

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization

International Bureau



(43) International Publication Date
6 December 2012 (06.12.2012)

(10) International Publication Number
WO 2012/164063 A1

(51) International Patent Classification: **C07K 16/28** (2006.01) [GB/GB]; Birchlea, the holdings, Balmedie, Aberdeenshire AB23 8WU (GB).

(21) International Application Number: PCT/EP2012/060345 (74) Agents: **CHAPMAN, Paul William** et al.; Kilburn & Strode LLP, 20 Red Lion Street, London, Greater London WC1R 4PJ (GB).

(22) International Filing Date: 1 June 2012 (01.06.2012) (81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(25) Filing Language: English (82) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV,

(26) Publication Language: English

(30) Priority Data: 1109238.4 1 June 2011 (01.06.2011) GB
61/492,005 1 June 2011 (01.06.2011) US

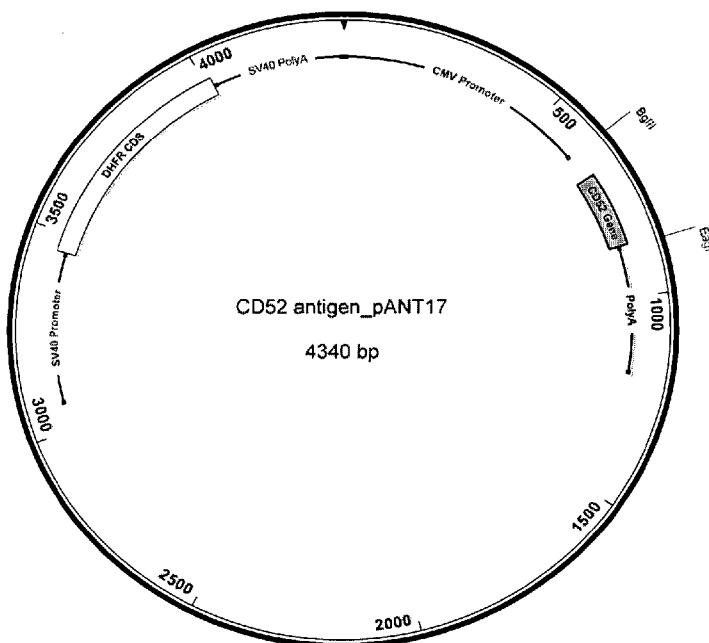
(71) Applicant (for all designated States except US): **ANTITOPE LTD** [GB/GB]; Babraham Institute, Babraham, Cambridgeshire CB2 4AT (GB).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **JONES, Timothy David** [GB/GB]; 27 Brick Row, Babraham, Cambridge, Cambridgeshire CB22 3AJ (GB). **HOLGATE, Robert George Edward** [GB/GB]; 96 Princess Mews, Royston, Hertfordshire SG8 9BL (GB). **CARR, Francis Joseph**

[Continued on next page]

(54) Title: HUMANISED ANTI-CD52 ANTIBODIES



(57) Abstract: The present invention relates to novel humanised antibodies against human CD52 and their use in methods of treating or preventing human diseases.

Figure 1a: Human CD52 expression vector



MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK,
SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ,
GW, ML, MR, NE, SN, TD, TG).

Published:

- *with international search report (Art. 21(3))*
- *with sequence listing part of description (Rule 5.2(a))*

HUMANISED ANTI-CD52 ANTIBODIES

The present invention relates to novel humanised antibodies against human CD52 and their use in methods of treating or preventing human diseases.

5

BACKGROUND OF THE INVENTION

CD52 is a glycosylated, glycosylphosphatidylinositol (GPI)-anchored cell surface protein found in abundance on a variety of normal and malignant lymphoid cells especially B and T cells (Gilleece et al, Blood 82 807-812 (1993); Hale et al, J Biol Regul Homeost Agents, 15 p386- 391 10 (2001); Rodig et al, Clin Cancer Res 12, p7174-7179 (2006)). CD52 is expressed at lower levels on myeloid cells such as monocytes, macrophages and dendritic cells (DC) with little expression found on mature natural killer (NK) cells, neutrophils, and hematological stem cells. CD52 is also produced by epithelial cells in the epididymis and duct deferens, and is acquired by sperm during passage through the genital tract (Hale et al, *ibid*; Domagala et al, Med Sci Monit 7 p325- 15 331 (2001)). The exact biological function of CD52 remains unclear but some evidence suggests that it may be involved in T cell migration and co-stimulation (Masuyama et al, J Exp Med 189 979-989 (1999); Watanabe et al, Clin Immunol 120 247-259 (2006)).

20 Campath-1H (alemtuzumab, Campath®, MabCampath®) is a humanised anti-human CD52 monoclonal antibody that exhibits potent *in vitro* antibody-dependent cell mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). CD52 is present on at least 95% of all human peripheral blood lymphocytes and monocytes/macrophages (Hale G. et al., The CAMPATH-1 antigen (CD52). *Tissue Antigens* 1990,35:118-127). Campath-1H recognizes an epitope which consists of the carboxy terminal four amino acids of the mature CD52 protein and 25 a portion of the negatively charged GPI anchor. Due to its significant cytotoxic effects, Campath-1H is capable of depleting CD52 positive cells *in vivo* and it is approved for front line and third line treatment of chronic lymphocytic leukemia (CLL). Campath-1H has been evaluated for its utility in the treatment of several autoimmune diseases, including rheumatoid arthritis, vasculitis, myositis, Wegener's disease and diabetes. The most advanced studies of 30 Campath-1H are in treating relapsing remitting multiple sclerosis (MS). These studies showed a significant improvement in time to relapse compared to human interferon beta-1a (Rebif® (i.e., interferon beta- Ia)).

35 A major limitation of Campath-1H is immunogenicity whereby antibodies are induced in up to 70% of patients (Therapeutic Monoclonal Antibodies: From Bench to Clinic, ed. Zhiqiang An (2009) ISBN: 978-0-470-11791-0). In order to improve the clinical utility of anti-CD52 antibodies, there is a major need for improved anti-CD52 antibodies which are not associated with significant immunogenicity in patients.

40 **SUMMARY OF THE INVENTION**

The invention relates to humanized immunoglobulins that have binding specificity for human CD52 (huCD52). The invention also provides humanised antibodies that bind to human CD52 with an equilibrium dissociation constant (Kd) of at least 10^{-8} M. The invention also provides humanised antibodies that specifically bind to human CD52 having an antibody heavy chain of 45 either IgG1, IgG2, IgG3 or IgG4, or using a mutated IgG constant region especially a constant region which enhances ADCC (antibody-dependant cellular cytotoxicity) or CDC (complement-dependant cytotoxicity). The invention also provides humanised antibodies wherein the antibody light chain is a kappa light chain. The humanised antibody can be encoded by human IgG heavy chain and human kappa light chain nucleic acids that encode protein sequences in their variable 50 regions as set forth in SEQ ID NO:20 through SEQ ID NO:28.

The present invention also provides humanised antibodies that specifically bind to human CD52 whereby the antibody variable regions have been selected or modified to exclude one or more human CD4+ T cell epitopes. The present invention also provides humanised antibodies that 55 specifically bind to human CD52 whereby the antibody variable regions have been formed primarily by fusing segments of sequences entirely derived from existing human antibody variable region sequences.

The present invention also provides humanised anti-CD52 antibodies of the invention 60 comprising heavy chain CDR1, CDR2, and CDR3 amino acid sequences, “RYGMS” (SEQ ID NO.5), “MMKTKGGRTYYPDSVKG” (SEQ ID NO.6) and “DGYY” (SEQ ID NO. 7), respectively, and light chain CDR1, CDR2, and CDR3 amino acid sequences, “KSSQSLLHSDGKTYLN” (SEQ ID NO.8), “LVSKLDS” (SEQ ID NO.9), and

“WQGTHLWT” (SEQ ID NO. 10), respectively. The present invention also provides
65 humanised anti-CD52 antibodies of the invention comprising heavy chain variable region amino acid sequences corresponding to SEQ ID NOS:20 to 24 for the heavy chain and SEQ ID NOS:25 to 28 for the light chain. In a preferred embodiment of the invention, a humanised anti-CD52 antibody of the present invention comprising heavy chain variable region amino acid sequences corresponding to SEQ ID NO:22 for the heavy chain and SEQ ID NO: 28 is provided.

70

Humanised antibodies of the present invention can be composed of any of the above CDR sequences SEQ ID NO.5 to SEQ ID NO.10 and minor variants of these CDR sequences where alterations of one or more amino acids does not significantly reduce binding to human CD52. Humanised antibodies can be created by joining together the CDR sequences with sequences
75 from human variable region frameworks where such framework sequences are derived from single or multiple other human antibody variable region framework sequences. Commonly such human variable region framework sequences will include one or more mutations which contribute to optimal or improved binding of the humanised antibodies to CD52. In a preferred embodiment of the present invention, such human variable region framework sequences in the
80 humanised antibodies are derived entirely from sequences in other human antibody variable regions as described in of EP1844074 . These sequences comprise joined segments of sequences from other human antibody variable regions, together with human constant regions. In particular, such humanised antibodies also contain CDR sequences derived entirely from CDR sequences in other human antibody variable regions including joined segments of CDR
85 sequences from other human CDRs together with human constant regions, thus creating humanised antibodies in which the variable region sequences are derived entirely from sequences in other human antibody variable regions together with human constant regions, this creating a “fully human” antibody.

90 The invention also provides humanised antibodies that specifically bind to human CD52, wherein said humanised antibody is produced by a prokaryotic or eukaryotic cell, especially from a mammalian cell line, especially CHO or NS0 cells. The invention also provides a humanised antibody that specifically binds to human CD52 that is a Fab fragment or a single chain Fv (scFv). The invention also provides multispecific proteins including at least one

95 humanised antibody from the sequences SEQ ID NOS:20 to 24 for the heavy chain and SEQ ID NOS:25 to 28 for the light chain whereby the multispecific protein specifically binds to human CD52 and, additionally, binds or interacts with one or more other molecules. Different antibodies or proteins may be included in each multispecific antibody can be linked to each other either covalently or non-covalently.

100

The invention provides a pharmaceutical composition comprising a humanised antibody (either as a proteinaceous antibody or a gene encoding the antibody) that specifically binds to human CD52 and a pharmaceutically acceptable carrier. The pharmaceutical composition can further comprise one or more chemotherapeutic agents either linked or unlinked to the humanised 105 antibody.

The invention provides a method for treatment of CLL and other leukemias; several autoimmune diseases including multiple sclerosis, rheumatoid arthritis, vasculitis, myositis, Wegener's disease and diabetes; and organ transplant rejection and graft-vs-host disease, in each case comprising 110 administering to the patient an effective dosage of a humanised antibody (either as a proteinaceous antibody or a gene encoding the antibody) that specifically binds to human CD52, wherein the antibody causes the destruction or apoptosis of CD52+ target cells such as B and T cells. In addition, the invention also provides a method for diagnosis of the above mentioned diseases, for example by administration of humanised antibody attached to a detectable label and 115 determination of binding of the humanised antibody *in vivo* to provide a basis for detection of CD52+ cells, for example in localised tumour masses or in inflammatory lesions. Alternatively the humanised antibodies of the present invention may be used for *in vitro* tests for CD52+ cells as a means for detection of disease and also for *in vitro* tests for antibodies which may bind to the humanised antibodies used therapeutically. Accordingly, such humanised antibodies of the 120 invention can be used as diagnostic or therapeutic agents *in vivo* and *in vitro*.

The humanised antibodies of the invention can encompass various antibody isotypes, or mixtures thereof, such as IgG1, IgG2, IgG3, IgG4, IgM, IgA1, IgA2, IgAsec, IgD, IgE or mutated forms 125 of these IgGs such as mutations which enhance binding to Fc receptors (for example, Horton et al., Blood 116 (2010) p3004-3012) or to complement (for example, Natsume et al., Cancer Res

68 (2008) p3863-3872). Typically humanised antibodies include IgG1 heavy chain constant regions and κ light chain constant regions. The humanised antibodies can be full-length (e.g. IgG1/ κ antibody) or can include only an antigen-binding portion (e.g., a Fab, F(ab')2, Fv or a scFv fragment).

130

Some humanised anti-CD52 antibodies of the present invention can be characterised by one or more of the following properties: a) specificity for human CD52 (specifically binding to human CD52); b) a binding affinity to human CD52 with an equilibrium dissociation constant (Kd) of at least 10^{-8} M.

135

In another aspect, the invention provides nucleic acid molecules encoding the humanised antibodies, or antigen-binding portions, of the invention. Accordingly, recombinant expression vectors that include the antibody-encoding nucleic acids of the invention, and host cells transfected with such vectors, are also encompassed by the invention, as are methods of making 140 the antibodies of the invention by culturing these host cells.

Anti-human CD52 humanised monoclonal antibodies of the invention, or antigen binding portions thereof (e.g., Fab), can be derivatised or linked to another functional molecule, e.g., another peptide or protein (e.g., a Fab' fragment). For example, an antibody or antigen-binding portion of the humanised antibodies of the invention can be functionally linked (e.g., by 145 chemical coupling, genetic fusion, noncovalent association or otherwise) to one or more other molecular entities. For example, the humanised anti-CD52 antibody, or antigen binding fragment thereof, can be conjugated to a therapeutic moiety, e.g., a cytotoxic drug, an enzymatically active toxin, or a fragment thereof, a radioisotope, a therapeutic nucleic acid, or a 150 small molecule anti-cancer drug. The antibodies of the invention can also be conjugated to cytotoxic pharmaceuticals, e.g., radiolabeled with a cytotoxic agents such as, e.g. 131I, or can be coupled to a ribosome inactivating protein, e.g. pseudomonas exotoxin (PE38 fragment, plant or bacterial toxins such as ricin, the α -chain of ricin, saporin, pokeweed antiviral protein, diphtheria toxin, or *Pseudomonas* exotoxin A (Kreitman and Pastan (1998) Adv. Drug Delivery Rev. 155 31:53.).

In another aspect, the present invention provides compositions, e.g., pharmaceutical and diagnostic compositions, comprising a pharmaceutically acceptable carrier and at least one humanised monoclonal antibody of the invention, or an antigen-binding portion thereof, which 160 specifically binds to human CD52. Some compositions may also comprise a combination of the humanised antibodies or antigen-binding portions of the invention. Such compositions may also comprise combinations with one or more other biologically active molecules as separate molecules, for example, a combination of at least one humanised monoclonal antibody of the invention and another biologically active molecule, or may combine combinations with one or 165 more other biologically active molecules in the same molecule, for example as a bispecific or multispecific molecule either as a combination of two or more humanised antibodies of the invention or as a combination with one or more other biologically active molecules.

For *in vivo* methods, the humanised antibodies, or antigen-binding portions thereof (or a 170 bispecific or multispecific molecule of the invention) can be administered to a human subject suffering from a disease related to CD52+ cells, or to a disease that can be ameliorated or prevented by treatment with the humanised antibodies of the invention.

Humanised monoclonal antibody compositions of the invention also can be administered in 175 combination with other known therapies, e.g., an anti-cancer therapy, a therapy for an autoimmune disease such as rheumatoid arthritis, or a therapy for multiple sclerosis. Accordingly, the invention provides a method for treating cancer or inflammatory diseases in a subject comprising administering a therapeutically effective amount of a pharmaceutical composition of a humanised antibody together with a pharmaceutical carrier to the subject.

180 In yet another aspect, the present invention provides a method using antibodies of the invention for detecting *in vitro* or *in vivo* the presence of human CD52 antigen in a sample, e.g., for diagnosing a human CD52-related disease. In some methods, this is achieved by contacting a sample to be tested, along with a control sample, with a humanised monoclonal antibody of the invention, or an antigen-binding portion thereof (or a bispecific or multispecific molecule), under 185 conditions that allow for formation of a complex between the antibody and human CD52. Complex formation is then detected (e.g., using an ELISA) in the test samples, and any

statistically significant increase in the formation of complexes between the test and control samples is indicative the presence of human CD52 antigen in the test sample.

190

It will be understood by those skilled in the art that the humanised antibodies of the present invention will have additional uses or compositions beyond those described herein, in all cases where the humanised antibody binds to human CD52 antigen whereby such uses and compositions shall be considered to be within the scope of the invention. It will be understood by those skilled in the art that the variable region sequences of the humanised antibodies of the present invention (SEQ ID NO:20 through SEQ ID NO:28) or CDRs of the humanised antibodies of the present invention (SEQ ID NO:5 through SEQ ID NO:10) may be subject to variations which do not significantly change the properties of the humanised antibodies of the present invention whereby such variants shall be considered to be within the scope of the invention. In addition, such variations either within the variable region or CDR sequences of the humanised antibodies should be considered to be within the scope of the present invention where such variations have significant homology to the humanised sequences of the present invention. For example, a variant nucleic acid may be determined to be within the scope of the invention where this includes sequences containing or substantially identical to SEQ ID NO:11 through SEQ ID NO:19 as determined by its ability to hybridise under stringent conditions to a nucleic acid of the present invention. In one embodiment, a nucleic acid sequence can be determined to be within the scope of the invention (e.g., is substantially identical to SEQ ID NO:11 through SEQ ID NO:19) by its ability to hybridise under stringent conditions to a nucleic acid within the scope of the invention (such as SEQ ID NO:11 through SEQ ID NO:19). The term “hybridise” refers to the binding, duplexing, or hybridising of a molecule to a particular nucleotide sequence under stringent hybridisation conditions when that sequence is present in a complex mixture (e.g. total cellular or library DNA or RNA), wherein the particular nucleotide sequence is detected at least at about 10 times background. Stringent hybridisation conditions will be selected, for example, to be 5-10°C lower than the thermal melting point (Tm) for the specific sequence at a defined ionic strength pH. It will also be understand that humanised antibodies of the present invention may be modified in the heavy chain constant regions in order to enhance ADCC and CDC. For enhancement of ADCC, fucose-depleted forms of the humanized antibodies may be produced by expression of the antibodies in certain mammalian cells including a variant CHO

line, Lec13 (Shields et al., *J Biol Chem* 277 (2002) p26733–26740), a rat hybridoma cell line, 220 YB2/0 (Shinkawa et al., *J Biol Chem* 278 (2003) p3466–3473), and a FUT8 (α-1,6-fucosyltransferase) knockout CHO cell line (Yamane-Ohnuki et al., *Biotechnol Bioeng* 87 (2004) p614–622). Alternatively mutations in the heavy chain constant regions may be used to enhance ADCC such as described by Shields et al., *J Biol Chem* 276 (2001) p6591-6604 and Lazar et al., *Proc Natl Acad Sci U S A* 2006; 103 (2006) p4005–4010. Alternatively mutations 225 in the heavy chain constant regions may be used to enhance CDC, for example using antibodies of mixed human IgG1 / IgG3 isotype (Natsume et al., *ibid*).

It will be understood by those skilled in the art, from precedent elsewhere especially from 230 clinical studies with Campath-1H (Zhiqiang An, *ibid*), that antibodies which bind to human CD52 antigen are fundamentally immunogenic in patients, probably due to the inherent cytotoxicity of anti-CD52 antibodies which acts as co-stimulatory signal to CD4+ T cell epitopes from the antibodies, thus resulting in CD4+ T helper cell responses and immunogenicity. It will therefore be understood by those skilled in the art that the antibodies of the present invention are 235 surprisingly devoid of such CD4+ T helper cell responses as determined by *in vitro* studies with human blood (cf Example 9) and that such anti-CD52 antibodies with low CD4+ T cell responses (<=4% T cell responses in human T cell assays) are novel.

BRIEF DESCRIPTION OF THE DRAWINGS

240

Within the figure legends, the nomenclature 2E8 or ANT01 is used interchangeably for mouse, chimeric or humanised antibodies derived from the 2E8 mouse monoclonal antibody.

Figure 1 shows the plasmid vectors used for expression of chimeric and humanised antibodies in 245 mammalian cells comprising pANT17 for heavy chains and pANT13 for light chains.

Figure 2 shows a flow cytometry analysis of binding of the 2E8 mouse monoclonal antibody to NS0 cells transfected with human CD52 compared to binding to NS0 CD52-. Staining was with anti-mouse IgG-PE conjugated antibody with signal derived from PE on the Y axis.

250

Figure 3 shows a flow cytometry analysis of binding of dilutions of chimeric 2E8 to Hut78 cells compared to Campath-1H. Staining was with anti-human IgG-PE conjugated antibody.

255 Figure 4 shows a competition flow cytometry analysis using Campath-1H-PE in competition with chimeric 2E8 and Campath-1H for binding to Hut78 cells.

Figure 5 shows the mean cytotoxicity from 5 human PBMC samples used as effector cells in an ADCC assay for chimeric 2E8 and Campath-1H with REH target cells.

260 Figure 6 – as Figure 5 except for 2 individual PBMC with dilutions of chimeric 2E8 and Campath-1H with high expressing REH target cells.

Figure 7 – as Figure 6 except using human complement for CDC assays with dilutions of chimeric 2E8 and Campath-1H and high expressing REH target cells.

265

Figure 8 shows a competition CD52 peptide ELISA for binding of humanised 2E8 variants in competition with biotinylated chimeric 2E8.

270 Figure 9 shows a flow cytometry analysis for binding of dilutions of humanised variants and Campath-1H to REH cells.

Figure 10 shows the mean cytotoxicity from 4 human PBMC samples used as effector cells in an ADCC assay for humanised 2E8 variants and Campath-1H with REH target cells.

275 Figure 11 shows the CDC for humanised 2E8 variants and Campath-1H with high CD52 expressing REH target cells and human complement.

Figure 12 shows a competition flow cytometry analysis using Campath-1H-PE in competition with chimeric 2E8, Campath-1H and selected variants for binding to REH cells.

280

Figure 13 shows the direct cytotoxic effects of anti-human CD52 antibodies on REH cells as measured by apoptosis and necrosis.

Figure 14 shows the Kaplan-Meier plot for SCID mice transplanted with Raji human Burkitt lymphoma cells following treatment with Campath-1H and the lead humanised 2E8 variant VH3/VK4 (V region SEQ ID NOS. 22 and 28).

EXAMPLES

Commercially available reagents referred to in the Examples were used according to manufacturer's instructions unless otherwise indicated. The source of cells identified in the Examples and throughout the specification by ECACC accession numbers is the European Collection of Cell Cultures (ECACC), Salisbury, England. Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Exemplary methods and materials are described below although methods and materials similar or equivalent to those described herein can also be used in the practice or testing of the present invention. The materials, methods, and examples are illustrative only and not intended to be limiting in scope.

EXAMPLE 1: GENERATION OF MOUSE MONOClonAL ANTIBODIES

CD52 peptide (GQNNTSQTSSPSC) was custom synthesised and conjugated to either KLH or BSA via a maleimidocaproyl-N-Hydroxysuccinimide linker (Mimotopes, Wirral, Cheshire UK) leaving the peptide N-terminus free. Raji and HuT78 cells were obtained from ECACC. CD52-expressing NSO cell lines were generated as follows: DNA encoding human CD52 (NCBI Reference Sequence: NM_001803.2) (full length sequence including the N-terminal signal peptide, the C-terminal displaced GPI-anchor signal peptide and the mature GPI-anchored surface peptide) was PCR amplified and subcloned into pANT antibody expression vectors (Figure 1a) via BglII and EagI sites. Transcription of the CD52 gene was under the control of the CMV I/E promoter (US5168062 and US5385839, University of Iowa). The pANT expression plasmid contained a mutant *dhfr* minigene (Simonsen & Levinson 1983, PNAS 80:2495-2499) under the control of a SV40 promoter and polyA sequence for selection in

eukaryotic cells as well as a β -lactamase (Ap^R) gene for prokaryotic selection and a pMB1 origin of replication for propagation in prokaryotic cells. The expression plasmid was propagated in *E. coli* XL1-blue (Stratagene Cat. No. 200130). Stable CD52 expressing cell lines were obtained by 315 transfecting NS0 cells by electroporation and placing cells under selection with 200nM methotrexate. Cells were grown and expanded then tested by flow cytometry for CD52 expression. High CD52 expressing cell lines were frozen down and used for immunising mice as described below.

320 Female Balb/c mice were primary immunised by intraperitoneal (i.p.) injection either of 50ug of CD52 peptide-KLH conjugate in Complete Freund's Adjuvant (CFA), or primary immunised with 1×10^6 RAJI cells expressing CD52 in phosphate-buffered saline (PBS). After four weeks, all mice were boosted by i.p. injection of 10^6 HUT-78 cells in PBS with an additional booster injection two weeks later. Four weeks later, all mice received a third boost with 3×10^6 NS0 325 cells expressing CD52 in PBS i.p. Two subsequent boosts of 10^7 NS0 cells expressing CD52 in PBS were injected i.p at two weekly intervals and some mice were given a further boost of 5ug of CD52 peptide-KLH.

330 Three days prior to myeloma fusion, the two mice showing the highest antibody titre were given an i.p. boost of 10^7 NS0 cells expressing CD52 in PBS. On the day of fusion, both mice were sacrificed, spleens were removed, and the cells from each entire spleen were pooled, washed in serum-free culture medium and split into two equal samples. Half of the spleen cells were fused to F0 myeloma cells and half were fused to P3X63Ag8U.1 myeloma cells by PEG-mediated fusion. Plates 1-4 contained the F0 fused cells and plates 5-8 contained the P3X63Ag8U.1 cells. 335 The complete fusion medium consisted of DMEM, 2% L-glutamine, 1% penicillin-streptomycin, 10% fetal bovine serum, 5% BriClone hybridoma cloning medium (National Institute for Cellular Biotechnology, Dublin, Ireland) and Hypoxanthine-Aminopterin-Thymidine (HAT). The resulting fusions were seeded into 96-well plates at 200ul per well. The remaining non-plated fused cells were stabilized in culture for up to three days, then frozen and stored in liquid 340 nitrogen. The plated fusion cells were cultured at 37°C in 5% CO₂ for two weeks, transferred to 96-well plates, and tested for the presence of secreted anti-CD52 antibodies using the CD52 peptide-KLH ELISA as described below. Cells from 24 immunopositive wells were expanded in

culture and tested for CD52-specific antibody by CD52 peptide ELISA, NS0-CD52 cell-based ELISA and by flow cytometry.

345

For the CD52 peptide ELISA, ELISA plates (VWR, Lutterworth, UK) were coated overnight at 4°C with 100ul/well of either CD52 peptide-KLH, CD52 peptide-BSA, KLH only or BSA only at 0.5µg/ml in PBS. Plates were washed and blocked with 150ul/well PBS containing 2% BSA. Cell culture supernatants or purified antibodies were diluted in PBS/2% BSA and 100ul added to 350 each plate followed by incubation for 1 hour at room temperature. Plates were washed three times with PBS-Tween (0.05%) and incubated for 1 hour with 100ul/well goat anti-mouse Ig (Fab-specific) conjugated to Horseradish Peroxidase (Sigma-Aldrich). Plates were washed three times with PBS-Tween following which SigmaFast OPD substrate (Sigma-Aldrich) was added and incubated at room temperature in the dark to allow colour to develop. The reaction was 355 stopped by adding 50 µl of 3M HCl. Plates were read at 490nm using a Dynex plate reader (Dynex, Worthing, UK). CD52 peptide-specific hybridomas were those that bound to CD52 peptide-KLH and CD52 peptide-BSA but not to either KLH only or BSA only

For the NS0-CD52 cell-based ELISA, 3x10⁵cells/well (NS0 wild-type or NS0 cells expressing 360 CD52) were plated out in a V-bottom 96 well plate. The plate was centrifuged, supernatants were removed and the plate blotted on absorbent paper. Hybridoma samples were diluted 1 in 2 in FACS buffer (D-PBS containing 1%BSA and 0.05% sodium azide) and 100µl transferred to each of two plates containing either NS0 (Plate 1) or NS0-CD52 (Plate 2) cells. After incubation at room temperature for 1hr, the plates were washed twice by centrifuging the plates and 365 resuspending the cells in 200µl FACS buffer between centrifuging. After centrifugation, cells were resuspended in 100µl FACS Buffer containing anti-mouse IgG (Fab specific) (Sigma) diluted 1:500. After incubation for 1hr at room temperature, plates were washed twice by centrifuging and resuspending the cells in PBS. After centrifugation, cells were resuspended in 50µl PBS and transferred to an ELISA plate. 100µl TMB substrate (Invitrogen) was added and incubated at room temperature in the dark to allow the colour to develop. The reaction was 370 stopped by adding 50 µl of 3M HCl. Plates were read at 450nm using Dynex plate reader. CD52-specific clones were those that bound to NS0-CD52 cells specifically when compared with NS0 wild-type cells.

375 For flow cytometry, 3×10^5 cells NS0-CD52 or wild-type NS0 were stained using a 1 in 2 dilution of anti-CD52 hybridomas antibodies together with a 1 in 100 dilution of anti-mouse IgG-PE conjugated antibody (Sigma). Mouse IgG (Sigma) was also included as a separate control for the different murine isotypes present within the hybridomas. Cells were stained for 1 hour at 4°C. An anti-mouse IgG-PE conjugated antibody only control was also included. Cells were washed 380 twice with FACS buffer and finally resuspended in FACS buffer and flow cytometry performed using a Beckton Dickinson FACSCalibur (Becton Dickinson, Oxford, UK). Instrument settings were determined by analysis of relevant isotype control antibodies.

385 From results of CD52 peptide ELISA, NS0-CD52 cell-based ELISA and flow cytometry, huCD52 specific hybridomas were cloned, expanded in culture, frozen as parental stocks and stored in liquid nitrogen. Each of the selected hybridomas was diluted in cloning medium and plated into 96-well plates at a cell density of one cell per three wells. Cloning medium consisted of DMEM, 2% L-glutamine, 1% penicillin-streptomycin, 10% fetal bovine serum, 5% BriClone 390 hybridoma cloning medium and hypoxanthine-thymidine (HT). Cultures were maintained at 37°C in 5% CO₂ for 2 weeks with the cloned cells receiving fresh medium after one week in culture. Two weeks after cloning, supernatants from all seeded wells were transferred to new 96-well plates and tested for the presence of anti-CD52 antibodies using the CD52 peptide ELISA and flow cytometry as described previously. Positive wells were expanded in culture and 395 retested. Positive cells were further expanded and tested for antibody isotype. Anti-CD52 positive subclones were frozen, stored in liquid nitrogen and used for monoclonal antibody production for further studies.

400 Monoclonal antibodies were isotyping using the Rapid ELISA Mouse Antibody Isotyping Kit (Perbio, Cramlington, UK). Antibodies were purified on a 1ml Protein A-sepharose column (GE Healthcare, Little Chalfont, UK). Prior to purification, both the tubing and the Protein A column were depyrogenated using 0.4M NaOH. The column was re-equilibrated with 20 column volumes of PBS pH 7.4. Hybridoma cell culture supernatants were harvested, adjusted to 1x PBS pH 7.4 using 10x PBS and filter sterilised. Filtered supernatant was pumped through on the Protein A-sepharose column at 0.5 ml/min. The column was washed with 1x PBS pH 7.4 and

405 IgG was eluted using sterile 0.1M Sodium Citrate pH3, with 0.9 ml fractions collected and neutralised with 0.1ml of sterile 1M Tris-HCl pH 9. Under sterile conditions, the product was buffer exchanged into PBS pH 7.4 to remove any elution buffer and concentrate the sample. After concentration, antibodies were quantified by OD280nm using an extinction coefficient, Ec (0.1%) of 1.4. Purified antibodies were analysed by SDS-PAGE using a Novex NuPAGE 410 electrophoresis system with 4-12% NuPage gel (Invitrogen, Paisley, UK) and MES running buffer. 1 µg of antibody was prepared with 4xNuPAGE sample buffer plus beta-mercaptoethanol and heated. The gel was stained with InstantBlue staining solution (Expedeon, Cambridge, UK) and molecular size were estimated by comparing stained bands to PageRuler™ Plus Prestained Protein Ladder (Fermentas, York, UK). Two bands were identified for each antibody with no 415 detectable contamination present. Purified antibodies were testing using the CD52 peptide flow cytometry as described above. From flow cytometry analysis (Figure 2), the lead monoclonal antibody designated 2E8 was shown to bind selectively to NS0-CD52 cells.

EXAMPLE 2 – VARIABLE REGION GENE SEQUENCING

420 Total RNA was extracted from 2E8 hybridoma cells using the RNAqueous-4PCR Kit (Ambion, Warrington, UK) and used to synthesis cDNA. Murine immunoglobulin heavy and kappa light chain variable (V) region fragments were amplified by PCR using degenerate mouse leader sequence primers (Sigma) and unique constant domain primers (Sigma) as shown in Table 1. The resulting PCR fragments were subcloned into the pGEM-T Easy I vector system (Promega, 425 Southampton, UK) and inserts were sequenced using the vector-specific primer, M13Forward (Sigma). All DNA sequencing was performed by Geneservice Ltd, Cambridge, UK). The resultant V region nucleotide sequences are shown as SEQ ID No.1 and SEQ ID No.2 and corresponding amino acid sequences as SEQ ID No.3 and SEQ ID No.4 for heavy and light chain V regions respectively.

430

Sequence	Name-Pool
ATGRASSTSKGGYTMARCTKGRTTT	MulgV _H 5'-A
ATGRAATGSASCTGGGTYWTYCTCTT	MulgV _H 5'-B
ATGGACTCCAGGCTAATTAGTTTCT	MulgV _H 5'-C
ATGGCTGTCYTRGBGCTGYTCYTCTG	MulgV _H 5'-C

ATGGVTTGGSTGTGGAMCTTGCYATTCCCT	MulgV _H 5'-C
ATGAAATGCAGCTGGRTYATSTTCTT	MulgV _H 5'-D
ATGGRCAGRCTTACWTYYCATTCCCT	MulgV _H 5'-D
ATGATGGTGTAAAGCTTCTGTACCT	MulgV _H 5'-D
ATGGGATGGAGCTRTATCATSYTCTT	MulgV _H 5'-E
ATGAAGWTGTGGBTRAACGGRT	MulgV _H 5'-E
ATGGRATGGASCKKIRTCTTMTCT	MulgV _H 5'-E
ATGAACTTGGGYSAGMTTGRRTT	MulgV _H 5'-F
ATGTAACGGGACTGAGCTGTGTAT	MulgV _H 5'-F
ATGAGAGTGCTGATTCTTTGTG	MulgV _H 5'-F
ATGGATTTGGGCTGATTTTTATTG	MulgV _H 5'-F
CCAGGGRCARKGGATARACIGRTGG	MulgV _H 3'-2
ATGRAGWCACAKWCYCAGGTCTT	MulgV _I 5'-A
ATGGAGACAGACACACTCCTGCTAT	MulgV _I 5'-B
ATGGAGWCAGACACACTSCTGYATGGT	MulgV _I 5'-C
ATGAGGRCCCTGCTCAGWTTTGGIWTCTT	MulgV _I 5'-D
ATGGGCWTCAAGATGRAGTCACAKWYYCWGG	MulgV _I 5'-D
ATGAGTGTGCYCACTCAGGTCTGGSGTT	MulgV _I 5'-E
ATGTGGGGAYCGKTTTYAMMCTTCAATTG	MulgV _I 5'-E
ATGGAAGCCCCAGCTCAGCTCTCTCC	MulgV _I 5'-E
ATGAGIMMKTCIMTCATTCYTG	MulgV _I 5'-F
ATGAKGTHCYCIGCTCAGTYCTIRG	MulgV _I 5'-F
ATGGTRTCCWCASCTCAGTTCTT	MulgV _I 5'-F
ATGTATATATGTTGGTGTATTCT	MulgV _I 5'-F
ATGAAGTTGCCTGTTAGGCTGTTGGGCT	MulgV _I 5'-G
ATGGATTWCARGTCAGATTWTCAAGCTT	MulgV _I 5'-G
ATGGTYCTYATVTCCTGCTGTTCTGG	MulgV _I 5'-G
ATGGTYCTYATVTRCTGCTGCTATGG	MulgV _I 5'-G
ACTGGATGGTGGGAAGATGGA	MulgV _I 3'-1

Table 1

435

Sequences of the 2E8 hypervariable regions (CDRs) were as follows;

SEQ ID NO. 5 CDRH1 RYGMS

440 SEQ ID NO. 6 CDRH2 MMKTKGGRTYYPDSVKG

SEQ ID NO. 7 CDRH3 DGYY

SEQ ID NO. 8 CDRL1 KSSQSLLHSDGKTYLN

SEQ ID NO. 9 CDRL2 LVSKLDs

SEQ ID NO. 10 CDRL3 WQGTHLWT

445

EXAMPLE 3 – GENERATION OF CHIMERIC ANTIBODY

The heavy and light chain V region sequences of the 2E8 monoclonal antibody were PCR amplified and subcloned into pANT antibody expression vectors (Figure 1b) with heavy and light chain V regions cloned into pANT17 and pANT13 respectively. Heavy chain V region genes were cloned into pANT17 via MluI and HindIII sites in frame with the human $\gamma 1$ heavy chain gene (G1m3 (G1m(f)) allotype) and light chain V region genes were cloned into pANT13 via BssHII and BamHI sites in frame with the human kappa light chain constant region gene (Km3 allotype). Transcription of both heavy and light chain genes was under the control of the CMV I/E promoter (US5168062 and US5385839, University of Iowa) and the pANT17 plasmid 450 contained a mutant *dhfr* minigene (Simonsen & Levinson 1983, PNAS **80**:2495-2499) under the control of a SV40 promoter and polyA sequence for selection in eukaryotic cells. Both pANT17 and pANT13 contained a β -lactamase (Ap^R) gene for prokaryotic selection and a pMB1 origin of replication for propagation in prokaryotic cells. All plasmids were propagated in *E. coli* XL1-blue (Stratagene Cat. No. 200130). Primers used to amplify the variable region genes for cloning 455 into the pANT expression vectors are shown in Table 2.

Sequence	Name
ctgttgtacgcgtgtccactccGAGGTGCACCTGATGGAG	2E8 VH 5'
ctgccccagaaagttaccTGAGGGAGACTGTGAGAGTG	2E8 VH 3'
ggctcccaaggcgcgcgtgtGATGTTTGATGACCCAGAC	2E8 VK 5'
gaattgcggatccaactgaggaagcaaagttaattctactcacgTTTGATTTCCAGTTGGTGCC	2E8 VK 3'

Table 2

465

The heavy and light chain expression constructs were then co-transfected either transiently into HEK293 cells by calcium phosphate-based transfection or stably transfected into NS0 cells by

electroporation. Secreted antibody was purified from the cell culture supernatants by Protein A chromatography. As shown in Figure 3 using flow cytometry analysis as detailed in Example 1, 470 dilutions of the 2E8 antibody exhibited an improved binding profile to CD52+ HuT78 cells compared to Campath-1H. As shown in Figure 4, the 2E8 antibody exhibited an improved competitive binding profile by flow cytometry analysis when competed with Campath-1H for binding to CD52+ HuT78 cells.

475 **EXAMPLE 4 – ANTIBODY-DEPENDENT CELL-MEDIATED CYTOTOXICITY (ADCC)**

ADCC assays were performed with the 2E8 monoclonal antibody as follows. Target cells (either REH or Raji cells) were harvested and preloaded with 25 μ M (final) Calcein-AM (Sigma). After 480 incubation with Calcein for 1hr at 37°C, cells were washed in media to remove unincorporated Calcein. 1x10⁴ target cells were added to a clear V-bottomed plate containing 2E8 or control antibodies at 50 μ g/ml final as in Figure 5 or as depicted in Figure 6, and incubated for 1hr to pre-opsonise the target cells. PBMCs (effector cells) were isolated from healthy community donor buffy coats (from blood drawn within 24 hours) obtained from the UK National Blood Transfusion Service (Addenbrooke's Hospital, Cambridge, UK) and according to approval 485 granted by Addenbrooke's Hospital Local Research Ethics Committee. PBMCs were isolated from buffy coats by Lymphoprep (Axis-Shield, Dundee, UK) density centrifugation. 5x10⁵ effector cells were added to each well of the plate containing target cells and antibody in a final volume of 250 μ l (50:1 ratio of effector to target cells). Samples were incubated for 4hr at 37°C / 5% CO₂. After 4 hours, Triton X-100 was added to the control wells containing cells (effector 490 and/or target cells) to establish the maximum lysis control. Following centrifugation, 150 μ l media was transferred from each well to a 96 well clear-bottom black-walled plate and the plate fluorescence was measured at 520nm. Results were expressed as:

495
$$\% \text{ Cytotoxicity} = \frac{(\text{test sample signal} - \text{background Calcein-AM release})}{(\text{maximum target cell lysis signal} - \text{background Calcein-AM release})} \times 100$$

Figure 5 shows the mean ADCC for PBMC from 5 human donors for chimeric 2E8 ('ANT01') and Campath-1H for target CD52+ REH cells showing a significantly enhanced ADCC for

500 chimeric 2E8. Subsequently a high CD52 expressing variant REH cell line was isolated by FACS which exhibited approximately 2x the binding of Campath-1H compared to the parent REH cells. Figure 6 shows a dilution series of chimeric 2E8 and Campath-1H for ADCC on high CD52+ REH cells using PBMC from two individual donors. This also shows the significantly enhanced ADCC profile for chimeric 2E8 compared to Campath-1H.

505

EXAMPLE 5 – COMPLEMENT-DEPENDENT CYTOTOXICITY (CDC)

CDC assays were performed on the 2E8 monoclonal antibody as follows. Target cells (either REH or Raji cells) were harvested and 5×10^4 cells/well were added to a black-walled, clear flat-bottomed 96 well plate. 2E8 or control antibodies for final concentrations as shown in Figure 7 510 were added together with either active or heat inactivated (@60°C for 30min) human serum (Pathway Diagnostics Ltd, Dorking, UK) per well (25% final serum concentration). Samples were incubated for 3hr at 37°C / 5% CO₂. After 3 hours, Triton X-100 was added to the control cell containing wells to establish the maximum lysis control. Prestoblue (10x) cell viability reagent (Invitrogen) was diluted with assay growth media and added to each well to obtain a 515 final 1 in 10 dilution of PrestoBlue. After incubation for 1hr at 37°C / 5% CO₂, the plate fluorescence was measured at 590nm. Results were expressed as:

$$\% \text{ Cell Viability} = \frac{(\text{Sample minus background release}) \times 100}{(\text{max readout (No lysis) minus background readout})}$$

520

$$(\text{max readout (No lysis) minus background readout})$$

Figure 7 shows a significantly enhanced CDC profile for chimeric 2E8 compared to Campath-1H.

525

EXAMPLE 6 – GENERATION OF HUMANISED ANTIBODIES

Humanised antibodies were generated using methods described in EP1844074 (Antitope Ltd). Structural models of the mouse 2E8 V regions were produced using Swiss PDB and analysed in order to identify important amino acids that were likely to be important for the CD52 binding 530 properties of the antibody ('constraining residues'). A database of human V region sequences was used to identify segments of human V region sequences containing each of the constraining

residues to be used in design of the humanised antibodies. Typically two or more alternative V region sequence segments were used to provide each constraining residue resulting in a large range of possible sequences of humanised anti-CD52 V region sequences for 2E8. These 535 sequences were then analysed for the prediction of non-germline MHC class II peptide binding by *in silico* analysis as described in Fothergill et al. (WO9859244, assignee Eclagen Ltd) and also for known CD4+ T-cell epitopes using databases including “The Immune Epitope Database and Analysis Resource”, <http://www.immuneepitope.org/>. V region sequences with predicted non-germline MHC class II binding peptides, or with significant hits against T cell epitope 540 databases were discarded. This resulted in a reduced set of V region sequences. Selected combinations of V region sequence segments were then combined to produce humanised heavy and light chain variable region amino acid sequences. Five heavy chains and four light chain sequences (designated VH1 to VH5, and V κ 1 to V κ 4 respectively) were selected for 2E8 (SEQ ID No.s 20 to 24 and 25 to 28 respectively).

545

DNA encoding humanised variant V regions were synthesised and subcloned into the expression vectors pANT17 and pANT13 (Figure 1) as described in Example 3. All combinations of humanised VH and V κ chains (i.e. a total of 20 pairings for 2E8) were transiently transfected into HEK293 and also transfected into NS0 cells, and antibody was purified by protein A 550 chromatography from the culture supernatants as described in Example 3.

EXAMPLE 7 – ANALYSIS OF HUMANISED ANTIBODIES

The binding of HEK-derived and NS0-derived 2E8 humanised variants to CD52 peptide was assessed in a competition ELISA against the parent chimeric antibody. The parental 2E8 555 chimeric antibody was biotinylated using Biotin TagTM Micro Biotinylation kit (Sigma–Aldrich). 96 well MaxiSorp plates (Nunc) were coated with 0.025 μ g/ml CD52 peptide-KLH in Dulbecco's PBS (PAA Laboratories, Yeovil, UK) (100 μ l final volume) at 4°C overnight. Plates were blocked with Dulbecco's PBS-2% BSA for 1 hour at room temperature. Plates were washed 3 times with wash buffer (0.05% Tween20 in Dulbecco's-PBS). Test humanised antibodies at 560 various concentrations were premixed with biotinylated parent chimeric antibody (0.035 μ g/ml final concentration) and then added to the CD52 peptide-KLH plate (100 μ l final volume). All samples were tested in duplicate. Plates were incubated for 1h at room temperature and washed

3 times with wash buffer. 100µl of a 1 in 1000 dilution of Streptavidin HRP (Sigma-Aldrich) was added and incubated for 1 hour at room temperature. Plates were washed 3 times with wash 565 buffer and 100µl of TMB substrate (Invitrogen) was added and incubated at room temperature in the dark to allow the colour to develop. The reaction was stopped by adding 50 µl of 3M HCl. Plates were read at 450nm using Dynex plate reader.

As shown in Figure 8, all lead humanised 2E8 variants displayed competitive binding profiles 570 similar to the parent chimeric antibody. Humanised variants were subsequently tested for binding by flow cytometry as detailed in Example 1, for ADCC as in Example 4, and for CDC as in Example 5. As shown in Figure 9, the humanised variants exhibited an improved binding profile by flow cytometry to Campath-1H for binding to REH cells. As shown in Figures 10 and 11, the humanised variants also exhibited improved ADCC and CDC profile using REH target cells for 575 ADCC at a target:effector cell ratio of 50:1 or the high CD52+ REH cell line for CDC (as in Example 4) compared to Campath-1H.

EXAMPLE 8 – GENERATION OF scFv's and Fab's

Humanised 2E8 variants from Example 6 were converted into scFv's and cloned into M13 phage 580 display vectors as described in Benhar I. and Reiter Y., Current Protocols in Immunology, Unit 10.19B, Wiley Online Library, May 2002 (<http://www.currentprotocols.com/protocol/im1019b>) using the pCANTAB5E vector RPAS Expression Module (Amersham Pharmacia Biotech, Little Chalfont, UK). Humanised VH and VK genes were amplified using primers which provided 585 terminal SfiI and NotI restriction sites, an internal Gly4Ser linker and a C terminal his6 tag. The scFv constructs were inserted into the pCANTAB5E vector as SfiI-NotI fragments and transformed into E.coli HB2151 resulting in scFv exported to the periplasm and partially to the growth medium. scFv's were purified from growth medium by nickel-chelate affinity chromatography using HIS-Select HF Cartridges (Sigma-Aldrich). Purified 2E8 scFv's were tested in a competition assay as detailed in Example 7 for binding to CD52 peptide and all 590 humanised scFvs exhibited competitive binding to CD52 peptide. Humanised 2E8 variants from Example 6 were also converted into Fab's using the method used for scFv's except that amplified humanised VH and VK genes were further amplified with CH1 and C κ constant region genes to form VH-CH1 and VK-C κ fragments which were further amplified with primers to join

these fragments with a 22 amino acid pelB leader sequence (Lei S.P. et al., J Bacteriol. 169 595 (1987) p4379–4383) between the upstream VH-CH1 and downstream VK-C κ gene fragments resulting in a dicistronic Fab gene. Fab's from humanised 2E8 variants were generated and purified as above for scFv's and tested in CD52 peptide competition assay as detailed in Example 7. All humanised Fab's exhibited competitive binding to CD52 peptide.

600 **EXAMPLE 9 – ANALYSIS OF CD4+ T CELL RESPONSES**

PBMCs were isolated from healthy community donor buffy coats (from blood drawn within 24 hours) obtained from the UK National Blood Transfusion Service (Addenbrooke's Hospital, Cambridge, UK) and according to approval granted by Addenbrooke's Hospital Local Research Ethics Committee. PBMCs were isolated from buffy coats by Lymphoprep (Axis-shield, 605 Dundee, UK) density centrifugation and CD8 $^{+}$ T cells were depleted using CD8 $^{+}$ RosetteSepTM (StemCell Technologies Inc, London, UK). Donors were characterized by identifying HLA-DR haplotypes using an HLA SSP-PCR based tissue-typing kit (Biotest, Solihull, UK). T cell responses to control antigens including the recall antigen tetanus toxin were also determined (KLH Pierce, Cramlington, UK and peptides derived from Influenza A and Epstein Barr 610 viruses). PBMC were then frozen and stored in liquid nitrogen until required.

To prepare monocyte derived dendritic cells (DC), 50 different donor PBMCs were selected to provide a distribution with frequencies of HLA-DR and HLA-DQ allotypes similar to the frequencies in the overall world population. PBMCs were revived in AIM-V® culture medium 615 and CD14 $^{+}$ cells isolated using Miltenyi CD14 Microbeads and LS columns (Miltenyi Biotech, Oxford, UK). Monocytes were resuspended in AIM-V® supplemented with 1000U/ml IL-4 and 1000U/ml GM-CSF (“DC culture media”) to 4-6x10 6 PBMC/ml and then distributed in 24 well plates (2ml final culture volume). Cells were fed on day 2 by half volume DC culture media change. By day 3, monocytes had differentiated to semi-mature DC which were pre-incubated 620 with either 40ug/ml of Campath-1H, chimeric 2E8 antibody, humanised 2E8 antibodies, 100 μ g/ml KLH or media only. Semi-mature DC were incubated with antigen for 24 hours after which excess test antibody was removed by washing the cells twice and resuspending in DC culture media supplemented with 50ng/ml TNF- α (Peprotech, London, UK). DCs were fed on day 7 by a half volume DC culture media (supplemented with 50ng/ml TNF α) change before

625 harvesting mature DC on day 8. The harvested mature DC were counted and viability assessed using trypan blue dye exclusion. The DC were then γ -irradiated (4000 rads) and resuspended at 2×10^5 cells per ml in AIM-V media before use in the ELISpot and proliferation assays. Additionally, on day 8, fresh CD4+ T cells were also prepared. To purify CD4+ T cells, PBMCs 630 were revived in AIM-V® culture medium and CD4⁺ cells isolated using Miltenyi CD4 Microbeads and LS columns (Miltenyi Biotech, Oxford, UK) and resuspended in AIM-V® media at 2×10^6 cells/ml.

On day 8, T cell proliferation assays were established whereby 1×10^5 autologous CD4⁺ T cells 635 were added to 1×10^4 humanised 2E8 or chimeric 2E8 antibody loaded DC (ratio of 10:1) in 96 well U-bottomed plates, with AIM-V® media added to a final volume 200ul/well). On day 14, assay plates were pulsed with 1uCi [3H] (Perkin Elmer, Beaconsfield, UK) per well in 25ul AIMV for 6 hours before harvesting onto filter mats (Perkin Elmer) using a TomTec Mach III (Hamden CT, USA) cell harvester. Counts per minute (cpm) for each well were determined by 640 Meltilex™ (Perkin Elmer) scintillation counting on a 1450 Microbeta Wallac Trilux Liquid Scintillation Counter (Perkin Elmer) in paralux, low background counting. Counts per minute for each antibody sample were normalised to the media only control.

For ELISpot assays, ELISpot plates (Millipore, Watford, UK) were coated with 100 μ l/well IL-2 645 capture antibody (R&D Systems, Abingdon, UK) in PBS. Plates were then washed twice in PBS, incubated overnight in block buffer (1% BSA (Sigma) in PBS) and washed in AIM V® medium. On day 8, 1×10^5 autologous CD4⁺ T cells were added to 1×10^4 antigen loaded DC (ratio of 10:1) in 96 well ELISpot plates. All preparations were tested in sextuplet cultures. For each donor PBMC, a negative control (AIM V® medium alone), no cells control and a PHA (10ug/ml) positive control were also included.

650

After a further 7 day incubation period, ELISpot plates were developed by three sequential washes in dH₂O and PBS prior to the addition of 100 μ l filtered biotinylated detection antibody (R&D Systems, Abingdon, UK) in PBS/1% BSA. Following incubation at 37°C for 1.5 hour, plates were further washed three times in PBS and 100 μ l filtered streptavidin-AP (R&D 655 Systems) in PBS/1% BSA was added for 1 hour (incubation at room temperature).

Streptavidin-AP was discarded and plates were washed four times in PBS. BCIP/NBT (R&D Systems) was added to each well and incubated for 30 minutes at room temperature. Spot development was stopped by washing the wells and the backs of the wells three times with dH₂O. Dried plates were scanned on an ImmunoscanTM Analyser and spots per well (spw) were determined using ImmunoscanTM Version 4 software.

For both proliferation and IL-2 ELISpot assays, results were expressed as a Stimulation Index (SI) defined as the ratio of cpm (proliferation assay) or spots (ELISpot assay) for the test antibody against a medium-only control using a threshold of SI equal to or greater than 2 (SI \geq 2.0) for positive T cell responses. The data showed that both Campath-1H and chimeric 2E8 antibody induced T cell responses in 10 or more of the 50 donor PBMCs tested (\geq 20%) whilst none of the humanised 2E8 antibodies induced T cell responses in more than 2 of 50 donors (\leq 4%) demonstrating the effectiveness of the humanisation process in removing T cell responses from the V regions.

670

EXAMPLE 10 – DIRECT CYTOXICITY ASSAY

Direct cytotoxic effects of anti-human CD52 antibodies were assessed using Annexin V / Propidium Iodide co-staining as markers of apoptosis and necrosis respectively. 1 \times 10⁵ REH cells were plated in the presence of 100 μ g/ml anti-human CD52 test antibodies or an isotype matched control antibody +/- 100 μ g/ml F(ab')² crosslinking antibody (Jackson ImmunoResearch, Cat no. 109-006-008) (600ul final vol). Cells were incubated for 72hrs before washing in PBS/2%BSA followed by co-staining with Annexin V/Propidium Iodide according to manufacturers recommended protocol (Invitrogen, Cat no. V13245). Scatterplots were generated using FACS analysis and divided into three regions for quantitation of live cells (unstained), apoptotic cells (FL1, Annexin V positive) and necrotic cells (FL1, Annexin V positive & FL3, Propidium Iodide positive). As shown in Figure 13, the humanised 2E8 antibodies exhibited increased apoptosis and necrosis compared to Campath-1H of REH target cells, with % necrotic cells of >40% from the humanised antibodies compared to 19.9% for Campath-1H.

685

EXAMPLE 11 - TUMOUR ANIMAL MODEL

A tumour animal model was used for *in vivo* analysis of anti-human CD52 antibodies in inhibiting tumour growth. In the model, Raji human Burkitt lymphoma cells were transplanted 690 into SCID mice and the animals treated with anti-human CD52 antibodies. 7 week old female Fox Chase SCID Mice (Charles River, Morrisville, North Carolina, USA) were injected with 1 x 10⁶ Raji cells (American Type Culture Collection, 0.2 mL cell suspension) via a bolus tail-vein (i.v.) injection. Anti-human CD52 test antibodies or an isotype matched control antibody were each administered intraperitoneally (i.p.) once daily on alternate days for seven doses, starting 695 three days after tumour cell injection. The dosing volume of 10 mL/kg (0.20 mL/20 g mouse) was scaled to the body weight of each animal, as determined twice weekly. The results shown in Figure 14 demonstrated an improved survival rate at both 1 and 10mg/kg doses by the lead VH3/VK4 anti-CD52 antibody (V region SEQ IDs: 22 and 28) compared to Campath 1H.

700

Sequences

>SEQ ID No. 1

2E8 Mouse VH DNA

705 GAGGTGCACCTGATGGAGTCTGGGGAGGCTTAGTGCAGCCTGGAGGGCCCTGAGACTCTCCTGTGC
AGCCTCTGGATTCACTTCACTAGGTATGGCATGTCTGGGTTGCCAGACTCCAGACAAGAGGCTGG
AGTTGGTCGCAATGATGAAAAGTAAAGGTGGTAGGACCTATTATCCAGACAGTGTGAAGGGCCGATTC
ACCATTCCAGAGACAATGCCAAGAAGTACCTGTACCTGCAAATGAGCAGTCTGAAGTCTGAGGACAC
AGCCATCTATTCTGTGCAAGTGAATGGTTACTACTGGGCCAAGGCACCACTCTCACAGTCTCCTCA

710

>SEQ ID No. 2

2E8 Mouse VK DNA

715 GATGTTTGATGACCCAGACTCCACTCACTTGTGGTAACCATTGGACAACCAGCCTCCATCTCTTGC
AACTCAAGTCAGAGCCTCTTACATAGTGTGGAAAGACATATTGAATTGGTTTACAGAGGCCAGG
CCAGTCTCAAAGGCCCTAATCTATCTGGTGTCTAACTGGACTCTGGAGTCCCTGACAGGTTCACTGG
CAGTGGATCAGGGACAGATTICACACTGAAAATCAGCAGAGTGGAGGCTGAGGATTGGAAATTATT
ATTGCTGGCAAGGTACACATTGTGGACGTTGGAGGCACCAAAGTGGAAATCAAA

720

>SEQ ID No. 3

2E8 Mouse VH amino acid

EVHLMESGGGLVQPGGSLRLSCAASGFTFSRYGMSWVRQTPDKRLELVAMMKTGGRTYYPDSVKGRFT
ISRDNAKNSLYLMQSSLKSEDTAIYFCASDGYYWGQGTTLVSS

725

>SEQ ID No. 4

2E8 Mouse VK amino acid

DVLMTQTPLTLSVTIGQPASICKSSQSLHSDGKTYLNWLLQRPGQSPKRILYLVSKLDGVPDRFTGSGS
GTDFTLKRVEAEDLGIYYCWQGTHLWTFGGGTKLEIK

730

>SEQ ID No. 5

2E8 VH CDR1 amino acid

RYGMS

>SEQ ID No. 6

2E8 VH CDR2 amino acid

MMKTGGRTYYPDSVKG

735

>SEQ ID No. 7

2E8 VH CDR3 amino acid

DGYY

740

>SEQ ID No. 8

2E8 VK CDR1 amino acid

KSSQSLHSDGKTYLN

745

>SEQ ID No. 9

2E8 VK CDR2 amino acid

LVSKLD

>SEQ ID No. 10

2E8 VK CDR3 amino acid

750 **WQGTHLWT****>SEQ ID No. 11****2E8 Humanised VH Variant 1 DNA**

755 GAGGTGCACCTGGTGAATCCGGCGGAGGACTGGTGCAGCCTGGCGGCTCCCTGAGACTGTCTTGCGCCGCTC
 CGGCTTCACCTCTCCAGATACGGCATGTCTGGTCCGACAGGCCCCCTGGCAAGGGCCTGGAACTGGTGGCCA
 TGATGAAGACCAAGGGCGGCAGAACCTACTACCCCGACTCCGTGAAGGGCCGGTTACCATCTCCCGGGACAAAC
 GCCAAGAACCTCCCTGTACCTGCAGATGTCTCCCTGAAGGCCGAGGACACCGCCATCTACTTTGCGCCTCCGA
 CGGCTACTACTGGGCCAGGGCACCACCGTGACCGTGTACATCA

760 **>SEQ ID No. 12****2E8 Humanised VH Variant 2 DNA**

765 GAGGTGCACCTGGTGAATCCGGCGGAGGACTGGTGCAGCCTGGCGGCTCCCTGAGACTGTCTTGCGCCGCTC
 CGGCTTCACCTCTCCAGATACGGCATGTCTGGTCCGACAGGCCCCCTGGCAAGGGCCTGGAACTGGTGGCCA
 TGATGAAGACCAAGGGCGGCAGAACCTACTACCCCGACTCCGTGAAGGGCCGGTTACCATCTCCCGGGACAAAC
 GCCAAGAACCTCCCTGTACCTGCAGATGAACCTCCCTGCAGGCCGAGGACACCGCCATCTACTTTGCGCCTCCGA
 CGGCTACTACTGGGCCAGGGCACCACCGTGACCGTGTACATCA

>SEQ ID No. 13**2E8 Humanised VH Variant 3 DNA**

770 GAGGTGCACCTGGTGAATCCGGCGGAGGACTGGTGCAGCCTGGCGGCTCCCTGAGACTGTCTTGCGCCGCTC
 CGGCTTCACCTCTCCAGATACGGCATGTCTGGTCCGACAGGCCCCCTGGCAAGGGCCTGGAACTGGTGGCCA
 TGATGAAGACCAAGGGCGGCAGAACCTACTACCCCGACTCCGTGAAGGGCCGGTTACCATCTCCCGGGACAAAC
 GCCAAGAACCTCCCTGTACCTGCAGATGAACCTCCCTGCAGGCCGAGGACACCGCCATCTACTACTGCGCCTCCGA
 CGGCTACTACTGGGCCAGGGCACCACCGTGACCGTGTACATCA

775

>SEQ ID No. 14**2E8 Humanised VH Variant 4 DNA**

780 GAGGTGCACCTGGTGAATCCGGCGGAGGACTGGTGCAGCCTGGCGGCTCCCTGAGACTGTCTTGCGCCGCTC
 CGGCTTCACCTCTCCAGATACGGCATGTCTGGTCCGACAGGCCCCCTGGCAAGGGCCTGGAACTGGTGGCCA
 TGATGAAGACCAAGGGCGGCAGAACCTACTACCCCGACTCCGTGAAGGGCCGGTTACCATCTCCCGGGACAAAC
 GCCAAGAACCTCCCTGTACCTGCAGATGAACCTCCCTGCAGGCCGAGGACACCGCCGTACTACTGCGCCTCCGA
 CGGCTACTACTGGGCCAGGGCACCACCGTGACCGTGTACATCA

785

>SEQ ID No. 15**2E8 Humanised VH Variant 5 DNA**

790 GAGGTGCACCTGGTGAATCCGGCGGAGGACTGGTGCAGCCTGGCGGCTCCCTGAGACTGTCTTGCGCCGCTC
 CGGCTTCACCTCTCCAGATACGGCATGTCTGGTCCGACAGGCCCCCTGGCAAGGGACTGGAATGGGTGGCCA
 TGATGAAGACCAAGGGCGGCAGAACCTACTACCCCGACTCCGTGAAGGGCCGGTTACCATCTCCCGGGACAAAC
 GCCAAGAACCTCCCTGTACCTGCAGATGAACCTCCCTGCAGGCCGAGGACACCGCCATCTACTACTGCGCCTCCGA
 CGGCTACTACTGGGCCAGGGCACCACCGTGACCGTGTACATCA

795

>SEQ ID No. 16**2E8 Humanised VK Variant 1 DNA**

GACGTGCTGATGACCCAGACCCCCCTGACCTGTCCGTGACCCCTGGGCCAGCCTGCCTCCATCTCTGCAAGTC
 CTCCTGCTGCACTCCGACGGCAAGACCTACCTGAACCTGGCTGAGCAGCGGCCTGGCCAGTCCCCCA
 AGCGGCTGATCTACCTGGTGTCCAAGCTGGACTCCGGCGTGGCCGACAGATTACCCGGCTCTGGCTCCGGCACC
 GACTTCACCTGAAGATCTCCGGGTGGAAGGCCGAGGACGTGGCATCTACTACTGCTGGCAGGGCACCCATCT
 GTGGACCTTCGGCGAGGCACAAAGGTGGAAATCAA

800

>SEQ ID No. 17

2E8 Humanised VK Variant 2 DNA

805 GACGTGCTGATGACCCAGACCCCCCTGACCCCTGTCCTGTGACCCCTGGGCCAGCCTGCCTCCATCTCCTGCAAGTC
CTCCCAGTCCTGCTGCACTCCGACGGCAAGACCTACCTGAACCTGGCTGCAGCAGCGGCCTGGCCAGTCTCCTC
GGCGGCTGATCTACCTGGTGTCCAAGCTGGACTCCGGCGTGCCTGACAGATTCACCGGCTCTGGCTCCGGCACC
GACTTCACCCCTGAAGATCTCCGGGTGGAAGCCGAGGACGTGGGCATCTACTACTGCTGGCAGGGCACCCATCT
GTGGACCTTCGGCGGAGGCACAAAGGTGAAATCAAA

>SEQ ID No. 18

2E8 Humanised VK Variant 3 DNA

810 GACGTGCTGATGACCCAGACCCCCCTGTCCTGTGACCCCTGGGCCAGCCTGCCTCCATCTCCTGCAAGTC
CTCCCAGTCCTGCTGCACTCCGACGGCAAGACCTACCTGAACCTGGCTGCAGCAGCGGCCTGGCCAGTCTCCTC
GGCGGCTGATCTACCTGGTGTCCAAGCTGGACTCCGGCGTGCCTGACAGATTCACCGGCTCTGGCTCCGGCACC
GACTTCACCCCTGAAGATCTCCGGGTGGAAGCCGAGGACGTGGGCATCTACTACTGCTGGCAGGGCACCCATCT
GTGGACCTTCGGCGGAGGCACAAAGGTGAAATCAAA

815

>SEQ ID No. 19

2E8 Humanised VK Variant 4 DNA

820 GACGTGGTGTGATGACCCAGACCCCCCTGTCCTGTGACCCCTGGGCCAGCCTGCCTCCATCTCCTGCAAGTC
CTCCCAGTCCTGCTGCACTCCGACGGCAAGACCTACCTGAACCTGGCTGCAGCAGCGGCCTGGCCAGTCTCCTC
GGCGGCTGATCTACCTGGTGTCCAAGCTGGACTCCGGCGTGCCTGACAGATTCTCCGGCTCTGGCTCCGGCACC
GACTTCACCCCTGAAGATCTCCGGGTGGAAGCCGAGGACGTGGGCATCTACTACTGCTGGCAGGGCACCCATCT
GTGGACCTTCGGCGGAGGCACAAAGGTGAAATCAAA

>SEQ ID No. 20

2E8 Humanised VH Variant 1 amino acid

825 EVHLVESGGGLVQPGGSLRLSCAASGFTSRYGMSWVRQAPGKGLELVAMMKTGGRTYYPPDSVKGRFTISRDNAKNS
LYLQMSSLKAEDTAIYFCASDGYYWGQGTTTVSS

>SEQ ID No. 21

2E8 Humanised VH Variant 2 amino acid

830 EVHLVESGGGLVQPGGSLRLSCAASGFTSRYGMSWVRQAPGKGLELVAMMKTGGRTYYPPDSVKGRFTISRDNAKNS
LYLQMNSLRAEDTAIYFCASDGYYWGQGTTTVSS

>SEQ ID No. 22

2E8 Humanised VH Variant 3 amino acid

835 EVHLVESGGGLVQPGGSLRLSCAASGFTSRYGMSWVRQAPGKGLELVAMMKTGGRTYYPPDSVKGRFTISRDNAKNS
LYLQMNSLRAEDTAIYYCASDGYYWGQGTTTVSS

>SEQ ID No. 23

2E8 Humanised VH Variant 4 amino acid

840 EVHLVESGGGLVQPGGSLRLSCAASGFTSRYGMSWVRQAPGKGLELVAMMKTGGRTYYPPDSVKGRFTISRDNAKNS
LYLQMNSLRAEDTAVYYCASDGYYWGQGTTTVSS

>SEQ ID No. 24

2E8 Humanised VH Variant 5 amino acid

845 EVHLVESGGGLVQPGGSLRLSCAASGFTSRYGMSWVRQAPGKGLEWVAMMKTGGRTYYPPDSVKGRFTISRDNAKNS
SLYLQMNSLRAEDTAIYYCASDGYYWGQGTTTVSS

850 >SEQ ID No. 25
2E8 Humanised VK Variant 1 amino acid
DVLMTQTPLTLSVTLGQPASICKSSQSLHSDGKTYLNWLQQRPGQSPKRLIYLVSKLDGVPDRFTGSGSGTDFTLKISR
VEAEDVGIYYCWQGTHLWTFGGGTKVEIK

855 >SEQ ID No. 26
2E8 Humanised VK Variant 2 amino acid
DVLMTQTPLTLSVTLGQPASICKSSQSLHSDGKTYLNWLQQRPGQSPRRLIYLVSKLDGVPDRFTGSGSGTDFTLKISR
VEAEDVGIYYCWQGTHLWTFGGGTKVEIK

860 >SEQ ID No. 27
2E8 Humanised VK Variant 3 amino acid
DVLMTQTPLTLSVTLGQPASICKSSQSLHSDGKTYLNWLQQRPGQSPRRLIYLVSKLDGVPDRFTGSGSGTDFTLKISR
VEAEDVGIYYCWQGTHLWTFGGGTKVEIK

865 >SEQ ID No. 28
2E8 Humanised VK Variant 4 amino acid
DVVMTQTPLTLSVTLGQPASICKSSQSLHSDGKTYLNWLQQRPGQSPRRLIYLVSKLDGVPDRFSGSGSGTDFTLKIS
RVEAEDVGIYYCWQGTHLWTFGGGTKVEIK

870

CLAIMS:

1. An anti-CD52 antibody comprising at least one, two, three, four or five CDR sequences selected from the group consisting of
 - 5 (i) CDRH1 comprising sequence RYGMS (SEQ ID NO:5)
 - (ii) CDRH2 comprising sequence MMKTKGGRTYYPDSVKG (SEQ ID NO:6)
 - (iii) CDRH3 comprising sequence DGYY (SEQ ID NO:7)
 - (iv) CDRL1 comprising sequence KSSQSLLHSDGKTYLN (SEQ ID NO:8);
 - 10 (v) CDRL2 comprising sequence LVSK LDS (SEQ ID NO:9) ; and
 - (vi) CDRL3 comprising sequence WQGTHLWT (SEQ ID NO:10).
2. An anti-CD52 antibody comprising one or more variable region sequences selected from the group consisting of SEQ ID NOS: 20-24 for the heavy chain variable region in combination with one or more sequences selected from the group consisting of SEQ ID NOS: 25-28 for the light chain variable region.
3. An anti-CD52 antibody comprising SEQ ID NO: 22 for the heavy chain variable region in combination with SEQ ID NO: 28 for the light chain variable region.
4. An anti-CD52 antibody of claims 1 to 3 which, when tested *in vitro* for induction of CD4+ helper T cell responses in 50 human blood samples with a distribution of HLA-DR allotypes from the human population, gives rise to <=4% of T cell responses.
- 25 5. The antibody of claims 1 to 4 wherein the variable region sequences are entirely derived from sequences in human antibody variable regions.
- 30 6. The antibody of claim 5 which is comprised of variable regions together with human constant regions.

7. The antibody of claim 6 where the human heavy chain constant region is either isotype IgG1, IgG2, IgG3 or IgG4, or a mutated IgG constant region, and the human light chain human constant region is isotype kappa.

5

8. The antibody of claim 6 where the human constant regions are IgG1 and kappa.

9. The antibody of claim 6 where the human constant regions are IgG4 and kappa.

10

10. The antibody of claims 1 to 5 where the antibody is a scFv or Fab.

11. The antibody of claims 1 to 10 which is a component of a multispecific protein which specifically binds to human CD52 and, additionally, binds or interacts with one or more other molecules.

15

12. A polynucleotide encoding an antibody of any of claims 1-10.

13. A vector comprising the polynucleotide of claim 12.

20

14. A vector of claim 13 wherein the vector is an expression vector.

15. A host cell comprising a vector of claims 13 or 14.

16. The host cell of claim 15 wherein the host cell is prokaryotic.

25

17. The host cell of claim 15, wherein the host cell is eukaryotic.

18. The host cell of claim 17 wherein the host cell is mammalian.

30

19. A composition comprising an anti-CD52 antibody of any of claims 1-11.

20. A composition comprising a polynucleotide of claim 12 or a vector of claims 13 or 14.

5 21. A method for treating a disease including CLL and other leukemias; autoimmune diseases including multiple sclerosis, rheumatoid arthritis, vasculitis, myositis, and diabetes; and organ transplant rejection and graft-vs-host disease comprising administering an effective amount of an antibody of any one of claims 1 to 11, a polynucleotide of any one of claims 12 to 14 or a composition of claim 19 or 20 to a 10 subject in need of such treatment.

22. The method of claim 21 which further comprises co-administering an effective amount of a chemotherapeutic agent.

15 23. The method of any of claims 21 or 22 which further comprises co-administering a pharmaceutical carrier.

24. A method for detecting the presence of human CD52 antigen in a sample using the antibody of any one of claims 1 to 11 for diagnosis of a human CD52-related disease.

20 25. A method for detecting the presence of human CD52 antigen in a sample using the polynucleotide or vectors of claims 12 to 14 for diagnosis of a human CD52-related disease.

25 26. An anti-CD52 antibody which, when tested *in vitro* for induction of CD4+ helper T cell responses in 50 human blood samples with a distribution of HLA-DR allotypes from the human population, gives rise to <=4% of T cell responses.

1/16

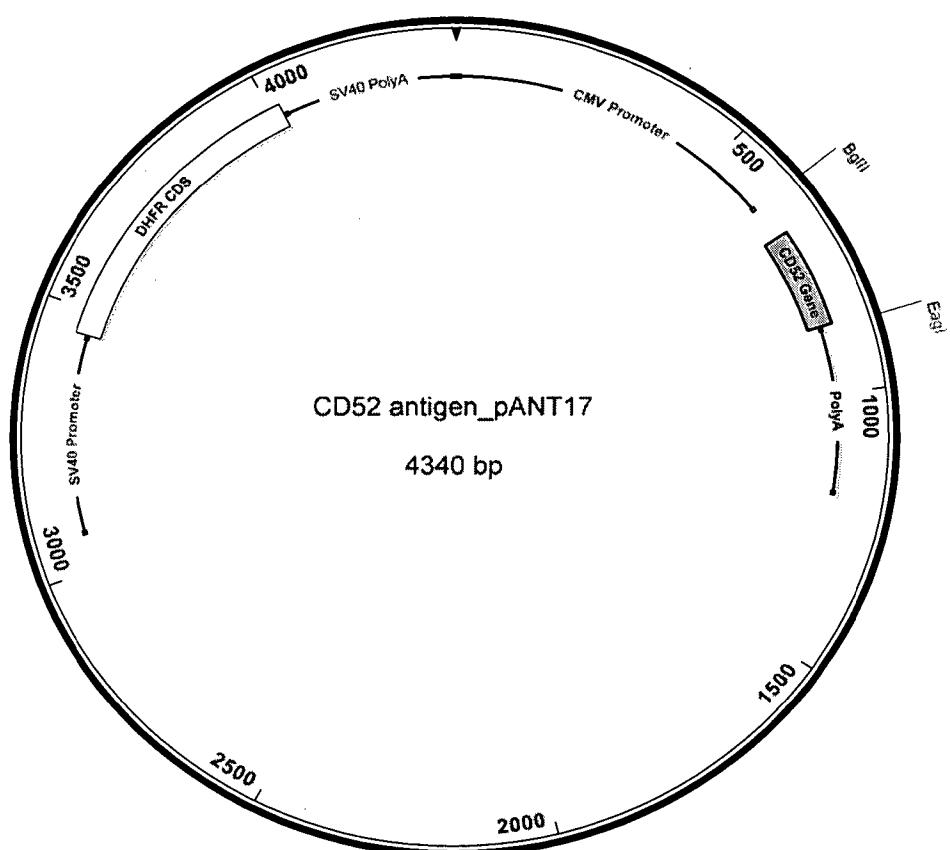


Figure 1a: Human CD52 expression vector

2/16

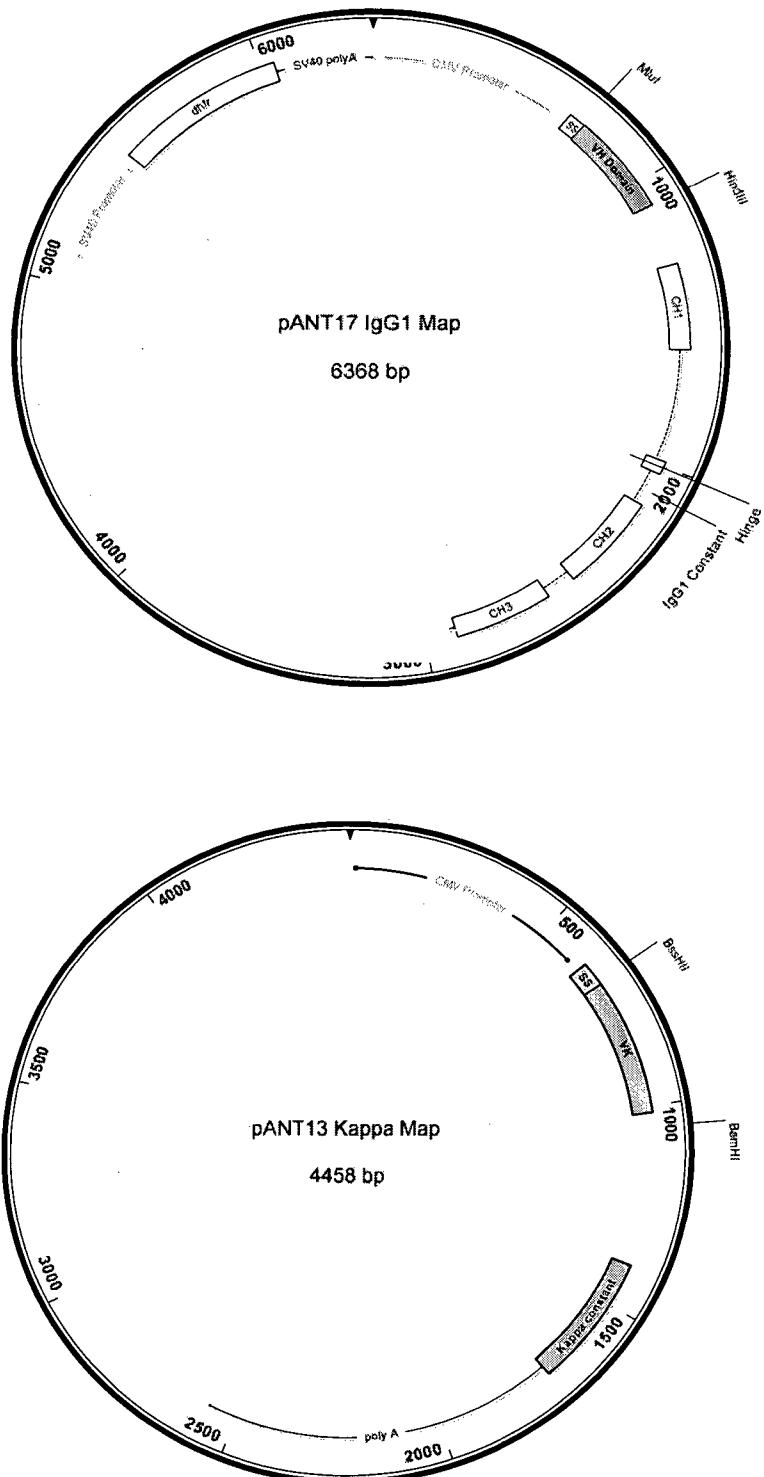
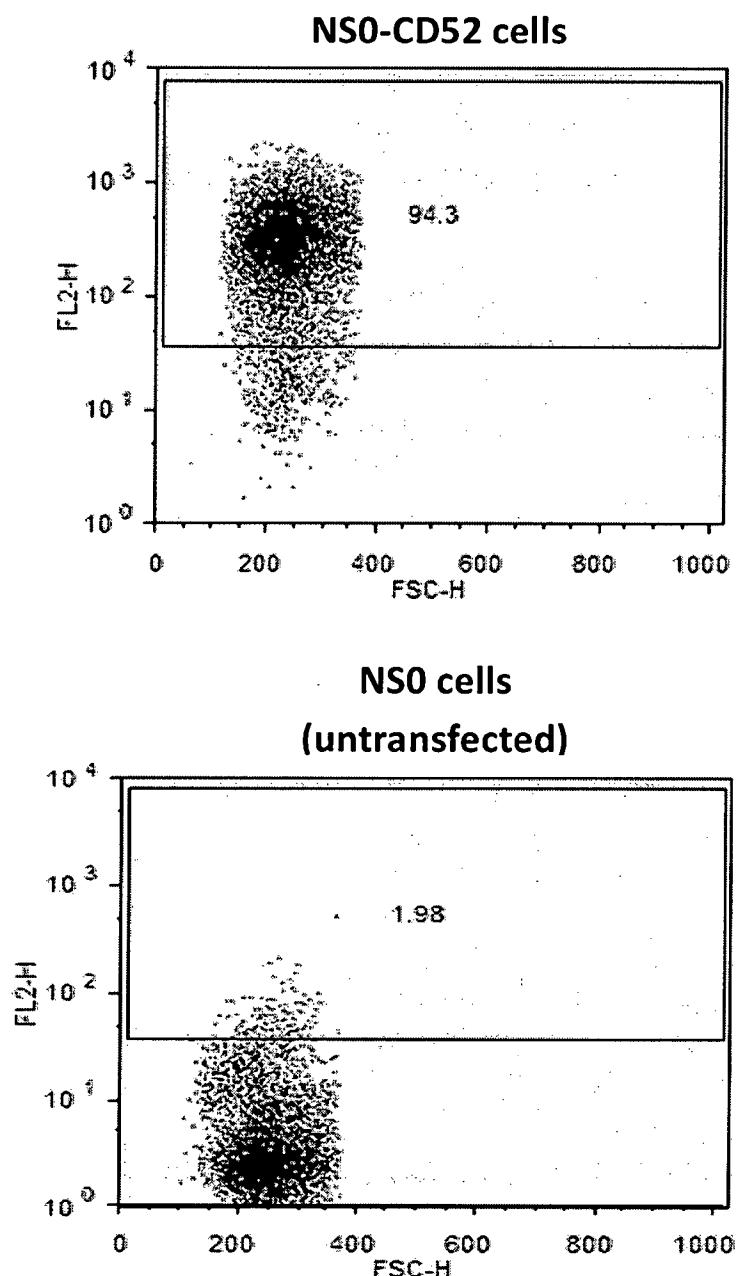


Figure 1b: Human IgG1 / kappa expression vectors

3/16

**Figure 2**

4/16

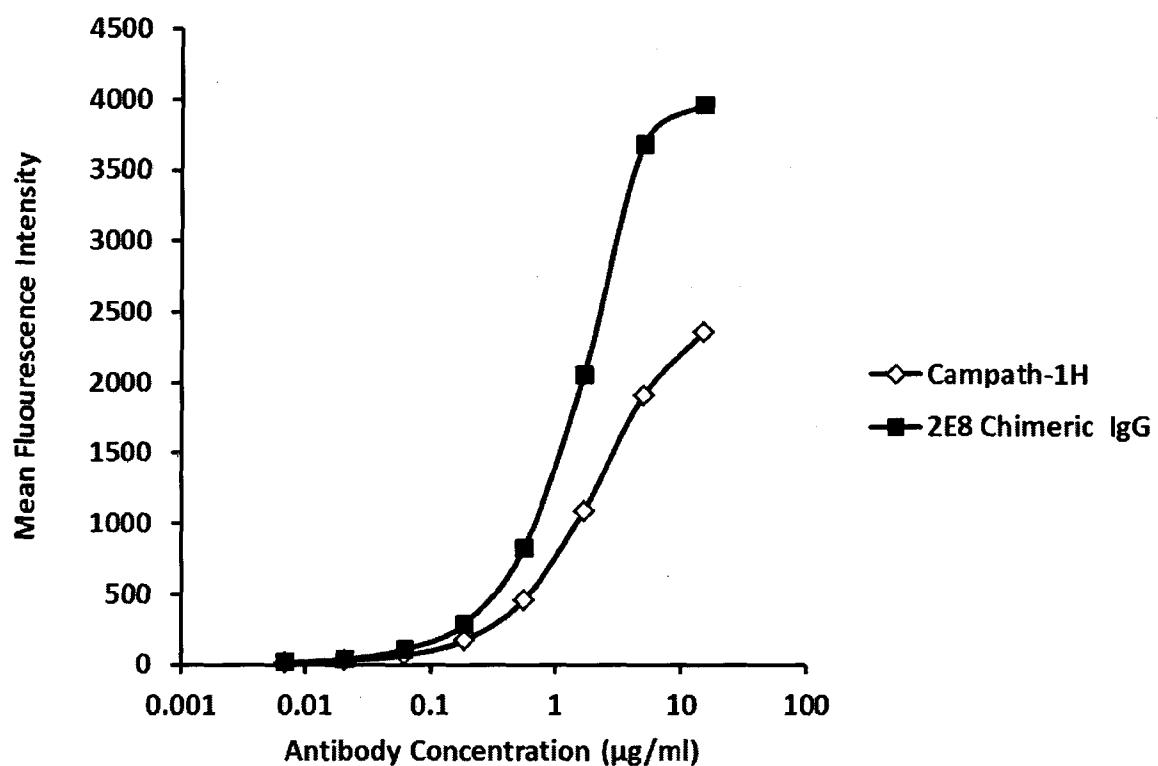


Figure 3

5/16

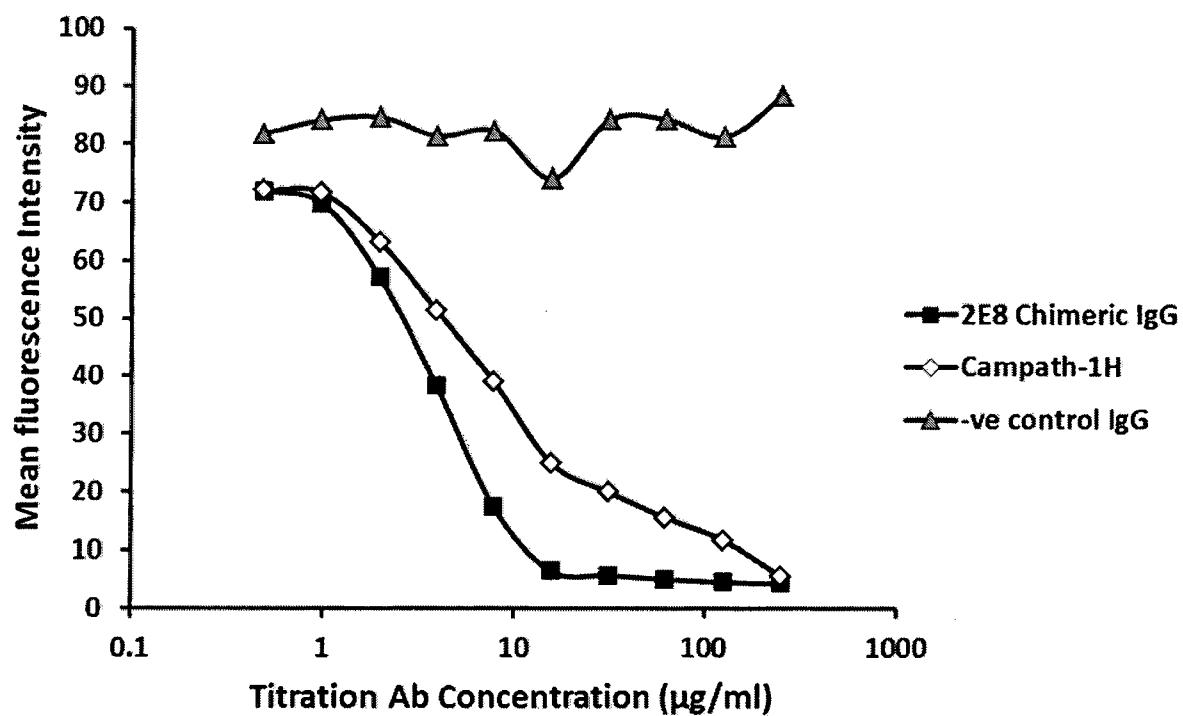
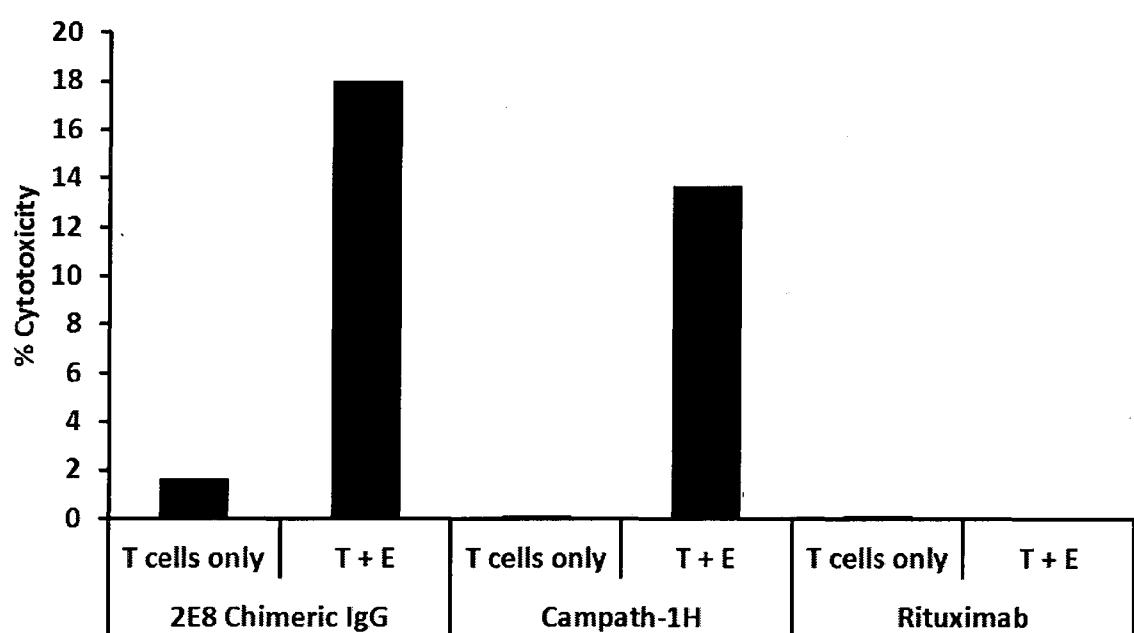


Figure 4

6/16



T = Target Cells (CD52+ REH)

E = Effector Cells (Human PBMC)

Figure 5

7/16

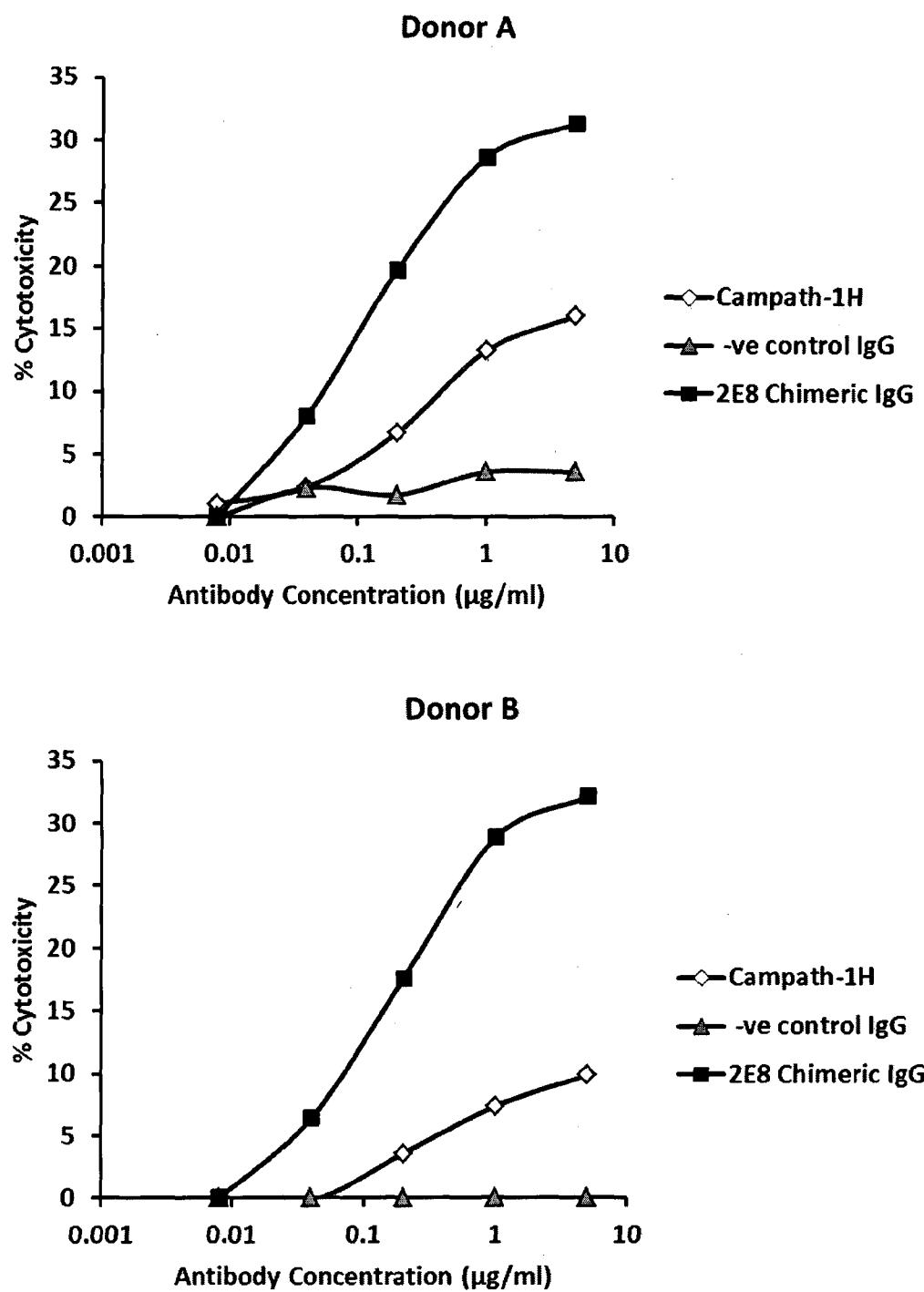
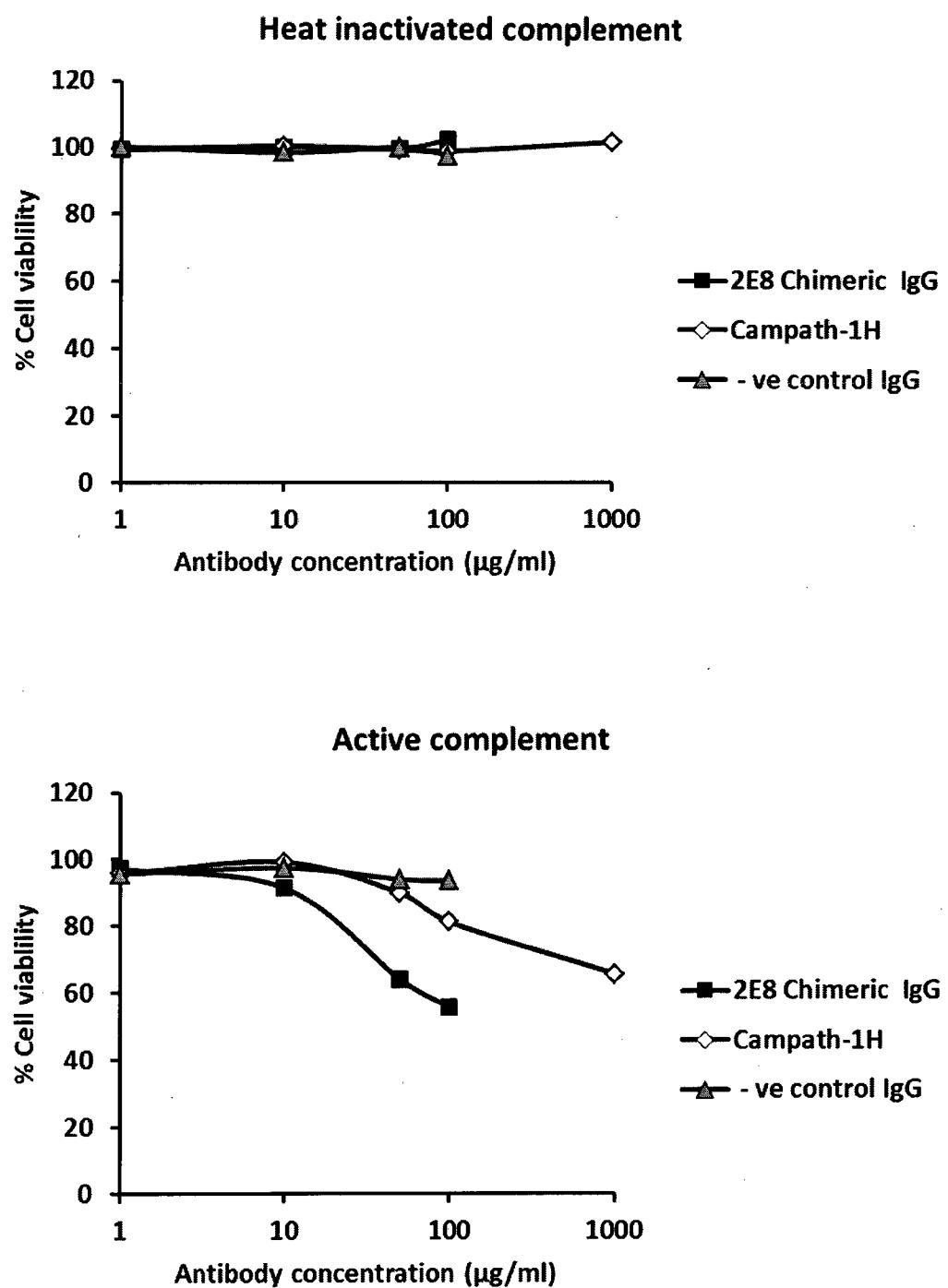


Figure 6

8/16

**Figure 7**

9/16

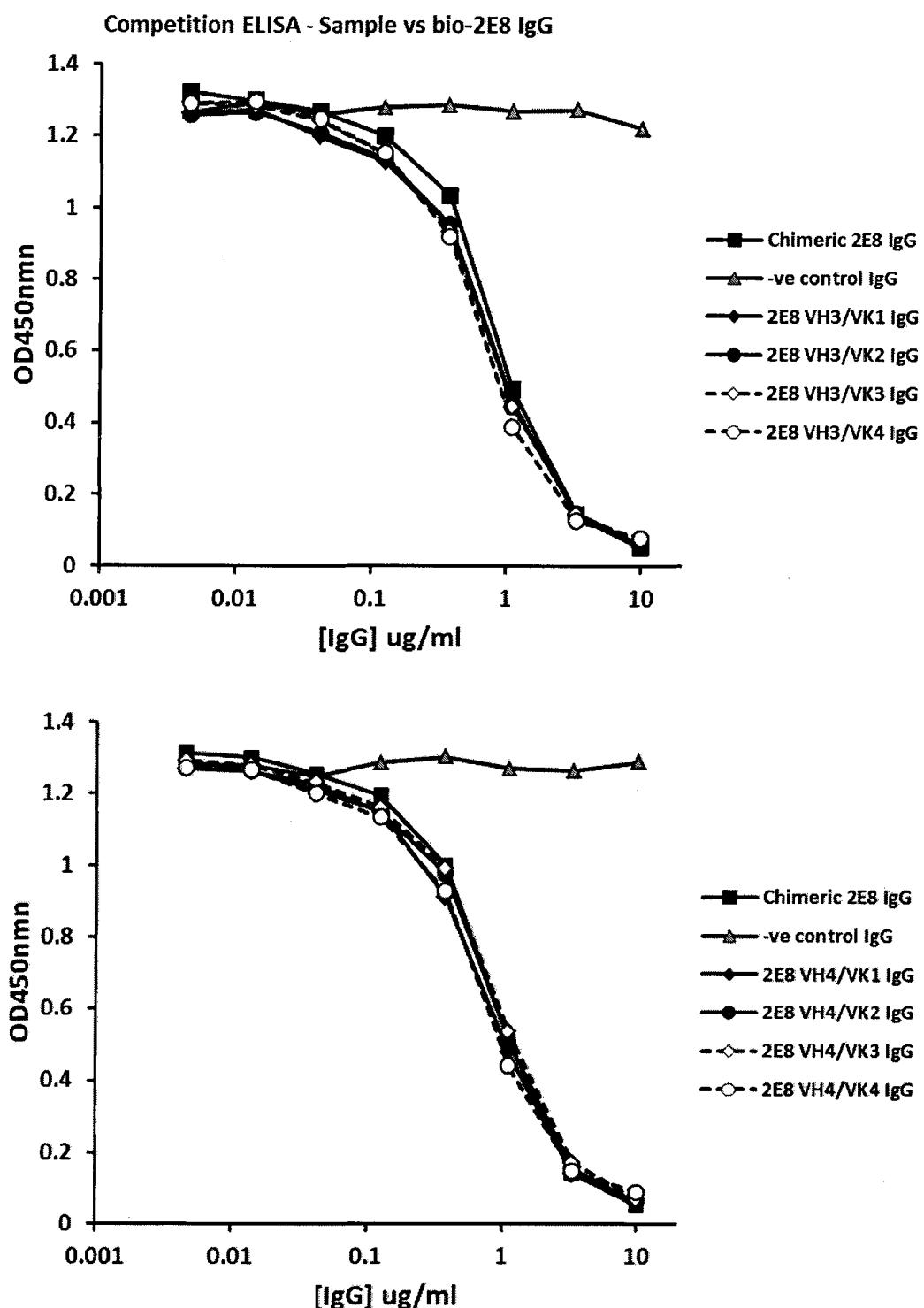


Figure 8

10/16

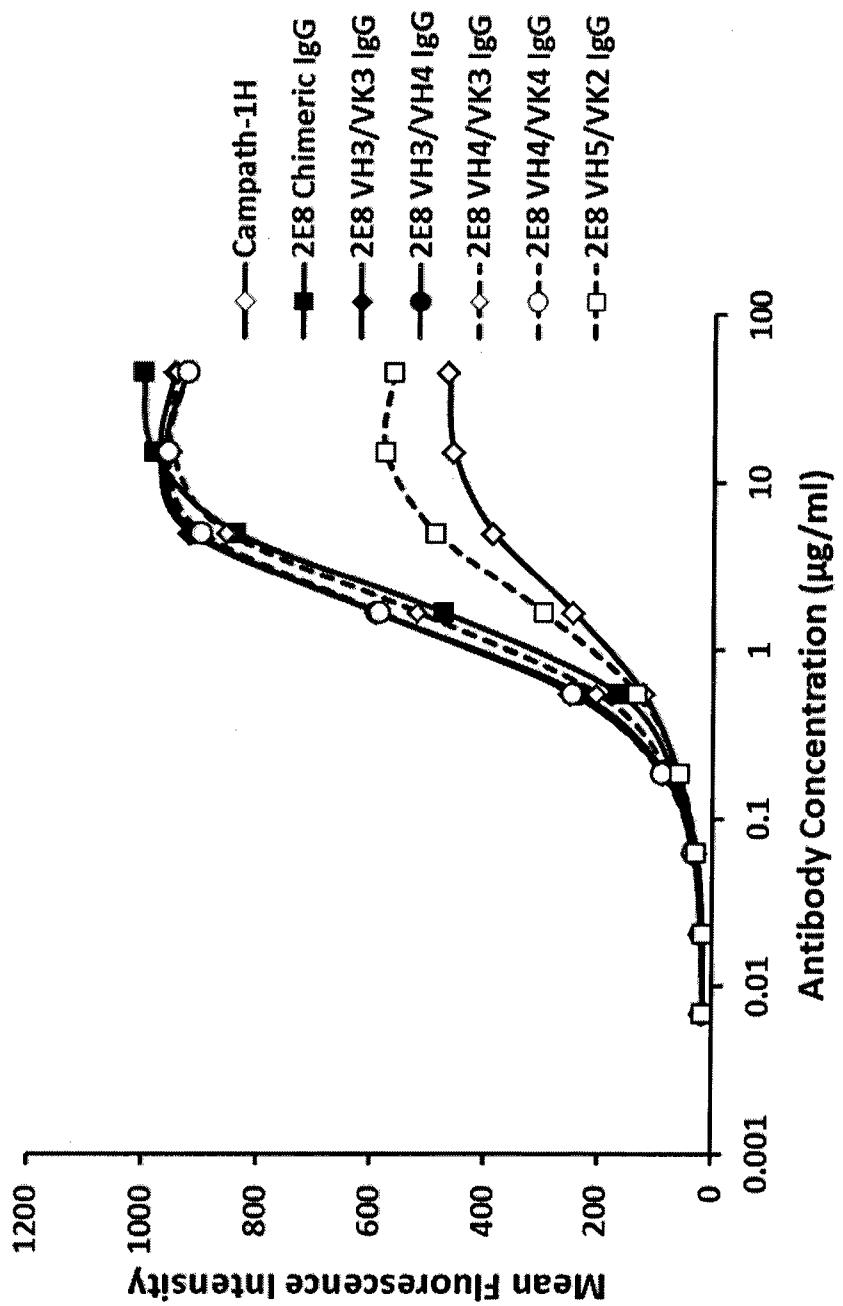


Figure 9

11/16

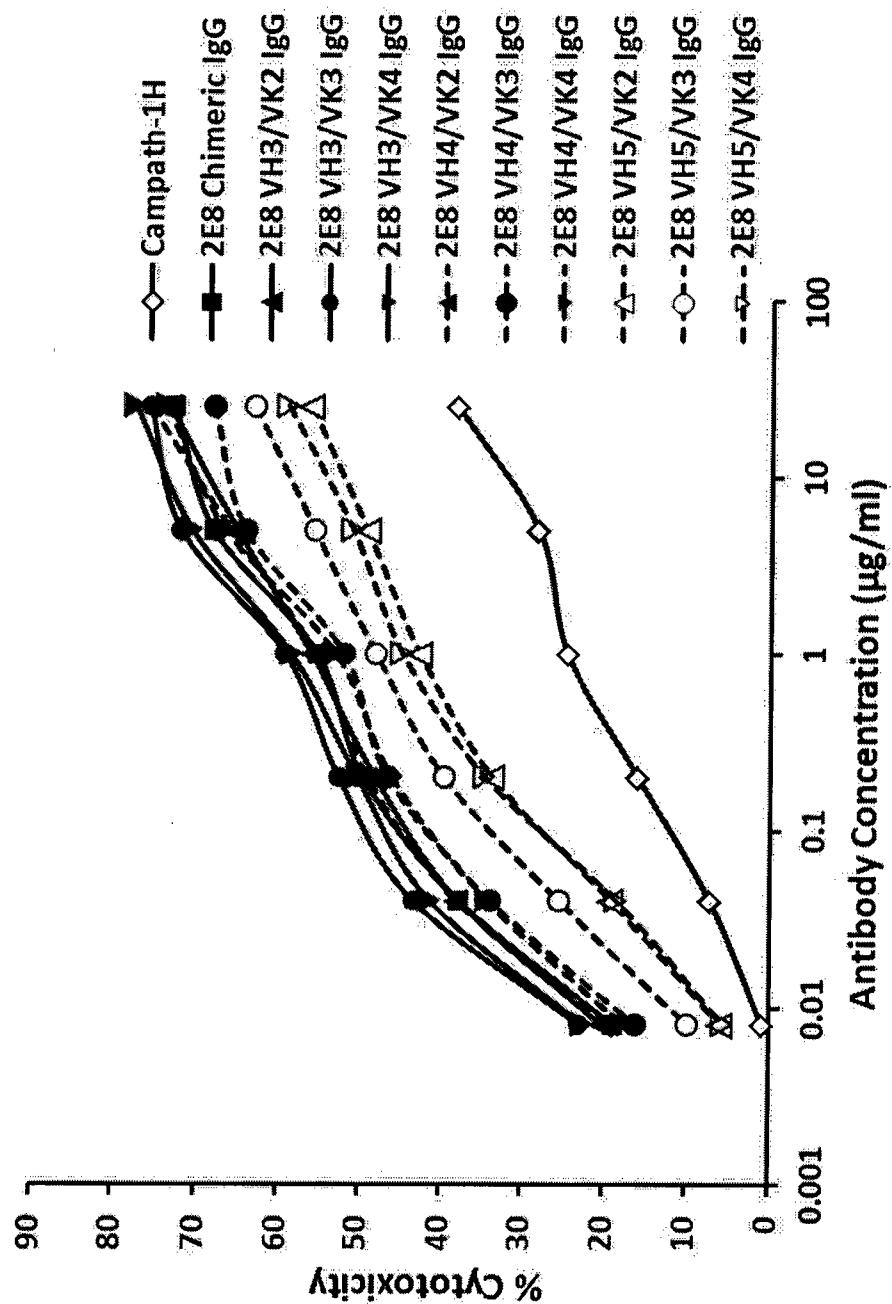


Figure 10

12/16

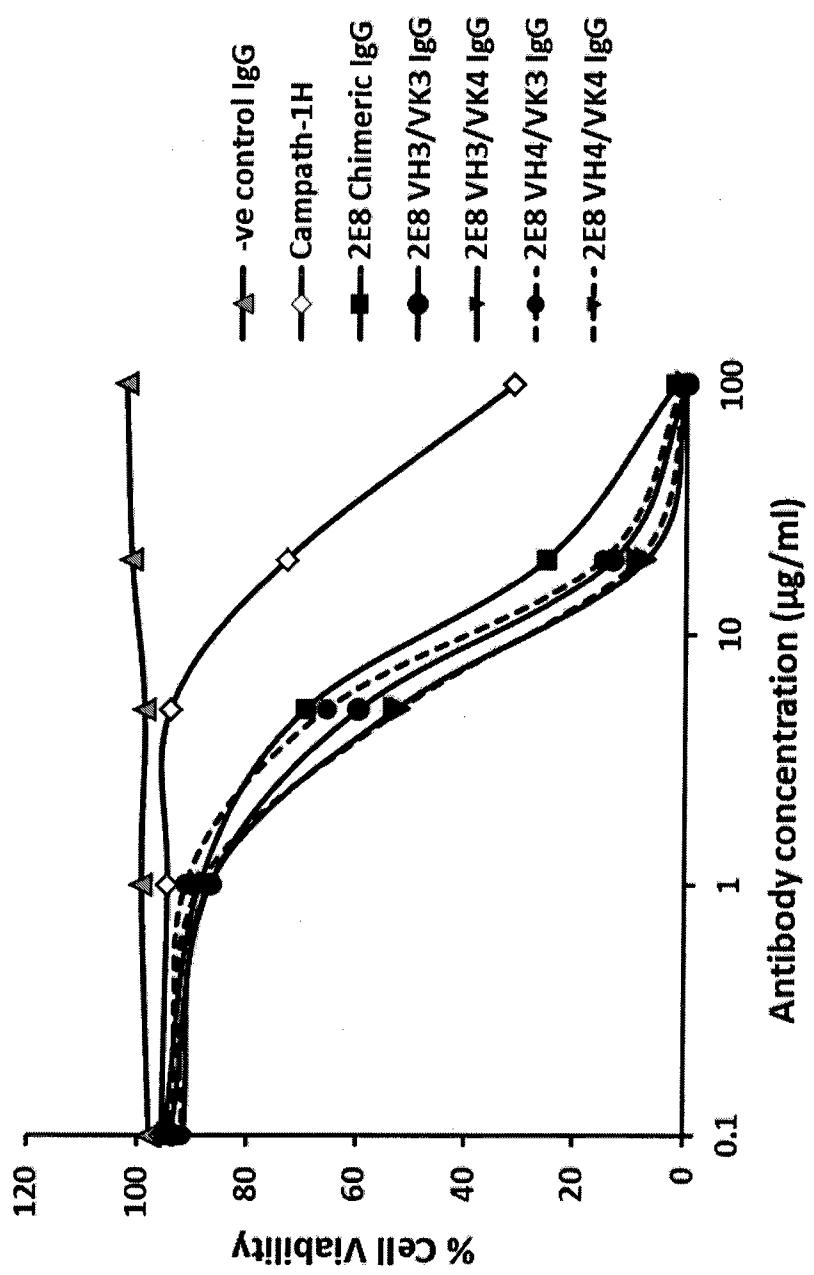


Figure 11

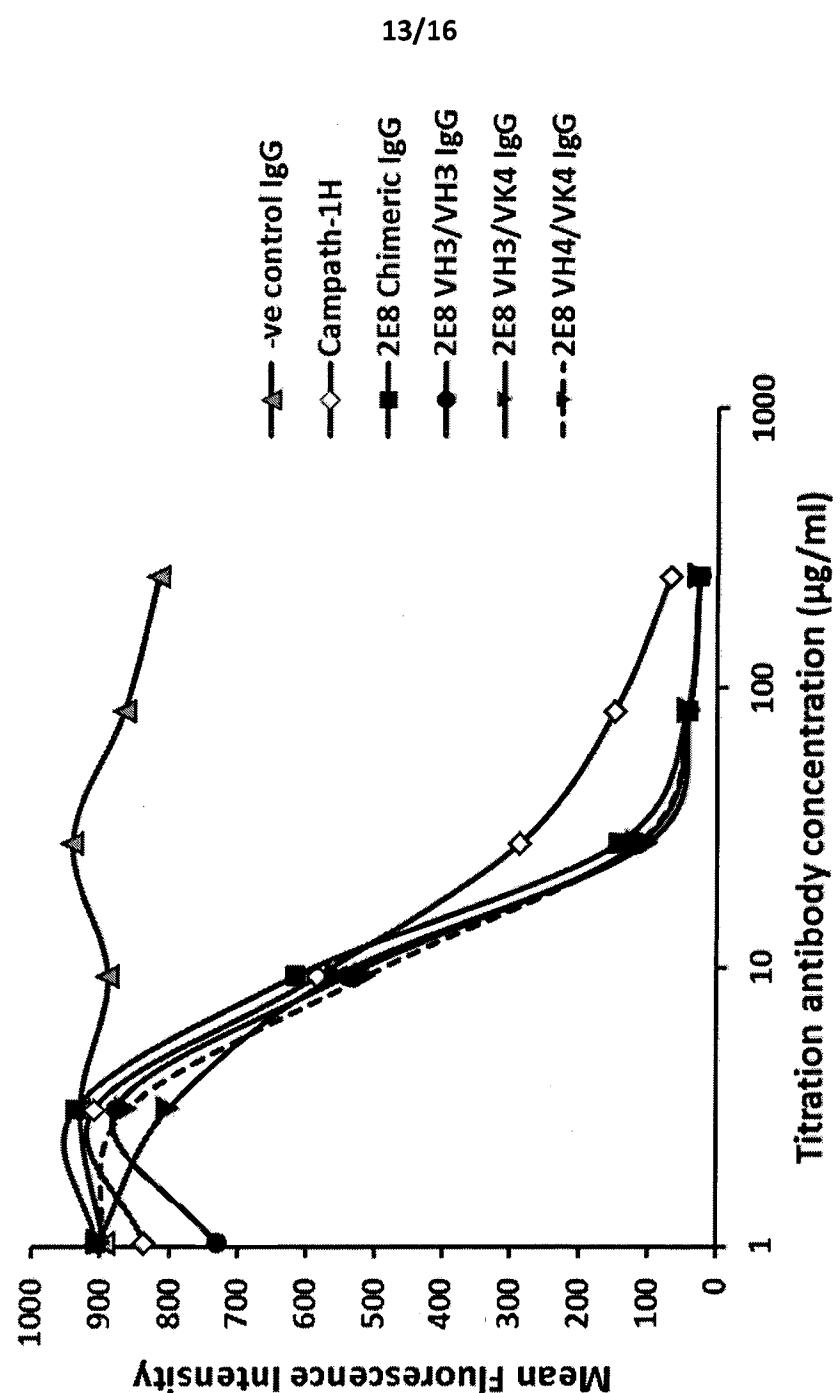
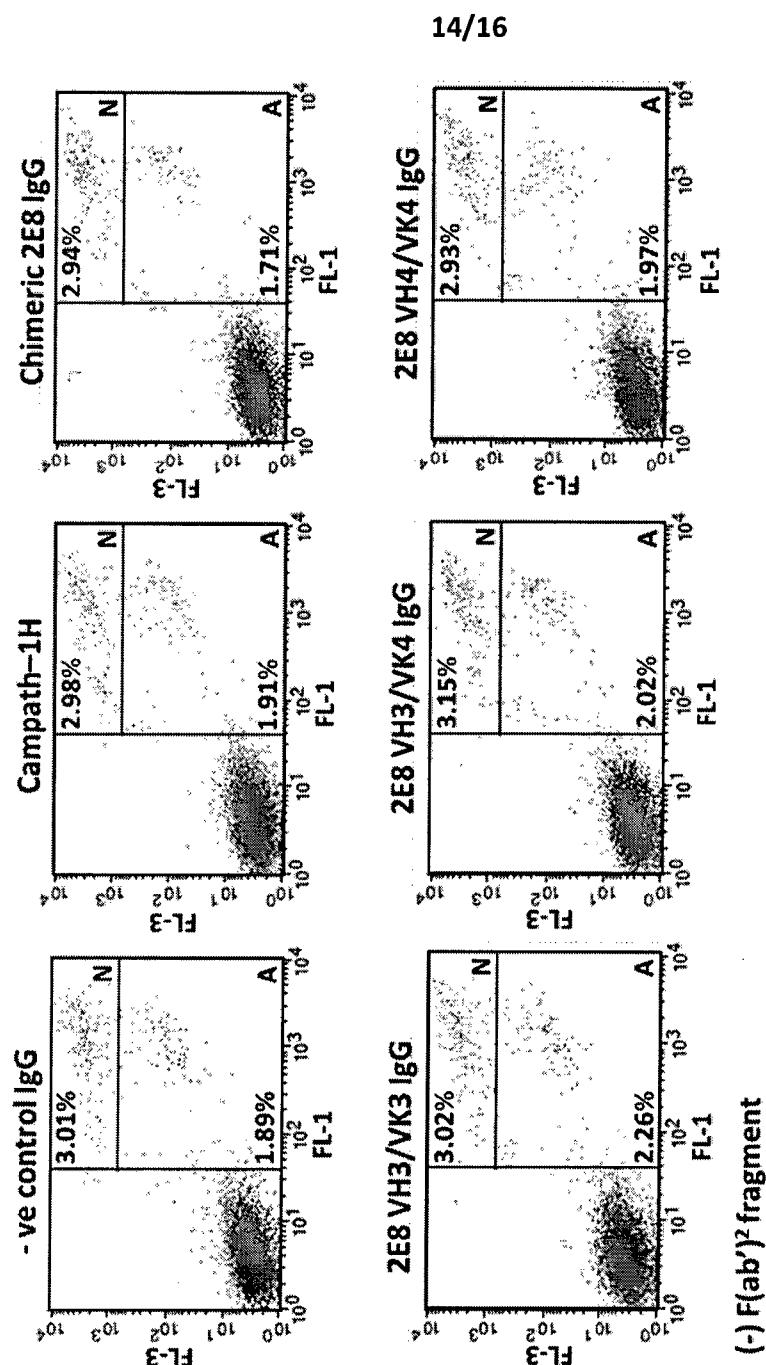


Figure 12

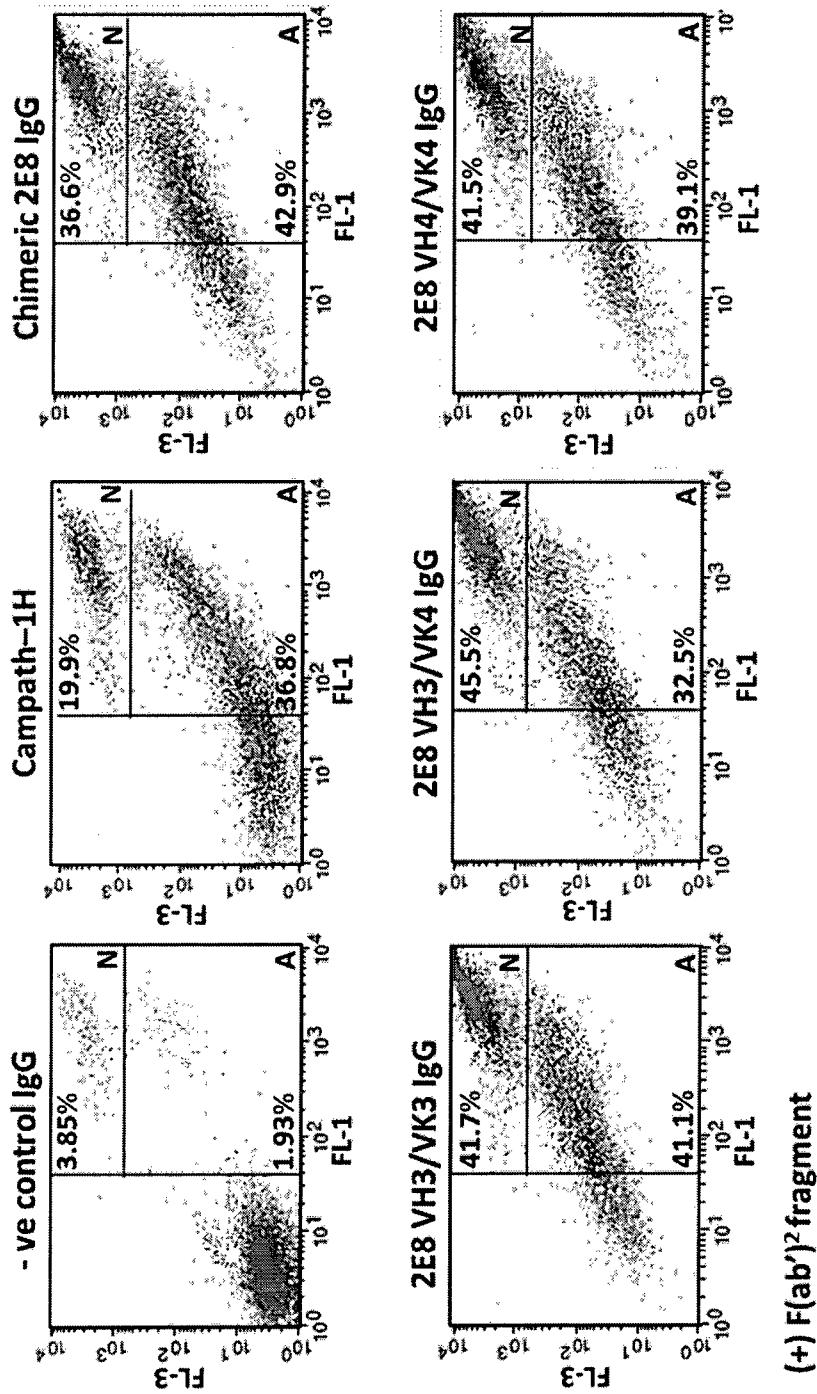


N – Necrosis – Cells strongly stained with both Annexin V and Propidium Iodide (FL1 and FL3 channels respectively)

A – Apoptosis – Cells positively stained with Annexin V with reduced Propidium Iodide stain

Figure 13: (-) $F(ab')^2$ fragment

15/16

Figure 13: (+) F(ab')² fragment

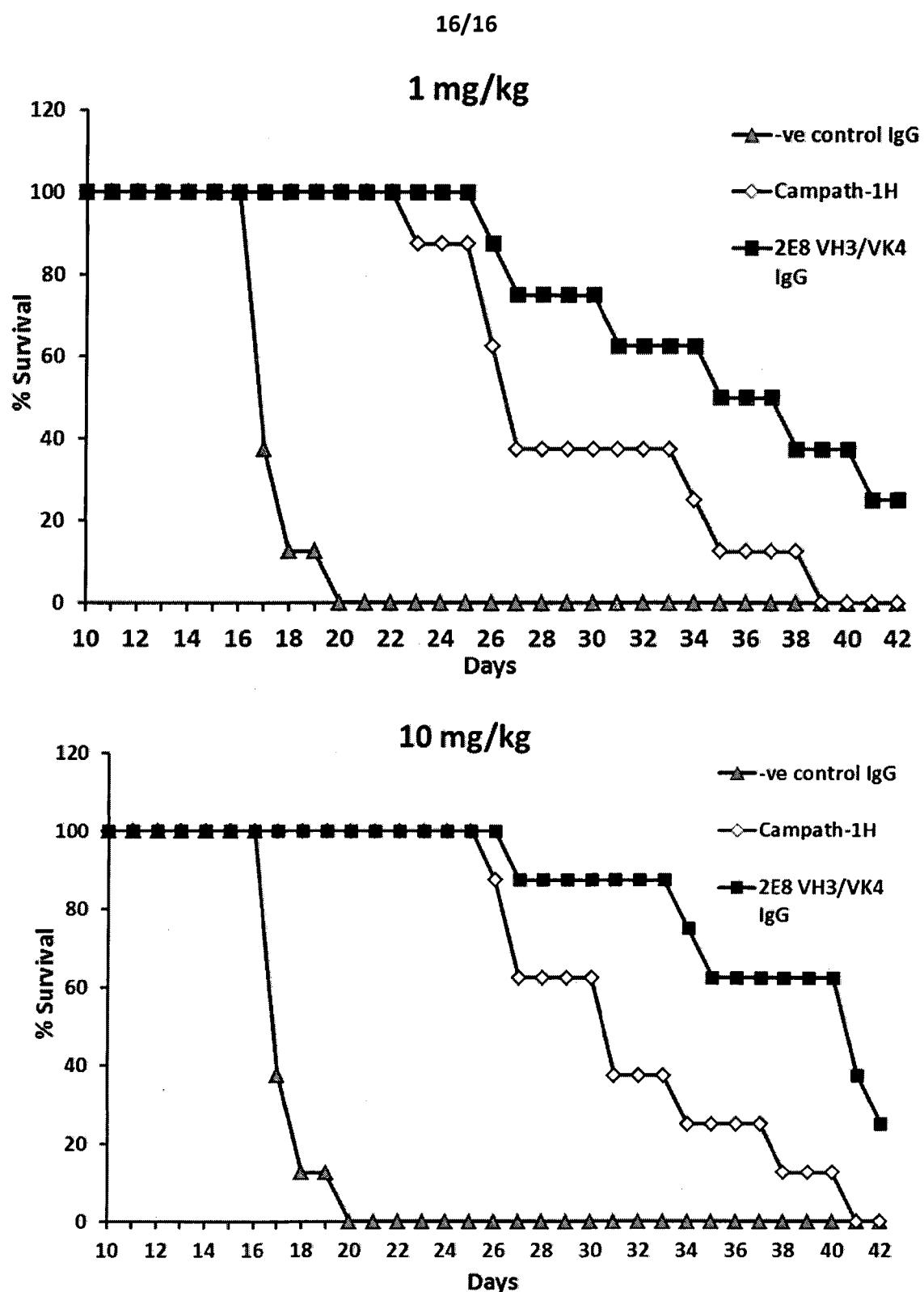


Figure 14

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/060345

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K16/28
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2010/132659 A2 (GENZYME CORP [US]; ROBERTS BRUCE L [US]; SHANKARA SRINIVAS [US]; BROND) 18 November 2010 (2010-11-18) example A11 ----- WO 2005/042581 A2 (BIOVATION LTD [GB]; CARR FRANCIS J [GB]; HAMILTON ANITA A [GB]) 12 May 2005 (2005-05-12) example A11 ----- CN 1 508 155 A (MA JING [CN]) 30 June 2004 (2004-06-30) example A11 -----	1-26 1-26 1-26



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier application or patent but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 August 2012	31/08/2012
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Authorized officer Fellows, Edward

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2012/060345

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO 2010132659	A2	18-11-2010	AR 076770 A1 AU 2010249003 A1 CA 2761800 A1 CN 102459628 A EP 2429582 A2 SG 176060 A1 TW 201043694 A US 2012100152 A1 WO 2010132659 A2	06-07-2011 03-11-2011 18-11-2010 16-05-2012 21-03-2012 29-12-2011 16-12-2010 26-04-2012 18-11-2010

WO 2005042581	A2	12-05-2005	AT 552276 T BR PI0416141 A CA 2544365 A1 CN 1898267 A DK 1694706 T3 EP 1694706 A2 ES 2384105 T3 JP 4804357 B2 JP 2007534304 A PT 1694706 E US 2005152898 A1 US 2008075715 A1 US 2008248529 A1 WO 2005042581 A2	15-04-2012 02-01-2007 12-05-2005 17-01-2007 16-07-2012 30-08-2006 29-06-2012 02-11-2011 29-11-2007 19-06-2012 14-07-2005 27-03-2008 09-10-2008 12-05-2005

CN 1508155	A	30-06-2004	NONE	
