Siglec-15/Sialic Acid Axis as a Central Glyco-Immune Checkpoint in Breast Cancer Bone Metastasis

Yixian Wang[1,§], Zhan Xu[2,§], Kuan-Lin Wu[1], Liqun Yu[2], Chenhang Wang[1], Haoxue Ding[1], Yang Gao[2], Han Sun[1], Yi-Hsuan Wu[2], Meng Xia[1], Yuda Chen[1], Han Xiao[1, 3, 4]

[1]Department of Chemistry, Rice University, 6100 Main Street, Houston, Texas, 77005
[2]Department of Molecular and Cellular Biology, Baylor College of Medicine, 1 Baylor Plaza, Houston, Texas, 77030
[3]Department of Biosciences, Rice University, 6100 Main Street, Houston, Texas, 77005
[4]Department of Bioengineering, Rice University, 6100 Main Street, Houston, Texas, 77005

[§] These authors contributed equally

* To whom correspondence should be addressed. Telephone: (731)-348-8239. Email: han.xiao@rice.edu

Supplementary contents

Supplementary Methods 3
Mice and cell lines 3
Lentivirus transduction of tumor cells 3
RT-PCR analysis 3
Flow cytometry 4
Quantification of IFN-γ levels in BICA 4
Supplementary Figures 5
Supplementary Figure. 1 Immunofluorescence staining of the patient sample (MDACC-35). 5
Supplementary Figure. 2 Immunofluorescence staining of the patient sample (MDACC-16). 6
Supplementary Figure. 3 Representative immunofluorescence staining of metastatic bone niche from LLC1 bone metastases bearing mouse 7
Supplementary Figure. 4 Representative immunofluorescence staining of metastatic bone niche from LLC1 bone metastases bearing mouse 8
Supplementary Figure. 5 Representative immunofluorescence staining of metastatic bone niche from LLC1 bone metastases bearing mouse 9
Supplementary Figure. 6 MS-Spec characterization of of 5G12. 10
Supplementary Figure. 7 SDS-PAGE gel of 5G12 11
Supplementary Figure. 8 Flow cytometric analysis of expression levels of Siglec-15 ligand mouse cell lines 12
Supplementary Figure. 9 Flow cytometric analysis of expression levels of Siglec-15 ligand human cell lines 13
Supplementary Figure. 10 Flow cytometric analysis of expression levels of Siglec-15 receptor mouse cell line 14
Supplementary Figure. 11 Flow cytometric analysis of expression levels of Siglec-15 receptor human cell lines 15
Supplementary Figure. 12 Representative gating strategy of flow cytometries 16
Supplementary Figure. 13 Expression levels of Siglec-15 ligand on common human and mouse cell lines. 17
Supplementary Figure. 14 Representative immunofluorescence staining of BIC 18
Supplementary Figure. 15 Representative pictures of 4T1 BICA lesion 19
Supplementary Figure. 16 Representative gating strategy of T cell markers. 20
Supplementary Figure. 17 Evaluation of cytotoxicity of 5G12 on EO771 and MDA-MB-231 21
Supplementary Figure. 18 Representative microCT slices from each treatment group. 22
Supplementary Figure. 19 Bioluminescence imaging of EMT-6 mode 23
Supplementary Figure. 20 Tumor growth curve of EMT-6 model 24
Supplementary Figure. 21 Body weight change of EMT-6 mode 25
Supplementary Figure. 22 Bioluminescence imaging of depletion study 26

Supplementary Methods

Mice and cell lines

C57BL/6 or Balb/c mice at ~6–8 weeks old were purchased from Jackson Laboratory. All mouse protocols were in accordance with NIH guidelines and were approved by the Institutional Animal Care and Use Committee of Rice University.
BT20, MDA-MB-231, HCC1954, MDA-MB-453, ZR-75, MDA-MB-361, MCF-7, BT474, SK-BR-3, MDA-MB-468, 4T1, 4T1-luc2, 4T1.2, EO771, PY8119, AT-3, LLC1, EMT-6, RAW264.7 cell lines were purchased from American Type Culture Collection and cultured according to the American Type Culture Collection instructions. Firefly luciferase and GFP-labeled EO771 and EMT-6 cell lines were generated as described below.

Lentivirus transduction of tumor cells

The Fluc-GFP fusion gene was cloned into expression vector pwpt-GFP (Addgene #12255) in place of the GFP gene in the backbone. Using Xtreme Gene HP DNA Transfection Reagent (Version 08, Roche), the pwpt-Fluc-GFP vector was transfected into 293T cells with pMD2.G(Addgene #12259) and psPAX2 (Addgene #12260) to package lentivirus. Lentiviralstocks were filtered by 0.45mm polyethersulfone membranes (VWR 28145–505). Cancer cells were incubated with Fluc-GFP lentivirus and 4mg/ml polybrene for 8 h. After a 72 h culture period, successfully labelled cells were isolated by FACS sorting of GFP-positive cells.

RT-PCR analysis

Total RNA was extracted from osteoclasts using TRIzol(T9424,Sigma) and was reverse-transcribed into cDNA using the PrimeScript RT master kit (RR036A, TakaRa Bio Inc., Shiga, Japan). Real-time reverse transcription PCR (RT-PCR) was per-formed using the Bio-Rad CFX96 system. The primer sets used were as follows: Oscar, 5’-CCTAGCCTCATACCCCCAG-3’ and 5’-CGTTGATCCCAGGA-GTCACAA-3’; Dc Stamp, 5’-GGGGACTTATGTGTTTCC-ACG-3’ and 5’-ACAAAGCAACAGACTCCCAAAT-3’; Ctsk,5’-GAAGAAGACTCACCAGAAGCAG-3’ and 5’-TCCAGG-TTATGGGCAGAGATT-3’; NFATc1, 5’-GACCCGGAGTT-CGACTTCG-3’ and 5’-TGACACTAGGGGACACATAA-CTG-3’; Siglec-15 5’-CAGCACCGAGATGTTGACGA-3’ and 5’-ACGATCGCTATGAGAGTCGC-3’; GAPDH 5’-AGGTCGGTGTGAACGGATTTG-3’ and 5’-TGTAGACCATGTAGTTGAGGTCA-3’.

Flow cytometry

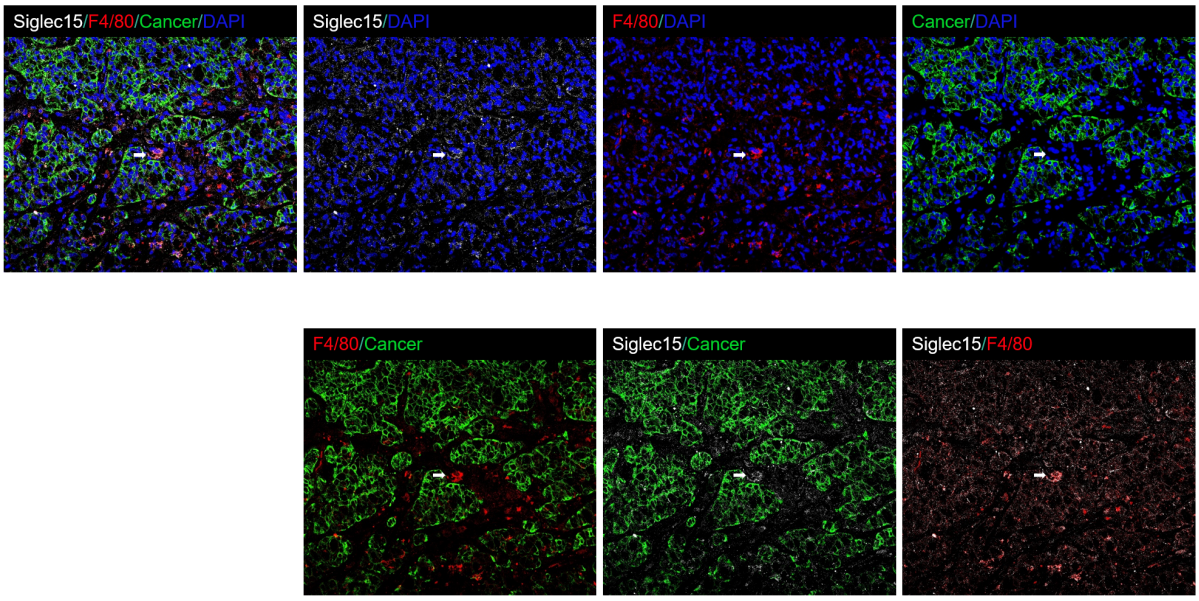
For detection of Siglec-15 ligand in human cancer cell lines, immune complexes were prepared by mixing 1 μg/mL of recombinant human Siglec-15 Fc chimera protein (R&D, 9227-SL-050) and 1 μg/mL of Alexa Fluor® 647 AffiniPure Goat Anti-Human IgG (Jackson ImmunoResearch Inc., 109-605-098) in 100 μL phosphate-buffered saline (PBS) containing 1% BSA for 1 h on ice. 1*10^6 cancer cells were suspended in the Siglec-15-Fc immune complexes and after incubation on ice for 30 min, the non-bound immune-complexes were washed off by centrifugation and cell pellets resuspended in 500 μL of 1% BSA PBS buffer. Alexa Fluor® 647 AffiniPure Goat Anti-Human IgG was used as a negative control.
For detection of Siglec-15 receptor in human cancer cell lines, 1*10^6 cells were incubated in 1% BSA PBS buffer 10 min at room temperature. Concentrated 5G12 stock was added to the buffer to give a 10 ug/ml 5G12 buffer. Unbound antibodies were washed off by centrifugation, and cell pellets were resuspended in 10 ug/ml Alexa Fluor® 647 AffiniPure Goat Anti-Human IgG antibody in 100 μL of 1% BSA PBS buffer and incubated for 30 min on ice. Cells were centrifuged and resuspended in 500 μL of 1% BSA PBS buffer. Alexa Fluor® 647 AffiniPure Goat Anti-Human IgG antibody was used as the negative control.
For detection of Siglec-15 ligand in mouse cancer cell lines, immune complexes were prepared by mixing 1 μg/mL of recombinant mouse Siglec-15 Fc chimera protein (R&D, 10101-SL-050) and 1 μg/mL of Alexa Fluor® 647 AffiniPure Goat Anti-Mouse IgG (Jackson ImmunoResearch Inc., 115-605-003) in 100 μL phosphate-buffered saline (PBS) containing 1% BSA for 1 h on ice. 1*10^6 cancer cells were suspended in the Siglec-15-Fc immune complexes and after incubation on ice for 30 min, the non-bound immune-complexes were washed off by centrifugation and cell pellets resuspended in 500 μL of 1% BSA PBS buffer. Alexa Fluor® 647 AffiniPure Goat Anti-Mouse IgG was used as a negative control.
For detection of Siglec-15 receptor in mouse cancer cell lines, 1*10^6 cells were incubated in 1% BSA PBS buffer 10 min at room temperature. Concentrated 5G12 stock was added to the buffer to give a 10 ug/ml 5G12 buffer. Unbound antibodies were washed off by centrifugation, and cell pellets were resuspended in 10 ug/ml Alexa Fluor® 647 AffiniPure Goat Anti-Human IgG antibody in 100 μL of 1% BSA PBS buffer and incubated for 30 min on ice. Cells were centrifuged and resuspended in 500 μL of 1% BSA PBS buffer. Alexa Fluor® 647 AffiniPure Goat Anti-Human IgG antibody was used as the negative control.
For sialidase control, 1*10^6 cells were incubated in 1% FBS PBS buffer containing home made STsialidase(0.3 ug/ml) for 1 hour at 37°C to remove the surface sialic acids.. The cells were washed three times and followed the procedure above.

Quantification of IFN-γ levels in BICA

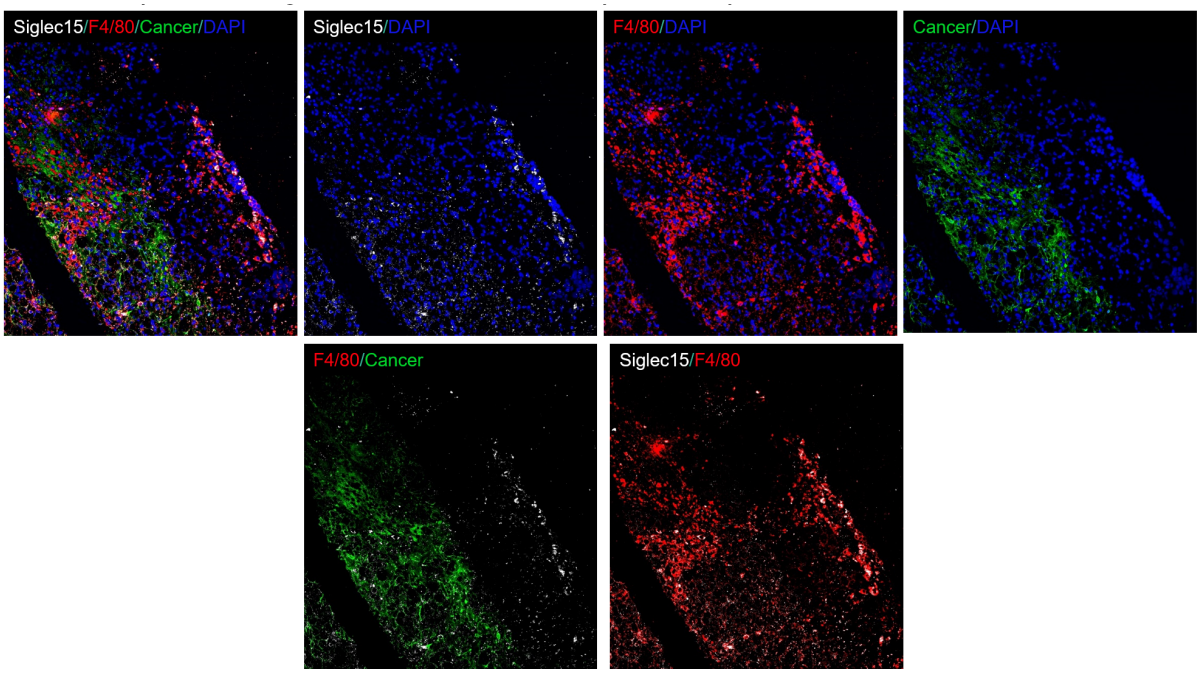
The BICA culture medium was collected and centrifuged at the desired time of BICA experiment. These culture supernates were stored at ≤20℃ and should be avoided repeated freeze-thaw cycles. The concentration of BICA-derived IFN-γ were measured by using Mouse IFN-gamma Quantikine ELISA Kit (R&D Systems, MIF00-1). The optical density of each well was read using

the accuSkan FC microplate reader according to the manual.

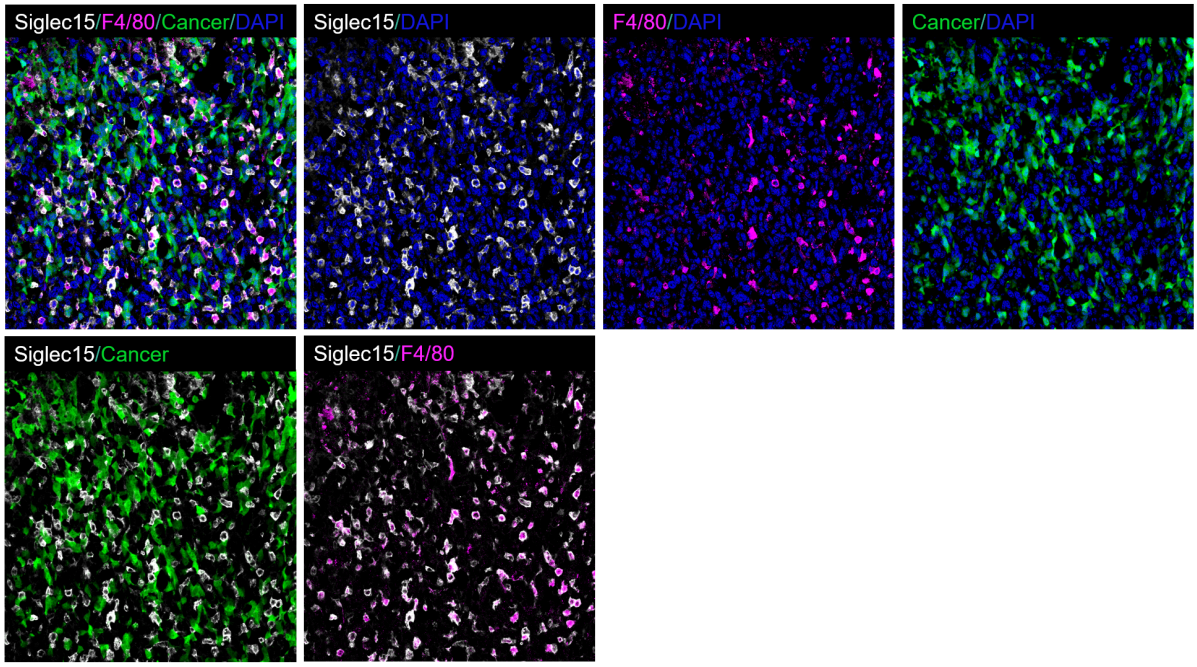
Supplementary Figures



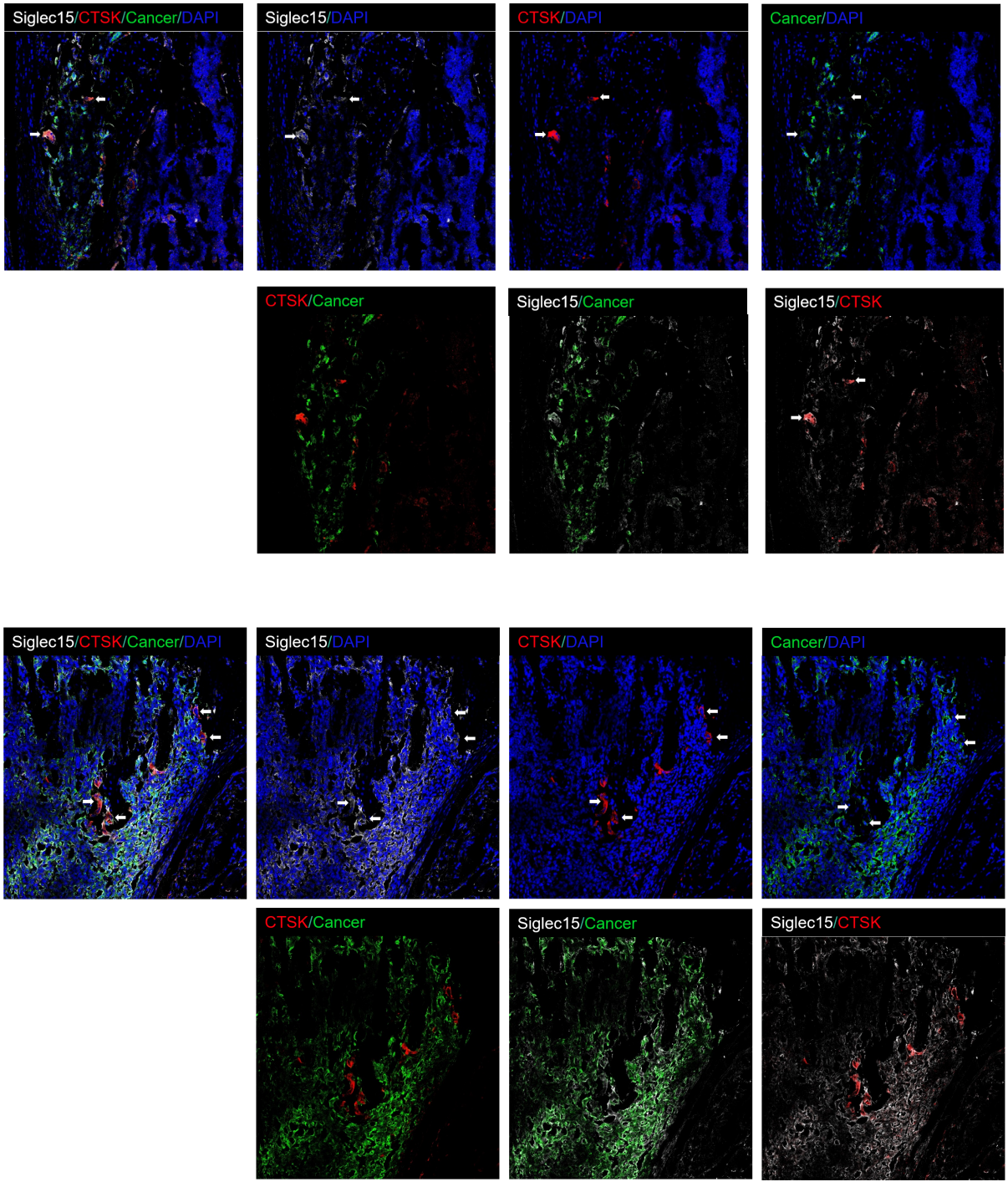
Supplementary Figure. 1 Immunofluorescence staining of the patient sample (MDACC-35). Representative immunofluorescence staining of patient samples from MD Anderson Cancer Center. Macrophage marker F4/80 (red), cancer (green), Siglec-15 (white) and nuclei (blue).



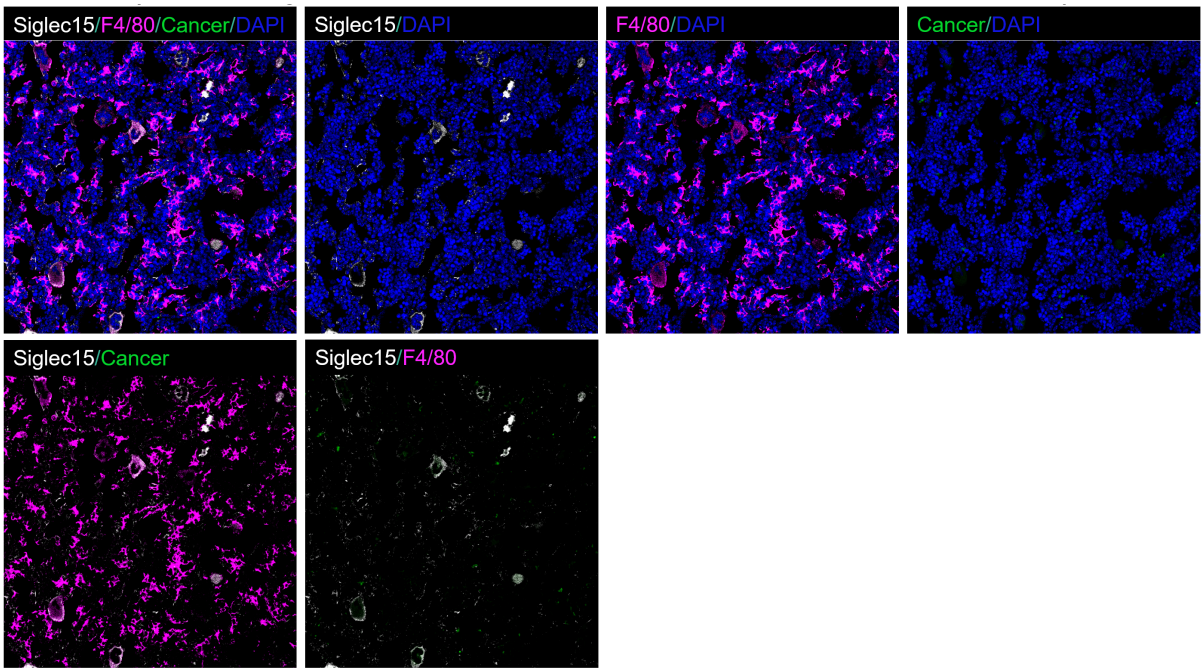
Supplementary Figure. 2 Immunofluorescence staining of the patient sample (MDACC-16). Representative immunofluorescence staining of patient samples from MD Anderson Cancer Center. Macrophage marker F4/80 (red), cancer (green), Siglec-15 (white) and nuclei (blue).



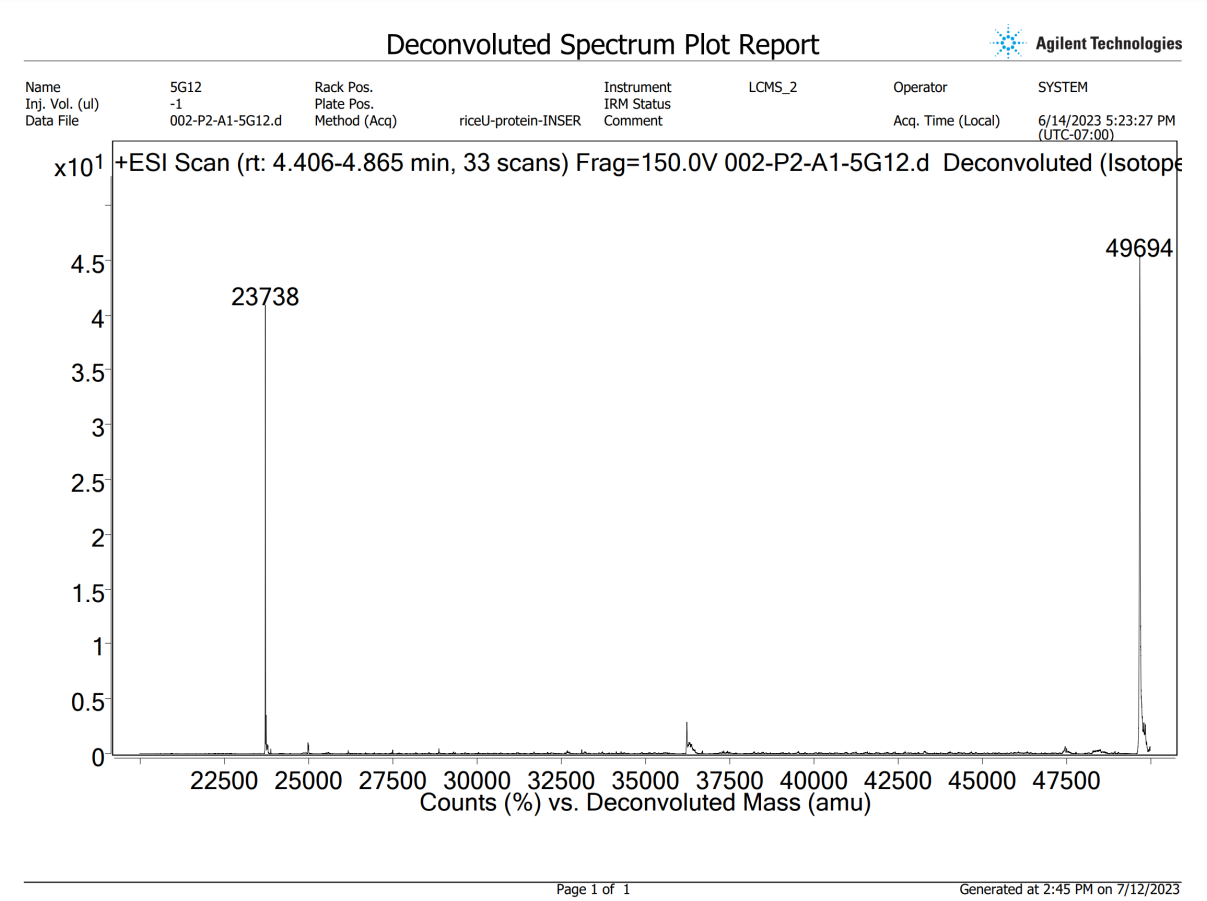
Supplementary Figure. 3 Representative immunofluorescence staining of metastatic bone niche from LLC1 bone metastases bearing mouse (tumor region). Macrophage marker F4/80 (red), cancer (green), Siglec-15 (white) and nuclei (blue).



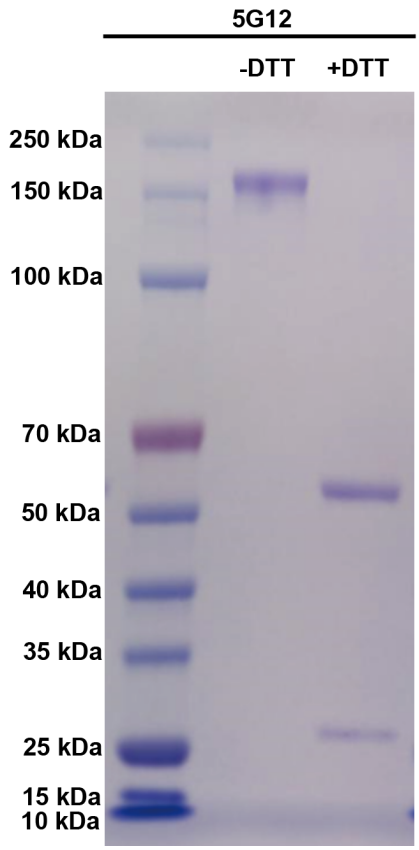
Supplementary Figure. 4 Representative immunofluorescence staining of metastatic bone niche from LLC1 bone metastases bearing mouse (tumor region). CstK (red), cancer (green), Siglec-15 (white) and nuclei (blue).



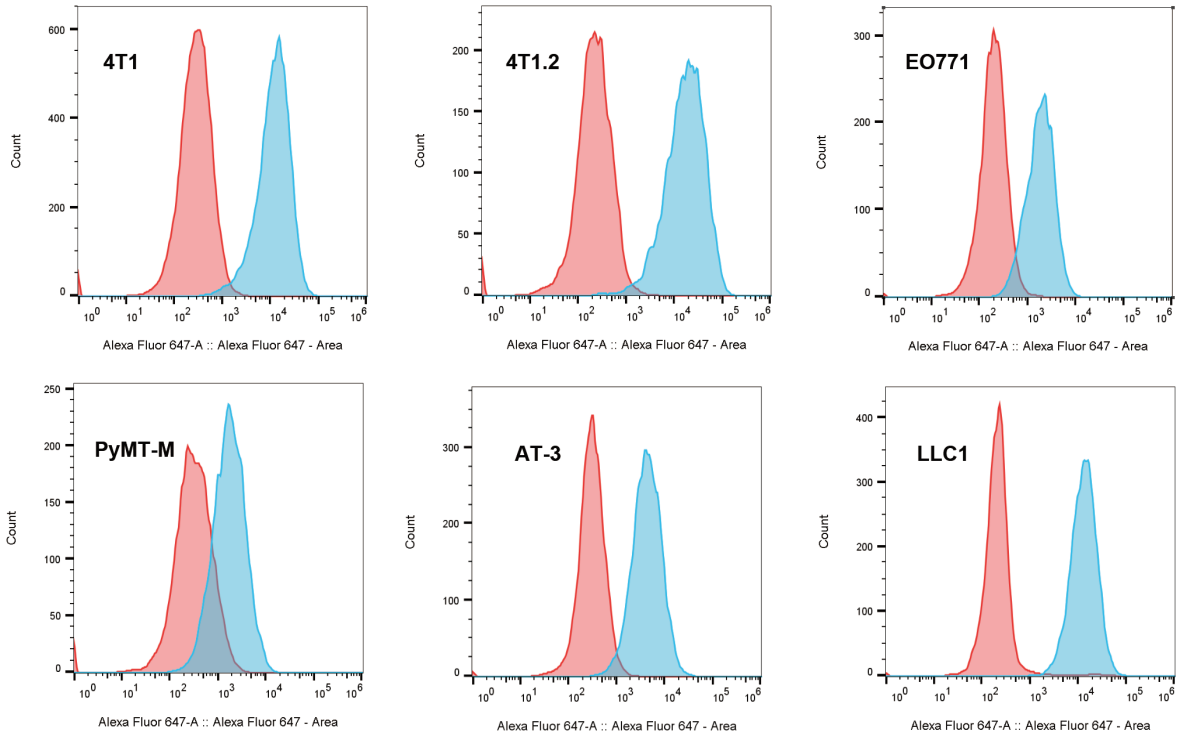
Supplementary Figure. 5 Representative immunofluorescence staining of metastatic bone niche from LLC1 bone metastases bearing mouse (health region). Macrophage marker F4/80 (red), cancer (green), Siglec-15 (white) and nuclei (blue).



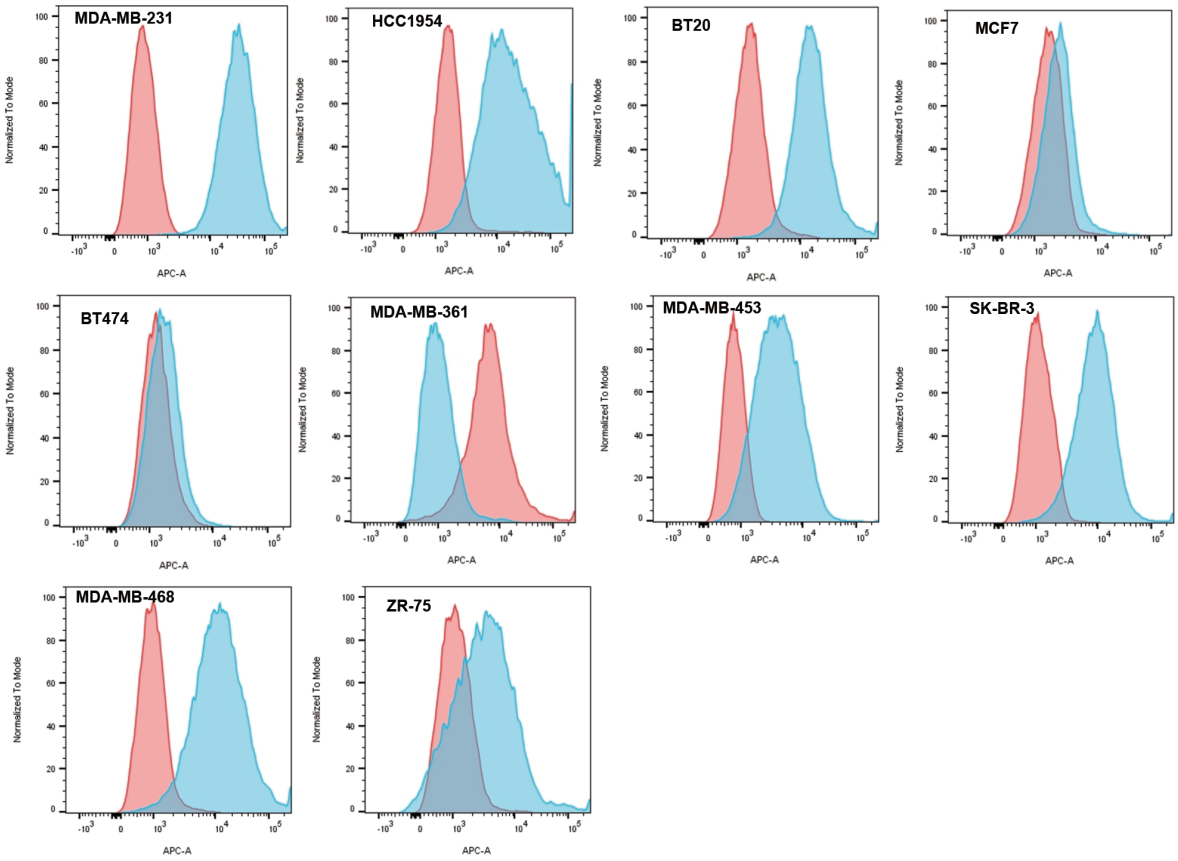
Supplementary Figure. 6 MS-Spec characterization of of 5G12.



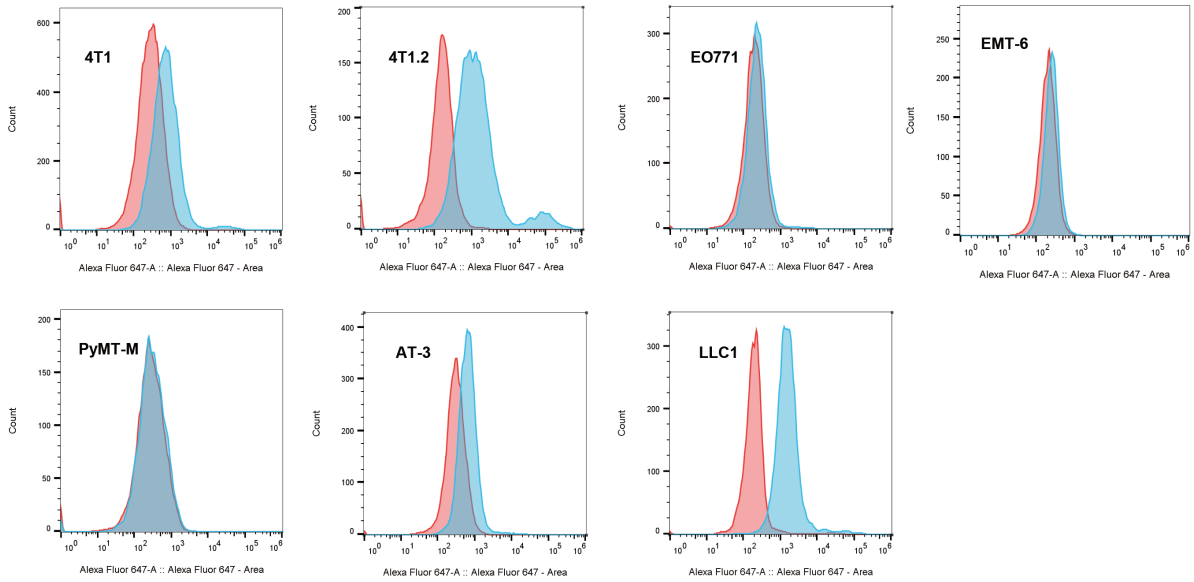
Supplementary Figure. 7 SDS-PAGE gel of 5G12, the gel was run in three replicas, for each column, ladder (left), 5G12 only (middle) and 5G12 with DTT (right).



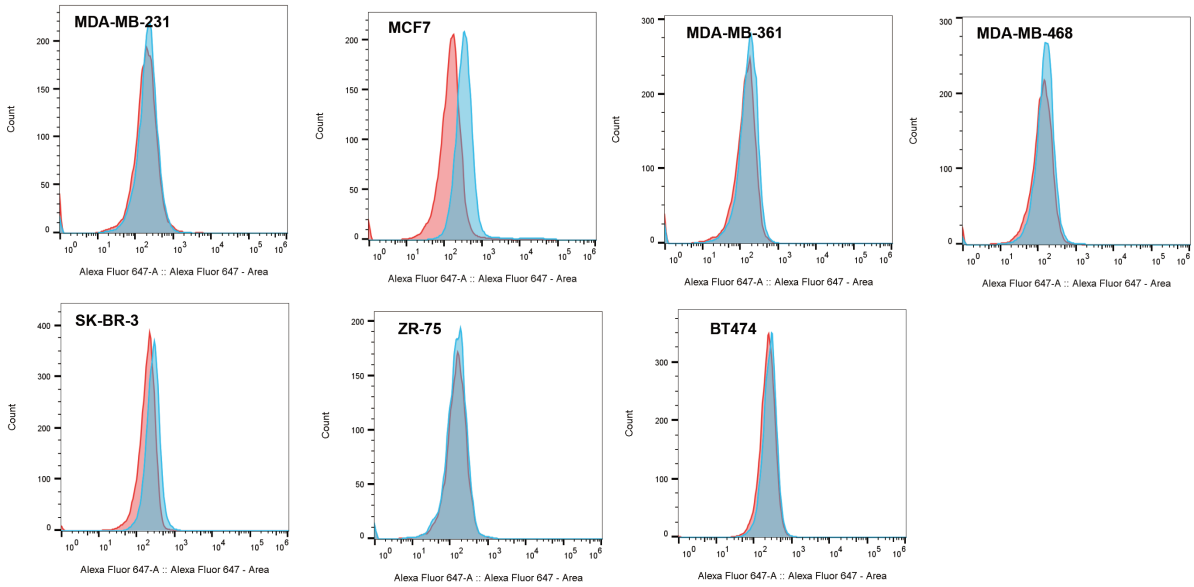
Supplementary Figure. 8 Flow cytometric analysis of expression levels of Siglec-15 ligand mouse cell lines. Negative control (red), experimental (blue).



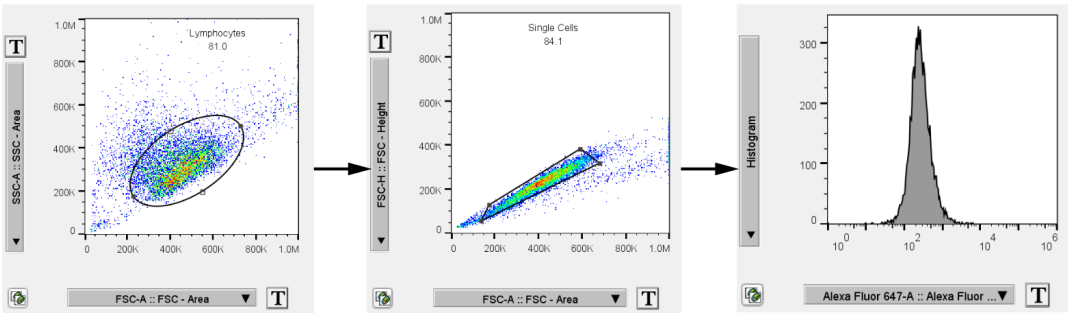
Supplementary Figure. 9 Flow cytometric analysis of expression levels of Siglec-15 ligand human cell lines. Negative control (red), experimental (blue).



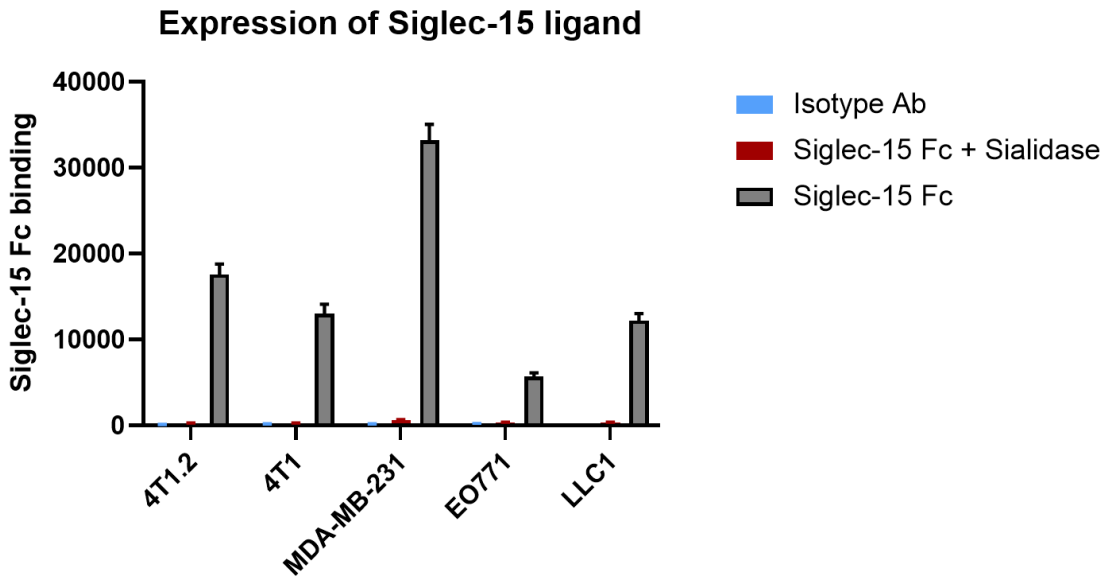
Supplementary Figure. 10 Flow cytometric analysis of expression levels of Siglec-15 receptor mouse cell lines. Negative control (red), experimental (blue).



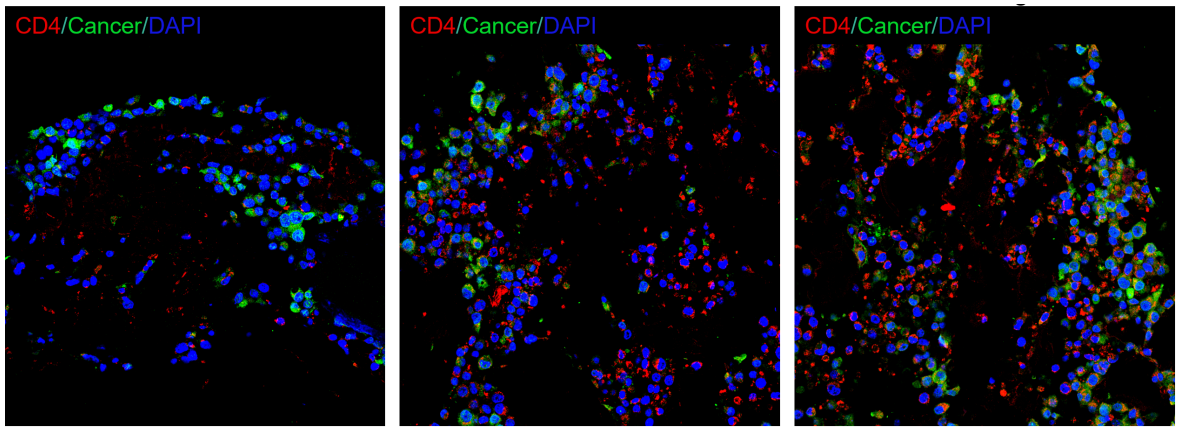
Supplementary Figure. 11 Flow cytometric analysis of expression levels of Siglec-15 receptor human cell lines. Negative control (red), experimental (blue).



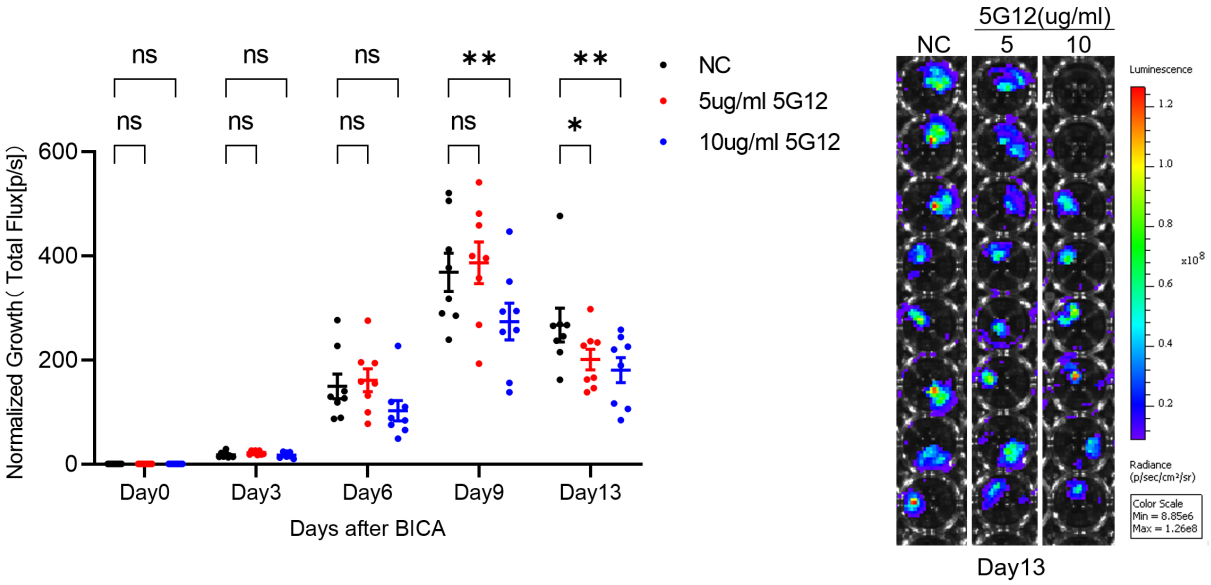
Supplementary Figure. 12 Representative gating strategy of flow cytometries above.



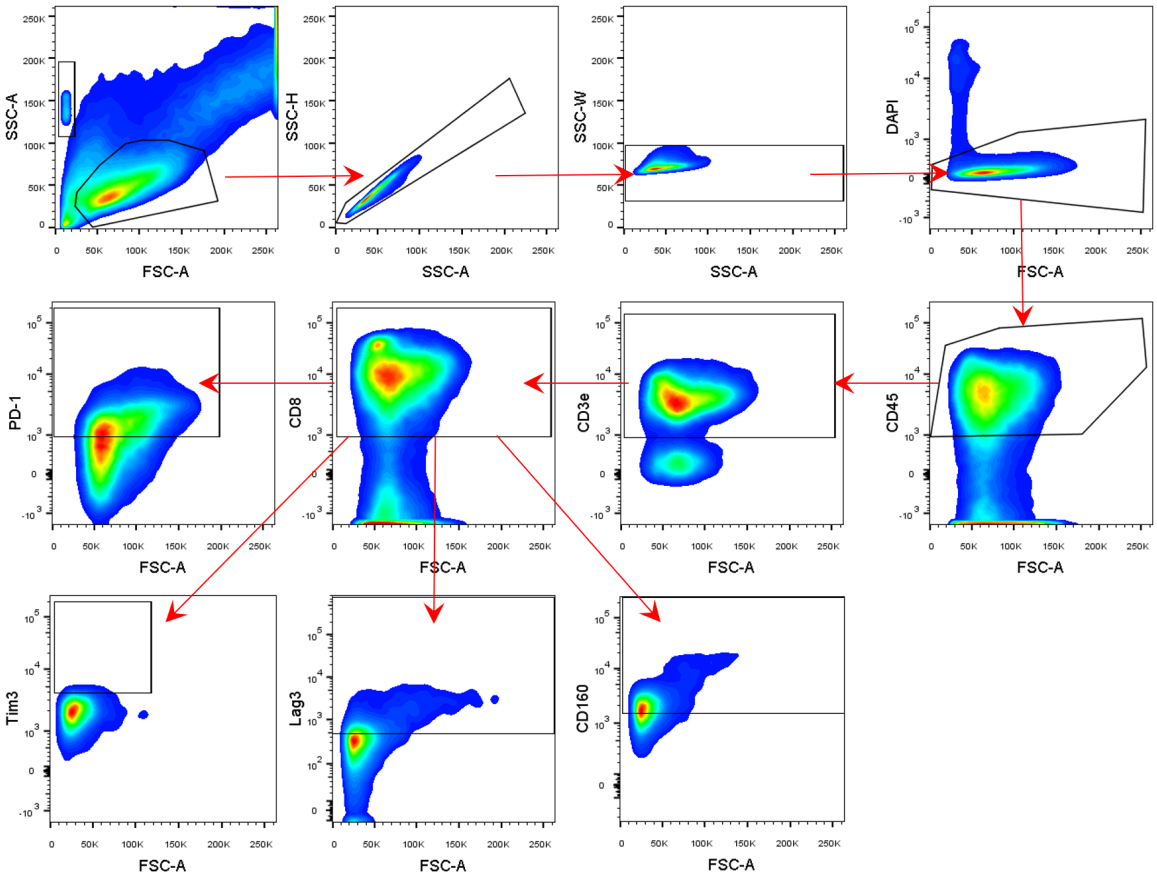
Supplementary Figure. 13 Expression levels of Siglec-15 ligand on common human and mouse cell lines.



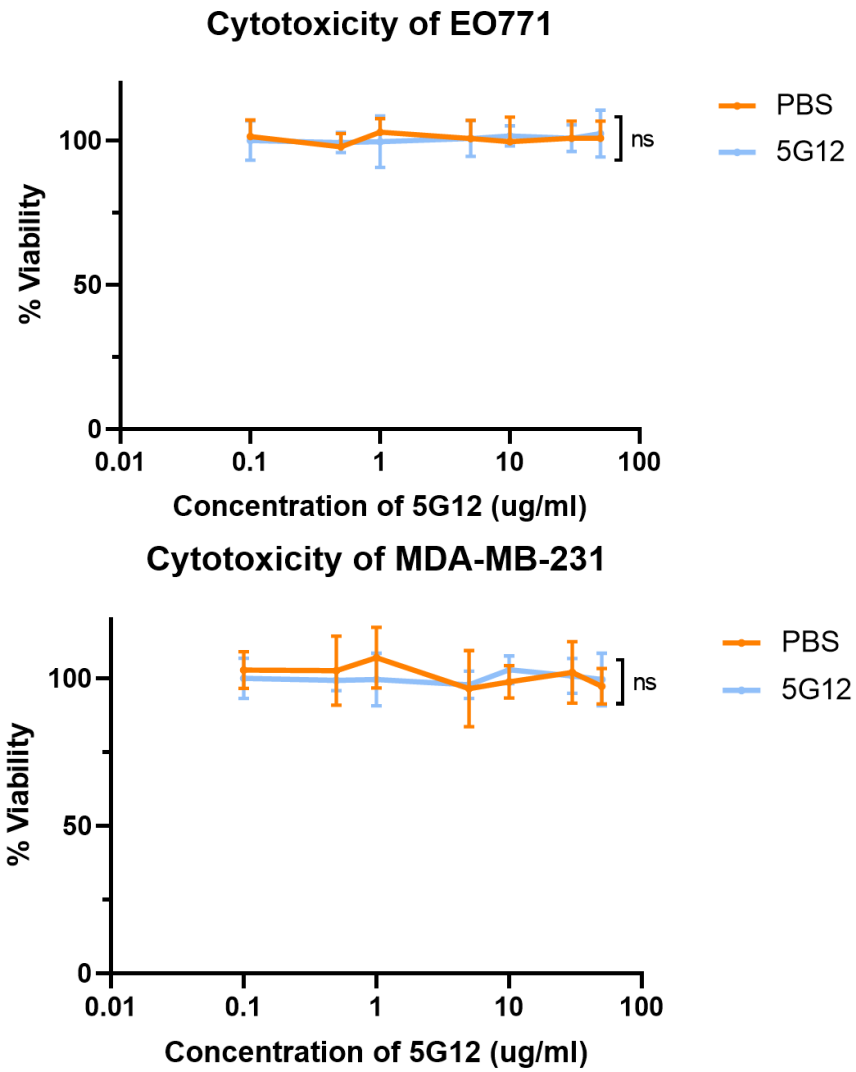
Supplementary Figure. 14 Representative immunofluorescence staining of BICA. $CD4^+$ T cell (red), cancer (green) and nuclei (blue). From left to right, no 5G12 treatment, 5 ug/ml 5G12 treatment, 10 ug/ml 5G12 treatment.



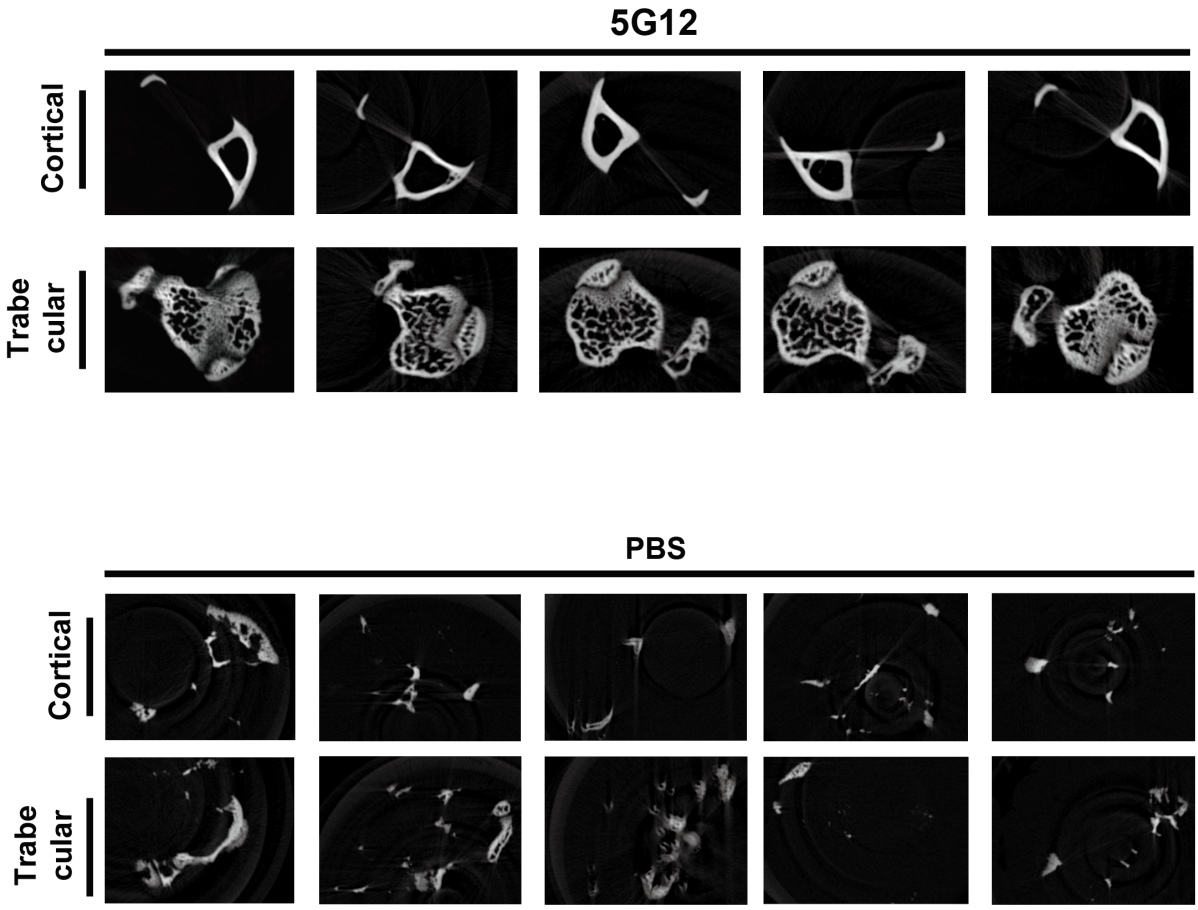
Supplementary Figure. 15 Representative pictures of 4T1 BICA lesions at day 13 culture with different concentrations of 5G12. Tumor lesion of BICA specimens with 5G12 treatment were quantificated by bioluminescence total flux at different time point and normalized to day 0. Data are mean ± s.e.m. (n= 8 bone fragments per group). P values by two-way ANOVA.



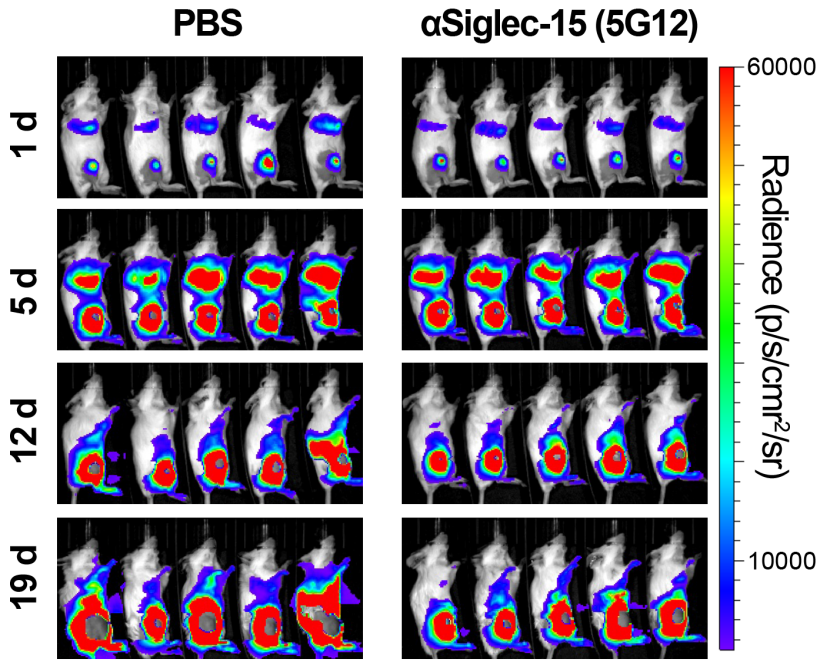
Supplementary Figure. 16 Representative gating strategy of T cell markers.



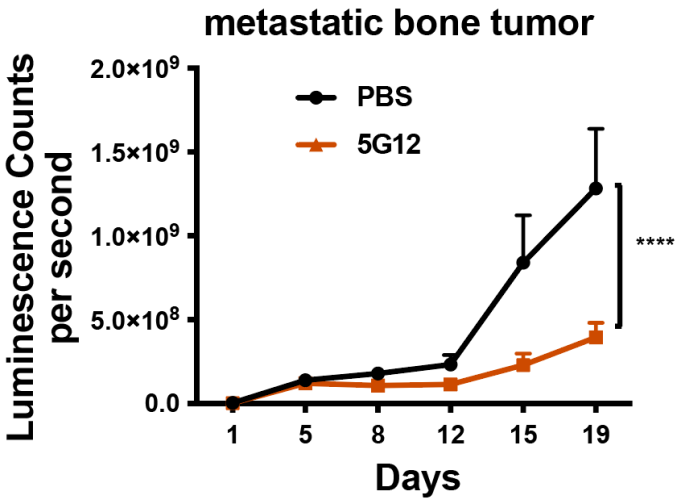
Supplementary Figure. 17 Evaluation of cytotoxicity of 5G12 on EO771 and MDA-MB-231. P value was calculated by unpaired t-test.



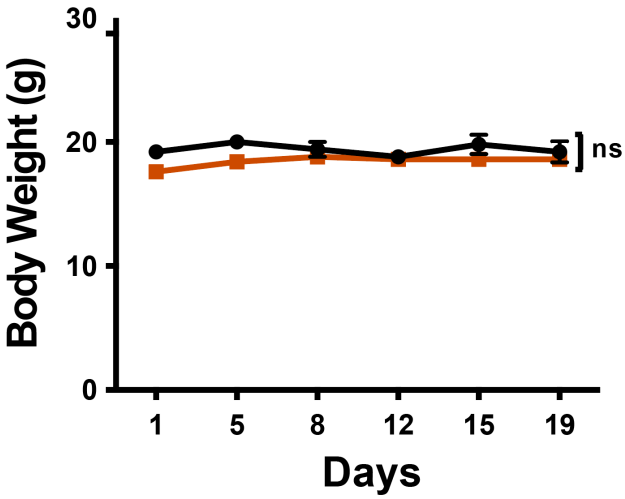
Supplementary Figure. 18 Representative microCT slices from each treatment group.



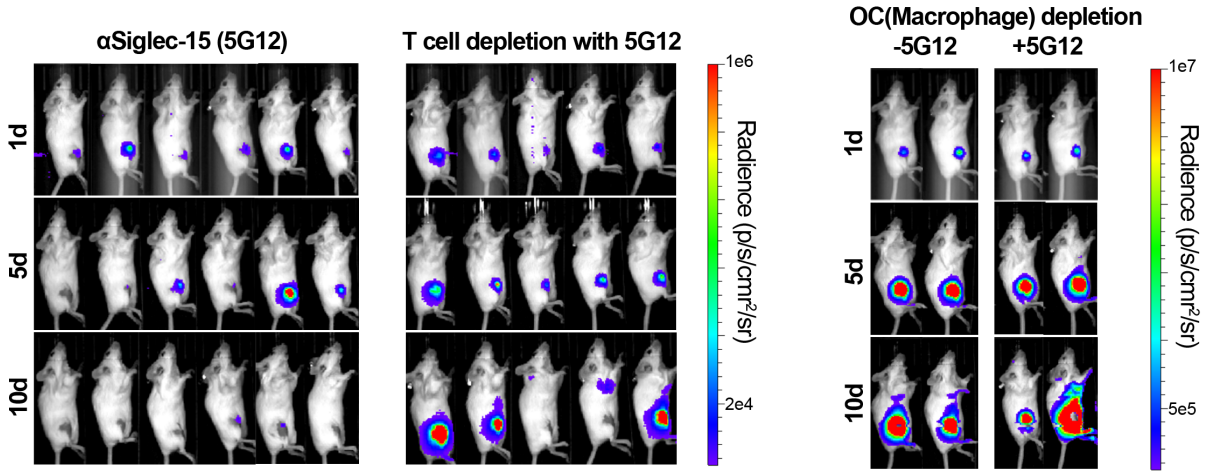
Supplementary Figure. 19 Bioluminescence imaging of EMT-6 model treated with 5G12. Luciferase and GFP labeled EMT-6 cells were para-tibia injected into the right hind limb and treated by PBS and 5G12 as described in Method. Tumor burden was monitored by weekly bioluminescence imaging.



Supplementary Figure. 20 Tumor growth curve of EMT-6 model treated with 5G12. Tumor growth curve measured by bioluminescence intensity and quantified by IVIS® system. Data are mean ± s.e.m. (n= 10 mice per group). P values by two-way ANOVA.



Supplementary Figure. 21 Body weight change of EMT-6 model treated with 5G12.



Supplementary Figure. 22 Bioluminescence imaging of depletion study. 4T1 cells were para-tibia injected into the right hind limb of no pre-treatment control, T cell depleted mice and macrophage depleted mice.