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(54) Title: HUMANIZED ANTI-TF-ANTIGEN ANTIBODIES

Figure 1

	FR1					CDR1					FR2					CDR2				
Kabat #	1	5	10	15	20	25	30	35	40	45	50	a	55	60	65					
mJAAF11	EVQLLES	GAEL	LAKPGASVK	MSCKA	SGYFTTTYWMH	WVKOR	PGQGLEWIG							FISPNTDYTEYNQKFRD						
H1	EVQLVES	GAEL	VKPGASVK	VSKCA	SGYFTTTYWMH	WVKOR	PGQGLEWIG							FISPNTDYTEYNQKFRD						
H2	EVQLLES	GAEL	LKPGASVK	VSKCA	SGYFTTTYWMH	WVKOR	APGQGLEWIG							FISPNTDYTEYNQKFRD						
H3	EVQLVES	GAEL	VKPGASVK	VSKCA	SGYFTTTYWMH	WVKOR	APGQGLEWIG							FISPNTDYTEYNQKFRD						

	FR3					CDR3					FR4			
Kabat #	70	75	80	a	b	c	83	85	90	95	100a	105	110	113
mJAAF11	KATLTADKSS	STTAYMQL	SSLTSED	SAVYYCA	RSFIGYNFD	FWGQGT	TLTVSS							
H1	RVLTADTSIS	TAYMEL	SLRSDDT	AVYYCA	RSFIGYNFD	FWGQGT	TLTVSS							
H2	RVLTADKSS	STAYMEL	SSLTSED	AVYYCA	RSFIGYNFD	FWGQGT	TLTVSS							
H3	KATLTADTSIS	TAYMEL	SLRSDDT	AVYYCA	RSFIGYNFD	FWGQGT	TLTVSS							

(57) Abstract: Provided are humanized monoclonal antibodies (mAbs) or fragments thereof that bind with specificity to the Thomsen-Friedenreich (TF) human tumor antigen. Three distinct variable heavy and three variable light chains are provided, and can be combined to make a total of twenty-five distinct heavy and light chain combinations. Methods of using the mAbs and fragments thereof for cancer therapy and diagnostic imaging are provided, as are methods for making the mAbs and fragments thereof. In vitro cell cultures that express the mAbs and fragments thereof, and kits are also provided.

HUMANIZED ANTI-TF-ANTIGEN ANTIBODIES**CROSS REFERENCE TO RELATED APPLICATIONS**

[0001] This application claims priority to U.S. provisional application no. 61/981,240, filed April 18, 2014, the disclosure of which is incorporated herein by reference.

5

FIELD

[0002] The present disclosure relates generally to humanized antibodies that recognize the Thomsen-Friedenreich (TF) human tumor antigen, and methods for using the monoclonal antibodies.

BACKGROUND

10 [0003] During carcinogenesis, alterations occur in the biosynthesis of carbohydrate structures on the cell surface, and several different carbohydrates linked either to proteins or to lipids have been recognized to be tumor-associated antigens. Thomsen-Friedenreich (TF) disaccharide (Gal β 1-3GalNAc α) is typically found O-linked to serine or threonine residues. TF-Ag (also known as T antigen) has been associated with several human carcinomas,
 15 including those found in the pancreas, colon, and breast, and on this basis has been referred to as a pan-carcinoma marker. TF-Ag is concealed from the immune system in normal adult tissues by extension with larger glycan chains. In cancer, the cellular glycosylation machinery may be disrupted leading to truncation of these chains and exposure of the TF antigen. New monoclonal antibodies and antibody fragments thereof which can target TF-Ag are desirable,
 20 and are provided by the present disclosure.

SUMMARY

[0004] The present disclosure comprises in various embodiments compositions and methods for therapy of TF+ cancers. In embodiments, the disclosure comprises partially humanized monoclonal antibodies (mAbs) or fragments thereof that bind with specificity to
 25 TF-Ag. The mAb or fragment thereof comprises a heavy chain and a light chain, wherein the heavy chain comprises a sequence selected from the group consisting of:

EVQLVESGAEVKKPGASVKVSCKASGYTFTTYWMHWVRQAPGQGLEWMG
 FISPNTDYTEYNQKFRDRVMTADTSISTAYMELSLRLSDDTAVYYCARSFIGYNFDF
 WGQGTLLTVSS (SEQ ID NO:7) (H1);

30 EVQLLESGLKPKPGASVKVSCKASGYTFTTYWMHWVRQAPGQGLEWMGF
 ISPNTDYTEYNQKFRDRVTLTADKSSSTAYMELSSLTSEDVAVYYCARSFIGYNFDF
 WGQGTTTVTVSS (SEQ ID NO:8) (H2); and

EVQLVESGAIEVKKPGASVKVSKASGYTFTTYWMHWVKQAPGQGLEWIGFI
SPNTDYTEYNQKFRDKATMTADTSISTAYMELSRLSDDTAVYYCARSFIGYNFDF
WGQGTTTLTVSS (SEQ ID NO:9) (H3), and combinations thereof.

[0005] The light chain comprises a sequence selected from the group consisting of:

5 DVVMTQSPLSLPVTLGQPASISCRSSQTIVYSNGNTYLEWFQQRPGQSPRLLIY
KVSNNRFSGVDPDRFSGSGSGTDFTLKISRVEAEDVGVYYCFQGSHVPFTFGSG
TKLEIK (SEQ ID NO:10) (L1);

L2DIVMTQTPLSLPVTLGQPASISCRSSQTIVYSNGNTYLEW
FQQRPGQSPRLLIYKVSNNRFSGVDPDRFSGSGSGTDFTLKISRVEAEDVGVYY
10 CFQGSHVPFTFGSG TKLEIK (SEQ ID NO:11) (L2); and

DVVMTQSPLSLPVTLGQPASISCRSSQTIVYSNGNTYLEWYLQRPQGSPRLLIY
KVSNNRFSGVDPDRFSGSGSGTDFTLKISRVEAEDVGVYYCFQGSHVPFTFGSG
TKLEIK (SEQ ID NO:12) (L3); and combinations thereof.

[0006] Additional versions H2, H3, L2 and L3 were made and tested. These include
15 the following:

[0007] Heavy variable region H2a:

QVQLVQSGAEVKKPGSSVKVSKASGYTFTTYWMHWVRQAPGQGLEWMG
FISPNTDYTEYNQKFRDRVTITADKSTSTAYMELSSLRSEDTAVYYCARSFIGYNFDF
WGQGTTVTVS (SEQ ID NO:13)

20 **[0008]** Heavy variable region H3a:

EGQLLESGLAKPGASVKMSCKASGYTFTTYWMHWVKRPGQGLEWIGFI
SPNTDYTEYNQKFRDKATLTADKSSTTAYMQLSSLTSDDSAVYYCARSFIGYNFDF
WGQGTTTLTVSS (SEQ ID NO:14)

[0009] Light variable region L2a:

25 DIVMTQSPLSLPVTGPGEASISCRSSQTIVYSNGNTYLEWYLQKPGQSPQLLIY
KVSNNRFSGVDPDRFSGSGSGTDFTLKISRVEAEDVGVYYCFQGSHVPFTFGSGTKVDIK
(SEQ ID NO:15)

[0010] Light variable region L3a:

ELVMTQTPLSLPVNLGDQASISCRSSQTIVYSNGNTYLEWYLQKPGQSPKLLI
30 YKVSNNRFSGVDPDRFSGSGSGTDFTLKISRVEADDLGVYYCFQGSHVPFTFGSGTKLEI
K (SEQ ID NO:16).

[0011] Combinations of heavy and variable chains included in this disclosure are: H1-
L1; H1-L2; H1-L2a; H1-L3; H1-L3a; H2-L1; H2-L2; H2-L2a; H2-L3; H2-L3a; H3-L1; H3-
L2; H3-L2a; H3-L3; H3-L3a; H2a-L1; H2a-L2; H2a-L2a; H2a-L3; H2a-L3a; H3a-L1;

H3a-L2; H3a-L2a; H3a-L3; and H3a-L3a.

[0012] In embodiments, the mAb comprises a human IgG constant region. In embodiments, the mAb or the TF-Ag binding fragment thereof is conjugated to an agent selected from the group consisting of chemotherapeutic drugs, toxins and radioactive isotopes

5 [0013] In another aspect, the disclosure comprises a method for prophylaxis and/or therapy of cancer in an individual, wherein the cancer comprises cancer cells expressing TF-Ag. The method comprises administering to the individual one or more mAbs or the fragments as described above, wherein the growth, or survival, or metastasis, or a combination thereof, of the cancer cells in the individual is inhibited subsequent to the
10 administration.

[0014] In another aspect, pharmaceutical compositions comprising the partially humanized mAb or fragment thereof are provided.

[0015] In another aspect the disclosure provides an *in vitro* cell culture, wherein cells in the cell culture express the partially humanized mAb or fragments thereof.

15 [0016] In another aspect, the disclosure provides polynucleotide sequences encoding the mAbs and TF-Ag binding fragments thereof, expression vectors comprising such polynucleotides, and *in vitro* cell cultures comprising such expression vectors. In embodiments, the mAb or TF-Ag binding fragment thereof is encoded by more than one expression vector.

20 [0017] In embodiments, methods of making the mAbs and TF-Ag binding fragments thereof are provided, and generally comprise expressing the mAbs or TF-Ag binding fragments, or combinations thereof in an *in vitro* cell culture, and separating the mAbs or TF-Ag binding fragments, or combinations thereof, from the cell culture.

[0018] Kits comprising the mAbs and TF-Ag binding fragments thereof are also
25 provided.

BRIEF DESCRIPTION OF THE FIGURES

[0019] **Figure 1** provides the amino acid sequence alignments of JAA-F11 heavy variable and the three VH variants (H1, H2, and H3) regions that are included in this disclosure. Bold indicates identical amino acids between JAA-F11 and the designed,
30 humanized H chains; bold and shaded amino acids indicate differences among the designed humanized chains and mouse JAA-F11. Alanine at position 72 shown in bold and italicized is a mouse residue that was retained to avoid steric clashes. The complementarity determining regions (CDRs) are italicized. Numbering is according to the Kabat system.

The mJAAF11 sequence is SEQ ID NO:17. The H1 sequence is SEQ ID NO:7; the H2 sequence is SEQ ID NO:8; the H3 sequence is SEQ ID NO:9.

[0020] **Figure 2** depicts amino acid sequence alignments of mJAA-F11 light variable (JAA-F11VH) and three VL variants (L1, L2, and L3) regions. Bold indicates mouse amino acids or amino acids identical to the mouse sequence, bold and shaded amino acids are differences among the designed humanized JAA-F11 variants and mouse JAA-F11. Leucine at position 51, shown as bold and italicized is a mouse residue that was retained to avoid steric clashes. The mJAAF11 sequence is SEQ ID NO:17. The L1 sequence is SEQ ID NO:10; the L2 sequence is SEQ ID NO:11; the L3 sequence is SEQ ID NO:12.

[0021] **Figure 3.** Determination of relative affinity using mJAA-F11 inhibition by ELISA to assess the binding of each hJAA-F11 and chimeric antibody to TF-Ag-BSA. Error bars represent ± 1 standard error.

[0022] **Figure 4.** Binding of hJAA-F11, chimeric and mouse JAA-F11 (50 $\mu\text{g/mL}$) to 10 breast cancer cell lines. The cell lines include triple negative (HCC 70, BT 549, MDA-MB-231, MDA-MB-468, DU 4475), ER/PR-positive (HCC 1419, AU 565, MDA-kb2), and HER2-positive (CAMA-1, HCC 1428). For all cell lines tested above, binding to hJAA-F11, chimeric or mouse JAA-F11 (at 50 $\mu\text{g/mL}$) was significantly higher ($p \leq 0.05$) (Student's T-test) than that of the TF-Ag negative control myeloma. Each error bar represents ± 1 standard deviation.

[0023] **Figure 5.** The chimeric antibody and the humanized JAA-F11 antibodies except for H3L3 caused a small ($\sim 6-11\%$) but statistically significant inhibition of tumor cell growth. No enhancement of cell proliferation was seen. No significant result was seen for any of the antibodies at 2 or 1 $\mu\text{g/mL}$.

[0024] **Figure 6A.** ADCC activity of mouse, chimeric, and 4 hJAA-F11 constructs against a TF-Ag positive human breast cancer cell line (BT 549). Results are presented as percent cell lysis in antibody treated cells compared to 100% lysis of cells. H2L2 and H3L3 showed statistically more ADCC activity than chimeric or mouse antibodies ($p < 0.05$) in all cases in the human cell line. This is representative average of all experiments on PBMCs from Donor # 1 and Donor # 2 (more than 3 independent experiments). Error bars represent ± 1 standard error.

[0025] **Figure 6B.** ADCC activity of mouse, chimeric, and 4 hJAA-F11 constructs against a TF-Ag positive human breast cancer cell line (BT 549) on PBMCs Donor #1.. Results are presented as percent cell lysis in antibody treated cells compared to 100% lysis of cells. H2L2 and H3L3 showed statistically more ADCC activity than chimeric or mouse

antibodies ($p<0.05$) in all cases in the human cell line. This is average of at least 3 independent experiments. Error bars represent ± 1 standard error.

[0026] **Figure 6C.** ADCC activity of mouse, chimeric, and 4 hJAA-F11 constructs against a TF-Ag positive human breast cancer cell line (BT 549) on PBMCs from Donor #2.

5 Results are presented as percent cell lysis in antibody treated cells compared to 100% lysis of cells. H2L2 and H3L3 showed statistically more ADCC activity than chimeric or mouse antibodies ($p<0.05$) in all cases in the human cell line. This is the average of at least 3 independent experiments. Error bars represent ± 1 standard error.

[0027] **Figure 6D.** ADCC activity of mouse, chimeric, and 3 hJAA-F11 constructs

10 against 4T1 TF-Ag positive mouse breast cancer cell line. Results are presented as percent cell lysis in antibody treated cells compared to 100% lysis of cells. H3L3 and chimeric showed statistically more ADCC activity than mouse antibody ($p<0.05$). Error bars represent ± 1 standard error. ADCC was performed only once with PBMCS from Donor #3.

[0028] **Figure 7.** Humanized, Chimeric and Mouse JAA-F11 do not induce

15 complement dependent cytotoxicity (CDC). CDC activity of mouse, chimeric, and 2 hJAA-F11 constructs against the human HCC 1428 breast cancer cell line were tested. Results are presented as percent cell lysis in antibody treated cells compared to 100% lysis of cells. Positive control: LDH positive cells included in the reagent kit showed lysis. Error bars represent ± 1 standard error.

20 [0029] **Figure 8.** Internalization of mouse, chimeric, and 4 hJAA-F11 antibodies

using an enzyme immunoassay to measure surface binding. Internalization was analyzed by incubation of mouse 4T1 breast cancer cells with antibodies at 37°C or 4°C. As expected, the pattern was opposite the ADCC pattern shown for H3L3 in Figure 20 but was unexpected for H2L2 which induced ADCC and internalized. Mouse, H2L2, chimeric Ab, H2L3, and H1L1

25 internalized significantly ($p<0.05$). Error bars represent ± 1 Standard error.

[0030] **Figure 9.** MicroPET images showing immunolocalization of hJAA-F11 antibody in 4T1 tumor bearing mouse.

[0031] **Figure 10.** MicroPET images showing localization of free ¹²⁴Iodine to the thyroid in 4T1 tumor bearing mouse.

30 DETAILED DESCRIPTION

[0032] The present disclosure comprises distinct humanized monoclonal antibodies (mAbs) and fragments thereof which specifically recognize TF-Ag, methods of making the mAbs, and methods of using the mAbs for prophylaxis and/or therapeutic purposes, and for

diagnostic imaging. The amino acid sequences of the mAbs provided by this disclosure were developed using a novel approach to change the amino acid sequences in the mAb produced by a hybridoma deposited under ATCC Catalog number CRL-2381, such that the framework regions of the mAbs incorporate both murine and human immunoglobulin (Ig) sequences in a guided way that maintains specificity and reduces immunogenicity. The hybridoma produces a mAb known as JAA-F11. Changes introduced into JAA-F11 sequence in the context of the murine framework regions result in an ensemble of three distinct heavy and three distinct light variable chains that can be combined to produce twenty five distinct mAbs that are suitable for combating any of variety of cancers that comprise cancer cells that express TF-Ag. Further, the mAbs have desirable characteristics that render them particularly suited for preferentially inducing distinct anti-cancer mechanisms in an individual. For example, depending on the choice of heavy and light chain, the disclosure includes providing for stimulating enhanced antibody-dependent cytotoxicity, or complement dependent cytotoxicity, or internalization of the mAb.

[0033] Embodiments of the disclosure include CDR sequences from the JAA-F11 mAb. These are: **VH chain:** CDR1: SGYTFTTYWMH; (SEQ ID NO:1); CDR2: FISPNTDYTEYNQKFRD; (SEQ ID NO:2); CDR3: RSFIGYNFDFWGQGT; (SEQ ID NO:3); and **VL chain:** CDR1: CRSSQTIVYSNGNTYLEW; (SEQ ID NO:4); CDR2: KVSNRFSGVPD; (SEQ ID NO:5); and CDR3: CFQGSHPFTGSG; (SEQ ID NO:6).

[0034] The CDR sequences are placed in the context of modified framework sequences. As a consequence, the mAbs and TF-Ag binding fragments thereof comprise a heavy chain selected from:

H1 - EVQLVESGAIEVKKPGASVKVSCKASGYTFTTYWMHWVRQAPGQGLEWMGFISPNTDYTEYNQKFRDRVTMTADTSISTAYMELSRLRSDDTAVYYCARSFIGYNFDFWGQGTLVTVSS (SEQ ID NO:7);

H2 - EVQLLESGAELKKPGASVKVSCKASGYTFTTYWMHWVRQAPGQGLEWMGFISPNTDYTEYNQKFRDRVTLTADKSSSTAYMELSSLTSEDVAVYYCARSFIGYNFDFWGQGTTVTVSS (SEQ ID NO:8);

H3 - EVQLVESGAIEVKKPGASVKVSCKASGYTFTTYWMHWVKQAPGQGLEWMGFISPNTDYTEYNQKFRDKATMTADTSISTAYMELSRLRSDDTAVYYCARSFIGYNFDFWGQGTTTLTVSS (SEQ ID NO:9) and combinations thereof;

and a light chain selected from the group consisting of:

L1 - DVVMTQSPLSLPVTLGQPASIS CRSSQTIVYSNGNTYLEW FQQRPGQSPRLLIY KVSNRFSGVPDRFSGSGSGTDFTLKISRVEAEDVGVYY CFQGSHPFTGSG

TKLEIK (SEQ ID NO:10);

L2 - DIVMTQTPLSLPVTLGQPASIS CRSSQTIVYSNGNTYLEW FQQRPGQSP
RLLIY KVSNRFSGVPDRFSGSGSGTDFTLKISRVEAEDVGVYY CFQGSHPFTFGSG
TKLEIK (SEQ ID NO:11);

5 **L3** - DVVMTQSPLSLPVTLGQPASIS CRSSQTIVYSNGNTYLEW
YLQRPGQSPRLLIY KVSNRFSGVPDRFSGSGSGTDFTLKISRVEAEDVGVYY
CFQGSHPFTFGSG TKLEIK (SEQ ID NO:12) and combinations thereof.

[0035] In addition to the foregoing sequences, the following heavy and light chains were made and tested in certain combinations:

10 **[0036]** Heavy variable region H2a:

QVQLVQSGAEVKKPGSSVKVCKASGYTFTTYWMHWVRQAPGQGLEWMG
FISPNTDYTEYNQKFRDRVITADKSTSTAYMELSSLRSEDVAVYYCARSFIGYNFDF
WGQGTTVTVS (SEQ ID NO:13)

[0037] Heavy variable region H3a:

15 EGQLLESGAELAKPGASVKMSCKASGYTFTTYWMHWVKKRPGQGLEWIGFI
SPNTDYTEYNQKFRDKATLTADKSSTTAYMQLSSLTSDDSAVYYCARSFIGYNFDF
WGQGTTLTVSS (SEQ ID NO:14)

[0038] Light variable region L2a:

DIVMTQSPLSLPVTGPGEASISCRSSQTIVYSNGNTYLEWYLQKPGQSPQLLIY
20 KVSNRFSGVPDRFSGSGSGTDFTLKISRVEAEDVGVYYCFQGSHPFTFGSGTKVDIK
(SEQ ID NO:15)

[0039] Light variable region L3a:

ELVMTQTPLSLPVNLGDQASISCRSSQTIVYSNGNTYLEWYLQKPGQSPKLLI
YKVSNRFSGVPDRFSGSGSGTDFTLKISRVEADDLGVYYCFQGSHPFTFGSGTKLEI
25 K (SEQ ID NO:16).

[0040] The disclosure encompasses mAbs and TF-Ag binding fragments thereof comprising amino acid sequences that comprise or consist of the foregoing sequences. In embodiments, the disclosure comprises mAbs and TF-Ag binding fragments thereof, wherein the framework and CDR sequences consist of SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, and SEQ ID NO:16.

[0041] Thus, the following 25 Heavy and Light chain combinations are encompassed by this disclosure: H1-L1; H1-L2; H1-L2a; H1-L3; H1-L3a; H2-L1; H2-L2; H2-L2a; H2-L3; H2-L3a; H3-L1; H3-L2; H3-L2a; H3-L3; H3-L3a; H2a-L1; H2a-L2; H2a-L2a; H2a-L3; H2a-

L3a; H3a-L1; H3a-L2; H3a-L2a; H3a-L3; and H3a-L3a. In embodiments, the disclosure includes mAbs and TF-Ag binding fragments thereof comprising a combination of H1L1, H2L2, H3L3, H2L3, H2a and L2a, and H3a and L3a. In this disclosure, in addition to the results for combinations of H and L chains as described below, H2a-L2a and H3aL3a were made and tested, and the results for these two combinations were similar to those reported for H2-L2 and H3-L3, respectively. The H2a-L2a and H3a-L3a combinations were made as a result of subcloning procedures, allowing for selection of additional subclones with further enhanced binding characteristics for TF-Ag, as determined by the highest reactivity in enzyme immunoassays (EIA). Of these, the best for internalization are H2L2 and H2aL2a, while the best for ADCC are H2L2, H2aL2a, H3L3 and H3aL3a.

[0042] Representative VH and VL sequences are further described by way of Figures 1 and 2. In particular, Figure 1 provides the amino acid sequence alignments of JAA-F11 heavy variable and the three VH variants (H1, H2, and H3) regions that are included in this disclosure. Bold indicates identical amino acids between JAA-F11 and the designed, humanized H chains; bold and shaded amino acids indicate differences among the designed humanized chains and mouse JAA-F11. Alanine at position 72 shown in bold and italicized is a mouse residue that was retained to avoid steric clashes. The CDRs are italicized. Numbering is according to the Kabat system. It will thus be recognized that, while the mAbs described herein are referred to as “humanized” they do comprise certain murine residues, and thus can be considered partially humanized.

[0043] Figure 2 depicts amino acid sequence alignments of mJAA-F11 light variable (JAA-F11VH) and three VL variants (L1, L2, and L3) regions. Bold indicates mouse amino acids or amino acids identical to the mouse sequence, bold and shaded amino acids are differences among the designed humanized JAA-F11 variants and mouse JAA-F11. Leucine at position 51, shown as bold and italicized is a mouse residue that was retained to avoid steric clashes. It will be apparent from Figures 1 and 2 that the presently provided, humanized H and L chains comprise differences from the JAA-F11 mouse antibody and differences among themselves, but certain murine amino acids have been retained to maintain the specificity of the humanized mAbs.

[0044] Fragments of the humanized mAbs described herein are also included in the invention. Examples of suitable antibody fragments include Fab, Fab', F(ab')₂, ScFv and Fv fragments. Various techniques have been developed for the production of antibody fragments and are included in the scope of this disclosure. In embodiments, the mAbs or fragments thereof are produced host cells by way of recombinant expression vectors. The

present disclosure includes all polynucleotide sequences encoding the amino acid sequences described herein, expression vectors comprising such polynucleotide sequences, and *in vitro* cell cultures comprising such expression vectors. In embodiments, the cell cultures are eukaryotic cells. In embodiments, the cell cultures are mammalian cells. In embodiments, the cells are CHO cells. Kits comprising the mAbs and/or TF-Ag binding fragments thereof, and/or cell cultures expressing the mAbs and/or TF-Ag binding fragments thereof, are provided by this disclosure. In general, the kits comprise one or more sealed containers that contain the mAbs and/or TF-Ag binding fragments thereof, or cells expressing them. Instructions for using the mAbs and/or TF-Ag binding fragments for therapeutic and/or imaging purposes can be included in the kits.

[0045] In embodiments, the disclosure includes a method of making the mAbs or TF-Ag binding fragments thereof, comprising culturing cells comprising an expression vector or other polynucleotide sequence encoding the mAbs or TF-Ag binding fragments thereof, allowing expression of the mAbs or TF-Ag binding fragments thereof, and separating the mAbs or TF-Ag binding fragments thereof from the cell culture. Nucleotide sequences encoding the mAbs or TF-Ag binding fragments thereof can be expressed using any suitable expression vector, many of which are known in the art and/or are commercially available. In one embodiment, the heavy and light chains are expressed on a single expression vector, such as a plasmid. In another embodiment, the heavy and light chains are expressed on distinct plasmids in the same cell, after which the expressed heavy and light chains form the conventional mAb architecture. The mAbs or TF-Ag binding fragments thereof can be isolated and/or purified using conventional techniques, given the benefit of the present disclosure.

[0046] In another aspect, the disclosure provides a method of inhibiting the growth of cancer cells in an individual, and/or inhibiting metastasis of cancer cells in an individual, which cancer cells express TF-Ag molecules. The method comprises administering to the individual a therapeutic amount of the humanized mAbs and/or fragments thereof, wherein the administration inhibits the growth and/or inhibits the metastasis of the TF-Ag expressing cancer cells. In embodiments, practicing the method of the invention reduced the volume of a tumor, and/or reduces the formation of metastatic foci, or secondary tumors. In embodiments, the method is provided for an individual in need thereof. In embodiments, the individual in need has been diagnosed with, is suspected of having, or is at risk for developing or having a recurrence of cancer. In embodiments, a therapeutically effective amount of a mAb or TF-Ag binding fragment thereof is used. The term “therapeutically

effective” as used herein means that the amount of the mAb or TF-Ag binding fragment thereof administered is of sufficient quantity to inhibit the growth, survival and/or metastasis of TF+ cancer cells.

[0047] In various embodiments, the humanized mAbs and/or fragments thereof can be conjugated to a chemotherapeutic agent to enable localization of the chemotherapeutic agent to cancer cells via binding to cells expressing TF-Ag. Chemotherapeutic agents useful in the generation of such antibody conjugates include enzymatically active toxins and fragments thereof. Suitable enzymatically active toxins include diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from *Pseudomonas aeruginosa*), ricin A chain, abrin A chain, modeccin A chain, alpha sarcin, Aleurites fordii proteins, dianthin proteins, *Phytolaca americana* proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin and the tricothecenes. The chemotherapeutic agents can be covalently attached to the mAb or TF-Ag binding fragment thereof by any suitable chemical conjugation approach. In embodiments, the chemotherapeutic agent can comprise a segment of a fusion protein with the mAb or TF-Ag binding fragment.

[0048] In another aspect the disclosure provides a method for identifying in an individual metastatic foci, tumors, or combinations thereof, wherein the metastatic foci or tumors comprise cells expressing TF-Ag. The method comprises the steps of administering to the individual humanized mAbs and/or fragments thereof, wherein the humanized mAbs and/or fragments thereof have been conjugated to a detectable label, and detecting the detectable label to identify metastatic foci, tumors, or combinations thereof. Thus, the humanized mAbs and/or fragments thereof may be conjugated to a detectable label, such as a radioactive agent. A variety of radioactive isotopes are available for conjugating to JAA-F11 mAbs such that cells to which the JAA-F11 mAbs bind may be imaged or selectively destroyed. For selective destruction of cells expressing TF-Ag, the JAA-F11 mAbs may be conjugated to a highly radioactive atom, such as In111, At211, I131, I125, Y90, Re186, Re188, Sm153, Bi212, P32, Pb212 and radioactive isotopes of Lu.

[0049] When the humanized mAbs and/or fragments thereof are used for identifying cells expressing TF-Ag in metastatic foci or in tumors, they may comprise a radioactive atom for scintigraphic studies, for example Tc99m (metastable technetium-99), I123, or a spin label for nuclear magnetic resonance (NMR) imaging (also known as magnetic resonance imaging, or “MRI”), such as I123, I131, I124, F19, C13, N15, O17 or Gadlinium (III) or Manganese (II).

[0050] Labeled humanized mAbs and/or fragments thereof can be injected into patients diagnosed with or suspected of having a metastatic disease to identify metastatic foci and/or tumors. Information from such imaging can be used for diagnosing or staging of the disease status of the patient. The label used can be selected in accordance with the imaging system to be used. For example, Indium111, Technetium99 or Iodine131 can be used for planar scans or single photon emission computed tomography (SPECT). Positron emitting labels such as Fluorine19 Iodine 123 and Iodine 124 can be used in positron emission tomography. Paramagnetic ions such as Gadolinium (III) or Manganese (II) can be used in magnetic resonance imaging (MRI). Localization of the label within a particular tissue of the individual permits localization of metastatic foci or tumors which comprise cells expressing TF-Ag. A concentration of label at a particular location greater than background permits identification of the presence of metastasized cells. In a preferred embodiment, after administration of labeled humanized mAbs and/or fragments thereof, a suitable period of time is allowed to pass such that unbound humanized mAbs and/or fragments thereof are cleared from the individual such that background label is greatly reduced.

[0051] Therapeutic formulations comprising conjugated or unconjugated humanized mAbs and/or fragments thereof may be prepared by mixing with pharmaceutically acceptable carriers, excipients or stabilizers (Remington's Pharmaceutical Sciences 16th edition, Osol, A. Ed. (1980)), in the form of lyophilized formulations or aqueous solutions. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrans; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (e.g. Zn-protein complexes); and/or non-ionic surfactants such as TWEENTM, PLURONICTM or polyethylene glycol (PEG). The humanized mAbs and/or fragments thereof may be combined with other chemotherapeutic agents in pharmaceutical compositions.

[0052] The humanized mAbs and/or TF-binding fragments thereof may be administered by any suitable means, including parenteral, subcutaneous, intraperitoneal, intrapulmonary, and intranasal. Parenteral infusions include intramuscular, intravenous, intraarterial, intraperitoneal, intralymphatic or subcutaneous administration. In addition, the humanized mAbs and/or fragments thereof may suitably be administered by pulse infusion, e.g., with declining doses of the antibody. Preferably, the dosing is given by injections, most preferably intravenous or subcutaneous injections, depending in part on whether the administration is brief or chronic. In certain embodiments, the humanized mAbs and/or fragments thereof are administered to an individual diagnosed with or suspected of having breast, colon, prostate, ovarian, bladder or other TF Ag+ cancers to inhibit metastasis or to inhibit the growth of the cancer cells.

[0053] Other compounds, such as chemotherapeutic agents, immunosuppressive agents and/or cytokines can also be administered. The combined administration can include co-administration, using separate formulations or a single pharmaceutical formulation, and can also include consecutive administration in either order, wherein preferably there is a time period while both (or all) active agents simultaneously exert their biological activities.

[0054] The humanized mAbs and/or fragments thereof may be administered to a human or other animal in accordance with the aforementioned methods of treatment in an amount sufficient to inhibit metastasis and/or growth of cells expressing TF-Ag. It will be recognized by one of skill in the art that the form and character of the pharmaceutically acceptable carrier or diluent is dictated by the amount of active ingredient with which it is to be combined, the route of administration and other well-known variables, such as the size of the individual and the stage of the disease.

Example 1

[0055] The following Example describes making and using humanized mAbs of this disclosure, and physical and functional properties of the mAbs.

[0056] Design and construction of humanized JAA-F11

[0057] After the sequences of the mouse JAA-F11 variable regions were verified, the CDRs were defined so as to maintain specificity and affinity for TF-Ag. The inclusive CDRs were defined according to both Kabat et al [139] and Chothia et al [141] based on sequence and structural variability, respectively. Two amino acids are considered to contact one another in a protein structure if both contain a pair of atoms separated by 6Å or less [138]. X-ray crystallography and computational carbohydrate threading studies on mouse JAA-F11

[142] identified amino acid residues within 5-6Å from the binding site. These amino acids as well as two cysteine residues at position L23 and L88 of the light chain that are important for conformational structure were included in the final inclusive definition of the CDRs used in JAA-F11 humanization.

5 **[0058]** Selection of human frameworks

[0059] Three different approaches were applied to select the human acceptor antibody framework for the heavy and light JAAF11 variable region chains. Three variants of the heavy chain (H1, H2, H3) and 3 variants of the light chain (L1, L2, L3) were designed. Any of the 3 heavy chains (VH) can pair with any of the 3 light chains (VL) to create a total of 9 possible hJAA-F11 VH/VL combination variants.

10 **[0060]** To create these variants, a BLAST search was performed separately comparing the VH and VL sequence of the mJAA-F11 (mouse antibody) against human immunoglobulin sequences. The top 10 most homologous human VH and VL sequences were aligned with the corresponding variable regions of mJAA-F11 using the Seaview Sequence Alignment program.

15 **[0061]** For the first variants, H1 and L1, the amino acid sequence of the framework regions were chosen based on the amino acid most often seen at each position in the top 10 most homologous human IgG variable sequences. For variants H2 and L2, the amino acid sequence of the framework regions were chosen as follows: if the amino acid in any of the top 10 human sequences matched the corresponding amino acid in the mouse immunoglobulin then that amino acid was chosen, in the remaining positions the amino acid most often seen in the human sequences was chosen. For the third variant, H3 and L3, the amino acid sequence of the framework region contained 3 mouse amino acids on either side of each CDR while the rest of the sequence is made up of amino acids most often seen in the top 10 human sequences.

20 **[0062]** A chimeric JAA-F11 was also constructed which consisted of the entire mouse JAA-F11 variable region attached to human constant regions. The chimeric was made to verify that the correct mouse variable regions have been cloned and sequenced and also to serve as a positive control when evaluating the humanized JAA-F11 antibodies. The chimeric is expected to maintain the same binding characteristics as the mouse JAA-F11, while having the human constant region.

25 **[0063]** Assessment of models of hJAA-F11

[0064] The originally proposed hJAA-F11 constructs were assessed for conformational effects on the binding site. The three heavy chain variants (H1, H2, H3) had

a replacement for alanine of arginine at position 72 that could potentially cause serious steric interaction with surrounding amino acids. A leucine at position 51 for the light chain variants (L1, L2, L3) was replaced with arginine which could result in steric clashes with surrounding amino acids side chains. The arginine residue was removed and replaced with the original mouse JAA-F11 framework residue, alanine 72 (VH) and leucine 51 (VL) as shown in Figures 1 and 2. The alignment comparisons between the mouse JAA-F11 heavy and light variable amino acid sequences and the hJAA-F11 constructs are as shown in Figures 1 and 2. Those skilled in the art will also recognize the distinctions in amino acid sequences presented in H2a, H3a, L2a, and L3a, as compared to the other H and L sequences.

10 [0065] Immunogenicity prediction of hJAA-F11 variants

[0066] The predicted immunogenicity using the T20 score of our humanized variants (CDR grafted) is anticipated to be very low (Table 1). T20 score is used to measure the “humanness” of monoclonal antibody variable region sequences. This scoring system was developed by Gao et al [*Monoclonal antibody humanness score and its applications*, 2013, BMC Biotechnology, 13:55], using a database of over 38,000 human antibody sequences. In this method, a protein BLAST of this database is performed and the test humanized Ab is compared against these human sequences. The humanized antibody is compared to the top 20 human Ab BLAST matches and scored for similarity to these sequences. The highest possible score is 100 (most human-like).

20 [0067] Validation of this method was shown when Gao et al tested this immunogenicity scoring method on more than 90 antibodies that have been used clinically in humans, and found that antibodies with T20 scores of FR & CDR sequences above 80 were not immunogenic while T20 scores for FR sequences only, which were above 85 were not immunogenic. Using the T20 values of the current hJAA-F11 variants, very low immunogenicity is expected in patients. This shows that the CDR-grafted antibodies are more human and expected to be less immunogenic than the chimeric, that the H1L1 variant is the most human of the CDR-grafted variants, and that all the hJAA-F11 variants are expected to have low immunogenicity.

[0068] **Table 1.** Assessment of immunogenicity of the hJAA-F11 constructs. * score > 80 for FR and CDR sequences is not immunogenic in humans. ** score > 85 for FR sequences only is not immunogenic in humans.

JAA-F11 variants	T20-score (FR & CDR)		T20-score (FR)	
	Heavy Chain	Light Chain	Heavy Chain	Light Chain
Chimeric	67	78	77	86
H1L1	83	88	97	96
H2L2	76	87	90	95
H3L3	78	86	90	93

[0069] Expression and production of humanized and chimeric JAA-F11 variants

5 [0070] The hJAA-F11 and chimeric VH and VL genes were cloned into two different mammalian expression vectors containing the human IgG1 heavy chain constant region (6307 pAH) and the human kappa light chain constant region (6714 pAN) respectively to produce plasmids containing complete IgG1 and kappa genes. The correct sequence and orientation of the each of cloned VH and VL regions in 6307 pAH and 6714 pAN expression
10 vectors were verified by sequencing.

[0071] After stable co-transfection of the two vectors into CHO-K1 cells, stable clones expressing hJAA-F11 or chimeric were selected on the basis of neomycin resistance using the antibiotic G418 and the expression of chimeric or hJAA-F11. Screening of the hJAA-F11 candidates was performed using an ELISA for anti-TF-Ag antibody as using
15 established techniques. The clones from co-transfection of each humanized JAA-F11 H/L combination variants and the chimeric JAA-F11 that showed the highest reactivity to TF-Ag were chosen for further characterizations as described herein. Humanized JAA-F11 constructs and chimeric JAA-F11 were purified from culture supernatants by protein A column chromatography individually. A chimeric antibody, as well as H1L1, H2L2, H3L3
20 and H2L3, H2aL3a, and H3aL3a, have been produced. Given the benefit of the present disclosure the skilled artisan can readily produce the remaining antibody H and L chain combinations that are encompassed in the present invention.

[0072] Chemical specificity analysis by glycan array

[0073] After the initial screening, the chemical specificity of the hJAA-F11 and
25 chimeric JAA-F11 variants were determined using a glycan array by the Consortium for Functional Glycomics. These data were compared to data obtained for mouse JAA-F11. The glycan array analysis is an indirect immunofluorescent method to determine glycan binding reactivity of lectins and antibodies. An earlier glycan array was utilized to demonstrate the chemical specificity of mouse JAA-F11. This method was used to analyze the reactivity of

the antibody candidates with 610 different glycans. The hJAA-F11 H1L1, H2L2, H3L3, and the chimeric antibody showed the same fine specificity of binding as the mouse antibody including the limitation of binding to the Gal β 1-3GalNAc- α (TF-Ag) linked structures, and the lack of binding with α 2-3 sialylated structures. The glycan array shows that out of 610
 5 glycans, the humanized antibodies, the chimeric, and mouse JAA-F11 only bind TF-Ag and four other TF-Ag containing saccharide structures. The four additional saccharides that mouse JAA-F11, the chimeric and hJAA-F11 constructs bind to out of the 440 or 610 saccharides tested should not be biologically problematic for tumor targeting with JAA-F11. Neu5Ac α 2-6(Gal β 1-3GalNAc β , GlcNAc β 1-6(Gal β 1-3)GalNAc β and Gal β 1-4GlcNAc β 1-
 10 6(Gal β 1-3)GalNAc β unsubstituted have only been found in excreted fluids, in cancer or other disease states. The Neu5Ac β 2-6 Gal β 1-3GalNAc is not a natural structure and is not in humans.

[0074] Specificity of the humanized JAA-F11 constructs appears to be the same or even improved when compared to the chimeric and mouse antibodies. For example, H2L3
 15 shows statistically ($p < 0.05$ by ANOVA) less binding with any addition to TF-Ag, and H1L1 and H2L2 do not permit a disaccharide to be added to the C-6 hydroxyl of the GalNAc and this is statistically different ($p < 0.05$) from the other antibodies.

[0075] The lack of binding with more than 600 negative saccharides on the array indicates likely targeting ability for all these antibodies. Important examples of closely
 20 related saccharides which do not bind are the Gal β 1-3GalNAc-beta linked structures, indicating that the antibody would bind to tumor tissues and not to the central nervous system GM1 ganglioside, the asialo-GM1 of NK cells, the GD1 of glycolipids or to asialo GM1 of peripheral nerve tissue (these are beta-linked). The well-known elongation of TF-Ag on normal tissues is carried out by β 1-3N-acetylglucosaminyl-transferase adding GlcNAc β 1-3
 25 on the Gal, forming GlcNAc β 1-3Gal β 1-3GalNAc α -Sp8, or by a 2-3 sialyltransferase forming Neu5Ac α 2-3Gal β 1-3GalNAc (sialyl-TF) or with a second sialyltransferase forming Neu5Ac α 2-3Gal β 1-3(Neu5Ac α 2-6)GalNAc (disialyl-TF) all of which do not bind the mouse, chimeric or the humanized JAA-F11. Thus normal tissue binding is not expected for JAA-F11 or the hJAA-F11 constructs.

[0076] Relative affinity analysis

[0077] The relative binding affinities of the four hJAA-F11 and chimeric antibodies to TF-Ag were determined by comparing the ability of the mouse antibody to compete with the humanized antibodies in an enzyme immunoassay. In this assay, the 3 μ g of each humanized Ab is mixed with serial dilutions of the mouse anti TF-Ag antibody (mJAA-F11).

Binding of the human anti-TF-Ag to the TF-Ag coated plate is measured using a species specific anti-human IgG. The amount of mouse mJAA-F11 required to inhibit 1 µg of hJAA-F11 to 50% is extrapolated and taken as a measure of relative affinity. The higher the amount of mouse antibody required for inhibition, the higher the relative affinity of the antibody.

- 5 **[0078]** The results are summarized in Figure 3 and Tables 2 and 3. Table 2 shows the half maximal inhibitory concentration of mouse JAA-F11 required (IC₅₀) by the different antibodies to compete with 1 µg of this humanized antibody. H2L2 is shown with the highest relative affinity to TF-Ag among the antibodies, with an IC₅₀ 2.31 µg of mouse antibody. Table 7 shows the p-values calculated for each antibody against each other ANOVA – Tukey
- 10 post-hoc test. It can be seen that this improved affinity difference of H2L2 antibody is statistically significant (p<0.05), when compare to each of the hJAA-F11 and chimeric antibody, except for H2L3 where it approached significance (p=0.057) (**Table 3**). H2L3 is not significantly different from H1L1, H2L2, H3L3 and chimeric antibody. The relative affinities of H1L1, H3L3 and the chimeric antibody are not significantly different from each
- 15 other.

[0079] **Table 2.** Relative affinity of hJAA-F11 and chimeric JAA-F11 antibodies to TF-Ag.

IC ₅₀ , Relative Affinity for TF-Ag: µg mJAA-F11 required for 50% inhibition of 1 µg of each hJAA-F11 or chimeric				
H1L1	H2L2	H2L3	H3L3	Chimeric
1.11 ± 0.18 ^a	2.31 ± 0.20	1.64 ± 0.39 ^a	1.28 ± 0.41 ^a	1.14 ± 0.30 ^a

^aThe average of three independent experiments is shown ± 1 SD.

- 20 **[0080]** **Table 3.** ANOVA analysis was performed on replicates of the IC₅₀ assay to compare the differences between the IC₅₀ of the different antibodies. P<0.05 significant (shaded).

P-value (Tukey)	H2L2	H2L3	H3L3	H1L1
H2L2		0.057	0.002	0.00
H2L3	0.057		0.330	0.071
H3L3	0.002	0.33		0.908
H1L1	0.00	0.071	0.908	

Chimeric	0.00	0.093	0.948	1.00
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[0081] Analysis of biological reactivity, specificity and activity of hJAA-F11

[0082] 3hJAA-F11 and chimeric JAA-F11 antibodies bind to human tumor cell lines.

[0083] Binding of hJAA-F11 and chimeric JAA-F11 antibodies to various human
5 breast tumor cell lines was assessed using whole cell ELISA. The TF-Ag positive mouse
mammary tumor 4T1 cell line served as a positive control while the P3-X63-Ag8 Myeloma
cell line served as the TF-Ag negative control. The assays were performed on three different
days and 3 to 4 cell lines were assayed at a time in quadruplicates with the control cell lines.
A cell line was considered positive if the relative binding due to hJAA-F11 or chimeric JAA-
10 F11 (at 50µg/mL) was significantly higher ($p \leq 0.05$) than that of the TF-Ag negative control
myeloma. Results are shown in Figure 4.

[0084] The cancer cell lines tested consist of the different subgroups of breast
cancers, hormone receptor positive or negative, Her2-positive or negative, and triple
negative. The estrogen receptor (ER) and progesterone receptor (PR) positive cell lines
15 tested were CAMA-1 and HCC-1428. Two Her2/neu receptor positive cell lines, HCC-1419
and AU-565 were tested. The triple negative breast cancer (TNBC) cell lines tested were
HCC-70, MDA-MB-231, MDA-MB-468, DU-4475, AND BT-549. One cell line, MDA-kb2,
expresses androgen receptor but is estrogen receptor negative. These breast cancer cell lines
have been examined previously using mouse JAA-F11 and all were positive for TF-Ag
20 expression. In this disclosure, results show that the hJAA-F11 and chimeric JAA-F11
antibodies bind to all 10 breast cancer cell lines examined, confirming the expression of TF-
Ag on these cell lines. Among the different breast cancer subgroups, currently there is no
targeted therapy for treating TNBC patients. The data obtained indicate a therapeutic role of
TF-Ag in targeting the aggressive TNBC and the use of hJAA-F11 antibodies to treat and
25 increase survival of breast cancer patients regardless of receptor status. Table 4 summarizes
the types of breast cancers and the expression of TF-Ag of each cell line tested.

[0085] **Table 4. Table of breast cancer cell lines tested in whole cell ELISA.**
Table shows hormone receptor, Her2/neu receptor, and expression of TF-Ag. Triple negative
(bold), ER/PR positive (*italics*), and HER2-positive (***bold and italics***). **MDA-kb2*** is
30 negative for estrogen receptor but expresses androgen receptor.

Cell Line	Estrogen Receptor	Progesterone Receptor	Her2/neu Receptor	TF-Ag Expression
HCC-70	-	-	-	+
BT-549	-	-	-	+
MDA-MB-231	-	-	-	+
MDA-MB-468	-	-	-	+
DU-4475	-	-	-	+
<i>CAMA-1</i>	+	+	-	+
<i>HCC-1428</i>	+	+	-	+
<i>HCC-1419</i>	-	-	+	+
<i>AU 565</i>	-	-	+	+
<i>MDA-kb2*</i>	-	-	-	+

[0086] Humanized JAA-F11 inhibits proliferation of cancer cells in vitro

[0087] Since some anti-TF-Ag antibodies have been shown to cause tumor cell proliferation, it was important to determine the effect of the different constructs on cell proliferation. The MTT assay which measures metabolic activity was chosen as a surrogate for the tritiated thymidine direct proliferative assay. The effect of the humanized, chimeric, and mouse JAA-F11 antibodies on cancer cell growth compared to a control without antibody was determined at 4 ug/ml of the antibodies by known methods. The data is shown in Figure 5. The chimeric antibody and the humanized JAA-F11 antibodies except for H3L3 caused a small (~6-11%) but statistically significant inhibition of tumor cell growth. No enhancement of cell proliferation was seen.

[0088] Humanized JAA-F11 induces ADCC

[0089] Antibody-dependent cell mediated cytotoxicity (ADCC) was performed on human breast tumor cell lines using the CytoTox 96 Non-Radioactive Cytotoxicity assay; Promega, Madison, WI) based on the lactate dehydrogenase (LDH) release assay according to standard techniques with the following adaptations. Fresh human peripheral blood mononuclear cells were isolated from EDTA anti-coagulated whole blood for use with the humanized antibody and 4T1 mouse breast cancer cells in an effector to target ratio of 100:1.

The release of LDH was used to quantify cytotoxicity.

[0090] Figure 6A shows the amount of ADCC facilitated by the mouse, chimeric, H1L1 and H3L3 antibodies compared on a human breast cancer cell lines, BT 549. The experiments were repeated more than three times. In all experiments, H2L2 and H3L3 showed significantly more ($p < 0.5$) ADCC than either the chimeric or the mouse antibody in the human cell lines. More than 20% of the TF-Ag bearing BT549 and HCC70 target cells were lysed by H2L2 and H3L3 antibody at 200 $\mu\text{g/mL}$ at a 100:1 effector to target (E:T) ratio. Likewise, in all experiments on the mouse 4T1 cell line, although all the antibodies tested induced less than 10% ADCC, H3L3 antibody showed significantly more ($p < 0.05$) ADCC than either the chimeric or the mouse antibody. This indicates that currently the H2L2 and H3L3 antibody is the best choice for immunotherapy. In contrast, mouse JAA-F11 did not show statistically significant ADCC capabilities in any of the tests. The ADCC results from three individual PBMCs donors are as shown in Figures 6B, 6C, and 6D.

[0091] Humanized JAA-F11 does not induce CDC

[0092] The ability of hJAA-F11 and chimeric antibodies to mediate complement dependent cytotoxicity (CDC) was determined by the lactate dehydrogenase (LDH) release assay using human breast cancer cell HCC-1428 cells as the target cells. The LDH positive control provided with the reagent kit was used as a positive control and showed lysis. The mouse JAA-F11, chimeric, H1L1, H3L3, H2L3 antibodies did not induce complement-dependent cytotoxicity as no lysis occurred as shown in Figure 7.

[0093] Humanized JAA-F11 internalizes into cancer cells

[0094] Internalization of humanized and chimeric JAA-F11 into 4T1 breast tumor cells was determined by two methods, an enzyme immunoassay with surface binding measured and compared after incubating the cells at either 4°C or 37°C, and an immunofluorescent microscopy method with LAMP-1 (lysosomal associated membrane protein, a lysosomal marker) and DAPI staining for nuclear staining. In the enzyme immunoassay, as shown in Figure 8, mouse, H2L2, chimeric, H2L3, and H1L1 antibodies internalized significantly with p-values of 0.001, 0.002, 0.001, 0.002, and 0.014 respectively. However, H3L3 antibody did not show significant internalization ($p=0.16$) and this is expected as H3L3 showed significantly more ($p < 0.5$) ADCC than the chimeric or the mouse JAA-F11.

[0095] In immunofluorescent experiments, in agreement with the enzyme immunoassay mouse, chimeric, H2L2, H2L3 antibodies showed internalization and co-localization with the LAMP-1 and H3L3 showed membrane staining that did not internalize.

This data confirmed that obtained using the surface enzyme immunoassay. One would expect that the antibodies that showed higher ADCC activity would show lower internalization since antibody presence on the cell surface is needed for ADCC function. Internalization assays have been performed on the hJAA-F11, chimeric and mJAA-F11. H3L3 showed agreement with this expectation, having low amounts of internalization but relatively high ADCC. H1L1, H2L3, chimeric and mJAA-F11 all showed statistically significant internalization and did not perform ADCC well. An unexpected result was observed for H2L2 antibody which induced high ADCC similar to H3L3 but showed the highest internalization percent activity compared to the chimeric and other humanized antibodies.

[0096] The immunofluorescent internalization experiment was initially performed using 5µg/mL of antibody and was repeated using antibody concentrations at 0.1µg/ml, and the antibodies that internalized did so even at this low concentration. The above data indicates that due to high percentage of internalization, and the rapid rate of this internalization, the chimeric, the H2L2 and H2L3 constructs have the potential to be used as antibody-drug conjugates. The glycan specificity, relative affinity internalization, ADCC, and CDC data are summarized in Table 5 below.

[0097] **Table 5.** Summary of internalization, ADCC and glycan specificity of mouse, humanized and chimeric JAA-F11.

JAA-F11 variants	ADCC	CDC	Internalization	Increased Specificity for TF-Ag	Affinity Rank
Mouse	No	No	Yes	No	
Chimeric	No	No	Yes	No	3
H1L1	Yes	No	No	Yes	3
H2L2	Yes	No	Yes	Yes	1
H2L3	No	No	Yes	Yes	2
H3L3	Yes	No	No	No	3

[0098] MicroPET Imaging

[0099] MicroPET imaging was performed serially on one mouse each that was injected with 124Iodine-hJAA-F11 H2L2 antibody, and with free 124Iodine. Imaging was performed at 24, 48, 72, 96, 168, and 192 hours. **Figure 9** shows the coronal view of a

mouse injected with ¹²⁴Iodine-hJAA-F11 H2L2 antibody at different time points, while Figure 10 is of a control mouse injected with free ¹²⁴Iodine. Uptake of the radiolabeled antibody by the tumor was observed at 24, 48, 72, and 96 hours after injection. Uptake of antibody was also seen in the spleen at 48 hours (Figure 9). The negative control mouse bearing the 4T1 TF-Ag positive tumor, which received free ¹²⁴Iodine only showed that there was no localization to any organs or tumor except to the thyroid throughout the study (Figure 10).

[0100] The fully murine JAA-F11 has potential for passive humoral immunotherapy and drug conjugate therapy in breast cancer patients. However, as the use of mouse antibodies in humans had been shown to be limited by the development of human anti-murine antibody (HAMA) responses and a short half-life in patients, humanization is desirable to decrease the immunogenicity of the mouse JAA-F11 antibody and also allow it to remain in circulation for a longer time.

[0101] It will be recognized from the foregoing description of results that this disclosure provides, among other aspects, a description of a novel approach to humanization of the mouse JAA-F11 mAb, and comparisons and contrasts between the mouse, chimeric and humanized JAA-F11 antibodies. The mJAA-F11 was humanized in part by CDR grafting. As will be recognized by those skilled in the art, traditionally, humanization by CDR-grafting uses a single human antibody acceptor framework [Jones, et al. Replacing the complementarity-determining regions in a human antibody with those from a mouse. 1986. Nature 321: 522– 525]. Thus, the human framework sequences is chosen from existing human germline genes. In contrast, in the present disclosure, three novel and different approaches were used to select the human acceptor antibody framework for the heavy and light JAAF11 variable region chains, generating three variants of the heavy chain (H1, H2, H3) and 3 variants of the light chain (L1, L2, L3). As discussed above, any of the 3 heavy chains (VH) can pair with any of the 3 light chains (VL) to create a total of 9 possible hJAA-F11 VH/VL combination variants. A chimeric JAA-F11 which has the whole mouse variable region and a human IgG1 and kappa constant regions was also generated, and used as a control.

[0102] One concern was whether the approach used in defining the CDRs and FR regions would reduce the immunogenicity of the humanized antibodies. The T20 score method developed by Gao et al [*Monoclonal antibody humanness score and its applications*. 2013. BMC Biotechnology, 13:55], and validated with analysis of 90 antibodies that have been utilized in patients, was used to predict the immunogenicity of the humanized JAA-

F11 variants. The T20 scores of the humanized JAA-F11 variants are all higher (improved) (>85) than the chimeric JAA-F11 (<85), indicating that humanization of heavy and light variable chains by all three of the present methods created hJAA-F11 variants that will be less immunogenic than the chimeric variant.

5 [0103] We humanized the mouse JAA-F11 and five antibodies have been produced using CHO-K1 cells, a chimeric and 4 hJAA-F11 constructs, H1L1, H2L2, H3L3 and H2L3. Thus, given the benefit of the present disclosure, the skilled artisan could make any use the other four possible VH and VL combinations.

[0104] Antibody humanization by CDR-grafting can cause a decrease in affinity or
10 loss of antigen binding, which may be due to the CDRs conformation and antigen binding site being negatively affected by changes in some framework amino acid residues in the humanized antibody. CDRs are made up of residues that interact with the framework regions and other CDRs. In addition to the interaction of CDR amino acids with sequentially nearby amino acids, some framework residues that can affect antigen binding directly or indirectly
15 include the Vernier zone residues and residues at the VL/VH interface. Vernier zone residues are residues in the β -sheet framework underlying the CDRs which provide a foundation for the conformation of loop structures. Vernier residues on the light chain are at position 2, 4, 35, 36, 46, 48, 49, 64, 67, 69, and 71, while on the heavy chain they are identified at position 2, 27, 28, 29, 30, 47, 48, 49, 67, 69, 71, 73, 78, 93, 94, and 103. Residues at the VL/VH
20 interface have been identified by Chothia and co-workers [Chothia C, et al. The packing of variable domains. 1989. J. Mol. Biol. 186:651-63], and are at position 34, 36, 38, 44, 46, 87, 89, 91, 96, and 98 of the light chain, and at position 35, 37, 39, 45, 47, 91, 93, 95, 100-100K, and 103 for the heavy chain.

[0105] We showed that the approaches used in this disclosure for selecting the human
25 FR and CDRs did not negatively alter the affinity of the humanized JAA-F11 for the TF-Ag, relative to the chimeric and mouse JAA-F11. When defining the CDRs, residues within 5 Å to 6 Å of the binding site had been included to ensure that affinity and specificity to TF-Ag is maintained. These residues are at position H31, H32, H33, H35, H50, H52, H53, H54, H95, H96, H97, H98, and H100 on the heavy chain, and at positions L27d, L28, L30, L32, L34,
30 L50, L89, L91, L92, and L96 on the light chain. However, from the relative affinity studies, whereas all the humanized antibodies maintain the affinity for the TF-Ag, we observed differences in affinity between the antibodies. The relative affinity studies revealed that H2L2 antibody is has the highest affinity antibody, followed by H2L3 antibody. H2L2 antibody has the highest relative affinity to TF-Ag among the antibodies, requiring over 2 mg

of mouse antibody for the inhibition of 1 mg of H2L2 antibody. The H2L3 antibody also had higher affinity than H1L1 and the chimeric antibody although not significantly, while the relative affinities of H1L1, H3L3 and the chimeric antibody to TF-Ag are not significantly different from each other.

- 5 [0106] These improvements in affinity can be attributed the changes in some residues between the different humanized variants. The differences between the variants are listed below in Table 6 and 6B.

Table 6A. Differences between heavy variable H1, H2, H3 and mouse sequences.

POSITION	H1	H2	H3	Mouse
5	Valine	Leucine	Valine	Leucine
11	Valine	Leucine	Valine	Leucine
38*	Arginine	Arginine	Lysine	Lysine
48***	Methionine	Methionine	Isoleucine	Isoleucine
66*	Arginine	Arginine	Lysine	Lysine
67***	Valine	Valine	Alanine	Alanine
69***	Methionine	Leucine	Methionine	Leucine
73**	Threonine	Lysine	Threonine	Lysine
75	Isoleucine	Serine	Isoleucine	Serine
82b	Arginine	Serine	Arginine	Serine
83	Arginine	Threonine	Arginine	Threonine
85	Aspartate	Glutamate	Aspartate	Glutamate
108*	Leucine	Threonine	Threonine	Threonine
109*	Valine	Valine	Leucine	Leucine
12	Lysine	Lysine	Lysine	Alanine
20	Valine	Valine	Valine	Methionine
40	Alanine	Alanine	Alanine	Arginine
76	Serine	Serine	Serine	Threonine
81	Glutamate	Glutamate	Glutamate	Glutamine

10 ** indicates Vernier zone position.

* indicates that this position is within 4 amino acids of our modified CDR.

Table 6B. Differences between light variable L1, L2, L3 and mouse sequences

POSITION	L1	L2	L3	Mouse
1	Aspartate	Aspartate	Aspartate	Glutamic Acid
2 **	Valine	Valine	Valine	Leucine
7	Serine	Threonine	Serine	Threonine
14	Threonine	Threonine	Threonine	Asparagine
17	Glutamine	Glutamine	Glutamine	Aspartic Acid
18	Proline	Proline	Proline	Glutamine
36***	Phenylalanine	Phenylalanine	Tyrosine	Tyrosine
37*	Glutamine	Glutamine	Leucine	Leucine
39*	Arginine	Arginine	Arginine	Lysine

45	Arginine	Arginine	Arginine	Lysine
81	Glutamic Acid	Glutamic Acid	Glutamic Acid	Aspartic Acid
83	Valine	Valine	Valine	Leucine

** indicates Vernier zone position.

* indicates that this position is within 4 amino acids of our modified CDR.

[0107] As mentioned above, framework residues affecting antigen binding include the

5 Vernier zone and VL/VH interface residues. When comparing the humanized constructs, the mAbs of this disclosure retain most of the Vernier zone and VL/VH interface residues that may be important for the binding site conformation, however some changes were made to create the less immunogenic humanized variants. The amino acids that were changed that were in the Vernier zone or in the VH/VL interface, and changes have been made to amino

10 acids within 4 amino acids (sequentially) to the CDRs, are as indicated by the asterisks. There are four Vernier zone positions on the heavy chain sequence that differ between the three constructs, positions 48, 67, 69, and 73. H2 retains mouse residues but differs between H1 and H3 constructs at eight positions 5, 11, 69, 73, 75, 82b, 83, and 85. Of these eight residues, two are Vernier zone residues, at positions 69 and 73. At the same time, H2 and H1

15 have substitutions at two Vernier zone positions, that is, at residue 48 and 67, which are maintained as mouse residues on H3. Since there is no difference in affinity between H1L1 and H3L3, this suggest that the Vernier zone positions at 48 and 67 may not likely contribute to the affinity difference as they are maintained in H3, but changed in H1. H2, however, retains the mouse Vernier zone residues at positions 69 and 73, unlike H1 and H3, suggesting

20 the changes at these two Vernier zone positions may contribute to the differences in affinity. These two Vernier zone residues that underlie the CDR2 may have enhanced H2 binding compared to H1 and H3. Of the amino acids different between the humanized constructs, positions 69 and 108 are the closest to the binding site. The amino acid in position 69 in H2 (like in the mouse antibody) is Leucine while both of the other humanized constructs have

25 Methionine. Of the differences between H2 and the mouse antibody, changes at amino acids 38, 48, 66, 67, and 109 are nearest the CDRs and also may affect the affinity.

[0108] Since H2L2 has a higher affinity than H2L3 the comparison of L2 and L3 may help further delineate the reason for this difference. There were no changes for the VL/VH residues and most of the Vernier zone residues on the light chain were maintained among the

30 3 humanized constructs, except for Vernier positions 2 and 36. Unlike L3, but like the mouse antibody, the higher affinity L2 has a Phenylalanine rather than Tyrosine at Vernier position

36. The amino acids at position 36 and 37 may influence the conformation in a favorable way, since they are within 5 Å of the binding site, and are part of the amino acids in positions 24-35 which were found to be important in the maintenance of the alpha linkage requirement at the reducing terminus of the GalNAc.

5 [0109] Many normal tissues have non-identical but very similar structures to TF-Ag such as the Galβ1-3GalNAc-beta linked structures that are normally found in the central nervous system, on NK cells, and peripheral nerve tissue, thus maintenance of fine specificity is the key feature in this humanization. The hJAA-F11 H1L1, H2L2, H3L3, and the chimeric antibody retained the same or improved fine specificity of antigen binding as compared to the
10 mouse antibody including the limitation of binding to the Galβ1-3GalNAc-α (TF-Ag) linked structures, and the lack of binding with α2-3 sialylated structures based on the glycan array. Specificity of the humanized JAA-F11 constructs appears to be the same or even improved when tested against over 600 different saccharides. The mouse JAA-F11 and all humanized constructs react with just 5 saccharides, TF-Ag and 3 trisaccharides with one additional sugar
15 1-6 linked to the GlcNAc (NeuAc in 2, and GlcNAc in the 3rd) or with one with 2 additional sugars GalGlcNAc linked 1-6 to the GalNAc. For example, H2L3 showed less binding on all 4 other saccharides that bind to JAA-F11, while H1L1 and H2L2 have no binding to the tetrasaccharide that binds to JAA-F11, when compared to the chimeric and mouse antibodies. Although these 4 other saccharides that bind to JAA-F11 are not known to be expressed on
20 normal tissues, increased specificity for the target structure is always desirable. This demonstrates the likely targeting ability for all these antibodies in human therapy. Since H2L3 has increased binding specificity as shown by less binding to 3 of the 4 saccharides compared to the other constructs, the difference between H2 and H3 may have cause this difference. The differences between variants that have already been discussed in the affinity
25 section may have been the reason for this improved specificity. H2L3 differs from H2L2 in its decreased binding to NeuAcalpha2-6(TF-Ag) and GlcNAcbeta2-6(TF-Ag).

[0110] Without intending to be constrained by theory, it is believed the reason H1L1 and H2L2 do not bind at all to the tetrasaccharide may have to do with a shared difference between these antibodies when compared to the H3L3 and mouse antibodies. H1 and H2
30 both have a Methionine at position 48, which is both a Vernier zone position and is sequentially within 4 amino acids of the CDR. This methionine is rather than the isoleucine of the mouse and H3 antibodies.

[0111] In the present disclosure, whole cell EIA results highlight the ability of the hJAA-F11 and chimeric JAA-F11 antibodies to bind triple-negative breast cancer cell lines.

The data obtained suggest a potential therapeutic role of TF-Ag in targeting all breast cancer but especially the aggressive triple-negative breast cancers (TNBC) as there are no targeted therapies for these types of cancers at present.

[0112] Some lectins and antibodies which bind TF-Ag cause increased proliferation of TF-Ag expressing tumor cells. This is thought to usually be linked to whether the lectin or antibody binds both the alpha and beta anomers at the reducing end (proliferation causing) or just the alpha anomer (inhibitory). While mouse JAA-F11 only binds the alpha anomer and did not cause proliferation of tumor cells, it is important to determine if the humanized constructs had this effect on tumor cells. Humanized JAA-F11 and chimeric antibodies do not cause proliferation of cancer cells in vitro, on the contrary, similar to the effect of mouse JAA-F11 a small (~6 – 11%) but significant inhibition of mouse 4T1 and human cancer cells growth was observed.

[0113] As ADCC is one of the major mechanisms by which antibodies eliminate tumor cells, and is important for an antibody to be utilized for cancer therapy, the ability of the humanized JAA-F11 variants to induce ADCC was examined. ADCC activity was seen with some but not all of the humanized constructs, and was seen with PBMCs of three individuals. Both H2L2 and H3L3 antibody induced significantly more ADCC than either the chimeric or the mouse antibody of both mouse 4T1 and human breast cancer cell lines, indicating that currently the H2L2 and H3L3 antibody may be the preferred choices for direct passive immunotherapy. The mouse JAA-F11 did not show any statistically significant ADCC abilities in any of the tests. Another effector function that antibodies used for killing tumor cells is CDC. None of the humanized, chimeric and the mouse JAA-F11 antibodies induced CDC, but this lack of CDC activity does not preclude its use as an immunotherapeutic, indeed Herceptin has been found to induce only a nominal amount of CDC, and this is not one of its modes of action. Data with Rituxan actually shows that complement binding inhibits NK cell binding and decreases efficacy.

[0114] Antibodies can potentially be used to carry drugs or toxins in the form of antibody-drug conjugates into the cell. Therefore, we assessed the ability of the humanized JAA-F11 variants to internalize after binding to TF-Ag on cancer cells. Mouse JAA-F11 had previously been shown to internalize within 1 hour. Using an enzyme immunoassay, we showed that mouse, H2L2, chimeric, H2L3, and H1L1 antibodies internalize significantly into mouse 4T1 breast cancer cells whereas H3L3 antibody did not show significant internalization. A live cell fluorescent microscopy method confirmed the results obtained from the enzyme immunoassay. We expected the antibodies that showed higher ADCC

activity would show lower internalization and results from both internalization assays show agreement with this expectation in that H3L3 antibody exhibited more ADCC than either mouse or chimeric antibodies but did not internalize much. However, H2L2, even though it performed well in the ADCC assay, internalizes well. The mechanism of how both of these
5 can occur with the same antibody is not yet understood, but one possibility is that different production rates in the CHO cells can result in different fucosylation rates, so if H2L2 is less fucosylated it may perform better at ADCC during the time it is on the cell surface even though it is internalized. These results show that the chimeric, the H2L2 and H2L3 constructs have the potential to be used as antibody-drug conjugates.

10 [0115] The mouse 124I-JAA-F11 antibody has been shown to localize to TF-Ag breast tumors in mice. The labeled JAA-F11 antibody remained bound to the 4T1 tumor for at least 20 days and 24 days for a human triple negative breast tumor implying that JAA-F11 could be used to find metastasis and to treat the TF-Ag bearing tumor. To test whether iodine-124 labeled humanized antibody will localize to human breast tumors in mice, the
15 humanized antibody with the highest affinity, H2L2 variant, (which internalizes and performs ADCC) was utilized. The imaging showed preferential tumor uptake, with uptake in the thyroid and in the spleen that was blocked in a later experiment with cold rabbit immunoglobulin. MicroPET imaging showed that the radiolabeled humanized antibody was taken up by the tumor within 24 hours and could be seen until 96 hours. Such
20 radiolocalization in patients could be used to find metastasis and also used to determine if there is any off target binding in a particular patient prior to either direct passive immunotherapy or antibody-drug conjugate therapy.

[0116] The Thomsen-Friedenreich Antigen (TF-Ag) is present in more than 80% of several human carcinomas including breast cancers. It has a functional role in tumor
25 adhesion and metastasis so the ability of an antibody to target TF-Ag suggests possible use as an immunotherapeutic, as an antibody-drug conjugate for killing cancer cells and for inhibiting metastasis. The JAA-F11 antibody with its unique high specificity for the TF-Ag holds great potential as a passive anti TF-Ag response for treating breast and other cancers. In our humanization approach, we have maintain this unique specificity and have likely
30 enhanced it with the humanized JAA-F11 variants.

Example 2

[0117] This Example provides a description of the materials and methods used to obtain the results described herein.

[0118] The JAA-F11 CDRs were previously predicted. We performed cloning and sequencing of mouse JAA-F11 antibody to confirm the amino acid sequences of both the heavy and light chain variable regions.

[0119] To have therapeutic benefits in humans, an antibody should maintain its specificity for its target antigen and at the same time not generate anti- mouse immune reactions. We selected the CDR grafting approach to retain the CDRs of the mouse JAA-F11 so as to maintain specificity and affinity for TF-Ag α . The CDRs were selected using Chothia and Kabat methods as well as the x-ray crystal structure and computational carbohydrate threading.

10 [0120] To choose a human acceptor antibody framework for each of the heavy and light JAAF-11 variable region chain, a Protein BLAST® (BLASTP) search was performed at blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins against the entire non-redundant human (Homo sapiens) Genbank database to identify the most homologous human antibodies to mouse JAA-F11. All non-Homo sapiens protein sequences, humanized antibodies and phage display sequences were eliminated from the BLASTP results. The resulting top ten most homologous sequences to each of the heavy and light variable chain sequence of JAA-F11 were selected as potential human acceptor antibody frameworks for humanized JAA-F11 (hJAA-F11). The SeaView Sequence Alignment program was used to align the ten potential human acceptor variable heavy or light frameworks sequences with mouse JAA-F11 variable heavy or light region sequences.

[0121] Three heavy (H1, H2, and H3) and three light (L1, L2, and L3) variable regions were constructed from the protein BLAST selection of the 10 most similar human immunoglobulin sequences. The final CDRs from mouse JAA-F11 were then grafted onto human framework regions by one of three different methods, creating 3 different heavy chains and 3 different light chains. In the first method, (a) Heavy H1 and Light L1 FR sequences were designed to contain the most frequently occurring human amino acid in each site among the top ten selected human FR sequences. In the second method, (b) Heavy H2 and Light L2 were designed to contain the most frequently occurring human amino acid among the top ten selected human FR sequences in each site, unless an amino acid was present in any of the top ten human FR that matched that seen in the mouse JAA-F11, then the amino acid was kept as in mouse JAA-F11. In the third method, (c) In Heavy H3 or Light L3, the three FR residues before and after the CDR1, CDR2 and CD3 of mouse JAA-F11 were maintained and the rest of the residues were the most frequently seen amino acids in the

human sequences as in variants H1 or L1. Any one of these designed heavy variable chains can be paired with any of the similarly designed light variable chain, eg. H1/L1, H1/L2, H1L3, H2L1, H2L3, etc.

[0122] A chimeric JAA-F11 was also constructed in which the entire mouse JAA-F11 variable region was attached to a human IgG1 constant region. This is to provide a baseline that should have the original specificity and affinity of the mouse antibody while having the human constant region, to be used as a positive control for evaluating the humanized variants.

[0123] Assessment of models of hJAA-F11

[0124] Various hJAA-F11 constructs were assessed for conformational effects. Briefly, the proposed heavy and light variable chain sequences of various hJAA-F11 constructs were aligned with the JAA-F11 sequence. Any amino acid residue in the framework for each humanized variant that could potentially cause serious steric interaction with surrounding amino acids was removed and replaced with the original mouse JAA-F11 framework residue.

[0125] Immunogenicity prediction of hJAA-F11 variants using T20 score.

[0126] Although humanized or fully human antibodies are considered to be non-immunogenic and safe for human use, immunogenicity of fully human and humanized antibodies have been reported in patients. To determine if the selected sequences were of low immunogenicity, an immunogenicity analysis was performed by the T20 score method. The T20 score analyzer, developed by Gao et al, calculates the "humanness" of monoclonal antibody variable region sequences using a database of over 38,000 human antibody sequences.

[0127] In this method, a protein BLAST search is performed and the test humanized Ab is first compared against all these human sequences. The humanized antibody is then compared to the top 20 human Ab BLAST matches and scored for similarity to these sequences. T20 score for humanized antibody is obtained from the average of the percent identities of the top 20 matched human sequences. The highest possible score is 100 (most human-like). As proof of concept for the relationship of this method to immunogenicity in the patient, Gao et al compared the in vivo in patient immunogenicity results of more than 90 antibodies that are either approved for clinical use or in various stages of clinical development (mouse, chimeric (from mouse), humanized (n=22), and fully human antibody sequences) with the T20 score, and found that Abs with T20 scores of FR & CDR sequences above 80 were not immunogenic, and using a T20 scores for the FR sequences only, those

above 85 were not immunogenic. The T20 scores for the humanized JAA-F11 variants were calculated using the T20 Cutoff Human Databases at abanalyzer.lakepharma.com/.

[0128] Codon optimization and gene synthesis. After the design of the humanized JAA-F11 VH and VL variants amino acid sequences, the corresponding nucleotide sequences were selected manually for codon usage optimal for *Cricetulus griseus* (CHO) cell protein production and synthesized and inserted into pUC57 plasmid, a commonly used cloning vector.

[0129] Sub-cloning and sequencing of hJAA-F11 and chimeric JAA-F11 variable heavy (VH) and light (VL) genes in mammalian expression vectors. The hJAA-F11 VH genes were sub-cloned and inserted into an expression vector (pAH6307) containing a human IgG1 heavy chain leader/constant region under the control of human cytomegalovirus (CMV) promoter and ampicillin (Amp) and histidinol dehydrogenase (hisD) cassettes. The VL genes were inserted into an expression vector (pAN 6714) containing a human kappa light chain leader/constant region under the control of the human CMV promoter and neomycin phosphotransferase (neoR) cassette. Similarly, the chimeric JAA-F11 VH and VL were expressed and used as positive control in subsequent analyses.

[0130] Expression and production of humanized and chimeric JAA-F11 variants.

[0131] Co-transfection into CHO-K1 cells.

[0132] Adherent Chinese hamster ovary (CHO-K1; ATCC #CCL-61, Manassas, VA) cells were cultured for 18 hours before transfection in Ham's F12 medium (Corning Cellgro, Manassas, VA) supplemented with 10% fetal calf serum (FCS; Hyclone) at 37°C and 5% carbon dioxide (CO₂) in humidified air and harvested at 50-80% confluency. The mammalian 6307 pAH (VH) and 6714 pAN (VL) expression vectors were co-transfected into CHO-K1 cells by electroporation (Gene Pulser System (Bio-Rad, Hercules, CA). Briefly, each plasmid (VH and VL) was linearized using the PvuI restriction enzyme (Promega, Madison, WI) and 5 µg of each plasmid was added to 5 x 10⁶ CHO-K1 cells in cold Ham's F12 medium (Corning Cellgro, Manassas, VA) in a total volume of 500 µL in a 0.4 cm electroporation cuvette (Bio-Rad, Hercules, CA). The cuvette containing the mixture was electroporated at 960 µF and 250 mV. The transfection mixture was then diluted with pre-warmed non-selective medium (Ham's F12 supplemented with 10% FCS) to a concentration of 1 x 10⁵ cells/mL. Two hundred microliters was then plated into 96-well tissue culture plate(s) (BD Bioscience, San Jose, California) at a density of 1 x 10⁴ cells per well. The transfected cells were incubated at 37°C and 5% CO₂ in a tissue culture incubator. At 72 hours, the non-selective medium from 96-well tissue culture plate was removed and replaced

with 200 μ l of Ham's F12 (plus 10% FCS) selective medium containing 700 μ g/ml G418 (Gibco, Life Technologies, Grand Island, NY), 5mM of histidinol (Sigma-Aldrich, St Louis, MO) and 10mM of HEPES buffer (Corning Cellgro, Manassas, VA) and colonies were allowed to grow for up to 3 weeks. CHO-K1 cells transfected with plasmids 6307 pAH and 6714 pAN will continue to grow, while untransfected CHO-K1 cells will not grow. Every 2-4 days for the next 14 to 21 days, the selective medium was replaced to remove debris of dead cells and colonies of resistant cells were allowed to grow in the selective medium.

[0133] Analysis of culture supernatants by enzyme linked immunoassay (ELISA)

[0134] Between 14 – 21 days after selection, CHO-K1 culture supernatants from the 96-well plate(s) were collected and analyzed for antibody by enzyme-linked immunoassay (ELISA). Briefly, 100 μ L of culture supernatants were added to Immulon 1 B-medium binding 96-well microtiter plate(s) (Thermo Scientific, Milford, MA) that had been coated with 1.25 μ g/mL of TF-Ag-BSA conjugate in coating buffer (0.1 M Na₂CO₃ at pH 9.6) and washed five times with TBS-brij (pH 7.2). A 1:500 dilution of purified mouse JAA-F11 (1 mg/mL) in sterile PBS was also added into 2-3 wells on each plate to serve as positive controls. After a 2-hour incubation at 37°C, the plates were washed five times and 100 μ L of anti-human IgG-Alkaline Phosphatase conjugate secondary antibody (Sigma-Aldrich, St Louis, MO) in 1% BSA-PBST buffer (1:10,000) was added into each well for transfected cells while a 1:1000 dilution of an anti-mouse IgG-Alkaline Phosphatase conjugate secondary antibody (Sigma-Aldrich, St Louis, MO) in 1% BSA-PBST buffer was used for the mouse JAA-F11 control well. After 1 hour incubation at room temperature, the plates were washed five times and then 100 μ L of phosphatase substrate (p-nitrophenyl phosphate (pNPP)) (Sigma-Aldrich, St Louis, MO) was added to each well. Plates were read at 405nm using a plate reader after an hour incubation at room temperature to screen for the presence of IgG antibodies to TF-Ag. As a blank, HAM's F12 (plus10% FCS) medium was used during the primary antibody incubation, followed by secondary antibody incubation and substrate incubation.

[0135] Generation of stable clones and production

[0136] After the initial screening for anti-TF-Ag antibody at 14-21 days after selection started, 10-12 clones with the highest absorbance readings were selected and transferred into 24-well tissue culture plates for expansion. The culture supernatants from these clones were then analyzed by ELISA. The 4 clones that gave the highest absorbance readings were transferred to T25 tissue culture flasks, expanded and screened again for anti-TF-Ag antibody by ELISA to ensure that the clones were still producing antibodies reactive

to TF-Ag. These 4 clones were then transferred to T75 tissue culture flasks, expanded, rescreened for anti-TF-Ag antibody and cells stored frozen in liquid nitrogen. The clone with the highest anti-TF-Ag result was then further expanded and passaged 2 – 3 times to ensure that the cell line is stable.

5 [0137] Stable individual cell clones for each hJAA-F11 heavy and light chain combination from the best clone obtained above were generated through subcloning by limiting dilution. Briefly, cells were grown to confluence in a T75 tissue culture flask, harvested and counted and seeded at a 0.3 cell/well dilution in each well of 96-well plates in the selective medium. The plates were examined carefully 7 – 10 days later under a
10 microscope and wells that showed a single focus of cells were marked and monitored until enough density of growth was seen. Supernatants from these marked wells were tested for anti-TF-Ag antibody by ELISA. The 4 clones that gave the highest absorbance readings were expanded as described above for obtaining the parental clones. The sub-clone that gave the highest absorbance was expanded and used for production of supernatant in T200 Nunc™
15 cell culture Tripleflasks (Thermo Fisher Scientific, Inc.).

[0138] For production in the Tripleflasks culture flasks for each humanized variant JAA-F11, cells were cultured for 3 weeks before 1 liter of culture supernatant was harvested. The clone producing the chimeric JAA-F11 was processed the same way as the humanized JAA-F11 variants.

20 [0139] Purification of humanized and chimeric JAA-F11 variants

[0140] Humanized and chimeric JAA-F11 variants supernatants were purified using Protein A-Sepharose® 4B, Fast Flow affinity column (Sigma-Aldrich, St Louis, MO). To prepare the Protein A column, a 1:1 suspension of resin in Buffer A (0.02 M NaH₂PO₄, 0.15 M NaCl, pH to 8.0) was poured into a column. After the column had settled, it was washed
25 with 20 column volumes (CV) of buffer A. One liter of CHO-K1 cell culture supernatant was centrifuged at 3500 rpm for 30 minutes to clear dead cells or debris and then filtered. The filtered supernatant was loaded onto the Protein A column and allowed to drip by gravity at a flow rate of 1 mL/min. The column was then washed with 10 CV of buffer A. The antibody was eluted from the protein A column using 3 CV of buffer B (0.2 M Na₂HPO₄, 0.1
30 M Citric Acid, pH 3.9). The eluate was neutralized carefully with 0.1M NaOH to minimize the effect of low pH on the antibody. The Protein A column was re-equilibrated with 20-30 CV of buffer A and stored at 2-8°C. Each column was used up to five times for the same antibody. The purified antibody was dialyzed overnight at 4°C (Slide-A-Lyzer Dialysis Cassette; Thermo Scientific) against either; 1X Phosphate Buffered Saline (PBS) or RPMI

media with or without phenol red as required for subsequent assays. After dialysis, the antibody was filtered with a 0.22 µm filter (Corning) and protein concentration determined by a Bio-Rad protein assay as detailed below. The antibody was stored at 4°C and checked for binding to TF-Ag using ELISA.

5 [0141] Bio-Rad Protein Assay

[0142] The antibody concentration was determined using the Bio-Rad Protein Assay which is based on the Bradford dye-binding method. Briefly, the dye reagent was diluted with distilled water at 1:4 ratio and filtered using Whatman filter paper #1. Serial dilutions of a known protein standard (1.44 mg/mL gamma globulin, Bio-Rad, Hercules, CA) and the
10 humanized antibody sample were prepared using 1X PBS buffer. 10 µL of each standard and sample solution were placed in triplicates into wells of a microtiter plate. Two hundred microliters of the diluted dye reagent was then added to all wells using a multichannel pipette and mixed thoroughly by pipetting up and down without creating bubbles. The plate was incubated at room temperature for at least 5 minutes to 1 hour. Absorbance was then read at
15 595 nm on the plate reader (Bio-Tek Instruments).

[0143] Analysis of Chemical Specificity and Affinity of humanized and chimeric JAA-F11 antibodies

[0144] Determination of chemical specificity

[0145] The chemical specificity of the various hJAA-F11 constructs and the chimeric
20 JAA-F11 was determined using the printed glycan array. This was compared to data obtained previously for mouse JAA-F11. The glycan array is an indirect immunofluorescent method, described on the Consortium for Functional Glycomics website. Each construct was analyzed for reactivity with 610 different glycans. Briefly, the printed array was incubated successively with the antibody, washed, and incubated with secondary antibody labeled with
25 FITC. After washing, the image was read in a Perkin Elmer Microscanarray XL4000 and a tiff file image stored and image analysis was performed using Imagene V.6 image analysis software. The relative binding of each binding glycan was expressed and normalized to the binding of the original glycan TF-Ag. Comparison and statistical analysis of the relative binding abilities were performed using ANOVA.

30 [0146] Determination of Relative Affinity

[0147] The relative affinity binding of the hJAA-F11 to TF-Ag was analyzed using a competitive inhibition ELISA with the mouse JAA-F11 antibody and chimeric JAA-F11 antibody. Briefly, 3 µg/mL hJAA-F11 or chimeric JAA-F11 was incubated in the presence of different concentrations of mouse JAA-F11 (10, 8, 6, 4, 2 ug/mL) on 1.25 ug/mL TF-Ag

coated 96-well plates (Thermo Scientific, Milford, MA). Bound antibody was detected by incubation with anti-human IgG secondary antibody and substrate and absorbance reading was obtained as described in these materials and methods. The amount of mouse antibody required to inhibit the binding of 3 µg/mL humanized or chimeric antibodies by 50% was determined and compared. The higher the amount of mouse antibody required for inhibition, the higher the relative affinity of the antibody. The relative affinity of each of the humanized antibodies and chimeric antibody was compared using ANOVA.

[0148] Analysis of biological efficacy of hJAA-F11

[0149] Assessment of binding to human breast tumor cell lines

[0150] Binding of hJAA-F11 to various human breast tumor cell lines was analyzed using whole cell ELISA. The TF-Ag bearing mouse mammary tumor 4T1 cell line served as a positive control while the P3-X63-Ag8 Myeloma (ATCC Number: CRL-1580) cell line which was the fusion partner for producing JAA F11 hybridoma served as the TF-antigen negative control. Positive binding was determined by comparing the reactivity of each antibody with each cell line to the reactivity of that antibody with the myeloma cell line using the student's T test. To standardize the data, binding to each cell line was expressed by dividing the absorbance reading of the test cell line by the absorbance reading of the myeloma cells.

[0151] Cell preparation

[0152] The 4T1 breast tumor and myeloma cell lines were harvested using a non-enzymatic Cellstripper (Mediatech, Inc. VA, USA). To prevent clumping of cells, media, buffer and reagents were pre-warmed before using. For adherent cells, the media was removed from the culture vessels and the cells were rinsed with 1X Dulbecco's Phosphate Buffered Saline (DPBS) without calcium and magnesium (Mediatech Inc., Cellgro). Five milliliters of Cell Dissociation Solution was added to each flask and then incubated at 37°C for 10 minutes. The flasks were tapped to dislodge the cells. For non-adherent myeloma cells, the flasks were tapped well and the culture supernatant was centrifuged at 1000 x G for 10 mins. The cell pellets were resuspended in Cell Dissociation Solution and incubated at 37°C for 10 mins. After incubation, 20 mL of 1X DPBS was added to the cells and pipetted repeatedly to remove clumps. The cell suspensions were then centrifuged at 1000 x G for 10 minutes. The supernatants were decanted and the pellets re-suspended in 5mL DPBS. The cells were counted using trypan blue stain and a hemocytometer. The cells were diluted to obtain 1×10^6 viable cells/ml. Two hundred microliters of the cell suspensions (2×10^5 cells) were placed in 5ml polystyrene tubes quadruplets. Two hundred microliters of 4%

formaldehyde solution was added to each tube and incubated for 20 mins at room temperature before being centrifuged at 1500 x G for 10 mins. The supernatant was decanted carefully in one throw, the cells were washed in DPBS followed by centrifugation and decanting. Two hundred microliters of PBS-tween 1% BSA (w/v) was added to each tube and stored at 4°C overnight or up to two weeks.

[0153] Enzyme Immunoassay on cells

[0154] Two hundred microliters of 50 µg/mL of the mouse JAA-F11, hJAA-F11 or chimeric JAA-F11 antibody was added to tubes containing the different cell lines tested in quadruplet and incubated at 37°C for two hours. One set of tubes treated with 200 µL of 1X PBS-0.1% Tween 20-1% BSA served as the negative control for each cell line tested. The tubes were washed three times with 3 mL of wash buffer (1X PBS Tween, no azide), then centrifuged for 10 minutes at 1500 x G. The supernatant was carefully decanted between each wash. Two hundred microliters of anti-mouse IgG (γ-chain-specific) horseradish peroxidase secondary antibody (1:1000, Sigma-Aldrich, St. Louis, MO) or anti-human IgG (γ-chain-specific) horseradish peroxidase secondary antibody (1:2000, Sigma Aldrich, St. Louis, MO) in PBS-Tween-1% BSA was added to respective tubes and then incubated for 1 hour at room temperature (RT). After incubation, the tubes were decanted and washed three times, and centrifuged at 1500 x G between washes. Two hundred microliters of O-phenylenediamine dihydrochloride substrate (OPD; Sigma, St. Louis, MO) solution was then added to each tube and incubated for 1 hour at room temperature. After incubation, the reaction was stopped by adding 100 µL of stop solution (1N H₂SO₄) and centrifuged for 10 minutes at 1500 x G. Next, 200 µL of supernatant was removed from each tube and transferred to respective wells in a microtiter plate. Absorbance was read at 490nm using the microplate reader (Microplate Autoreader, Model EL311, Bio-Tek Instruments, Inc.) and unreacted OPD substrate was used as blank. For the different cell lines, each respective average blank (tubes with only PBS-Tween-1%BSA) was deducted from their respective average OD to get the final optical reading. Each experiment was repeated 3 times.

[0155] Effects of hJAA-F11 on cancer cell proliferation in vitro

[0156] To examine the effects of the humanized and chimeric JAA-F11 antibodies on cancer cell growth, in vitro 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (thiazoyl blue, MTT) proliferation analyses were performed. 4T1 mouse breast tumor cells and human breast tumor cells were seeded at 1 x 10⁴ cells/well in 10 replicates in 96-well plates in the presence of varying amounts of JAA-F11, humanized and chimeric JAA-F11 (4, 2, and 1 µg/mL). When plated at this cell density, the cells are in the linear portion of their

growth curves at 72 hours. Cells grown in culture medium without antibodies served as normal growth controls. Culture medium alone was used as a blank. After 68 hours of cell growth at 37°C, 10 µL of the tetrazolium salt MTT (5mg/ml) was added to each well, and the plates were returned to the incubator for another 4 hours. At the end of incubation, the resulting formazan product in each well was solubilized by adding 120 µL of dimethyl sulfoxide (DMSO, Fisher Scientific), and the absorbance of each well was then measured at 570 nm (Microplate Autoreader, Model EL311, Bio-Tek Instruments, Inc.).

[0157] Antibody-dependent cellular cytotoxicity assay

[0158] To examine the effector functions of the hJAA-F11 antibodies, antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) analyses were performed.

[0159] ADCC was determined by the lactate dehydrogenase (LDH) release assay (CytoTox 96 Non-Radioactive Cytotoxicity assay; Promega, Madison, WI) using human peripheral blood mononuclear cells (PBMC) as effector cells and human breast tumor cell lines as target cells at an effector to target (E:T) ratio of 100:1.

[0160] The PBMCs were prepared from whole blood by density gradient centrifugation using Ficoll-Paque. Whole blood collected in purple-top EDTA vacutainers was mixed with an equal volume of pre-warmed sterile DPBS. Twenty milliliters of diluted blood was then gently layered onto 15 mL of Ficoll-Paque Plus (GE Healthcare) in 50 mL conical tubes. The samples were centrifuged at 1300 rpm for 30-40min at room temperature with no brake. Next, the PBMC layer was collected and sterile PBS was added to the PBMCs for a total volume of 40mL. The mixture was then centrifuged at 1000 rpm for 10min at 18° to 22°C to remove any contaminating Ficoll and platelets/plasma proteins. The supernatant was discarded, the cells were resuspended in fresh sterile PBS, and the centrifugation step was repeated. The cells were resuspended in RPMI 1640 culture media (10% FCS) and counted using hemocytometer and trypan blue.

[0161] Target cells (1 x 10⁴; 30µl) and PBMCs (1 x 10⁶; 30µl) were added into 96-well U-bottomed plates and incubated with hJAA-F11 or chimeric antibodies (200 µg/mL; 30µL) for 17 hours at 37°C in a tissue culture incubator. Forty-five minutes before completion of the 17 hours incubation, 10 µL of lysis solution (x10) was added to the wells containing the target cell maximum LDH release control (target cells and medium) and the volume correction control (medium alone). At the end of incubation, the plate was centrifuged for 4 minutes at 1000 rpm. Fifty microliters aliquots were then transferred from all wells to a fresh flat-bottomed 96-well plate. Next, 50 µL of reconstituted substrate mix

was added to each of these wells. The plate was covered with foil and incubated for 30 minutes at room temperature. The reaction was stopped with the addition of 50 µL of stop solution to each well, and absorbance was recorded at 490 nm using a microplate reader (MicroplateAutoreader, Model EL311, Bio-Tek Instruments, Inc.). Cytotoxicity was calculated using the formula:

$$\text{Cytotoxicity (\%)} = 100 \times [(E - SE) / (M - SE)],$$

where for each condition the absorbance of the substrate is measured. The conditions are as follows; E is the experimental well, SE is the spontaneous release without the antibody control (target cells incubated with effector cells and PBS), M is the maximum release determined by the target cells lysed with 10X lysis solution.

[0162] Complement dependent cytotoxicity (CDC) assay

[0163] CDC was determined by the lactate dehydrogenase (LDH) release assay (CytoTox 96 Non-Radioactive Cytotoxicity assay; Promega, Madison, WI) using HCC 1428 breast tumor cells (ATCC® CRL-2327, Manassas, VA) as the target cell population. A LDH positive sample that contains bovine heart LDH, provided with the reagent kit was used as a positive control. After comparing rabbit, baby rabbit and guinea pig complement for background levels of cytotoxicity, lyophilized guinea pig serum (CL3112, Cedarlane Laboratories, Burlington, NC) was used as the source of the complement and reconstituted as per manufacturer's instructions. The optimal cell concentration used in the assay was 1 x 10⁴ cells/50 µL per well. The antibodies used were dialyzed against phenol red free-RPMI media. For experimental wells, 50 µl of HCC 1428 cell suspension, 20 µl of complement dilution (final dilution at 1:20), and 30 µL of antibody (final concentration at 100 µg/ml) were mixed in each well in quadruplicate sets in a round-bottom 96-well culture plate. Included were control wells containing target cells and complement without antibody to control for any cytotoxicity mediated by the serum used as the complement source and the spontaneous release of LDH. The plate was centrifuged at 1000 rpm for 4 minutes and then incubated for 2 hours at 37°C with 5% carbon dioxide. Forty-five minutes before completion of the 2 hour incubation, 10 µL of lysis solution (x10) was added to the wells containing the target cell maximum LDH release control and the volume correction control.

[0164] At the end of incubation, the plate was centrifuged for 4 minutes at 1000 rpm. Fifty microliters aliquots were then transferred from all wells to a fresh flat-bottomed 96-well plate. Next, 50 µL of reconstituted substrate mix was added to each well. The plate was covered with foil and incubated for 30 minutes at room temperature. The reaction was stopped with the addition of 50 µl of stop solution to each well, and absorbance was then

recorded at 490 nm using a microplate reader (MicroplateAutoreader, Model EL311, Bio-Tek Instruments, Inc.). Cytotoxicity was calculated using the formula:

$$\text{cytotoxicity (\%)} = 100 \times [(E - SE)] / [(M - SE)],$$

where for each condition the absorbance of the substrate is measured. The conditions are as follows; E is the absorbance of the experimental well, SE is the spontaneous release with complement but without the antibody (target cells incubated with complement and media), and M is that of target cells lysed with 10X lysis solution.

[0165] Cell Internalization Assays

[0166] Internalization of humanized and chimeric JAA-F11 into 4T1 breast tumor cells was analyzed by two methods, an enzyme immunoassay with surface binding measured at 2 temperatures of incubation, 4°C and 37°C, and an immunofluorescent microscopy method.

[0167] Enzyme immunoassay method of Internalization

[0168] The mouse 4T1 breast cancer cell line was tested for internalization of JAA-F11, hJAA-F11 or chimeric JAA-F11. Five hundred thousand (5×10^5) cells were seeded in all six wells of a 6-well plate and were grown to confluence. For each antibody tested, two plates were prepared. Media was removed from each plate and 1 mL of antibody (200 µg/mL) was added to 3 wells of each plate, and 1 mL of PBS dilution was added to the remaining 3 wells. One set of plates was incubated at 37°C to allow internalization and the other plate was incubated at 4°C. After the 1 hour incubation, media was removed and the wells were washed 4 times with 1 mL of Phenol Red Free RPMI media. Next, 1 mL of 2% paraformaldehyde was added to each well and the plates were incubated at room temperature for 20 minutes to fix the cells. The plates were then washed 4 times with Phenol Red Free RPMI media. Next, one mL of anti-mouse or anti-human IgG (γ -chain-specific) alkaline phosphatase secondary antibody (1:5000, Sigma, St. Louis, MO) in 1% BSA/PBS was added and the plates were incubated at 37°C for 1 hour. The plates were then washed 4 times with Phenol Red Free RPMI media and 1 mL of p-nitrophenyl phosphate substrate (pNPP) was added and incubated for 1 hour in the dark. After the 1 hr incubation, 200 µL from each well was transferred to respective wells of a 96-well plate and absorbance was recorded at 405 nm using a microplate reader (MicroplateAutoreader, Model EL311, Bio-Tek Instruments, Inc.). Unreacted substrate was used as a blank. The triplicate wells were averaged, the average optical density of the media alone blank was subtracted from the wells containing antibody. Percent internalization was calculated using the formula:

$$\% \text{ internalization} = 100 * [1 - (37^\circ\text{C sample} - 37^\circ\text{C blank}) / (4^\circ\text{C sample} - 4^\circ\text{C blank})]$$

[0169] Immunofluorescent microscopy method

[0170] In this method, 4T1 breast cancer cells were seeded on coverslips in the wells of two 6-well tissue culture plates at a density of 3×10^5 cells in 10% FCS RPMI 1640 media and incubated at 37°C and 5% CO₂ for 24 hours. Media was removed from the plates and 5 1.5 mL of test antibodies (5 µg/mL) diluted in serum-free RPMI 1640 media was added to respective wells. Both plates were placed at 4°C for 20 minutes to allow for surface binding of antibodies. After the 20 minute incubation, the coverslips were transferred from one of the plates into a new 6-well plate. Antibody dilutions were removed from the second plate and pre-warmed serum-free RPMI 1640 media was added and the plate was incubated at 37°C for 10 1 hour in a tissue culture incubator. The coverslips from the first plate were washed twice with ice-cold 5%BSA/PBS, rinsed once with 1X PBS and were then fixed with 4% paraformaldehyde solution (Affymetrix) for 15 min at room temperature. After the 1 hour incubation at 37°C, coverslips from the second plate were transferred to a new 6-well plate, washed, rinsed and fixed.

15 [0171] Following three washes in 1X PBS, cells in both plates were permeabilized with 0.1% Triton X-100, 0.1% sodium deoxycholate in PBS for 10 min at room temperature. Next, cells were rinsed three times with 1X PBS and then incubated in 5% BSA/PBS for 30 minutes at room temperature. Coverslips were then incubated with rabbit anti-lysosomal membrane protein 1 (LAMP1; Abcam, 24170) antibody at dilutions of 1 µg/mL in 5% 20 BSA/PBS for 1 hour at room temperature. Coverslips were then rinsed three times with 1X PBS and incubated with anti-mouse IgG-Alexa 647 and anti-rabbit IgG Alexa 488 (mouse JAA-F11 coverslips) and anti-human IgG-Alexa 647 and anti-rabbit IgG Alexa 488 (chimeric and hJAA-F11) secondary antibodies (Molecular Probes, Invitrogen), at a dilution of 1:500 in 5% BSA/PBS. The anti-rabbit IgG Alexa 488 secondary antibody was used to detect the 25 anti-LAMP 1 antibody. The coverslips were incubated for 1 hour at room temperature in the dark. Following incubation, cells were rinsed three times with 1X PBS in the dark. Coverslips were placed with cells facing down on Slowfade Gold reagent (Molecular Probes, Life Technologies) with DAPI media on microscopic slides and sealed with nail polish. Cells were analyzed with AxioImager fluorescence microscope (Zeiss). Images were captured and 30 analyzed using AxioVision Release 4.8.2 software.

[0172] In vivo studies to test the efficacy of humanized JAA-F11 to detect TF-Ag bearing breast tumors

[0173] Using the H2L2 humanized JAA-F11 antibody obtained from the above in vitro biological studies, immunolocalization studies were performed in mice to test whether

iodine-124 labeled humanized antibody will localize to TF-Ag breast tumors in the mice.

[0174] [124]Iodine labeling of hJAA-F11 (H2L2) antibody

[0175] [124]Iodine labeling of the humanized JAA-F11 antibody was carried out using the Bolton Hunter method. Before iodination, the humanized antibody was first modified using a water-soluble Bolton Hunter reagent (Sulfo-SHPP)(Thermo Scientific, Rockford IL, USA). Briefly, 1.9 mg of hJAA-F11 antibody was dissolved in Modification Buffer (200 mM borate buffer, pH 9.0). Five mg of the water-soluble Bolton Hunter reagent was dissolved in 1 mL Modification Buffer immediately before use. A 100 μ L of the water-soluble Bolton Hunter reagent solution was then added to the antibody sample and incubated on ice for 3 hours with periodic mixing. Non-reacted water soluble Bolton Hunter reagent was removed by dialyzing against phosphate-buffered saline (PBS: 0.1 M sodium phosphate, 150 mM sodium chloride). At this intermediate step the humanized antibody contains a linker attached to some of the lysines of the antibody, and it is stable for 2 weeks.

[0176] The modified hJAA-F11 antibody was then labeled using the Chizzonite indirect labeling method [158]. Briefly, a Pierce pre-coated iodination tube (Thermo Fisher, Rockford, IL, USA) was prewetted with 1 mL of High Tris Iodination buffer (0.125 M Tris-HCl, pH 6.8, 0.15 M NaCl. The buffer was decanted and then 100 μ L of HighTris Iodination Buffer was added directly to the bottom of the tube, followed by the addition of 280 μ L of (4.59 milliCurie) of sodium 124I iodide in 0.02 M NaOH solution (IBA Molecular, Richmond, VA). An initial count was taken using a radioisotope calibrator CRC 12 (Capintec). The pH of the mixture was measured to ensure that it was at pH 7, neutral. After 6 minutes activation at room temperature, the activated iodide was added to the previously modified huJAA-F11 solution. After a 9 minutes incubation period, 50 μ L of Scavenging Buffer (10 mg tyrosine/mL in Tris Iodination Buffer; 25 mM Tris, pH 7.5, 0.4 M NaCl) and the mixture was incubated for 5 minutes. The purpose of the scavenging buffer is to remove free iodine that reacts with the tyrosine in the buffer.

[0177] A 1 mL of Tris/NaCl/EDTA buffer (25 mM Tris-HCl, pH 7.5, 0.4 M NaCl, 5 mM EDTA, 0.05% sodium azide) was then added to the reaction mixture. The sample was then added to a 10 mL desalting column that has been pre-equilibrated with Tris/NaCl/EDTA buffer. The sample tube was washed with 0.5 mL Tris/NaCl/EDTA buffer and the wash added to the column. The sample was eluted in fifteen fractions of 500 μ L each using Tris/NaCl/EDTA buffer, and was tested for radioactivity. Radiolabelling efficiency was determined on the fraction with the highest activity by high performance liquid chromatography (HPLC). The radiolabeled antibody was injected into the animals within 2

hours of labeling. To ensure that the labeled humanized JAA-F11 maintains its TF-Ag reactivity, a radioimmunoassay was also carried out.

[0178] Animals and tumor models

[0179] Animals in this study were housed and utilized in accordance with the
5 Institutional Animal Care and Use Committees (IACUC) regulations. All protocols were approved by the IACUC of the University at Buffalo. 4T1 mouse breast cancer cells were implanted in 7-8 weeks old female Balb/C mice by injecting 5×10^4 cells in 0.1 mL D-PBS subcutaneously under one of the right nipples. The mice were divided into 2 groups and injected with [124I]-hJAA-F11 (n=13) and free 124I24 (n=7) 10–14 days after tumor
10 implantation by tail vein and then subjected to biodistribution studies and micro-PET imaging. One mouse from each of the 2 groups were imaged and followed throughout the study. All mice received 0.2g/L potassium iodide water after injection with labeled antibody and throughout the whole study as a thyroid-blocking regime.

[0180] Biodistribution studies

15 [0181] Mice were sacrificed by injecting 0.1 mL Sodium Pentobarbital (Fatal Plus) intraperitoneally at 72, 96, 168, and 192 hours after injection of the radiolabeled antibody. At each time point, three mice that received the labeled antibody and two mice with free iodine were sacrificed. Blood, muscle, spleen, lungs, kidneys, heart, liver, small and large intestines, stomach, brain, skin, tumor tissue, bone, tail, esophagus, thyroid and ovaries were
20 harvested and placed into pre-weighed 5 ml polypropylene tubes. These tubes were re-weighed to obtain actual weight of each tissue/organ. All the tubes were capped and radioactivity was measured using a gamma counter. Radioactivity uptake for each tissue was calculated as the percentage of the injected dose per gram of tissue (%ID/g) as per the following formula as in Appendix 2.:

25 [0182] micro-PET imaging

[0183] Localization of the labeled antibody in one mouse each from the two groups was determined through microPET imaging using the microPET camera, Focus 120® (Siemens Concorde Microsystems) at 24, 48, 72, 96, 168, and 192 hours after injection using known techniques. Briefly, before scanning, mice were anesthetized with O2/isoflurane (1%-
30 3% isoflurane) and then imaged in the prone position in the gantry of the microPET scanner. The emission scan window was set between 350 and 750 keV. The scan was performed for 30 minutes for each mouse.

[0184] Radioimmunoassay

[0185] To determine the immunoreactivity of the radiolabeled antibody, a

radioimmunoassay (RIA) was performed. A microtiter plate was coated with 100 μ L of 1.25 μ g/mL TF-Ag-BSA conjugate in coating buffer. The buffer was removed from the wells and washed with 1%BSA/PBS-Tween. One hundred microliters of serial dilutions of radiolabeled hJAA-F11 in 1%BSA/PBS-Tween were added to the wells and allowed to bind
5 at room temperature for 1 hour. After incubation, the unbound antibody was removed by washing the wells with 1%BSA/PBS-Tween three times manually using a multi-channel pipette. The bound hJAA-F11 was removed from the plate by incubating with 200 μ L of 1M Acetic acid/0.15M NaCl buffer (pH 2.4) for 30 minutes at room temperature. After the incubation, 100 μ L solution from each well was added into separate polypropylene test tubes
10 and the radioactivity was measured using a gamma counter.

[0186] While the invention has been described through specific embodiments, routine modifications will be apparent to those skilled in the art and such modifications are intended to be within the scope of the present invention.

What is claimed is:

1. A partially humanized monoclonal antibody (mAb) or fragment thereof that binds with specificity to TF-Ag, the monoclonal antibody or fragment thereof comprising a heavy chain and a light chain, wherein the heavy chain comprises a sequence selected from the group consisting of:

a) the sequence consisting of:

EVQLVESGAIEVKKPGASVKVSKASGYTFTTYWMHWVRQAPGQGLEWMGFISPNT
DYTEYNQKFRDRVTMTADTSISTAYMELSRLRSDDTAVYYCARSFIGYNFDFWGQG
10 TLVTVSS (SEQ ID NO:7) (H1);

b) the sequence consisting of:

EVQLLESGLAEKKKPGASVKVSKASGYTFTTYWMHWVRQAPGQGLEWMGFISPNT
DYTEYNQKFRDRVTLTADKSSSTAYMELSSLTSEDVAVYYCARSFIGYNFDFWGQG
TTVTVSS (SEQ ID NO:8) (H2);

c) the sequence consisting of:

EVQLVESGAIEVKKPGASVKVSKASGYTFTTYWMHWVKQAPGQGLEWIGFISPNT
DYTEYNQKFRDKATMTADTSISTAYMELSRLRSDDTAVYYCARSFIGYNFDFWGQG
TTLTVSS (SEQ ID NO:9) (H3),

d) the sequence consisting of:

20 QVQLVQSGAEVKKPGSSVKVSKASGYTFTTYWMHWVRQAPGQGLEWMGFISPNT
DYTEYNQKFRDRVTITADKSTSTAYMELSSLRSEDVAVYYCARSFIGYNFDFWGQGT
TVTVS (SEQ ID NO:13) (H2a);

e) the sequence consisting of:

EGQLLESGLAEKAPGASVKMSCKASGYTFTTYWMHWVKKRPGQGLEWIGFISPNTD
25 YTEYNQKFRDKATLTADKSSSTAYMQLSSLTSDDSAVYYCARSFIGYNFDFWGQGT
TLTVSS (SEQ ID NO:14) (H3a); and

combinations thereof;

and wherein the light chain comprises a sequence selected from the group consisting of:

d) the sequence consisting of:

DVVMVTQSPLSLPVTLGQPASISCRSSQTIVYSNGNTYLEWFQQRPGQSPRLLIYKVS
NRFSGVPDRFSGSGSGTDFTLKISRVEAEDVGVYYCFQGSHVPFTFGSG TKLEIK (SEQ
ID NO:10) (L1);

e) the sequence consisting of:

DIVMTQTPLSLPVTLGQPASISCRSSQTIVYSNGNTYLEWFQQRPGQSPRLLIYKVSNR
FSGVPDRFSGSGSGTDFTLKISRVEAEDVGVYYCFQGSHVPFTFGSG TKLEIK (SEQ
ID NO:11) (L2);

f)) the sequence consisting of:

5 DVVMTQSPLSLPVTLGQPASISCRSSQTIVYSNGNTYLEWYLQRPQGQSPRLLIYKVSNR
RFSGVDPDRFSGSGSGTDFTLKISRVEAEDVGVYYCFQGSHVPFTFGSG TKLEIK (SEQ
ID NO:12) (L3);

g) the sequence consisting of:

10 DIVMTQSPLSLPVTLPGEASISCRSSQTIVYSNGNTYLEWYLQKPGQSPQLLIYKVSNR
FSGVPDRFSGSGSGTDFTLKISRVEAEDVGVYYCFQGSHVPFTFGSGTKVDIK (SEQ
ID NO:15) (L2a);

h) the sequence consisting of:

ELVMTQTPLSLPVNLGDQASISCRSSQTIVYSNGNTYLEWYLQKPGQSPKLLIYKVSNR
RFSGVDPDRFSGSGSGTDFTLKISRVEADDLGVYYCFQGSHVPFTFGSGTKLEIK (SEQ
15 ID NO:16) (L3a);

and combinations thereof.

2. The mAb of claim 1, wherein the mAb comprises a human IgG constant region.

20 3. The mAb of claim 1, wherein the mAb or the fragment thereof is conjugated to an
agent selected from the group consisting of chemotherapeutic drugs, toxins and radioactive
isotopes.

4. A method for prophylaxis and/or therapy of cancer in an individual, wherein the
25 cancer comprises cancer cells express TF-Ag, the method comprising administering to the
individual one or more mAbs or the fragments thereof of claim 1, wherein the growth, or
survival, or metastasis, or a combination thereof, of the cancer cells in the individual is
inhibited subsequent to the administration.

30 5. The method of claim 4, wherein the mAb comprises a human IgG constant region.

6. The method of claim 4, wherein the mAb or the fragment thereof is conjugated to an
agent selected from the group consisting of chemotherapeutic drugs, toxins and radioactive
isotopes.

7. A pharmaceutical composition comprising the partially humanized mAb or fragment thereof according to claim 1.
- 5 8. An *in vitro* cell culture, wherein cells in the cell culture express the partially humanized mAb or fragment thereof according to claim 1.

1/7

Figure 1

	<u>FR1</u>				<u>CDR1</u>				<u>FR2</u>				<u>CDR2</u>			
Kabat #	1	5	10	15	20	25	30	35	40	45	50	a	55	60	65	
mJAAF11	EVQL	L	ESGAEL	L	A	KPGASVK	M	SCKA	SGYTFTTYWMH	WV	K	Q	R	P	GQGLEWIG	FISPNTDYTEYNQKFRD
H1	EVQL	V	ESGAEL	V	K	KPGASVK	V	SCKA	SGYTFTTYWMH	WV	R	Q	A	P	GQGLEWIMG	FISPNTDYTEYNQKFRD
H2	EVQL	L	ESGAEL	L	K	KPGASVK	V	SCKA	SGYTFTTYWMH	WV	R	Q	A	P	GQGLEWIMG	FISPNTDYTEYNQKFRD
H3	EVQL	V	ESGAEL	V	K	KPGASVK	V	SCKA	SGYTFTTYWMH	WV	K	Q	A	P	GQGLEWIG	FISPNTDYTEYNQKFRD

	<u>FR3</u>				<u>CDR3</u>				<u>FR4</u>																																											
Kabat #	70	75	80	a	b	c	83	85	90	95	100a	105	110	113																																						
mJAAF11	KATLT	ADKSS	T	T	A	Y	M	Q	L	S	S	L	T	S	E	D	S	A	V	Y	Y	C	A	R	S	F	I	G	Y	N	F	D	F	W	G	Q	G	T	T	L	T	V	S	S								
H1	R	V	T	M	T	A	D	T	S	I	S	T	A	Y	M	E	L	S	R	L	R	S	D	D	T	A	V	Y	Y	C	A	R	S	F	I	G	Y	N	F	D	F	W	G	Q	G	T	L	V	T	V	S	S
H2	R	V	T	L	T	A	D	K	S	S	S	T	A	Y	M	E	L	S	S	L	T	S	E	D	T	A	V	Y	Y	C	A	R	S	F	I	G	Y	N	F	D	F	W	G	Q	G	T	T	V	T	V	S	S
H3	KAT	M	T	A	D	T	S	I	S	T	A	Y	M	E	L	S	R	L	R	S	D	D	T	A	V	Y	Y	C	A	R	S	F	I	G	Y	N	F	D	F	W	G	Q	G	T	T	L	T	V	S	S		

Figure 2

	<u>FR1</u>					<u>CDR1</u>					<u>FR2</u>					<u>CDR2</u>																																															
Kabat#	1	5	10	15	20	25	a	bce	30	35	40	45	50	55	60																																																
JAAF11	EL	V	M	T	Q	T	P	L	S	P	V	N	L	G	D	Q	A	S	I	S	CR	S	S	Q	T	I	V	Y	S	N	G	N	T	Y	L	E	W	Y	L	Q	K	P	G	Q	S	P	K	L	L	I	Y	K	V	S	N	R	F	S	G	V	P	D	
L1	D	V	V	M	T	Q	S	P	L	S	P	V	T	L	G	Q	P	A	S	I	S	CR	S	S	Q	T	I	V	Y	S	N	G	N	T	Y	L	E	W	F	Q	R	P	G	Q	S	P	R	L	L	I	Y	K	V	S	N	R	F	S	G	V	P	D	
L2	D	I	V	M	T	Q	T	P	L	S	P	V	T	L	G	Q	P	A	S	I	S	CR	S	S	Q	T	I	V	Y	S	N	G	N	T	Y	L	E	W	F	Q	R	P	G	Q	S	P	R	L	L	I	Y	K	V	S	N	R	F	S	G	V	P	D	
L3	D	V	V	M	T	Q	S	P	L	S	P	V	T	L	G	Q	P	A	S	I	S	CR	S	S	Q	T	I	V	Y	S	N	G	N	T	Y	L	E	W	Y	L	Q	R	P	G	Q	S	P	R	L	L	I	Y	K	V	S	N	R	F	S	G	V	P	D

	<u>FR3</u>					<u>CDR3</u>					<u>FR4</u>																																			
Kabat#	61	65	70	75	80	85	90	95	100	a																																				
JAAF11	R	F	S	G	S	G	S	G	T	D	F	T	L	K	I	S	R	V	E	A	D	D	L	G	V	Y	C	F	Q	G	S	H	V	P	F	T	F	G	S	G	T	K	L	E	I	K
L1	R	F	S	G	S	G	S	G	T	D	F	T	L	K	I	S	R	V	E	A	E	D	V	G	V	Y	C	F	Q	G	S	H	V	P	F	T	F	G	S	G	T	K	L	E	I	K
L2	R	F	S	G	S	G	S	G	T	D	F	T	L	K	I	S	R	V	E	A	E	D	V	G	V	Y	C	F	Q	G	S	H	V	P	F	T	F	G	S	G	T	K	L	E	I	K
L3	R	F	S	G	S	G	S	G	T	D	F	T	L	K	I	S	R	V	E	A	E	D	V	G	V	Y	C	F	Q	G	S	H	V	P	F	T	F	G	S	G	T	K	L	E	I	K

Figure 3

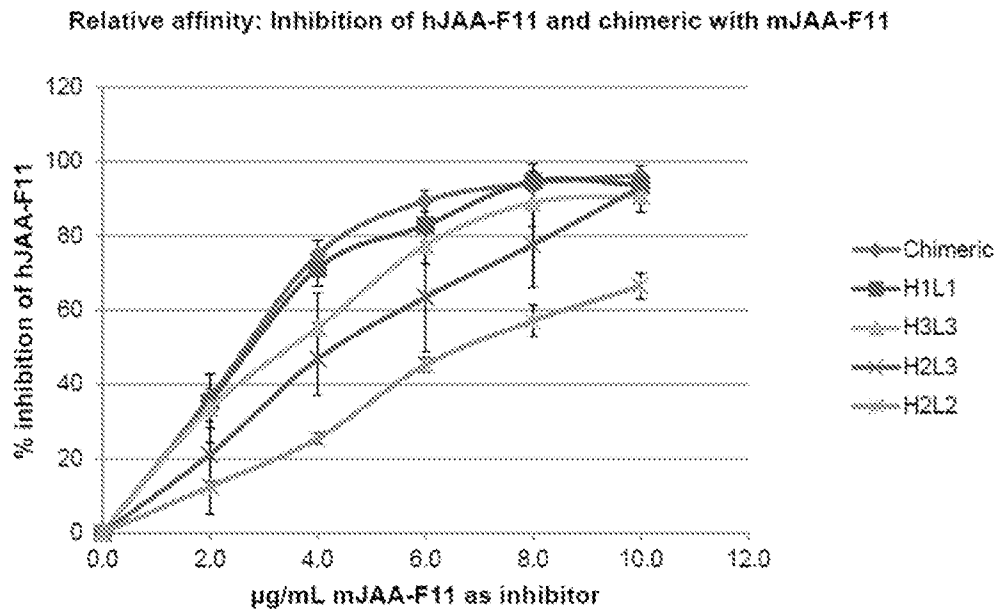


Figure 4

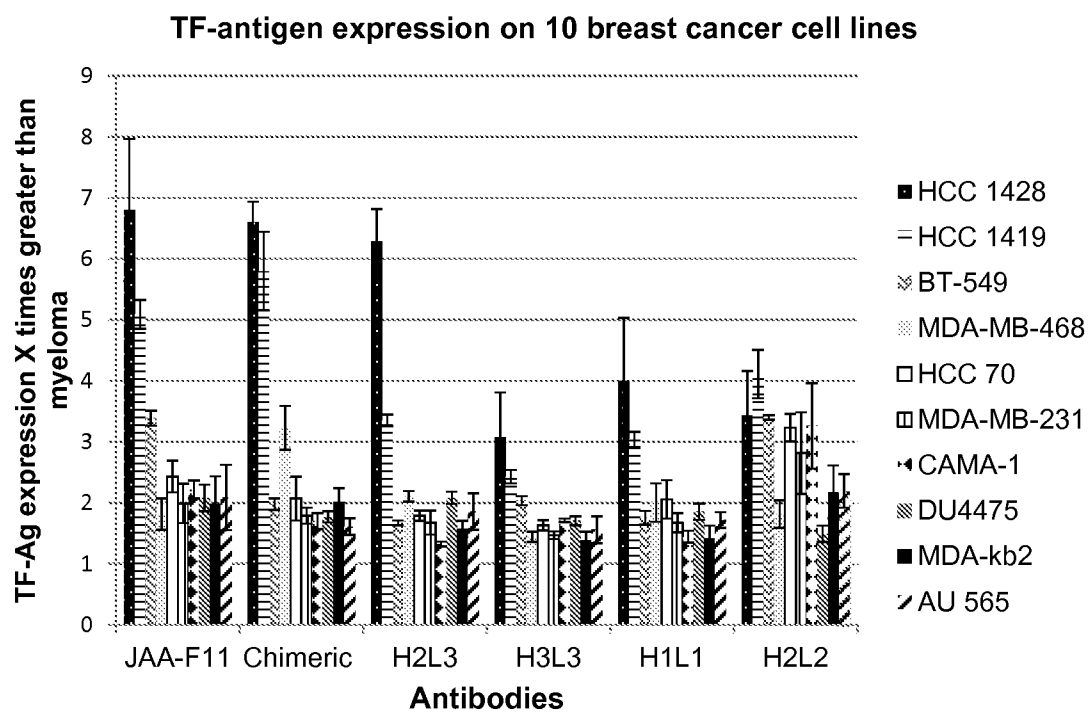


Figure 5

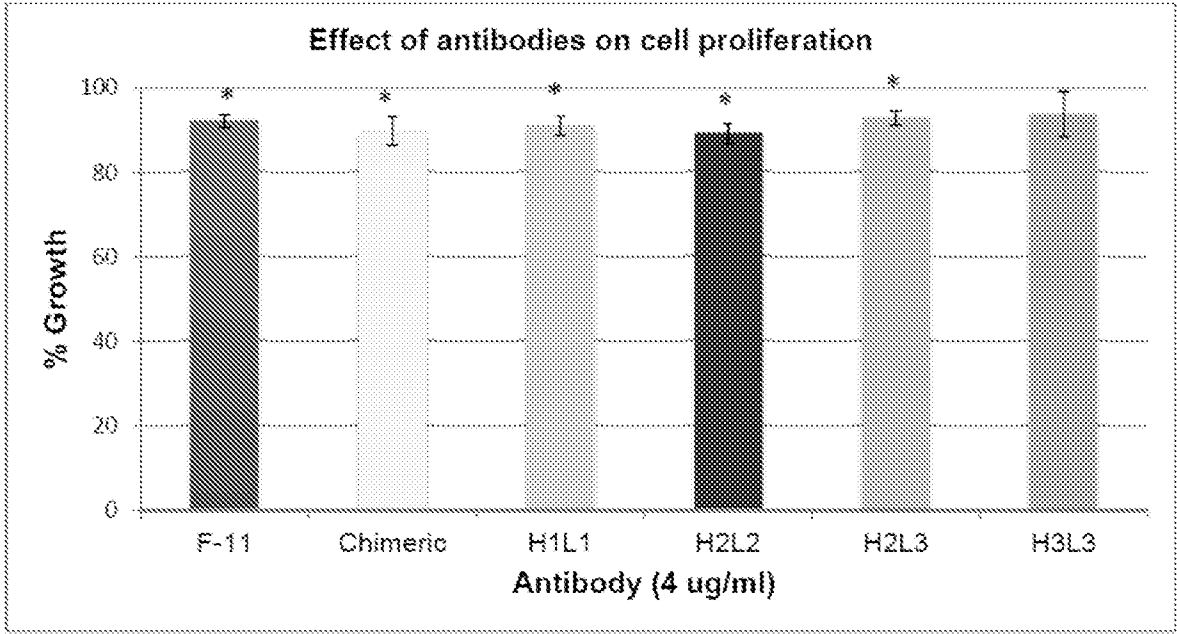


Figure 6A

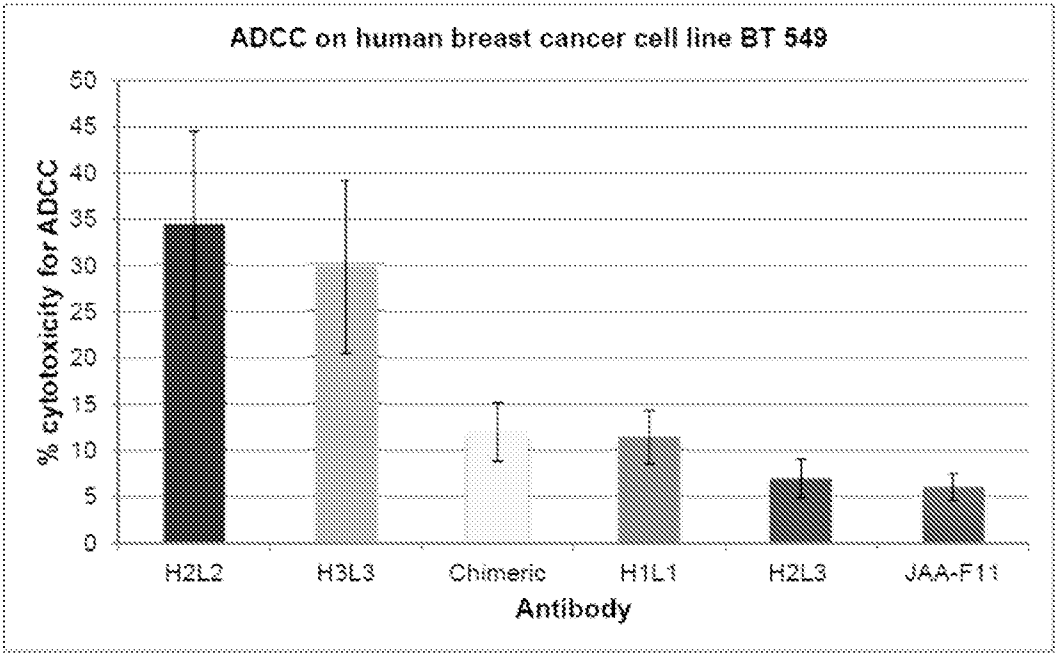


Figure 6B

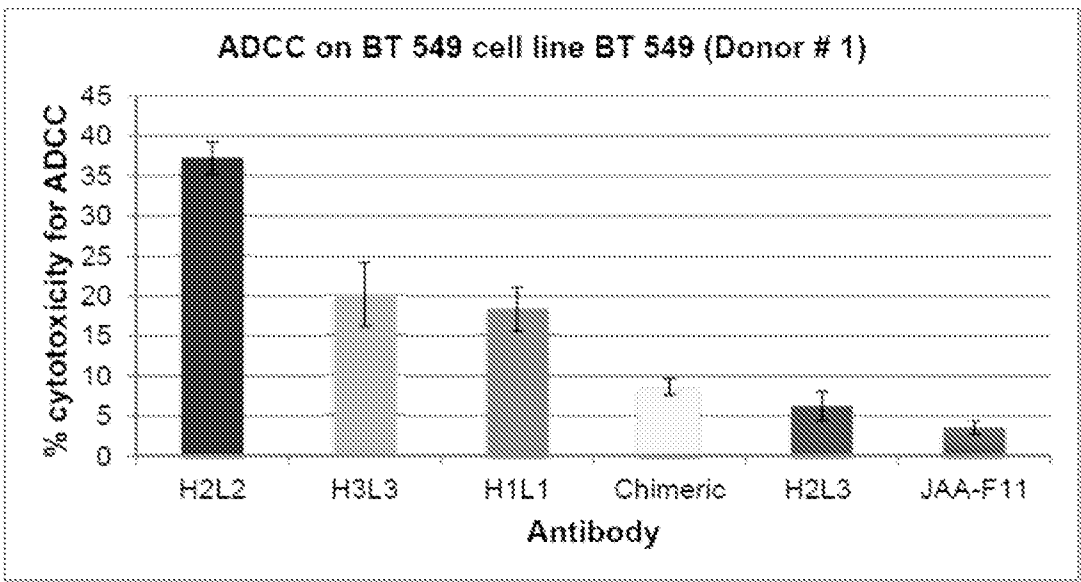


Figure 6C

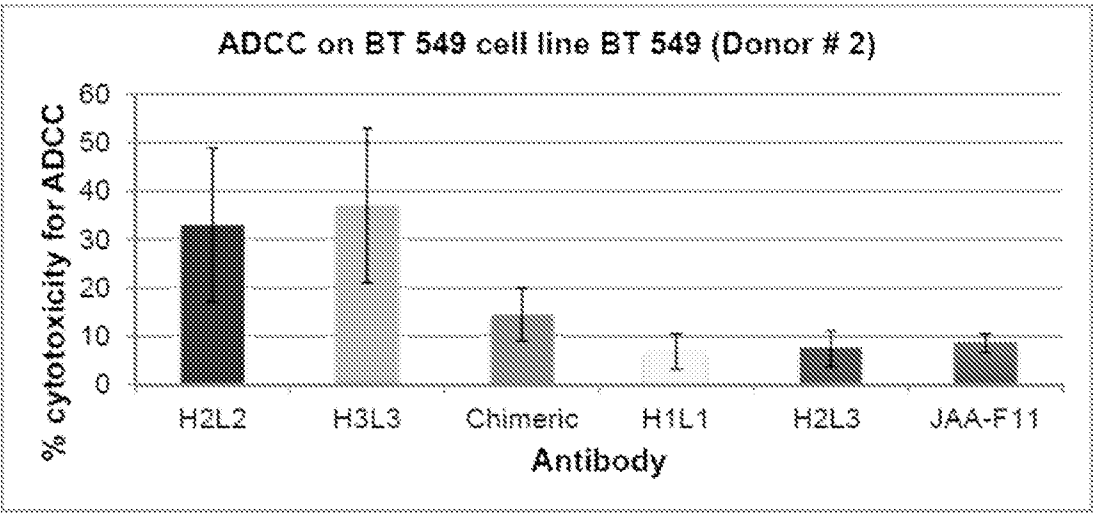


Figure 6D

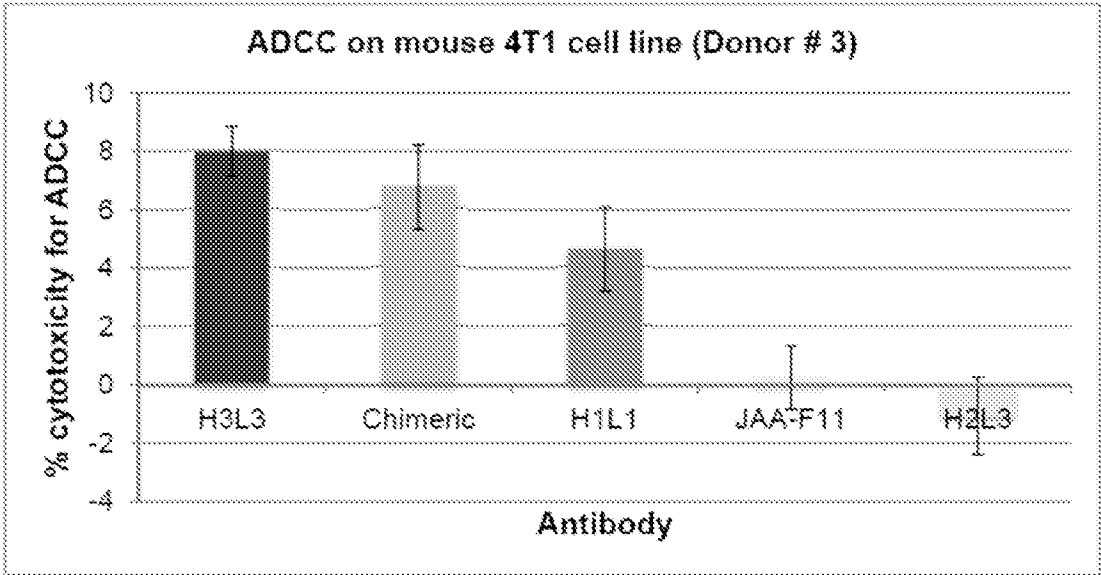


Figure 7

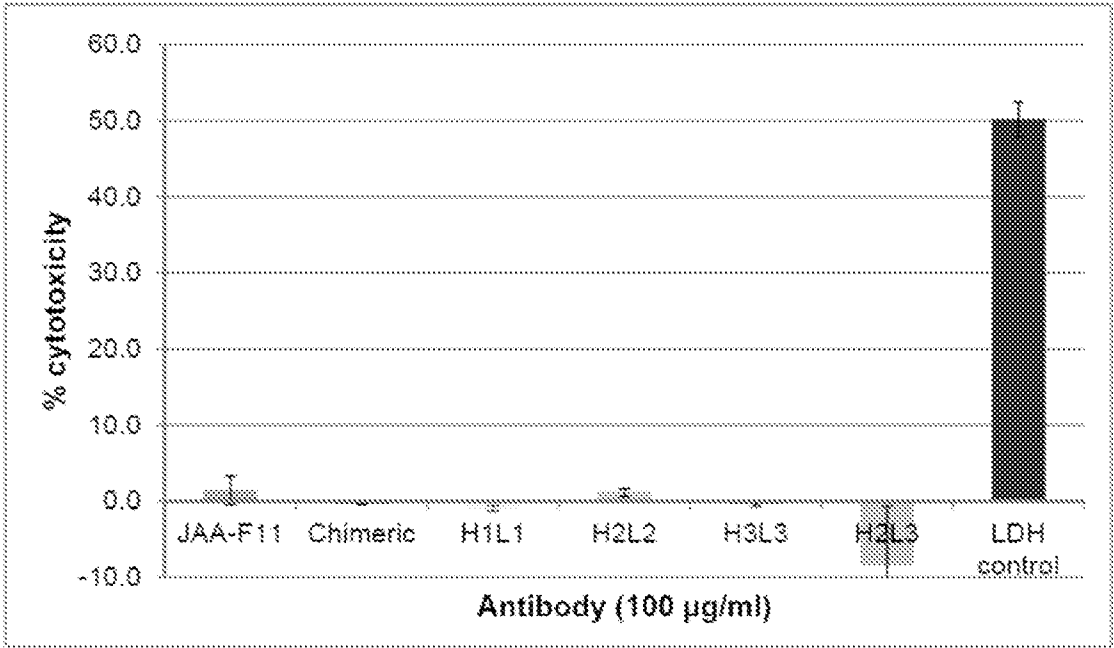


Figure 8

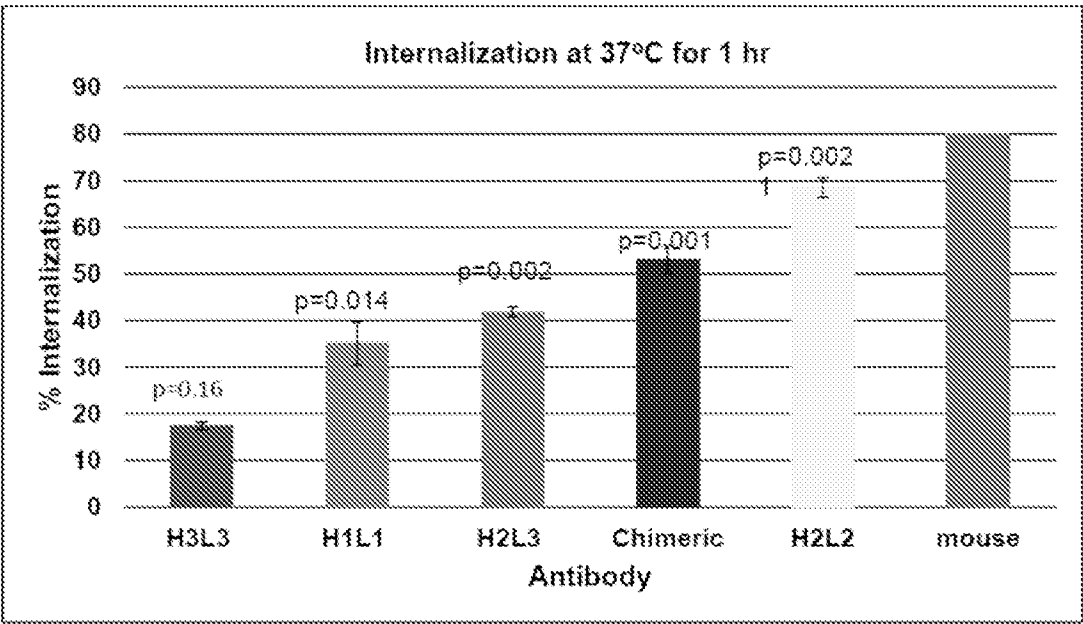


Figure 9

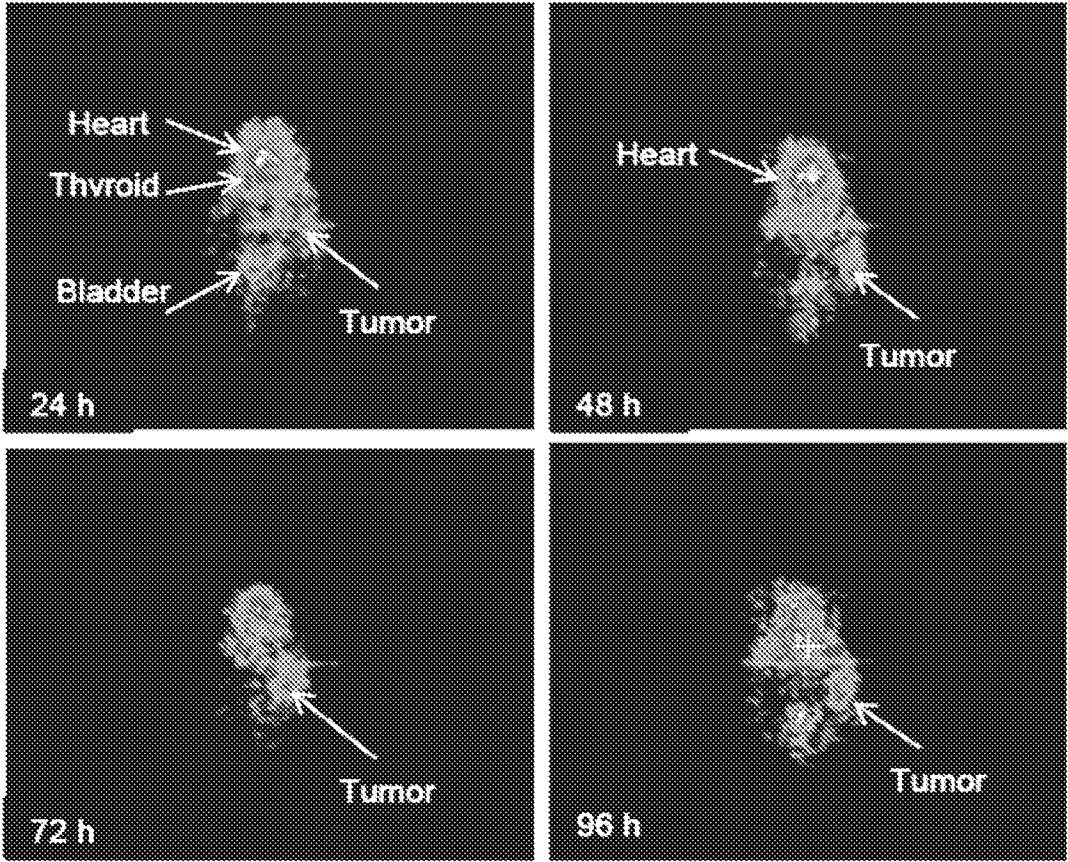


Figure 10

