxel (Taxol®) was from Fresenius Kabi. Cetuximab (anti-human EGFR chimeric IgG1 antibody) was from Merck. Matrigel (10 mg/ml,

#354234) was purchased from Corning. Collagenase IV (#C5138) and DNase I (#11284932001) were from Sigma-Aldrich (St Louis, MO). Mouse Fc Block (#130-097-575) and the fluorescent-conjugated antibody against CD27 (clone REA499, #130-114-163 were from Miltenyi Biotec (Bergisch Gladbach, Germany). Viakrome IR 808 (#C36628) was from Beckman Coulter (Brea, CA). The fluorescent-conjugated antibodies against CD45 (clone 30-F11, #103149), CD19 (clone 6D5, #115507), F4/80 (clone BM8, #123109), NKp46 (clone 29A1.4, #137606), CD11b (clone M1/70, #101237), and granzyme B (clone QA16A02, #372215) were from Biolegend (San Diego, CA). The antibodies against CD3 (clone 17A2, #555275), CD11c (clone HL3, #553802) and CD107a (clone 1D4B, #565533) were from BD Biosciences (Franklin Lakes, NJ). The fixation/permeabilization kit (#00-5523-00) was from Invitrogen. The anti-human Fc goat polyclonal antibody conjugated to HRP (A0170) and 3(4,5-dimethyl-thiazol-2-yl)2,5-diphenol tetrazolium bromide (MTT) were from Sigma Aldrich (Saint-Louis, MO). The HRP-conjugated anti-mouse F(ab)2 (#115-036-072) was from Jackson ImmunoResearch (West Grove, PA). The substrate reagent for HRP was purchased from Bio-Techne (R&D Systems, #DY999). The APC-Vio770-labeled anti-CD19, VioGreen-labeled anti-CD45, PE-Vio770-labeled anti-CD56, PE-labeled anti-CD69 antibodies and 7AAD were from Miltenyi Biotec. (Germany). Calcein (sc-203865) was from Santa Cruz Biotechnology (Dallas, TX). The anti-asialo GM1 antibody (clone Poly21460, #146002) was from BioLegend (San Diego, CA). The fluorescent-conjugated antibodies against Ly6G (clone 1A8, #127621), B220 (clone RA3-6B2, #103241), CD11c (clone N418, #117329) and F4/80 (clone QA17A29, #157307) were from BioLegend (San Diego, CA). The fluorescent-conjugated antibody against I-A/I-E (clone 2G9, #743876) was from BD Biosciences (Franklin Lakes, NJ). The ACK (Ammonium-Chloride-Potassium) lysis buffer (#A1049201) was from Gibco. Precision count beads (#424902) were from BioLegend (San Diego, CA).

Cell lines, conditioned medium, and western blotting, ELISA, and immunoprecipitation

The MDA-MB-231 (TNBC) cell line was previously described[3]. The SUM159 cell line was from Asterand (Bioscience, UK). MDA-MB-231 cells were cultured in DMEM with 10% fetal calf serum (FCS, Eurobio), and SUM159 cells in RPMI with 10% FCS. NK92 hCD16a 158V and NK92 hCD16a 158F cells were kindly provided by Beatrice Clémenceau and Henri Vié (INSERM U892, Nantes,

France)[4]. These two NK cell lines were cultured in RPMI1640/GlutaMAX with 10% decomplemented FCS, 1% penicillin-streptomycin supplemented with 100 IU/mL IL-2 (Peprotech, #200-02). The human CAF1 (hCAF1)[5] cell line (breast cancer-associated fibroblasts, CAF) was kindly provided by Olivier de Wever (Ghent University, Ghent, Belgium).The E0771Luc mouse cell line, transformed to constitutively express luciferase as reporter, was kindly provided by Dr. C-L. Tomasetto (IGBMC, Strasbourg, France). E0771Luc cells were cultured in RPMI1640 with 10% FCS and 10 mM Hepes, pH=7.5. To produce conditioned medium, cells grown to 90% confluence were incubated in the absence of FCS for 24 h or in the presence of FCS for 48 h. Then, conditioned medium was centrifuged at 800 x g for 5 min. Cell lysates were prepared in lysis buffer (50 mM Hepes pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl2, 1 mM EGTA) containing cOmplete™ Protease Inhibitor Cocktail (Roche, Switzerland) and centrifuged at 13,000 x g for 15 min. For western blotting experiments, proteins (10-30 µg) from cell lysates and conditioned media (40 µl) were separated on 13.5% SDS-PAGE and analyzed by immunoblotting using standard techniques. For the sandwich ELISA against human cath-D, 96-well plates were coated with the M2E8 antibody in PBS (250 ng/well) at 4°C overnight. After blocking non-specific sites with PBS/0.1% Tween-20/1% BSA, cath-D-containing conditioned medium with FCS from MDA-MB-231 cells was added at 4°C for 2h. After washes in PBS/0.1% Tween 20, serial dilutions of F1, the F1M1, F1M1-Fc^-, or F1M1-Fc^+ antibodies were added at 4°C for 2h, and interaction revealed with an anti-human Fc antibody conjugated to HRP for 1h at 37°C (1/2000; 355ng/well). For the sandwich ELISA against mouse cath-D, 96-well plates were coated with the anti-cath-D antibody (#AF1029, Bio-Techne) in PBS (100 ng/well) at 4°C overnight. After blocking non-specific sites with PBS/0.1% Tween-20/1% BSA, cath-D-containing supernatants from E0771Luc cells in the presence of serum were added at 37°C for 2h. After washes in PBS/0.1% Tween 20, serial dilutions of F1 or F1M1 in PBS were added at 37°C for 2h, followed by washes in PBS/0.1% Tween 20, and incubation with a HRP-conjugated anti-mouse F(ab')2 (1/2000) at 37°C for 2h. For immunoprecipitation, conditioned medium without FCS from MDA-MB-231 cells was adjusted to different pH and incubated with 2.5 µg of F1M1, F1M1-Fc^-, or F1M1-Fc^+ at 4°C overnight, and subsequently with 50 µl of 10% protein A-Sepharose, at 4°C on a shaker for 2h. Sepharose beads

were washed four times with PBS, boiled in SDS sample buffer for 5min (95°C), and analyzed by SDS-PAGE and immunoblotting.

Fluorescence microscopy

Paraffin-embedded PDX1995, PDX3977 or MDA-MB-231 tumor sections were deparaffined, rehydrated, rinsed, and saturated in PBS with 5% FCS at 4°C overnight. Sections were incubated with 0.4 µg/ml anti-cath-D mouse monoclonal antibody (clone C-5) followed by incubation with a Cy3-conjugated anti-mouse IgG (1/500). Nuclei were stained with 0.5 µg/ml Hoechst 33342. Sections were then imaged with a 63X Plan-Apochromat objective on z stacks with a Zeiss Axio Imager light microscope.

Immunohistochemistry

For cath-D immunostaining in TNBC samples from patients, tumor tissue sections were incubated with 0.4 µg/ml anti-human cath-D mouse monoclonal antibody (clone C-5) for 20 min after heat-induced antigen retrieval with the PTLink pre-treatment (Dako) and the High pH Buffer (Dako) and endogenous peroxidase quenching with Flex Peroxidase Block (Dako). After two rinses in EnVision™ Flex Wash buffer (Dako), sections were incubated with an HRP-labeled polymer coupled to a secondary anti-mouse antibody (Flex® system, Dako) for 20 min, followed by incubation with 3,3'-diaminobenzidine as chromogen. Sections were counterstained with Flex Hematoxylin (Dako) and mounted after dehydration. For human cath-D immunostaining in MDA-MB-231 tumor xenografts, tumor sections were incubated with 6.5 ng/ml anti-human cath-D mouse monoclonal antibody (clone CT19, SIGMA) after heat-induced antigen retrieval with the PT-Link pre-treatment device (Dako) and the High pH Buffer (Dako). Endogenous peroxidase was quenched with Flex Peroxidase Block (Dako). After two rinses in EnVisionTM Flex Wash buffer (Dako), the primary antibody was bridged to the anti-rabbit HRP-labeled polymer (Envision® system, Dako) using a rabbit anti-mouse antibody (ab133469, abcam). Sections were analyzed independently by two experienced pathologists, both blind. Cath-D signal was scored in cancer cells according to the staining intensity (0: none, 1: low, 2: moderate, 3: high).

Surface plasmon resonance

Surface plasmon resonance experiments were performed at 25°C using a Biacore T200 apparatus (Cytiva) in PBS supplemented with 0.05% P20 surfactant (ThermoFisher). The anti-His antibody (R&D Systems) was immobilized on a CM5S dextran sensor chip (Cytiva) by amine coupling according to the manufacturer's instructions. The human receptor proteins FcγRIIIA/hCD16a 158V and FcγRIIIA/hCD16a 158F (R&D Systems) were captured at 170 RU. All kinetic measurements were performed by single-cycle kinetic titration. Five increasing concentrations of these analytes (3.7, 11, 33, 100, 300 nM) were injected onto the captured hCD16a 158V and hCD16a 158F at a flow rate of 100 µl/min (injection time of 60ms), followed by a dissociation phase of 600ms after the final injection. Regeneration was done using 10mM glycine-HCl buffer, pH 1.5. All curves were analyzed with the Biacore T200 BiaEvaluation software 3.2 (Cytiva) after double-referencing subtraction. Because of the binding kinetics complexity, the two-state fitting model was chosen to allow comparing the KD values of the different antibodies with the two receptors[6].

Study approvals

Mouse experiments were performed in compliance with the French regulations and ethical guidelines for experimental animal studies in an accredited establishment (Agreement No. #31135-2021042212479661). The study approval for PDXs was previously published[7]. For immunohistochemistry (IHC), TNBC biopsy samples were provided by the biological resource center (Biobank number BB-0033-00059) after approval by the Montpellier Cancer Institute Institutional Review Board, following the French national Ethics and Legal dispositions for patients’ information and consent. For TNBC cytosols, patient samples were processed according to the French Public Health Code (law n°2004-800, articles L. 1243-4 and R. 1243-61), and the biological resources center has been authorized (authorization number: AC-2008-700; Val d’Aurelle, ICM, Montpellier) to deliver human samples for scientific research. All patients were informed before surgery that their surgical specimens might be used for research purposes. For human primary NK cell isolation and expansion, this work benefited from umbilical cord blood units and John De Vos’ expertise (head of the Biological Resource

Center Collection of the University Hospital of Montpellier, http://www.chu-montpellier.fr/en/platforms; BIOBANQUES Identifier: BB-0033-00031).

***In vivo* experiments**

In monotherapy experiments, MDA-MB-231 cells (2× 10^6; mixed 1:1 with Matrigel) or SUM159 cells (5x10^6; mixed 1:1 with Matrigel) were injected subcutaneously in 6-week-old female athymic mice (NU(NCr)-Foxn1^{nu}) (Charles River Laboratory, France). For PDX models, approximately 5x5x5 mm of B1995 and B3977 tumor fragments were transplanted subcutaneously in 5-week-old female Swiss nude mice (NU(Ico)-Foxn1^{nu}) (Charles River Laboratory, France). When xenografts reached a volume of ~50 mm^3 (MDA-MB-231 and SUM159 cell xenografts) and ~100mm^3 (PDXs), tumor-bearing mice were randomized and treated with F1M1, F1M1-Fc^-, F1M1-Fc^+, or rituximab (15 mg/kg for all) by intraperitoneal injection (ip) three times per week. In other experiments, when tumors reached a volume of about 50 mm^3, tumor-bearing mice were randomized and treated with paclitaxel (1, 4, or 7 mg/kg), or saline by intraperitoneal injection once a week. In combined treatments, when MDA-MB-231 cell xenografts reached a volume of ~50 mm^3, tumor-bearing mice were randomized and treated with paclitaxel (1 or 4 mg/kg) or saline by ip once per week or/and F1M1-Fc^+ (15 mg/kg), or rituximab (15 mg/kg) by ip three times per week. When SUM159 cell xenografts reached a volume of ~50 mm^3, tumor-bearing mice were randomized and were treated with enzalutamide (30mg/kg) or DMSO in corn oil (per os, five times per week) or/and with F1M1-Fc^+ (15 mg/kg), or with rituximab (15 mg/kg) by ip twice per week. For the oral treatment, 5 µl of enzalutamide in DMSO (120 µg/µl) or DMSO alone was added to 145 µl of corn oil and given by oral gavage. Tumors were measured using a caliper and volume was calculated with the formula V = (tumor length × tumor width^2)/2, until the tumor volume reached 1000-2000 mm^3, depending on the experiment. In the NK cell depletion experiments, athymic mice were treated with anti-asialo GM1 antibodies (50µL) by ip two times per week. When tumor volume reached 50 mm^3, tumor-bearing mice were randomized and treated with F1M1-Fc^+ or rituximab (15 mg/kg for both) by ip three times per week.

In vivo immunophenotyping of NK cell depletion in spleen and blood

Spleens were crushed. Both spleens and blood samples were lysed with ACK lysis buffer for 10 min at room temperature. Cells were washed and resuspended in FACS buffer (PBS pH 7.2, 1% decomplemented FCS, 2mM EDTA, and 0.02% sodium azide). Cells were blocked with FACS Buffer containing 1% (v/v) mouse Fc block for 30 min and stained with fluorescent-conjugated antibodies against the cell surface markers CD45, CD3, B220, Ly6G, NKp46, CD11c, I-A/I-E (MHC-II), CD11b and F4/80 for 1h. After fixation with 1% paraformaldehyde in PBS, cell samples were analyzed by flow cytometry using a Beckman and Coulter Cytoflex flow cytometer and FlowJo 10.8.1. Living immune cells were defined as Viakrome IR808$^-$ CD45$^+$ cells. Neutrophils were defined as CD45$^+$CD3$^-$Ly6G$^+$ cells. B cells were defined as CD45$^+$CD3$^-$Ly6G$^-$B220$^+$ cells. NK cells were defined as NKp46$^+$ cells within the gate excluding CD3$^+$ T and NK T cells, B220$^+$ B cells and Ly6G$^+$ neutrophils. Dendritic cells were defined as CD45$^+$CD3$^-$Ly6G$^-$B220$^-$NKp46$^-$CD11c$^+$MHC-II$^+$ cells. Macrophages were defined as CD11b$^+$ F4/80$^+$ cells in the gate excluding all other immune cells.

Blood counts

20µL of blood was collected from the tail vein of mice in EDTA tubes (EDTA K3E, 200µL, Kabe Labortechnik). Blood samples were analyzed with the scil Vet abc Plus+ system (scil Animal Care Co).

Quantitative RT-PCR

Reverse transcription of total RNA was performed at 37°C using the Moloney murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA) and random hexanucleotide primers (Promega, Madison, WI). Real-time quantitative PCR analyses were performed on a Light Cycler 480 SYBR Green I master and a Light Cycler 480 apparatus (both from Roche Diagnostics, Indianapolis, IN). The PCR product integrity was verified by melting curve analysis. Quantification data were normalized to the amplification data for the reference gene encoding ribosomal protein S9 (*Rps9*). The sequences of the primers for *Eomes*, *Tnfa, Gzmb, Prf1* and *Rps9* are in the online supplemental table 1.

Cell viability assay

8 x10^3 MDA-MB-231 cells were seeded in 96-well plates in DMEM/10% FCS. When cells reached 60-70% of confluence, they were serum-deprived for 24h to induce synchronization, and then were incubated or not with paclitaxel in DMEM/2% FCS for 48h. Cell survival was evaluated with the mitochondrial dehydrogenase enzymatic assay, using MTT (Sigma) and determination of OD_{540nm}.

Human primary NK cell isolation, expansion, and characterization

Umbilical cord blood mononuclear cells were isolated through density gradient centrifugation using Histopaque-1077 (Sigma). Briefly, blood samples were diluted (1:1 ratio) with RPMI, then layered above 10mL of Histopaque in a 50mL conical tube. After centrifugation at 400xg for 30min, the white layers of mononuclear cells were collected and washed. Using the EasySep Human CD3 Positive Isolation kit (StemCell Technologies), the $CD3^+$ cell fraction (T and NK T cells) from the mononuclear cell samples was depleted to better culture NK cells. After depletion verification by flow cytometry, NK cells were cultured with γ-irradiated PLH feeder cells at 1:4 (NK cell: feeder cell) ratio, IL-2 (100 IU/mL) and IL-15 (5 ng/mL) for 20 days. Feeder cells and cytokines were refreshed every 3-4 days. To monitor expansion, NK cells were immunophenotyped. Briefly, 2.10^5 cells were stained with APC-labeled anti-CD3, VioBlue-labeled anti-CD16, APC-Vio770-labeled anti-CD19, VioGreen-labeled anti-CD45, PE-Vio770-labeled anti-CD56, PE-labeled anti-CD69 antibodies and with 7AAD at 4°C in the dark for 20min. After two washes, cells were analyzed using a Gallios flow cytometer (Beckman Coulter) and the FlowJo V10 software.

ADCC by live cell imaging

MDA-MB-231 cells (5 x 10^3; target) were labeled with calcein 2µM for 30 min and then seeded on Ultra Low Attachment 96-well plates (#7007, Corning). After 72h, MDA-MB-231 cell spheroids were first incubated with F1M1-Fc^+, F1M1-Fc^-, or cetuximab at 37 °C for 30min and then with NK92 $hCD16a^+$ 158V cells (effector) at an effector:target ratio of 20:1 for 24 h. Cells were scanned every hour for 24h and green fluorescence intensity was measured using the Sartorius IncuCyte® device by live imaging using user-informed algorithms that are part of the IncuCyte™ software package.

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On line supplemental legends to figures

Online supplemental figure 1. Representative images of TNBC tissue sections showing cath-D expression

Cath-D was monitored in a TNBC biopsy by IHC using an anti-cath-D (C-5) antibody. Scale bar, 50 µm (left panel). Higher magnifications of the boxed region showing extracellular cath-D expression (right panel). Arrows show cath-D localization at the cancer cell surface.

Online supplemental figure 2. Generation of the VH- and VL-aglycosylated anti-cath-D F1M1 antibody that binds to secreted cath-D and inhibits TNBC MDA-MB-231 cell xenograft growth

(A) Coomassie blue staining of F1 and F1M1 in denaturing and non-denaturing conditions. The IgG1 F1 and F1M1 (2.5 µg) were separated on 13.5% SDS-PAGE followed by Coomassie blue staining.

(B) Binding of F1M1 to human cath-D secreted from MDA-MB-231 cells. Sandwich ELISA in which human cath-D from conditioned medium of MDA-MB-231 cells was added to wells pre-coated with the anti-human cath-D M2E8 mouse monoclonal antibody in the presence of increasing concentrations of the IgG1 F1M1. F1M1 binding to secreted human cath-D was revealed with an anti-human Fc HRP-conjugated antibody. The EC_{50} values are shown.

(C) Binding of F1 and F1M1 to mouse cath-D secreted from E0771Luc cells. Sandwich ELISA in which mouse cath-D from conditioned medium of E0771Luc cells was added to wells pre-coated with the anti-mouse cath-D AF1029 antibody in the presence of increasing concentrations of the IgG1 F1 or F1M1. Binding of F1 and F1M1 to secreted mouse cath-D was revealed with an anti-mouse F(ab')2 HRP-conjugated antibody. The EC_{50} values are shown.

(D) Tumor growth. MDA-MB-231 cells were subcutaneously injected in nude mice. When tumor volume reached 50 mm^3, mice were treated with F1 (n=7), F1M1 (n=7), or rituximab (Ctrl; n=7) (15 mg/kg) three times per week for 36 days. Tumor volume (in mm^3) is shown as the mean ± SEM. ***P <0.001 for F1 versus Ctr; **P=0.003 for F1M1 versus Ctrl, P= 0.378 F1 versus F1M1 (mixed-effects ML regression test).

Online supplemental figure 3. Characterization of the anti-cath-D F1M1, F1M1-Fc$^-$, and F1M1-Fc$^+$ antibodies

(A) Coomassie staining of the F1M1, F1M1-Fc$^-$, and F1M1-Fc$^+$ antibodies in denaturing conditions. F1M1, F1M1-Fc$^-$, and F1M1-Fc$^+$ (3.5µg) were analyzed on 15% SDS PAGE followed by Coomassie staining.

(B) Immunoprecipitation of pro-cath-D by F1M1, F1M1-Fc$^-$ and F1M1-Fc$^+$ at acidic pH. Conditioned medium of MDA-MB-231 cells was immunoprecipitated with F1M1, F1M1-Fc$^-$, or F1M1-Fc$^+$ (2.5µg) at different pH values (7.5, 6.5, 6.0 and 5.5). Cath-D was detected by immunoblotting using an anti-cath-D (H-75) antibody. *Mr*, relative molecular mass (kDa).

Online supplemental figure 4. ADCC induction with the anti-cath-D F1M1-Fc$^+$, F1M1 and F1M1-Fc$^-$ antibodies in the presence of primary human NK cells

(A) Characterization of primary human NK 4928 V/F cells. Primary human NK cells from the 4928 V/F donor were immunophenotyped at day 17 of expansion. Shown are the percentages of CD3$^-$ CD19$^-$ negative cells (left panel) and of CD3$^-$ CD19$^-$ CD56$^+$ cells (middle panel), and also CD16a$^+$ expression in CD3$^-$ CD19$^-$ CD56$^+$ cells (right panel).

(B) ADCC activity against MDA-MB-231 cells in the presence of human primary NK cells in response to F1M1-Fc$^-$, F1M1 or F1M1-Fc$^+$. MDA-MB-231 cells (target) were pre-incubated with F1M1-Fc$^-$, F1M1, F1M1-Fc$^+$, or with cetuximab (Cetux; positive control) at 100 µg/ml (666 nM) at 37°C for 30 min, followed by incubation with primary human NK cells (human NK V/F; hCD16a V/F allotype; effector) at an effector:target ratio of 3:1 for 24h. ADCC of MDA-MB-231 cells was measured as described in Figure **2C.** ****P <0.0001 for F1M1-Fc$^+$ versus F1M1, ****P <0.0001 for F1M1-Fc$^+$ versus F1M1-Fc$^-$, *P=0.046 for F1M1 versus F1M1-Fc$^-$ (one-way ANOVA).

(C) Characterization of primary human NK 4924 V/V cells. Primary human NK cells from the 4924 V/V donor were immunophenotyped at day 17 of expansion as described in **(A).**

(D) Blocking cath-D binding to M6P receptors in MDA-MB-231 cells affects F1M1-Fc$^+$-induced ADCC in the presence of human primary NK cells. MDA-MB-231 cells (target) were pre-incubated with M6P (10 mM) or G6P (10 mM) for 24h. Then, ADCC was evaluated in the presence of primary

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human NK cells (human NK V/V; hCD16a V/V allotype; effector) at an effector:target ratio of 3:1 after incubation with F1M1-Fc^{+} at 100µg/ml (666 nM) and M6P (10 mM) or G6P (10 mM), as described in **figure 2F**. Cetuximab (Cetux), positive control (100µg/ml). F1M1-Fc^{-}, negative control (100µg/ml). ****P <0.0001 for F1M1-Fc^{+} versus F1M1-Fc^{+} + M6P, ****P <0.0001 for F1M1-Fc^{+} + G6P versus F1M1-Fc^{+} + M6P (one-way ANOVA).

Online supplemental figure 5. Fc-mediated cytotoxicity of F1M1-Fc^{+} in spheroids by live imaging.

(A) Representative images of calcein-labeled MDA-MB-231 cell spheroids. MDA-MB-231 cells were labeled with calcein (2µM) and co-cultured or not with hCD16 158V-expressing NK92 cells. Representative images obtained using IncuCyte® after 20h of co-culture. Bars, 800 µm.

(B) Green fluorescence intensity measurement by live imaging. Green fluorescence intensity of calcein-labeled MDA-MB-231 cells was measured using IncuCyte® by live imaging at different time points of incubation with F1M1-Fc^{-}, F1M1-Fc^{+}, or cetuximab (Cetux, positive control) at 100µg/ml or buffer (Ctrl-, negative control) in the presence of hCD16V-expressing NK92 cells at a ratio of 20:1. Values are the mean ± SD (n=3 spheroids/group).

(C) Green fluorescence intensity at 24h. Values are the mean ± SD (n=3 spheroids/group). ***P=0.001 for F1M1-Fc^{+} versus Ctrl-, ***P=0.0004 for F1M1-Fc^{+} versus F1M1-Fc^{-}, P=0.7962 for F1M1-Fc^{-} versus Ctrl^{-}.

Online supplemental figure 6. Cath-D expression in MDA-MB-231 cell xenografts of mice treated with F1M1-Fc^{-}, F1M1, or F1M1-Fc^{+}

(A) Representative images of MDA-MB-231 cell xenograft sections showing cath-D expression

Cath-D was monitored in MDA-MB-231 cell xenografts from mice treated with F1M1-Fc^{-}, F1M1, or F1M1-Fc^{+} (from **figure 4B**) (n=9 for F1M1-Fc^{-}; n=8 for F1M1; n=5 for F1M1-Fc^{+}; n=9 for Ctrl) by IHC using an anti-cath-D (clone CTD19) antibody. Scale bar, 50 µm.

(B) Quantification of cath-D expression in MDA-MB-231 cell xenograft sections. (n=9 for F1M1-Fc^{-}; n=8 for F1M1; n=5 for F1M1-Fc^{+}; n=9 for Ctrl). P=0.8815, Kruskal-Wallis test.

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(C) Cath-D expression in MDA-MB-231 cell xenografts. Cath-D expression was determined in MDA-MB-231 cell xenografts from mice treated with F1M1-Fc$^-$, F1M1, or F1M1-Fc$^+$ (from **figure 4B**) (n=3 for F1M1-Fc$^-$; n=3 for F1M1; n=3 for F1M1-Fc$^+$; n=3 for Ctrl). Whole cytosols (10 µg proteins) were immunoblotted with the mouse monoclonal (#610801) (to detect mature cath-D) and rabbit polyclonal (H-75) (to detect pro-cath-D) anti-cath-D antibodies. HSC70 (clone B-6) was used as loading control. *Mr*, relative molecular mass (kDa).

Online supplemental figure 7. Percentage of living cells in MDA-MB-231 cell xenografts from mice treated with F1M1-Fc$^-$, F1M1, or F1M1-Fc$^+$ at day 48. The percentage of living cells from MDA-MB-231 cell xenografts from mice treated with F1M1-Fc$^-$, F1M1, or F1M1-Fc$^+$ at day 48 (from figure **4B**) was quantified by FACS (n=7 for F1M1-Fc$^-$; n=7 for F1M1; n=9 for F1M1-Fc$^+$; n=8 for Ctrl). P=0.6280 for all groups (Kruskal-Wallis), P= 0.4088 for F1M1-Fc$^+$ versus Ctrl, P= 0.3969 for F1M1 versus Ctrl, P= 0.2810 for F1M1-Fc$^-$ versus Ctrl, P=0.5859 for F1M1-Fc$^+$ versus F1M1-Fc$^-$, P=0.7365 for F1M1-Fc$^+$ versus F1M1, P>0.9999 for F1M1-Fc$^-$ versus F1M1 (Mann-Whitney t-test). Data are the mean ± SEM.

Online supplemental figure 8. Linear regression analysis of CD107a$^+$ NK cells or granzyme B$^+$ NK cells and volumes of MDA-MB-231 cell xenografts from mice treated with F1M1-Fc$^-$, F1M1, or F1M1-Fc$^+$.

(A) Linear regression analysis of CD107a$^+$ NK cells and tumor volumes. The percentage of CD107a$^+$ degranulating NK cells was quantified by FACS and expressed relative to all NKp46$^+$ NK cells. Tumor volumes are those from **figure 4B** (n=7 for F1M1-Fc$^-$; n=7 for F1M1; n=9 for F1M1-Fc$^+$; n=8 for Ctrl). R^2 =0.1703; *P=0.0211; n=31 mice (all treatment groups).

(B) Linear regression analysis of granzyme B$^+$ NK cells and tumor volumes. The percentage of GZMB$^+$ activated NK cells was quantified by FACS and expressed relative to all NKp46$^+$ NK cells. Tumor volumes are those from **figure 4B**. GZMB, granzyme B. (n=7 for F1M1-Fc$^-$; n=7 for F1M1; n=9 for F1M1-Fc$^+$; n=8 for Ctrl). R^2 =0.3085; **P=0.0012; n=31 mice (all treatment groups).

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Online supplemental figure 9. Quantification of granzyme B, perforin, and TNFα mRNA expression in MDA-MB-231 cell xenografts of mice treated with F1M1-Fc$^-$, F1M1, or F1M1-Fc$^+$.

(A) Quantification of granzyme B *(Gzmb)* mRNA expression. Total RNA was extracted from MDA-MB-231 cell xenografts from figure **4B** at treatment end and granzyme B expression level was analyzed by RT-qPCR. Relative fold change was normalized to *Rps9* expression and expressed as fold change relative to control. Data are the mean ± SEM (n=8 for F1M1-Fc$^-$; n=8 for F1M1; n=8 for F1M1-Fc$^+$; n=7 for Ctrl). *P*=0.1975 for all groups (Kruskal-Wallis). *P*= 0.3969 for F1M1-Fc$^+$ versus Ctrl, **P*=0.0207 for F1M1-Fc$^+$ versus F1M1-Fc$^-$, *P*=0.2345 for F1M1-Fc$^+$ versus F1M1, *P*=0.3823 for F1M1 versus F1M1-Fc$^-$ (Mann-Whitney t-test).

(B) Quantification of perforin (*Prf1*) mRNA expression, done as described in panel (**A**). *P*=0.0638 for all groups (Kruskal-Wallis). *P*= 0.2319 for F1M1-Fc$^+$ versus Ctrl, **P*= 0.0379 for F1M1-Fc$^+$ versus F1M1-Fc$^-$, **P*=0.0148 for F1M1-Fc$^+$ versus F1M1, *P*=0.7984 for F1M1 versus F1M1-Fc$^-$ (Mann-Whitney t-test).

(C) Quantification of TNFα (*Tnf*) mRNA expression, done as described in (**A**). ***P*=0.0038 for all groups (Kruskal-Wallis). ***P*= 0.0059 for F1M1-Fc$^+$ versus Ctrl, *P*= 0.0541 for F1M1 versus Ctrl, ***P*=0.0030 for F1M1-Fc$^+$ versus F1M1-Fc$^-$, **P*=0.0214 for F1M1 versus F1M1-Fc$^-$, *P*=0.7984 for F1M1 versus F1M1-Fc$^+$ (Mann-Whitney t-test).

Online supplemental figure 10. Depletion of NK cells with the anti- αGM1 antibody in nude mice.

(A) Treatment schedule. Mice were treated by ip injection of saline (control) or anti-asialo GM1 antibody (αGM1) (50µl) (n=4 by group) at day 0 and day 3. Peripheral blood was collected 6 days before the first injection, and 1 day after the second injection (day 4). Mice were sacrificed at day 7 after the first injection and blood samples and spleens were collected.

(B) Percentage of NK cells in peripheral blood before and after αGM1 treatment. The percentage of NK cells in peripheral blood samples of nude mice treated with αGM1 was quantified by FACS at day 1 and 4. NK cells were defined as CD45$^+$ CD3$^-$ Ly6G$^-$ B220$^-$ NKp46$^+$ cells (n=4).

(C) NK cell gating. NK cells were defined as described in **(B).** A representative gate from the same mouse before (day 6 before the first injection) (left panel) and at day 4 (right panel) after αGM1 treatment is shown.

(D) Immune cell populations in blood. The percentage of B cells, neutrophils and NK cells in blood samples of nude mice treated or not with αGM1 was quantified by FACS at day 4 (n=4 for both groups). Neutrophils were defined as $CD45^{+}$ $CD3^{-}$ $Ly6G^{+}$ cells. B cells were defined as $CD45^{+}$ $CD3^{-}$ $Ly6G^{-}$ $B220^{+}$ cells. NK cells were defined as $CD45^{+}$ $CD3^{-}$ $Ly6G^{-}$ $B220^{-}$ $NKp46^{+}$ cells.

(E) Immune cell populations in spleen. The percentage of B cells, neutrophils, NK cells, dendritic cells and macrophages from spleens of nude mice treated or not with αGM1 was quantified by FACS at day 7 (n=4 for both groups). B cells, neutrophils and NK cells are defined as described in **(D).** Dendritic cells were defined as $CD45^{+}$ $CD3^{-}$ $Ly6G^{-}$ $B220^{-}$ $NKp46^{-}$ $MHC\text{-}II^{+}$ $CD11c^{+}$ cells. Macrophages were defined as $CD45^{+}$ $CD3^{-}$ $Ly6G^{-}$ $B220^{-}$ $NKp46^{-}$ $MHC\text{-}II^{-}$ $CD11c^{-}CD11b^{+}$ $F4/80^{+}$ cells.

Online supplemental figure 11. Efficacy of NK cell depletion with the αGM1 antibody in MDA-MB-231 cell xenografted nude mice treated with F1M1-Fc^{+}.

(A) Depletion of NK cells in peripheral blood. The percentage of NK cells from peripheral blood of nude mice treated with F1M1-Fc^{+} or rituximab in the presence or absence of αGM1 was quantified by FACS at day 45 (n=7 for F1M1-Fc^{+}; n=6 for F1M-Fc^{+} + αGM1; n=8 for Ctrl; n=7 for Ctrl + αGM1). NK cells were defined as $CD45^{+}$ $CD3^{-}$ $Ly6G^{-}$ $B220^{-}$ $NKp46^{+}$ cells.

(B) Depletion of NK cells in spleen. The percentage of NK cells in the spleen of nude mice treated with F1M1-Fc^{+} or rituximab in the presence or absence of αGM1 was quantified by FACS at day 48 (n=8 for F1M1-Fc^{+}; n=8 for F1M-Fc^{+} + αGM1; n=7 for Ctrl; n=8 for Ctrl + αGM1). NK cells were defined as $CD45^{+}$ $CD3^{-}$ $Ly6G^{-}$ $B220^{-}$ $NKp46^{+}$ cells.

Online supplemental figure 12. Analysis of neutrophils and blood counts in MDA-MB-231 cell xenografted nude mice treated with F1M1-Fc^{+}.

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(A) Neutrophils in peripheral blood. The percentage of neutrophils in peripheral blood samples of nude mice treated with F1M1-Fc^+ or rituximab (control, Ctrl) was quantified by FACS at day 45 (n=7 for F1M1-Fc^+; n=8 for Ctrl). Neutrophils were defined as $CD45^+$ $CD3^-$ $Ly6G^+$ cells.

(B) Blood counts. Blood samples from mice treated with F1M1-Fc^+ or rituximab (Ctrl) were analyzed using the scil Vet abc Plus+ system at day 48 (scil Animal Care Co) (n=8 for F1M1-Fc^+; n=8 for Ctrl). White blood cells (top left panel), platelets (top right panel), red blood cells (bottom left panel) and hemoglobin (bottom right panel) were quantified.

Online supplemental figure 13. Effect of paclitaxel on *in vitro* and *in vivo* cancer cell growth and on cath-D expression in MDA-MB-231 cells.

(A) Concentration-response curve of paclitaxel in MDA-MB-231 cells. MDA-MB-231 cells were incubated or not with increasing concentrations of paclitaxel for 2 days and then, cell viability was evaluated with the MTT assay. Results are expressed as the percentage of cell viability inhibition relative to control (cells without treatment). Values are the mean ± SD (n=6). The IC_{50} value is shown. PTX, paclitaxel.

(B) Effect of paclitaxel on cath-D expression level. MDA-MB-231 cells in DMEM/2% FCS were incubated or not with 5 nM PTX for 48h. Conditioned medium (40 µl) and cell lysates (20µg) were analyzed by 13.5% SDS-PAGE and immunoblotting with anti-cath-D (#610801) (to detect cellular cath-D) and anti-cath-D (H-75) (to detect secreted pro-cath-D) antibodies. β-actin, loading control. *Mr*, relative molecular mass (kDa). PTX, paclitaxel.

(C) Dose-dependent effect of paclitaxel on the growth of MDA-MB-231 cell xenografts. MDA-MB-231 cells were subcutaneously injected in nude mice. When tumor volume reached 50 mm^3, mice were treated with PTX at different concentrations (1 mg/kg: n=3; 4 mg/kg: n=3; and 7 mg/kg: n=3), or NaCl (Ctrl; n=4) once per week for 55 days. Tumor volume (in mm^3) is shown as the mean ± SEM. P=0.209 for PTX (1 mg/kg) versus Ctrl, **P=0.007 for PTX (4 mg/kg) versus Ctrl, and ***P<0.001 for PTX (7mg/kg) versus Ctrl (mixed-effects multiple linear regression test). PTX, paclitaxel.

Online supplemental figure 14. Expression of androgen receptor (AR) and cath-D in SUM159 cells.

(A) Expression of AR and cath-D in SUM159 cells. Conditioned medium (40 µl) and cell lysates (20µg) from SUM159 cells were analyzed by 13.5% SDS-PAGE and immunoblotting with anti-AR (D6F11) (left panel), anti-cath-D (#610801) (to detect cellular cath-D), and anti-cath-D (H-75) (to detect secreted pro-cath-D) (right panel) antibodies. Tubulin, loading control. β-actin, loading control. *Mr*, relative molecular mass (kDa). AR, androgen receptor.

(B) Effect of enzalutamide on cath-D expression and secretion in SUM159 cells. SUM 159 cells were incubated with DMSO (Ctrl) or enzalutamide (20 µM; Enza) in the absence of FCS for 24h. Cell extracts (25µg proteins) and conditioned media (40 µl) were immunoblotted for cellular cath-D (#610801) and secreted cath-D (H-75) detection, respectively. β-actin, loading control. *Mr*, relative molecular mass (kDa).

(C) Effect of F1M1-Fc^{+} on AR expression. SUM 159 cells were incubated with DMSO (Ctrl) or F1M1-Fc^{+} (50 µg/ml=333 nM) in the absence of FCS for 24h. Cell extracts (25 µg proteins) were immunoblotted with an anti-AR antibody (D6F11). β-actin, loading control. 110K, 110-kDa. *Mr*, relative molecular mass (kDa).

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