Supplementary Methods

Cell lines, transfection and culture

Human *HER2*-amplified breast cancer BT474 cells (ATCC) were cultured in RPMI 1640 supplemented with 10% FBS (Atlanta Biologicals, Flowery Branch, GA, USA). Human *HER2*-amplified MDA-MB-361 cells that harbor an activating *PIK3CA* mutation (ATCC) were cultured in DMEM/F12 supplemented with 10% FBS. All cell lines were authenticated before use and after completion of the studies by IDEXX laboratories (North Grafton, MA). BT474 and MDA-MB-361 cells were authenticated in June 2013 and October 2013 respectively. The genetic profiles for all samples were identical to the genetic profile reported for each cell line. Authentication was also confirmed at the end of the studies in February 2015. Both samples were identical to the genetic profiles reported for each cell line. BT474 and MDA-MB-361 cells were transduced with an expression cassette encoding Gaussia luciferase (Gluc) and green fluorescent protein (GFP), as previously described[1,2]. GFP positive cells were sorted with a FACS Aria cell sorter (BD Biosciences, San Jose, CA, USA). BT474-Gluc and MDA-MB361-Gluc cells were cultured in RPMI 1640 supplemented with 10% FBS (Atlanta Biologicals, Flowery Branch, GA, USA) and DMEM/F12 supplemented with 10% FBS, respectively.

Tumor growth

Tumor growth was determined by measuring the Gluc activity in the blood as previously described[3]. Blood Gluc activity was measured with a Promega Glomax 96 microplate luminometer (Fisher Scientific, Waltham, MA) To assess

tumor size, *in vivo* imaging was performed through a cranial window using a small animal ultrasonography device (Vevo 2100, FujiFilm VisualSonics Inc., Toronto, Canada).

***In Vitro* Cell Viability Assay**

3,000 BT474-Gluc cells were seeded in a 96-well plate and allowed to adhere overnight. Cells were treated with trastuzumab or T-DM1 at different concentrations. 72h after treatment cell growth was measured using the CellTiter-Glo Luminescent Cell Viability Assay according to the manufacturer's protocol (Promega, Madison, WI).

Western Blotting

Tumor tissues were lysated in 1% Nonidet P-40 lysis buffer (15mM Tris, 10mM EDTA, 10mM EGTA) with protease and phosphatase inhibitors, and incubated at 4°C for 15min. Lysates were centrifuged at 14,000rpm (~18,000*g*) for 10min at 4°C. Approximately 40 µg of protein from tumor lysates were loaded on SDS/PAGE, separated by electrophoresis and transferred to nitrocellulose membranes. Non-specific binding was blocked by 5% non-fat milk for 1h. Membranes were incubated overnight at 4°C with primary antibodies and were followed by anti-rabbit HRP secondary (Cell Signaling 1:1000). The membranes were developed with low sensitivity Amersham ECL Western Blotting Analysis System (GE Healthcare, MMP-14) or high- sensitivity Pierce ECL 2 Western Blotting Substrate (Thermo Scientific, iNOS) and exposed to film. Membranes were probed for β-actin (Sigma, A5441, 1:5000) as loading control.

FACS analysis of immune cells

Mice (N=4) were anesthetized and perfused with PBS to avoid contamination from circulating immune cells. Tumors were harvested in cold HBSS (Corning Cellgro, Manassas, VA) and digested at 37°C for 15min in complete DMEM (ATCC) containing collagenase type 1A (1.5mg/ml, Sigma) and hyaluronidase (1.5mg/ml, Sigma). The cell suspension was filtered through a 70µm cell strainer, washed and re-suspended in flow buffer (PBS containing 1% fetal calf serum). One-to-two million cells were incubated with rat anti-mouse CD16/32 (eBioscience, San Diego, CA, Clone 93) to block non-specific staining. The cells were stained with appropriate antibodies from BD Pharmingen: anti-CD45 (30-F11, PE/CY7), anti-F4/80 (MB8, PE), anti-CD11b (M1/70, APC/CY7), anti-Gr1 (R86-8C5, APC), anti-CD19 (ID3, APC) and anti-NK1.1 (PK136, PE). After washing, cells were filtered through a 40µm cell strainer and 7-AAD (7-Aminoactinomycin D, eBioscience) was added to eliminate dead cells. The data was acquired on an LSR-II flow cytometer and assessed with FACSDiva software (BD Biosciences).

EdU proliferation assay

Tumor bearing mice (N=5-6) were injected 2h prior to sacrifice i.p. with EdU labelling agent (Click-iT EdU Alexa Fluor 647 Imaging Kit, catalog no. C10340, Invitrogen, Woburn, MA). Paraffin embedded tissue sections were mounted onto glass slides and staining of EdU positive nuclei was performed according to manufacturer’s instructions.

Apoptosis staining and analysis

Paraffin embedded tissue (N=5-6), fixed 3h after IgG, trastuzumab or T-DM1 injection, was sectioned and stained for cleaved caspase 3 by IHC (Cell Signaling, Rabbit mAb Cat#9664, 1:50) or DNA fragmentation by direct TUNEL method (ApopTag® Peroxidase In Situ Apoptosis Detection Kit, #S7100, Millipore). Staining was performed according to the manufacturer instructions. Sections were counterstained with DAPI. Samples were imaged by confocal microscopy and analyzed using ImageJ software (version 1.48u, U. S. National Institutes of Health, Bethesda, Maryland, USA). The area of necrosis was measured with Image J on merged 20x magnification images of H & E stained tumor sections.

Immunostaining for vessel penetration

Brains were fixed 3h after drug injection (N=4). OCT embedded tissue was sectioned (10μm) and immunostained for CD31 (Millipore, MAB1398Z, mouse mAb, 1:200) and human IgG (Invitrogen, Cat# A-21091, 1:100). Slides were imaged by confocal microscopy (Olympus America, Center Valley, PA). The penetration distance of antibodies from vessels was characterized as previously described[4]. Briefly, vessels were segmented based on intensity and minimum size thresholds semi-automatically with a custom MATLAB algorithm. The presence of IgG was evaluated at steps of 2-μm up to 40-μm from the vessel perimeter. The profile of signal decay was fit to an exponential decay function to determine the exponential decay constant. This constant, which is the characteristic penetration length, was compared between treatment groups.

Immunohistochemistry.

Paraffin-embedded primary breast cancer and matched brain metastases were cut into 4-µm thick serial sections, dewaxed with xylene, rehydrated with an ethanol gradient and washed with distilled water. Antigen retrieval was performed by submerging sections in 0.5 M EDTA antigenic retrieval buffer or citrate antigen retrieval buffer (pH 6), and microwaving for 20 m. Sections were then treated with 3% hydrogen peroxide solution to quench endogenous peroxidase activity. Sections were washed with TBST, blocked with 5% normal goat serum and then incubated overnight at 4°C with anti-HER2 (CST, 1:200 dilution). The following day, anti-rabbit secondary antibody polymer (Dako) was added and incubated for 30 m at room temperature. Sections were then washed with TBST to remove any unbound antibody and developed with DAB (Dako). Next, the sections were counterstained with hematoxylin and blued in Scott's tap water substitute (Electron Microscopy Sciences). Finally, the samples were dehydrated and made transparent, sealed and examined.

Optical frequency domain imaging and perfused vessel analysis

In vivo imaging of perfused vessels and analysis of the vascular fraction, vessel density and diameter was performed using the optical frequency domain imaging (OFDI) as previously described[5] (Supplementary Movie S1). 8-week-old, female nude mice (N=4) bearing cranial windows and BT474-Gluc cells were randomized in three treatment groups (control IgG, trastuzumab or T-DM1 15mg/kg) when tumors reached a size of about 5-6 mm^3. OFDI assessment was performed twice per week for a period of two weeks. The construction, design

and algorithms used to derive OFDI images are described in detail by Vakoc et al.[6].

One micron toluidine blue sections and transmission electron microscopy

Tissue samples were placed into electron microscopy fixative (2.5% glutaraldehyde, 2.0% paraformaldehyde. 0.025% calcium chloride in a 0.1M sodium cacodylate buffer, pH 7.4) immediately after excision and allowed to fix for 3 hours at room temperature. The fixative was replaced with cacodylate buffer and the tissues stored at 4ºC until further processing in a Leica Lynx™ automatic tissue processor. One micron thick sections were cut using glass knives and a Sorvall MT-1 (Dupont) ultramicrotome, and floated on water droplets on glass slides. The slides were dried in a humidity chamber on a warm hot plate. Toluidine blue stain (0.5% toluidine blue in aqueous 0.5% sodium borate) was pipetted over the sections and placed onto the hot plate until a slight gold rim could be seen around the stain droplet. The sections were rinsed in a stream of distilled water, dried, cover slipped and examined by light microscopy.

Representative tissues were chosen, and the blocks trimmed accordingly. Thin sections were cut using a diamond knife and an LKB 2088 ultramicrotome and placed on copper grids. Sections were stained with lead citrate and examined in a FEI Morgagni transmission electron microscope. Images were captured with a AMT (Advanced Microscopy Techniques) 2K digital CCD camera.

Gene microarray analysis

BT474-Gluc brain tumors were snap-frozen 48h post treatment with trastuzumab or T-DM1 (15mg/kg), then cryopulverized and immediately lysed in Buffer RLT

supplemented with beta-mercaptoethanol. RNA was extracted using the RNeasy Mini kit (Qiagen Valencia, CA) with on-column DNaseI digestion. After initial quality control, RNA was subjected to in-solution DNaseI treatment and purified using the RNeasy MinElute Kit (Qiagen). Final determination of RNA quality was performed using the Agilent Bioanalyzer. Labeling was performed using the Nugen Pico WTA & Biotin Module Kit. Biotin-labeled samples were hybridized to GeneChip® Human Gene 2.0 ST Arrays (PC043) according to the manufacturer's instructions. Raw data from the gene arrays arrays were normalized on the transcript level, using the robust multi-array average summary measure[7] implemented in Bioconductor's oligo package[8] and are available at Gene Expression Omnibus under accession number GSE69042. We used Significance Analysis of Microarrays[9] and Bioconductor's limma package[10] to test for differential gene expression, considering the main array content only. To test our hypothesis that TDM-1 induces mitotic catastrophe, we assembled a custom gene set, associated with mitotic catastrophe (spindle assembly checkpoint, centrosomes and G2/M arrest). Gene Set Enrichment analysis was performed using gene set permutation and signal-to-noise ratio as a ranking metric.

References

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2. Wurdinger T, Badr C, Pike L, et al. A secreted luciferase for ex vivo monitoring of in vivo processes. *Nat Methods*. 2008;5(12):171-173.
3. Kodack DP, Chung E, Yamashita H, et al. Combined targeting of HER2 and VEGFR2 for effective treatment of HER2-amplified breast cancer brain metastases. *Proc Natl Acad Sci U S A*. 2012;109(45):E3119-127.
4. Tong RT, Boucher Y, Kozin SV, et al. Vascular normalization by vascular endothelial growth factor receptor 2 blockade induces a pressure gradient across the vasculature and improves drug penetration in tumors. *Cancer Res.* 2004;64(11):3731-3736.
5. Ager EI, Kozin SV, Kirkpatrick ND, et al. Blockade of MMP14 activity in murine breast carcinomas: implications for macrophages, vessels, and radiotherapy. *J Natl Cancer Inst*. 2015;107(4):pii:djv017.
6. Vakoc BJ, Lanning RM, Tyrrell JA, et al. Three-dimensional microscopy of the tumor microenvironment in vivo using optical frequency domain imaging. *Nat Med.* 2009;15(10):1219-1223.
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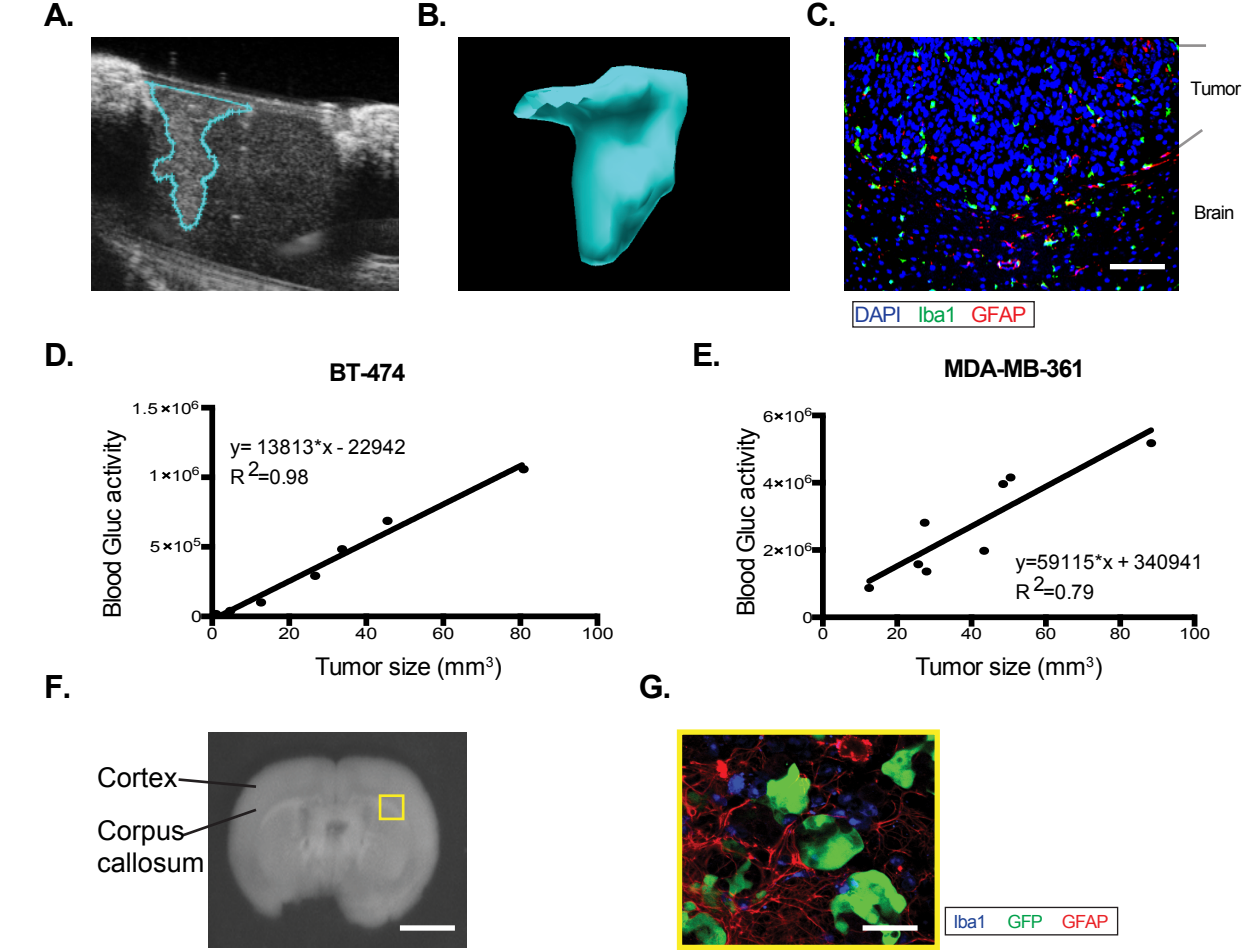
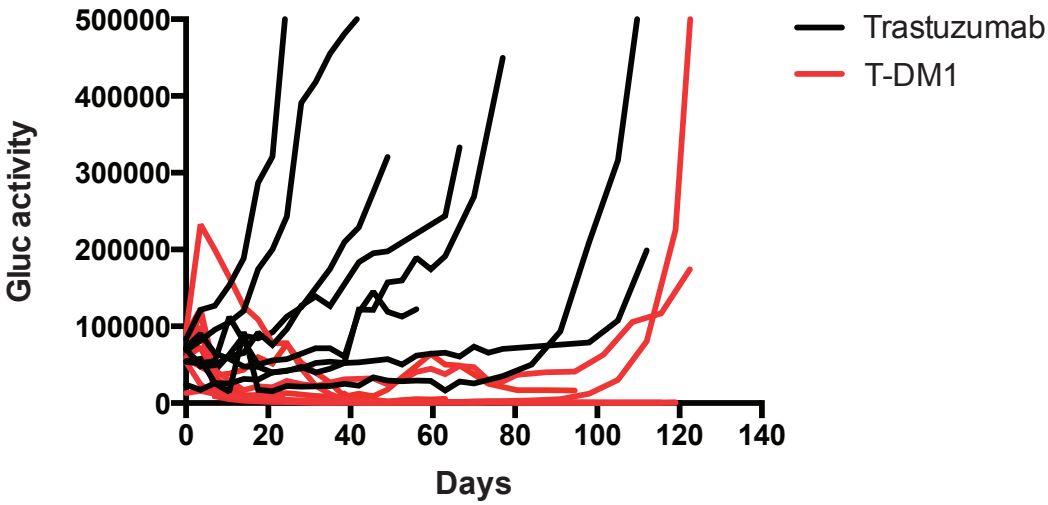
Supplementary Figure 1. Preclinical model: Blood Gaussia luciferase activity correlates with breast cancer brain metastasis volume, and the brain metastasis microenvironment can be replicated in whole-brain organotypic slice cultures. *In vivo* imaging (A) and tumor volume determination (B) using small animal ultrasonography through a cranial window. (C) Representative IF image of the model showing a peri- and intratumoral enrichment of activated astrocytes (GFAP) and microglia (Iba1), (scale bar: 100µm). The correlation between blood Gluc activity and tumor volume, as measured by ultrasonography-based imaging for (D) BT474-Gluc and (E) MDA-MB-361-Gluc tumors. Gluc activity is measured as Relative Light Units per sec (RLU/s). (F-G) Representative images of untreated organotypic brain slice culture of BT474-Gluc cells. (F) Bright field image of an organotypic brain slice culture. The yellow square indicates the site of implantation of tumor cells, scale bar: 0.5mm. (G) Confocal microscopy imaging of a brain slice organotypic culture. (Green) GFP positive BT474-Gluc tumor cells, (Red) GFAP positive astrocytes, (Blue) Iba1 positive microglia. Scale bar: 50µm.

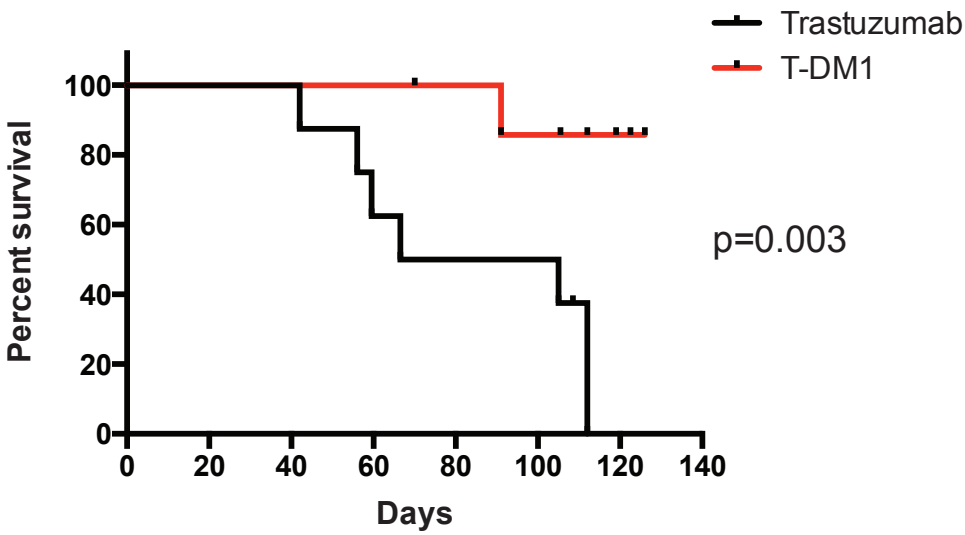

A.
B.
C.
Tumor
Brain
DAPI Iba1 GFAP
D.
BT-474
Blood Gluc activity
1.5×10^6
1×10^6
5×10^5
0
y= 13813*x - 22942
R 2=0.98
0
20
40
60
80
100
Tumor size (mm3)
E.
MDA-MB-361
Blood Gluc activity
6×10^6
4×10^6
2×10^6
0
y=59115*x + 340941
R 2=0.79
0
20
40
60
80
100
Tumor size (mm3)
F.
Cortex
Corpus callosum
G.
Iba1 GFP GFAP

Supplementary Figure 2. Differential response of BT474-Gluc brain metastases to trastuzumab and T-DM1 after intra-carotid injection of tumor cells. (A) Growth kinetics of BT474-Gluc brain lesions during treatment with trastuzumab or T-DM1 (15mg/kg). Blood Gluc activity (Relative Light Units per sec, RLU/s) was measured over time. (B) Survival analysis of mice from (A) (N=8).

A.

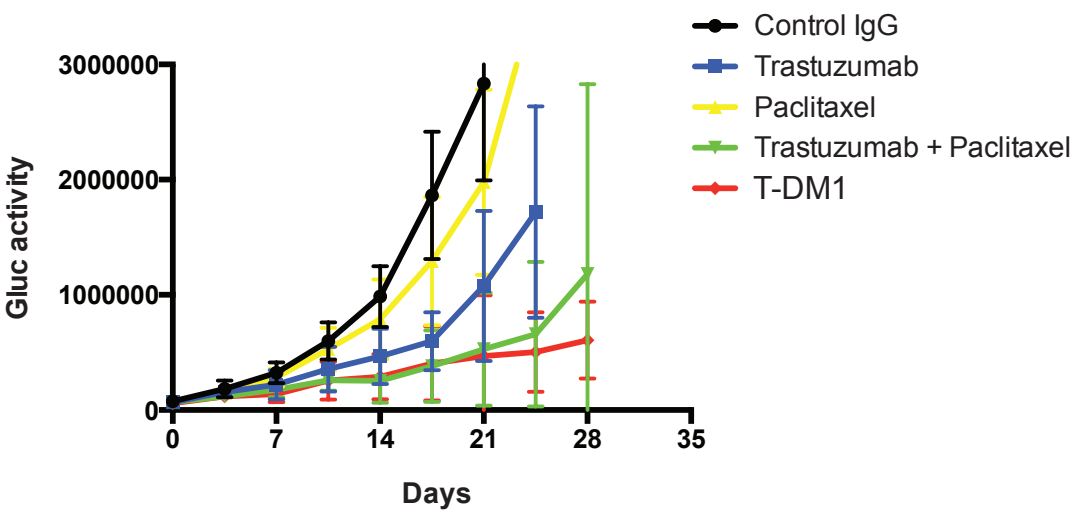

Gluc activity
500000
400000
300000
200000
100000
0
0
20
40
60
80
100
120
140
Days
Trastuzumab
T-DM1

B.

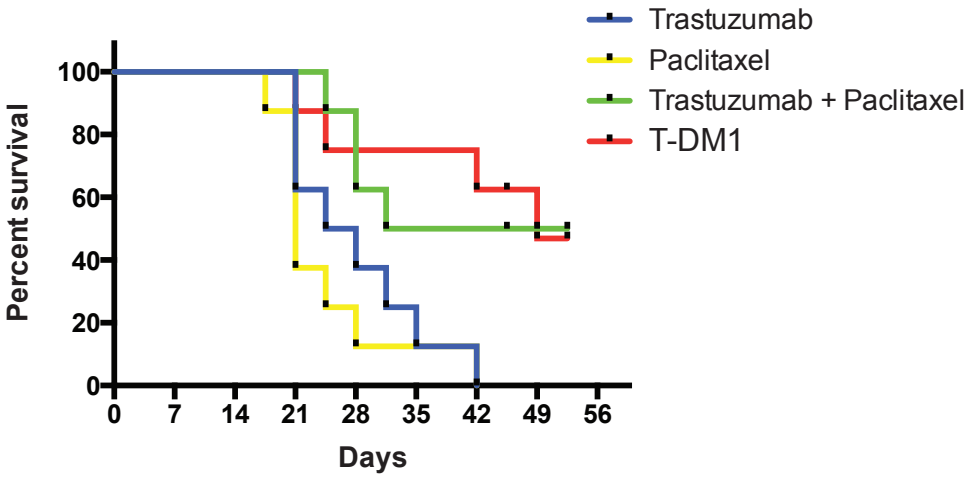

Percent survival
100
80
60
40
20
0
0
20
40
60
80
100
120
140
Days
Trastuzumab
T-DM1
p=0.003

Supplementary Figure 3. Efficacy of T-DM1 compared to trastuzumab plus paclitaxel against BT474-Gluc brain metastases. (A) BT474-Gluc tumors growing in the brain were treated with trastuzumab (6mg/kg), paclitaxel (6.5mg/kg), trastuzumab plus paclitaxel or T-DM1 (3.6mg/kg) and blood Gluc activity (Relative Light Units per sec, RLU/s) was measured over time. Nonspecific human IgG was used as control at 6mg/kg (N=8). (B) Survival analysis of mice from (A). (C) Tumor growth of BT474-Gluc brain metastases that progressed under treatment with trastuzumab (6mg/kg) plus paclitaxel (6.5mg/kg). Black: all mice were treated with trastuzumab plus paclitaxel until the endpoint. Red: treatment was switched on day 14 from trastuzumab plus paclitaxel to T-DM1 (3.6mg/kg) (N=4). (D) Survival analysis from (C).

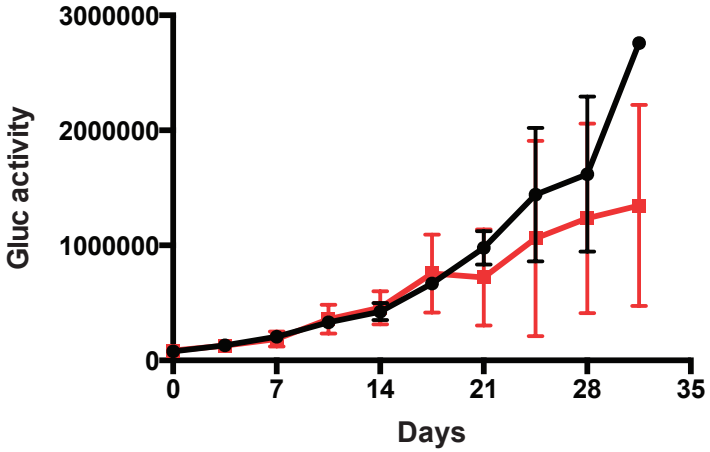
A.



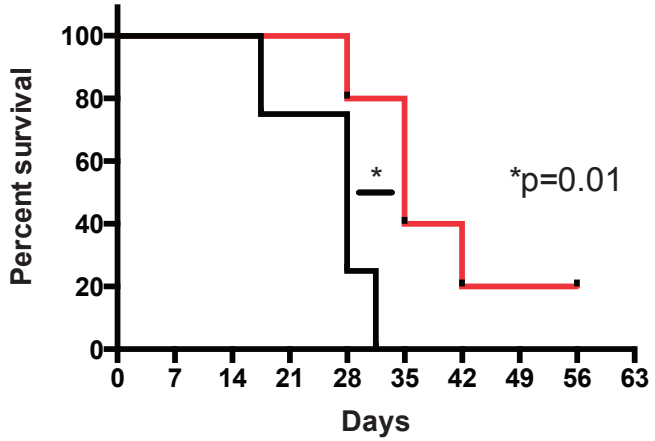
B.



C.



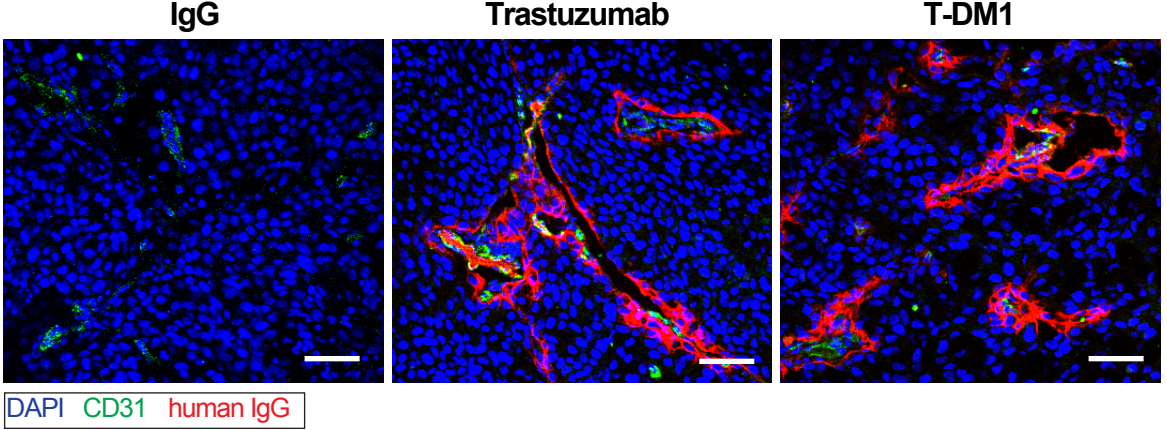
D.



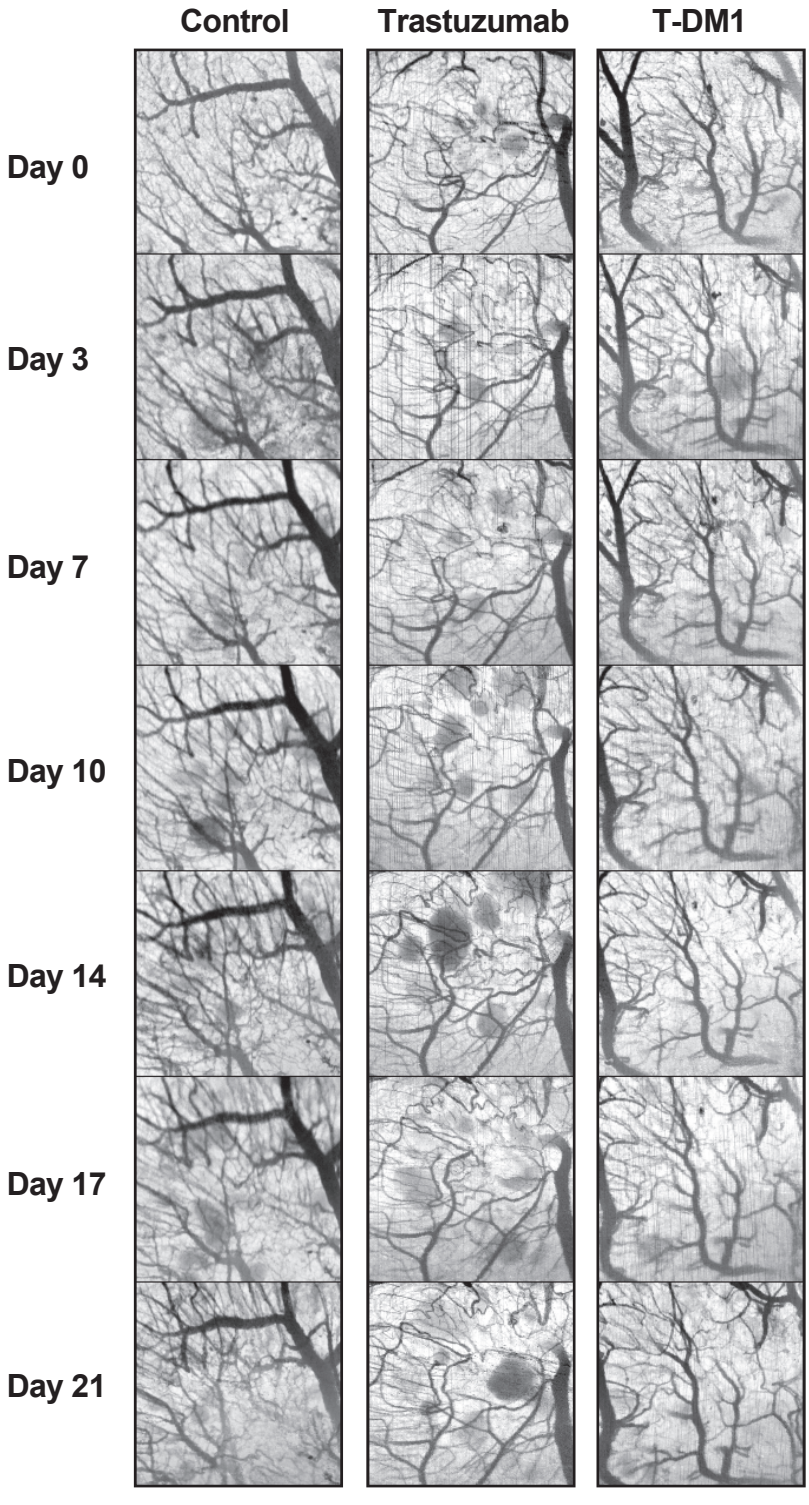
Trastuzumab + Paclitaxel

Trastuzumab + Paclitaxel followed by T-DM1

Supplementary Figure 4. Early distribution of T-DM1 and trastuzumab in HER2-positive breast cancer brain lesions. Immunofluoresence for human IgG1 and CD31 from BT474-Gluc brain metastases, collected 2h after a single treatment with control IgG, trastuzumab or T-DM1 (15mg/kg). Representative images, scale bars: 100μm.

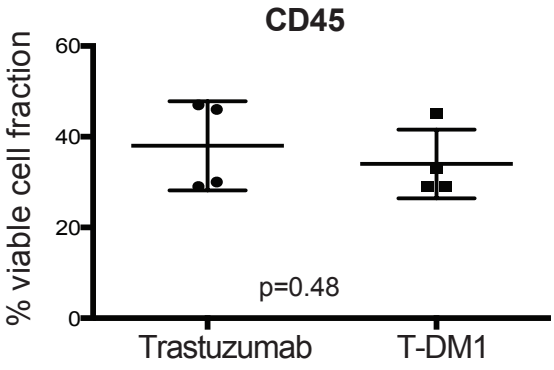

IgG
Trastuzumab
T-DM1
DAPI CD31 human IgG

Supplementary Figure 5. Vascular morphology parameters as determined with optical frequency domain imaging (OFDI). Representative OFDI images of the vasculature of BT474-Gluc brain metastases treated with control IgG, trastuzumab or T-DM1 (15mg/kg) over time.

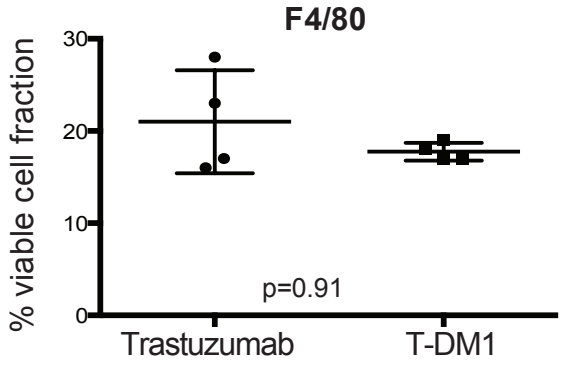

Control
Trastuzumab
T-DM1
Day 0
Day 3
Day 7
Day 10
Day 14
Day 17
Day 21

Supplementary Figure 6. The efficacy of T-DM1 is independent of immune cell infiltration. (A-E) FACS analysis for various myeloid and lymphoid immune cell populations from BT474-Gluc brain metastases after 4 weekly treatments with trastuzumab or T-DM1 (15mg/kg) (N=4).

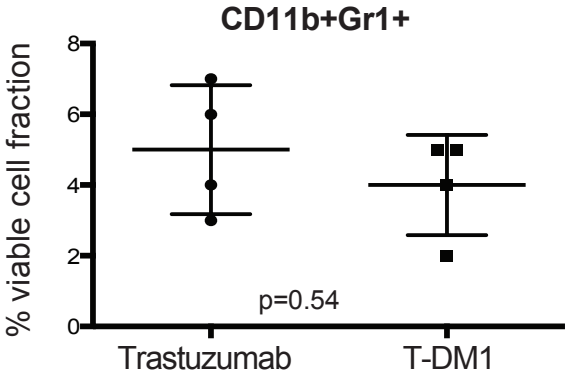
A.


CD45
% viable cell fraction
60
40
20
0
p=0.48
Trastuzumab
T-DM1

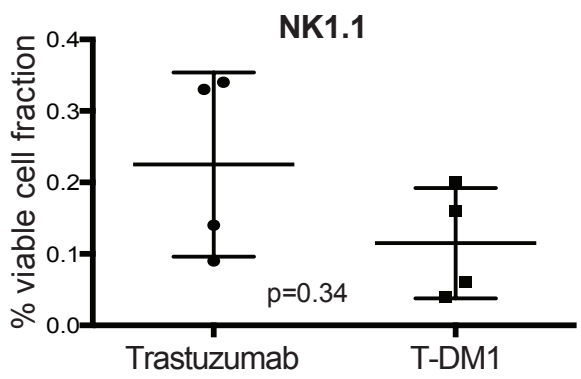
B.


F4/80
% viable cell fraction
30
20
10
0
p=0.91
Trastuzumab
T-DM1

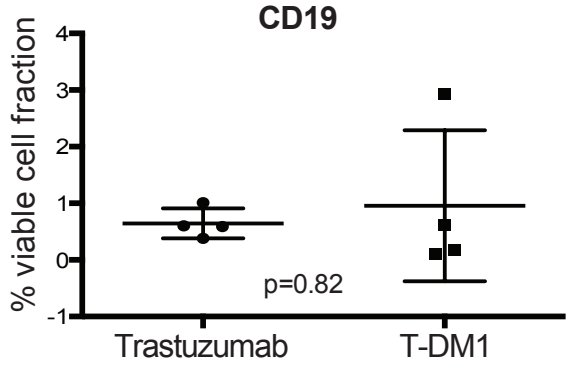
C.


CD11b+Gr1+
% viable cell fraction
8
6
4
2
0
p=0.54
Trastuzumab
T-DM1

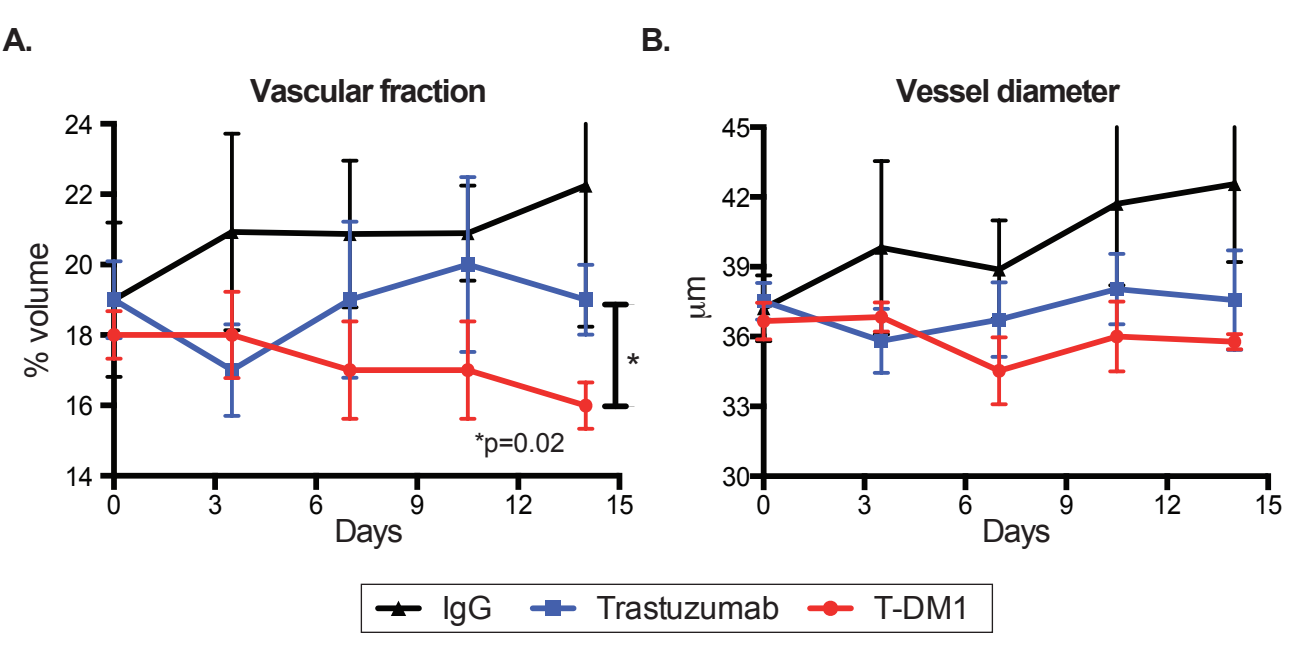
D.


NK1.1
% viable cell fraction
0.4
0.3
0.2
0.1
0.0
p=0.34
Trastuzumab
T-DM1

E.


CD19
% viable cell fraction
4
3
2
1
0
-1
p=0.82
Trastuzumab
T-DM1

Supplementary Figure 7. T-DM1 affects the vasculature of HER2-positive tumors growing in the brain microenvironment. (A) Vascular fraction and (B) vessel diameter as measured by OFDI in BT474-Gluc brain metastases during weekly treatment with trastuzumab, T-DM1 or control (N=4).


A.
Vascular fraction
% volume
24
22
20
18
16
14
0
3
6
9
12
15
Days
*
*p=0.02
B.
Vessel diameter
µm
45
42
39
36
33
30
0
3
6
9
12
15
Days
IgG
Trastuzumab
T-DM1

Supplemetary Movie S1. Representative OFDI-based imaging of the brain vasculature of a mouse bearing a BT474-Gluc tumor in the left frontal lobe over time.