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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
6 April 2017 (06.04.2017)

WIPO | PCT

(10) International Publication Number
WO 2017/055542 Al

(51) International Patent Classification:
C07K 16/28 (2006.01)

(21) International Application Number:
PCT/EP20 16/0734 13

(22) International Filing Date:
30 September 2016 (30.09.201 6)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
15 188067. 1 2 October 201 5 (02. 10.2015) EP

(71) Applicant *(for all designated States except DJ, KW, US):* **F. HOFFMANN-LA ROCHE AG** [CH/CH]; Grenzacher-strasse 124, 4070 Basel (CH).

(71) Applicant *(for US only):* **HOFFMANN-LA ROCHE INC.** [US/US]; Great Notch, 150 Clove Road, 8th Floor, Little Falls, New Jersey 07424 (US).

(72) Inventors: **DUERR, Harald;** Gustav-Meyrink-StraBe 7a, 8231 9 Starnberg (DE). **FENN, Sebastian;** Am Alpenblick 16, 82547 Achmuehle/Eurasburg (DE). **GOEPFERT, Ulrich;** Reindl 10b, 82377 Penzberg (DE). **IMHOF-JUNG, Sabine;** Egenhofenstr. 26 b, 82152 Planegg (DE). **KLEIN, Christian;** Chapfstrasse 26B, 8906 Bonstetten (CH). **LARFVTERE, Laurent;** Endelhauserstrasse 22, 80686 Munich (DE). **MOLHOJ, Michael;** Fachnerstr. 7A, 80686 Munich (DE). **REGULA, Joerg Thomas;** Innerkoflerstr. 17 B, 81377 Munich (DE). **RUEGER, Petra;** Auf der Leiten 4, 82377 Penzberg (DE). **SCHAEFER, Wolfgang;** Tannhaeuserring 190, 68199 Mannheim (DE).

(74) Agents: **SKOLAUT, Alexander** et al; Roche Diagnostics GmbH, Patent Department (LPP.....6164), P.O.Box 11 52, 82372 Penzberg (DE).

(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, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, 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.

(84) 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, ST, 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, 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, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

— *with sequence listing part of description (Rule 5.2(a))*

WO 2017/055542 A1

(54) Title: BISPECIFIC ANTI-HUMAN CD20/HUMAN TRANSFERRIN RECEPTOR ANTIBODIES AND METHODS OF USE

(57) Abstract: Herein are provided bispeciiic anti-human CD20/human transferrin receptor antibodies and methods of using the same.

BISPECIFIC ANTI-HUMAN CD20/HUMAN TRANSFERRIN RECEPTOR ANTIBODIES AND METHODS OF USE

FIELD OF THE INVENTION

The present invention relates to bispecific antibodies against human CD20 and human transferrin receptor, methods for their production, pharmaceutical compositions containing these antibodies, and uses thereof.

BACKGROUND

Lymphocytes are one of several populations of white blood cells. They specifically recognize and respond to foreign antigen. The three major classes of lymphocytes are B lymphocytes (B-cells), T lymphocytes (T-cells) and natural killer (NK) cells. B lymphocytes are the cells responsible for antibody production and provide humoral immunity. B-cells mature within the bone marrow and leave the marrow expressing an antigen-binding antibody on their cell surface. When a naive B-cell first encounters the antigen for which its membrane-bound antibody is specific, the cell begins to divide rapidly and its progeny differentiate into memory B-cells and effector cells called "plasma cells". Memory B-cells have a longer life span and continue to express membrane-bound antibody with the same specificity as the original parent cell. Plasma cells do not produce membrane-bound antibody but instead produce secreted form of the antibody. Secreted antibodies are the major effector molecules of humoral immunity.

The CD20 antigen (also called human B-lymphocyte-restricted differentiation antigen, Bp35) is a hydrophobic transmembrane protein with a molecular weight of approximately 35 kDa located on pre-B and mature B lymphocytes (Valentine et al, J. Biol. Chem. 264 (1989) 11282-11287; and Einfeld et al, EMBO J. 7 (1988) 711-717). The antigen is also expressed on greater than 90 % of B-cell non-Hodgkin's lymphomas (NHL) (Anderson et al, Blood 63 (1984) 1424-1433), but is not found on hematopoietic stem cells, pro-B-cells, normal plasma cells or other normal tissues (Tedder et al, J. Immunol. 135 (1985) 973-979). CD20 is thought to regulate an early step(s) in the activation process for cell cycle initiation and differentiation (Tedder et al, supra) and possibly functions as a calcium ion channel (Tedder et al, J. Cell. Biochem. 14D (1990) 195).

Given the expression of CD20 in B-cell lymphomas, this antigen has been a useful therapeutic target to treat such lymphomas. Given the expression of CD20 in B-cell lymphomas, this antigen can serve as a candidate for "targeting" of such lymphomas. In essence, such targeting can be generalized as follows: antibodies specific to the CD20 surface antigen of B-cells are administered to a patient. These anti-CD20 antibodies specifically bind to the CD20 antigen of (ostensibly) both normal and malignant B-cells; the antibody bound to the CD20 surface antigen may lead to the destruction and depletion of neoplastic B-cells. Additionally, chemical agents or radioactive labels having the potential to destroy the tumor can be conjugated to the anti-CD20 antibody such that the agent is specifically "delivered" to the neoplastic B-cells. Irrespective of the approach, a primary goal is to destroy the tumor; the specific approach can be determined by the particular anti-CD20 antibody which is utilized and, thus, the available approaches to targeting the CD20 antigen can vary considerably. For example, the rituximab (RITUXAN®) antibody which is a genetically engineered chimeric murine/human monoclonal antibody directed against human CD20 antigen (commercially available from Genentech, Inc., South San Francisco, California, USA) is used for the treatment of patients with relapsed or refractory low-grade or follicular, CD20 positive, B-cell non-Hodgkin's lymphoma. Rituximab is the antibody referred to as "C2B8" in US 5,736,137 and in US 5,776,456. In vitro mechanism of action studies have demonstrated that RITUXAN® binds human complement and lyses lymphoid B-cell lines through complement-dependent cytotoxicity (CDC) (Reff et al., Blood 83 (1994) 435-445). Additionally, it has significant activity in assays for antibody-dependent cellular cytotoxicity (ADCC). In vivo preclinical studies have shown that RITUXAN® depletes B-cells from the peripheral blood, lymph nodes, and bone marrow of cynomolgus monkeys, presumably through complement and cell-mediated processes (Reff et al, Blood 83 (1994) 435-445). Other anti-CD20 antibodies indicated for the treatment of NHL include the murine antibody Zevalin™ which is linked to the radioisotope, Yttrium-90 (IDEC Pharmaceuticals, San Diego, CA, USA), Bexxar™ which is a another fully murine antibody conjugated to 1-131 (Corixa, WA, USA).

CD20 is also a useful target antigen for treating autoimmune diseases. Rituximab has also been studied in a variety of non-malignant autoimmune disorders, in which B-cells and autoantibodies appear to play a role in disease pathophysiology (see e.g. Edwards et al., Biochem. Soc. Trans. 30 (2002) 824-828). Rituximab has been reported to potentially relieve signs and symptoms of, for example, rheumatoid arthritis (RA) (Leandro et al, Ann. Rheum. Dis. 61 (2002) 883-888; Edwards et al, Arthritis Rheum. 46 (Suppl. 9) (2002) S46; Stahl et al, Ann. Rheum. Dis. 62 (Suppl. 1) (2003) OP004; Emery et al, Arthritis Rheum. 48 (2003) S439), lupus (Eisenberg, Arthritis. Res. Ther. 5 (2003) 157-159; Leandro et al., Arthritis Rheum. 46 (2002) 2673-2677; Gorman et al, Lupus, 13 (2004) 312-316), immune thrombocytopenic purpura (D'Arena et al., Leuk. Lymphoma 44 (2003) 561-562; Stasi et al, Blood 98 (2001) 952-957; Saleh et al, Semin. Oncol. 27 (Suppl. 12) (2000) 99-103; Zaia et al, Haematologica 87 (2002) 189-195; Ratanatharathorn et al, Ann. Int. Med. 133 (2000) 275-279), pure red cell aplasia (Auner et al, Br. J. Hematol. 116 (2002) 725-728); autoimmune anemia (Zaja et al., Haematologica 87 (2002) 189-195 (erratum appears in Haematologica 87 (2002) 336), cold agglutinin disease (Layios et al, Leukemia 15 (2001) 187-188; Berentsen et al, Blood 103 (2004) 2925-2928; Berentsen et al, Br. J. Hematol. 115 (2001) 79-83; Bauduer, Br. J. Hematol. 112 (2001) 1083-1090; Damiani et al, Br. J. Hematol. 114 (2001) 229-234), type B syndrome of severe insulin resistance (Coll et al, N. Engl. J. Med. 350 (2004) 310-311, mixed cryoglobulinemia (De Vita et al., Arthritis Rheum. 46 Suppl. 9 (2002) S206/S469), myasthenia gravis (Zaja et al, Neurology 55 (2000) 1062-1063; Wylam et al, J. Pediatr. 143 (2003) 674-677), Wegener's granulomatosis (Specks et al., Arthritis & Rheumatism 44 (2001) 2836-2840), refractory pemphigus vulgaris (Dupuy et al, Arch. Dermatol. 140 (2004) 91-96), dermatomyositis (Levine, Arthritis Rheum. 46 (Suppl. 9) (2002) S1299), Sjogren's syndrome (Somer et al., Arthritis & Rheumatism 49 (2003) 394-398), active type-II mixed cryoglobulinemia (Zaja et al, Blood 101 (2003) 3827-3834), pemphigus vulgaris (Dupay et al., Arch. Dermatol. 140 (2004) 91-95), autoimmune neuropathy (Pestronk et al, J. Neurol. Neurosurg. Psychiatry 74 (2003) 485-489), paraneoplastic opsoclonus-myoclonus syndrome (Pranzatelli et al. Neurology 60 (Suppl. 1) (2003) P05.128:A395), and relapsing-remitting multiple sclerosis (RRMS) (Cross et al. (abstract) "Preliminary results from a phase II trial of Rituximab in MS" Eighth Annual Meeting of the Americas Committees for Research and Treatment in Multiple Sclerosis, (2003) 20-21).

Publications concerning therapy with rituximab include: Perotta and Abuel, Blood 10 (1998) (part 1-2) 88B; Perotta et al, Blood 94 (1999) 49 (abstract); Matthews, R., Ann. Rheum. Di's, supra; Leandro et al., Arthritis and Rheumatism 44(9): S370 (2001); Leandro et al, Arthritis and Rheumatism 46 (2002) 2673-2677; Weide et al, Lupus 12 (2003) 779-782; Edwards and Cambridge, Rheumatology 40 (2001) 205-211; Cambridge et al, Arthritis Rheum. 46 (Suppl. 9) (2002) S1350; Edwards et al., Arthritis and Rheumatism 46 (2002) S197; Levine and Pestronk, Neurology 52 (1999) 1701-1704; De Vita et al, Arthritis & Rheum. 46 (2002) 2029-2033; Hidashida et al., Annual Scientific Meeting of the American College of Rheumatology; Oct 24-29; New Orleans, LA 2002; Tuscano, J., Annual Scientific Meeting of the American College of Rheumatology; Oct 24-29; New Orleans, LA 2002; Martin and Chan, Immunity 20 (2004) 517-527; Silverman and Weisman, Arthritis and Rheumatism 48 (2003) 1484-1492; Kazkaz and Isenberg, Current opinion in pharmacology 4 (2004) 398-402; Virgolini and Vanda, Biomedicine & pharmacotherapy 58 (2004) 299-309; Klemmer et al., Arthritis and Rheumatism 48 (2003) 9,S (SEP) S624-S624; Kneitz et al, Immunobiology 206 (2002) 519-527; Arzoo et al., Annals of the Rheumatic Diseases 61 (2002) p922-924; Comment in Ann. Rheum. Dis. 61 (2002) 863-866; "Future Strategies in Immunotherapy" by Lake and Dionne, in Burger's Medicinal Chemistry and Drug Discovery (2003 by John Wiley & Sons, Inc.); Liang and Tedder, Wiley Encyclopedia of Molecular Medicine, Section: CD20 as an Immunotherapy Target, 2002 entitled "CD20"; Appendix 4A entitled "Monoclonal Antibodies to Human Cell Surface Antigens" by Stockinger et al., Eds: Coligan et al., in Current Protocols in Immunology (2003 John Wiley & Sons, Inc.); Penichet and Morrison, "CD Antibodies/molecules: Definition; Antibody Engineering" in Wiley Encyclopedia of Molecular Medicine Section: Chimeric, Humanized and Human Antibodies; posted online 15 January, 2002; Specks et al, Arthritis & Rheumatism 44 (2001) 2836-2840; Koegh et al, "Rituximab for Remission Induction in Severe ANCA-Associated Vasculitis: Report of a Prospective Open-Label Pilot Trial in 10 Patients", American College of Rheumatology, Session Number: 28-100, Session Title: Vasculitis, Session Type: ACR Concurrent Session, Primary Category: 28 Vasculitis, Session 10/18/2004 (http://www.abstractsonline.com/viewer/SearchResults.asp); Eriksson, Kidney and Blood Pressure Research 26 (2003) 294; Jayne et al, Kidney and Blood Pressure Research, 26 (2003) 294; Jayne, poster 88 (11th International Vasculitis and ANCA workshop), 2003 American Society of Nephrology; Stone and Specks in the Clinical Trial Research Summary of the 2002-2003 Immune Tolerance Network, http://www.immunetolerance.org/reseaTclT/autoimmune/trial s/stone.html; Leandro et at, Arthritis Rheum. 48 (Suppl. 9) (2003) SI 160.

Patents and patent publications concerning CD20 antibodies include US 5,776,456, US 5,736,137, US 5,843,439, US 6,399,061, US 6,682,734, US 2002/0197255 Al, US 2003/0021781 Al, US 2003/0082172 Al, US 2003/0095963 Al, US 2003/0147885 Al; US 6,455,043; WO 00/09160; WO 00/27428;

WO 00/27433; WO 00/44788; WO 01/10462; WO 01/10461; WO 01/10460; US 2001/0018041 Al, US 2003/0180292 Al, WO 01/34194; US 2002/0006404; WO 02/04021; US 2002/0012665 Al; WO 01/74388; US 2002/0058029 Al; US 2003/0103971 Al; US 2002/0009444 Al; WO 01/80884; WO 01/97858; US 2002/0128488 Al; WO 02/34790; WO 02/060955; WO 02/096948; WO 02/079255; US 6,171,586 Bl; WO 98/56418; WO 98/58964; WO 99/22764; WO 99/51642; US 6,194,551 Bl; US 6,242,195 Bl; US 6,528,624 Bl; US 6,538,124; WO 00/42072; WO 00/67796; WO 01/03734; US 2002/0004587 Al; WO 01/77342; US 2002/0197256; US 2003/0157108 Al; US 6,565,827 Bl; US 6,090,365 Bl; US 6,287,537 Bl; US 6,015,542; US 5,843,398; US 5,595,721; US 5,500,362; US 5,677,180; US 5,721,108; US 6,120,767; US 6,652,852 Bl; US 6,410,391 Bl; US 6,224,866 Bl; WO 00/20864; WO 01/13945; WO 00/67795; US 2003/0133930 Al; WO 00/74718; WO 00/76542; WO 01/72333; US 6,368,596 Bl; US 6,306,393; US 2002/0041847 Al; US 2003/0026801 Al; WO 02/102312; US 2003/0068664; WO 03/002607; WO 03/049694; US 2002/0009427 Al; US 2003/0185796 Al; WO 03/061694; US 2003/0219818 Al; US 2003/0219433 Al; WO 03/068821; US 2002/0136719 Al; WO 2004/032828; WO 2004/035607; US 2004/0093621; US 5,849,898; EP 0,330,191; US 4,861,579; EP 0,332,865; WO 95/03770; US 2001/0056066; WO 2004/035607; WO 2004/056312; US 2004/0093621; WO 2004/103404. Publications concerning CD20 antibody include: Teeling, J., et al, Blood 10 (2004) 1182.

WO 2014/033074 relates to blood brain barrier shuttles that bind receptors on the blood brain barrier and methods of using the same. Low affinity blood brain barrier receptor antibodies and uses therefor are reported in WO 2012/075037. WO 2014/189973 relates to anti-transferrin receptor antibodies and methods of their use. A blood brain barrier shuttle module comprising a brain effector entity, a linker and one monovalent binding entity which binds to a blood brain barrier receptor was reported in WO 2015/101588. WO 2010/033587 concerns methods for treating progressive multiple sclerosis in a patient, and an article of manufacture with instructions for such use. A method of treating, arresting or preventing a disease responsive to treatment with an anti-CD20 antibody in a patient suffering therefrom, comprising administering to the patient at least one sub-depleting dose of antiCD20 antibody was reported in WO 2012/096924. Hawker, K., et al. (Ann. Neurol. 66 (2009) 460-471) reported about the results of a randomized double-blind placebo-controlled multicenter trial of Rituximab in patients with primary progressive multiple sclerosis.

STJMMARY

One aspect as reported herein is a bispecific antibody comprising

a) one (full length) antibody comprising two pairs each of a (full length) antibody light chain and a (full length) antibody heavy chain, wherein the binding sites formed by each of the pairs of the (full length) heavy chain and the (full length) light chain specifically bind to a first antigen, and

b) one additional Fab fragment, wherein the additional Fab fragment is fused to any C-terminus of one heavy chain of the (full length) antibody, wherein the binding site of the additional Fab fragment specifically binds to a second antigen,

wherein each of the (full length) antibody light chains comprises in the constant light chain domain (CL) at position 123 the amino acid residue arginine (instead of the wild-type glutamic acid residue; E123R mutation) and at position 124 the amino acid residue lysine (instead of the wild-type glutamine residue; Q124K mutation) (numbering according to Kabat),

wherein each of the (full length) antibody heavy chains comprises in the first constant heavy chain domain (CHI) at position 147 an glutamic acid residue (instead of the wild-type lysine residue; K147E mutation) and at position 213 an glutamic acid residue (instead of the wild-type lysine amino acid residue; K213E mutation) (numbering according to Kabat EU index),

wherein the additional Fab fragment specifically binding to the second antigen comprises a domain crossover such that the constant light chain domain (CL) and the constant heavy chain domain 1 (CHI) are replaced by each other, and

wherein the first antigen is human CD20 and the second antigen is human transferrin receptor.

In one embodiment the additional Fab fragment is fused to the C-terminus of the heavy chain by a peptidic linker.

In one embodiment the N-terminus of the heavy chain variable domain of the Fab fragment is fused to the C-terminus of the (full length) heavy chain or the C-terminus of the peptidic linker.

In one embodiment

a) the (full length) heavy chain that is fused to the additional Fab fragments has as C-terminal (heavy chain) amino acid residues the tripeptide LSP wherein the proline thereof is directly fused to the first amino acid residue of the additional Fab fragment or of the peptidic linker via a peptide bond, and

b) the (full length) heavy chain that is not fused to the additional Fab fragments has as C-terminal (heavy chain) amino acid residues the tripeptide LSP, or SPG, or PGK.

In one embodiment the (full length) antibody is

a) a full length antibody of the human subclass IgGl,

b) a full length antibody of the human subclass IgG4,

c) a full length antibody of the human subclass IgGl with the mutations L234A, L235A and P329G,

d) a full length antibody of the human subclass IgG4 with the mutations S228P, L235E and P329G,

e) a full length antibody of the human subclass IgGl with the mutations L234A, L235A and P329G in both heavy chains and the mutations i) T366W, and ii) S354C or Y349C, in one heavy chain and the mutations i) T366S, L368A, and Y407V, and ii) Y349C or S354C, in the respective other heavy chain,

f) a full length antibody of the human subclass IgG4 with the mutations S228P, L235E and P329G in both heavy chains and the mutations i) T366W, and ii) S354C or Y349C, in one heavy chain and the mutations i) T366S, L368A, and Y407V, and ii) Y349C or S354C, in the respective other heavy chain,

g) a full length antibody of the human subclass IgGl with the mutations L234A, L235A, P329G, I253A, H310A and H435A in both heavy chains and the mutations i) T366W, and ii) S354C or Y349C, in one heavy chain and the mutations i) T366S, L368A, and Y407V, and ii) Y349C or S354C, in the respective other heavy chain, or

h) a full length antibody of the human subclass IgGl with the mutations L234A, L235A, P329G, M252Y, S254T and T256E in both heavy chains and the mutations i) T366W, and ii) S354C or Y349C, in one heavy chain and the mutations i) T366S, L368A, and Y407V, and ii) Y349C or S354C, in the respective other heavy chain, or

i) a full length antibody of the human subclass IgGl with the mutations L234A, L235A, P329G, H310A, H433A and Y436A in both heavy chains and the mutations i) T366W, and ii) S354C or Y349C, in one heavy chain and the mutations i) T366S, L368A, and Y407V, and ii) Y349C or S354C, in the respective other heavy chain.

In one embodiment the additional Fab fragment is fused to the C-terminus of the heavy chain comprising the mutation T366W, or to the C-terminus of the heavy chain comprising the mutations T366S, L368A, and Y407V.

In one embodiment

the (full length) antibody is of the human subclass IgGl with the mutations L234A, L235A and P329G in both heavy chains and the mutations T366W and S354C in one heavy chain and the mutations T366S, L368A, Y407V and Y349C in the respective other heavy chain, and

the additional Fab fragment is fused to the C-terminus of the heavy chain comprising the mutation T366W, or to the C-terminus of the heavy chain comprising the mutations T366S, L368A, and Y407V.

In one embodiment the bispecific antibody comprises

i) a light chain that has a sequence identity to SEQ ID NO: 01 of 70 % or more,

ii) a heavy chain that has a sequence identity to SEQ ID NO: 02 of 70 % or more,

iii) a light chain that has a sequence identity to SEQ ID NO: 03 of 70 % or more, and

iv) a heavy chain Fab fragment that has a sequence identity to SEQ ID NO: 04 of70 % or more,

wherein

SEQ ID NO: 01 has the amino acid sequence DIVMTQTPLSLPVTPGEPASISCRSSKSLLHSNGITYLYWYLQKPGQSPQLLIYQMSNLVSGVPDRFSGSGSGTDFTLKISRVEAEDVGVYYCAQNLELPYTFGGGTKVEIKRTVAAPSVFIFPPSDRKLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC,

SEQ ID NO: 02 has the amino acid sequence QVQLVQSGAEVKKPGSSVKVSCKASGYAFSYSWINWVRQAPGQGLEWMGRIFPGDGDTDYNGKFKGRVTITADKSTSTAYMELSSLRSEDTAVYYCARNVFDGYWLVYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVEDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDEKVEPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPREPQVCTLPPSRDELTKNQVSLSCAVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLVSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG,

SEQ ID NO: 03 has the amino acid sequence AIQLTQSPSSLSASVGDRVTITCRASQSISSYLAWYQQKPGKAPKLLIYRASTLASGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQNYASSNVDNTFGGGTKVEIKSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSC, and

SEQ ID NO: 04 has the amino acid sequence QSMQESGPGLVKPSQTLSLTCTVSGFSLSSYAMSWIRQHPGKGLEWIGYIWSGGSTDYASWAKSRVTISKTSTTVSLKLSSVTAADTAVYYCARRYGTSYPDYGDASGFDPWGQGTLVTVSSASVAAPSVFIFPPSDEQLKS

GTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTY
SLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC.

One aspect as reported herein is a bispecific antibody comprising a (full length) light chain that has the amino acid sequence of SEQ ID NO: 01, a (full length) heavy chain that has the amino acid sequence of SEQ ID NO: 02, a (full length) light chain that has the amino acid sequence of SEQ ID NO: 03, and an antibody Fab fragment comprising the amino acid sequences of SEQ ID NO: 04.

In one embodiment the bispecific antibody is monoclonal.

One aspect as reported herein is a bispecific antibody comprising

a) a first and a second Fab fragment, wherein each binding site of the first and the second Fab fragment specifically bind to a first antigen,

b) a third Fab fragment, wherein the binding site of the third Fab fragment specifically binds to a second antigen, and wherein the third Fab fragment comprises a domain crossover such that the variable light chain domain (VL) and the variable heavy chain domain (VH) are replaced by each other, and

c) an Fc-region comprising a first Fc-region polypeptide and a second Fc-region polypeptide,

wherein the first and the second Fab fragment each comprise a heavy chain fragment and a full length light chain,

wherein the C-terminus of the heavy chain fragment of the first Fab fragment is fused to the N-terminus of the first Fc-region polypeptide,

wherein the C-terminus of the heavy chain fragment of the second Fab fragment is fused to the N-terminus of the variable light chain domain of the third Fab fragment and the C-terminus of the heavy chain constant domain 1 of the third Fab fragment is fused to the N-terminus of the second Fc-region polypeptide,

wherein each of the full length light antibody chains of the first and second Fab fragment comprises in the constant light chain domain (CL) at position 123 the amino acid residue arginine (instead of the wild-type glutamic acid residue; E123R mutation) and at position 124 the amino acid residue lysine (instead of the wild-type glutamine residue; Q124K mutation) (numbering according to Kabat),

wherein each of the heavy chain fragments of the first and second Fab fragment comprises in the first constant heavy chain domain (CHI) at position 147 an glutamic acid residue (instead of the wild-type lysine residue; K147E mutation) and at position 213 an glutamic acid residue (instead of the wild-type lysine amino acid residue; K213E mutation) (numbering according to Kabat EU index),

wherein the first antigen is human CD20 and the second antigen is human transferrin receptor.

In one embodiment the first and the second Fc-region polypeptide is

a) of the human subclass IgGl ,

b) of the human subclass IgG4,

c) of the human subclass IgGl with the mutations L234A, L235A and P329G,

d) of the human subclass IgG4 with the mutations S228P, L235E and P329G,

e) of the human subclass IgGl with the mutations L234A, L235A and P329G in both Fc-region polypeptides and the mutations T366W and S354C in one Fc-region polypeptide and the mutations T366S, L368A, Y407V and Y349C in the respective other Fc-region polypeptide,

f) of the human subclass IgG4 with the mutations S228P, L235E and P329G in both Fc-region polypeptides and the mutations T366W and S354C in one Fc-region polypeptide and the mutations T366S, L368A, Y407V and Y349C in the respective other Fc-region polypeptide,

g) of the human subclass IgGl with the mutations L234A, L235A, P329G, 1253A, H310A and H435A in both Fc-region polypeptides and the mutations T366W and S354C in one Fc-region polypeptide and the mutations T366S, L368A, Y407V and Y349C in the respective other Fc-region polypeptide, or

h) of the human subclass IgGl with the mutations L234A, L235A, P329G, M252Y, S254T and T256E in both Fc-region polypeptides and the mutations T366W and S354C in one Fc-region polypeptide and the mutations T366S, L368A, Y407V and Y349C in the respective other Fc-region polypeptide, or

i) of the human subclass IgGl with the mutations L234A, L235A, P329G, H310A, H433A and Y436A in both Fc-region polypeptides and the mutations T366W and S354C in one Fc-region polypeptide and the mutations T366S, L368A, Y407V and Y349C in the respective other Fc-region polypeptide.

In one embodiment one of the chains of the third Fab fragment is fused to the Fc-region polypeptide comprising the mutation T366W, or to the Fc-region polypeptide comprising the mutations T366S, L368A, and Y407V.

In one embodiment the bispecific antibody comprises

i) a light chain that has a sequence identity to SEQ ID NO: 14 of at least 70 %, or at least 80 %, or at least 90 %, or 95 % or more,

ii) a heavy chain that has a sequence identity to SEQ ID NO: 15 of at least 70 %, or at least 80 %, or at least 90 %, or 95 % or more,

iii) a crossed antibody chain that has a sequence identity to SEQ ID NO: 16 of at least 70 %, or at least 80 %, or at least 90 %, or 95 % or more, and

iv) a modified heavy chain that has a sequence identity to SEQ ID NO: 17 of at least 70 %, or at least 80 %, or at least 90 %, or 95 % or more,

wherein

SEQ ID NO: 14 has the amino acid sequence DIVMTQTPLSLPVTPGEPASISCRSSKSLLHSNGITYLYWYLQKPGQSP QLLIYQMSNLVSGVPDRFSGSGSGTDFTLKISRVEAEDVGVYYCAQN LELPYTFGGGTKVEIKRTVAAPSVFIFPPSDRKLKSGTASVVCLLNNF

YPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKAD YEKHKVYACEVTHQGLSSPVTKSFNRGEC,

SEQ ID NO: 15 has the amino acid sequence QVQLVQSGAEVKKPGSSVKVSCKASGYAFSYSWINWVRQAPGQGLE WMGRIFPGDGDTDYNGKFKGRVTITADKSTSTAYMELSSLRSEDTAV YYCARNVFDGYWLVYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSG GTAALGCLVEDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSS VVTVPSSSLGTQTYICNVNHKPSNTKVDEKVEPKSCDKTHTCPPCPAP EAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYV DGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVS NKALGAPIEKTISKAKGQPREPQVCTLPPSRDELTKNQVSLSCAVKGF YPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLVSKLTVDKSRWQQ GNVFSCSVMHEALHNHYTQKSLSLSPG,

SEQ ID NO: 16 has the amino acid sequence QSMQESGPGLVKPSQTLSLTCTVSGFSLSSYAMSWIRQHPGKGLEWI GYIWSGGSTDYASWAKSRVTISKTSTTVSLKLSSVTAADTAVYYCAR RYGTSYPDYGDASGFDPWGQGTLVTVSSASVAAPSVFIFPPSDEQLKS GTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTY SLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC, and

SEQ ID NO: 17 has the amino acid sequence QVQLVQSGAEVKKPGSSVKVSCKASGYAFSYSWINWVRQAPGQGLE WMGRIFPGDGDTDYNGKFKGRVTITADKSTSTAYMELSSLRSEDTAV YYCARNVFDGYWLVYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSG GTAALGCLVEDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSS VVTVPSSSLGTQTYICNVNHKPSNTKVDEKVEPKSCDGGGGSGGGGS AIQLTQSPSSLSASVGDRVTITCRASQSISSYLAWYQQKPGKAPKLLIY RASTLASGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQNYASSNV DNTFGGGTKVEIKSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYF PEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTY ICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLFPPK PKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPR EEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALGAPIEKTISK AKGQPREPQVYTLPPCRDELTKNQVSLWCLVKGFYPSDIAVEWESNG QPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEA LHNHYTQKSLSLSPG.

One aspect as reported herein is a bispecific antibody comprising two full length light chains that each has the amino acid sequence of SEQ ID NO: 14, a full length heavy chain that has the amino acid sequence of SEQ ID NO: 15, a crossed antibody chain that has the amino acid sequence of SEQ ID NO: 16, and a modified heavy chain that has the amino acid sequence of SEQ ID NO: 17.

In one embodiment the bispecific antibody is monoclonal.

One aspect as reported herein is an isolated nucleic acid encoding the bispecific antibody as reported herein.

One aspect as reported herein is a host cell comprising the nucleic acid as reported herein encoding the bispecific antibody as reported herein.

One aspect as reported herein is a method of producing a bispecific antibody as reported herein comprising the following steps:

a) culturing the host cell as reported herein so that the bispecific antibody is produced, and

b) recovering the bispecific antibody from the cell or the cultivation medium and thereby producing the bispecific antibody as reported herein.

One aspect as reported herein is an immunoconjugate comprising the bispecific antibody as reported herein and a cytotoxic agent.

One aspect as reported herein is a pharmaceutical formulation comprising the bispecific antibody as reported herein and a pharmaceutically acceptable carrier.

One aspect as reported herein is the antibody as reported herein for use as a medicament.

One aspect as reported herein is the antibody as reported herein for the treatment of cancer.

One aspect as reported herein is the bispecific antibody as reported herein for use in treating B-cell proliferative disease.

One aspect as reported herein is the bispecific antibody as reported herein for use in inhibiting growth of tumor cells expressing CD20. In one embodiment the inhibiting is in the brain.

One aspect as reported herein is the bispecific antibody as reported herein for use in treating carcinoma. In one preferred embodiment the carcinoma is carcinoma of/in the brain.

One aspect as reported herein is the bispecific antibody as reported herein for use in treating lymphoma. In one preferred embodiment the lymphoma is primary central nervous system lymphoma (PCNSL).

One aspect as reported herein is the bispecific antibody as reported herein for use in treating an autoimmune disease. In one embodiment the autoimmune disease is multiple sclerosis. In one preferred embodiment the autoimmune disease is secondary progressive multiple sclerosis.

One aspect as reported herein is the bispecific antibody as reported herein for use in depleting tumor cells expressing CD20. In one embodiment the depleting is in the brain.

One aspect as reported herein is the bispecific antibody as reported herein for use in depleting circulating B-cells expressing CD20. In one embodiment the depleting is in the brain.

One aspect as reported herein is the bispecific antibody as reported herein for use in depleting brain sequestered B-cells expressing CD20.

One aspect as reported herein is the use of the bispecific antibody as reported herein in the manufacture of a medicament.

In one embodiment the medicament is for the treatment of a proliferative type disease. In one embodiment the proliferative type disease is a B-cell proliferative disease. In one embodiment the proliferative type disease is B-cell lymphoma. In one preferred embodiment the proliferative type disease is primary central nervous system lymphoma.

In one embodiment the medicament is for the treatment of a tumor.

In one embodiment the medicament is for the treatment of human carcinoma.

In one embodiment the medicament is for the treatment of an autoimmune disease. In one embodiment the autoimmune disease is selected from the group consisting of inflammatory responses such as inflammatory skin diseases including psoriasis and dermatitis (e.g. atopic dermatitis); systemic scleroderma and sclerosis; responses associated with inflammatory bowel disease (such as Crohn's disease and ulcerative colitis); respiratory distress syndrome (including adult respiratory distress syndrome; ARDS); dermatitis; meningitis; encephalitis; uveitis; colitis; glomerulonephritis; allergic conditions such as eczema and asthma and other conditions involving infiltration of T-cells and chronic inflammatory responses; atherosclerosis; leukocyte adhesion deficiency; rheumatoid arthritis; systemic lupus erythematosus (SLE); diabetes mellitus (e.g. Type I diabetes mellitus or insulin dependent diabetes mellitus); multiple sclerosis; Reynaud's syndrome; autoimmune thyroiditis; allergic encephalomyelitis; Sjogren's syndrome; juvenile onset diabetes; and immune responses associated with acute and delayed hypersensitivity mediated by cytokines and T-lymphocytes typically found in tuberculosis, sarcoidosis, polymyositis, granulomatosis and vasculitis; pernicious anemia (Addison's disease); diseases involving leukocyte diapedesis; central nervous system (CNS) inflammatory disorder; multiple organ injury syndrome; hemolytic anemia (including, but not limited to cryoglobinemia or Coombs positive anemia); myasthenia gravis; antigen-antibody complex mediated diseases; anti-glomerular basement membrane disease; anti-phospholipid syndrome; allergic neuritis; Graves' disease; Lambert-Eaton myasthenic syndrome; pemphigoid bullous; pemphigus; autoimmune polyendocrinopathies; Reiter's disease; stiff-man syndrome; Bechet disease; giant cell arteritis; immune complex nephritis; IgA nephropathy; IgM polyneuropathies; immune thrombocytopenic purpura (ITP) or autoimmune thrombocytopenia. In one embodiment the medicament is for treatment of multiple sclerosis. In one preferred embodiment the medicament is for the treatment of secondary progressive multiple sclerosis.

In one embodiment the medicament is for depleting tumor cells expressing CD20. In one embodiment the depleting is in the brain.

In one embodiment the medicament is for depleting circulating B-cells expressing CD20. In one embodiment the depleting is in the brain.

In one embodiment the medicament is for depleting brain sequestered B-cells expressing CD20.

One aspect as reported herein is a method of treating an individual having a proliferative type disease comprising administering to the individual an effective amount of the bispecific antibody as reported herein. In one embodiment the proliferative type disease is a B-cell proliferative disease.

One aspect as reported herein is a method of treating an individual having carcinoma comprising administering to the individual an effective amount of the bispecific antibody as reported herein. In one preferred embodiment the carcinoma is carcinoma of/in the brain.

One aspect as reported herein is a method of treating an individual having lymphoma comprising administering to the individual an effective amount of the bispecific antibody as reported herein. In one preferred embodiment the lymphoma is primary central nervous system lymphoma (PCNSL).

One aspect as reported herein is a method of treating an individual having an autoimmune disease comprising administering to the individual an effective amount of the bispecific antibody as reported herein. In one embodiment the autoimmune disease is multiple sclerosis. In one preferred embodiment the autoimmune disease is secondary progressive multiple sclerosis.

One aspect as reported herein is a method for inhibiting growth of tumor cells expressing CD20 in an individual comprising administering to the individual an effective amount of the bispecific antibody as reported herein to inhibit growth of tumor cells expressing CD20. In one embodiment the inhibiting is in the brain.

One aspect as reported herein is a method for depleting tumor cells expressing CD20 in an individual comprising administering to the individual an effective amount of the bispecific antibody as reported herein to deplete tumor cells expressing CD20. In one embodiment the inhibiting is in the brain.

One aspect as reported herein is a method for depleting circulating B-cells expressing CD20 in an individual comprising administering to the individual an effective amount of the bispecific antibody as reported herein to deplete circulating B-cells expressing CD20. In one embodiment the inhibiting is in the brain.

One aspect as reported herein is a method for depleting brain sequestered B-cells expressing CD20 in an individual comprising administering to the individual an effective amount of the bispecific antibody as reported herein to deplete brain sequestered B-cells expressing CD20.

One aspect as reported herein is a method of treating multiple sclerosis in a human comprising administering to the human a therapeutically effective amount of an antibody as reported herein which binds to human CD20 and depletes B-cells, and wherein the antibody is not conjugated with a cytotoxic agent.

DETAILED DESCRIPTION OF EMBODTMENTS OF THE TNVENTTON

The knobs into holes dimerization modules and their use in antibody engineering are described in Carter P.; Ridgway J.B.B.; Presta L.G.: Immunotechnology, Volume 2, Number 1, February 1996 , pp. 73-73. The additional disulfide bridge in the CH3 domain is reported in Merchant, A.M., et al, Nat. Biotechnol. 16 (1998) 677-681.

General information regarding the nucleotide sequences of human immunoglobulins light and heavy chains is given in: Kabat, E.A., et al, Sequences of Proteins of Immunological Interest, 5th ed., Public Health Service, National Institutes of Health, Bethesda, MD (1991).

As used herein, the amino acid positions of all constant regions and domains of the heavy and light chain are numbered according to the Kabat numbering system described in Kabat, et al, Sequences of Proteins of Immunological Interest, 5th ed., Public Health Service, National Institutes of Health, Bethesda, MD (1991) and is referred to as "numbering according to Kabat" herein. Specifically, the Kabat numbering system (see pages 647-660) of Kabat, et al, Sequences of Proteins of Immunological Interest, 5th ed., Public Health Service, National Institutes of Health, Bethesda, MD (1991) is used for the light chain constant domain CL of kappa and lambda isotype, and the Kabat EU index numbering system (see pages 661-723) is used for the constant heavy chain domains (CHI, Hinge, CH2 and CH3, which is herein further clarified by referring to "numbering according to Kabat EU index" in this case).

T. DEFTNTTTONS

The "blood-brain-barrier" or "BBB" refers to the physiological barrier between the peripheral circulation and the brain and spinal cord which is formed by tight junctions within the brain capillary endothelial plasma membranes, creating a tight barrier that restricts the transport of molecules into the brain, even very small molecules such as urea (60 Daltons). The BBB within the brain, the blood-spinal-cord barrier within the spinal cord, and the blood-retinal-barrier within the retina are contiguous capillary barriers within the CNS, and are herein collectively referred to an the blood-brain-barrier or BBB. The BBB also encompasses the blood-CSF barrier (choroid plexus) where the barrier is comprised of ependymal cells rather than capillary endothelial cells.

The terms "anti-human CD20 antibody" and "an antibody specifically binding to human CD20" refer to an antibody that is capable of binding human CD20 with sufficient affinity such that the antibody is useful as a diagnostic and/or therapeutic agent in targeting CD20.

Thus, the term also encompasses antibodies that bind to a shortened fragment of human CD20.

Examples of antibodies which bind the CD20 antigen include: "C2B8" which is now called "rituximab" ("RITUXAN®") (US 5,736,137); the yttrium-[90]-labeled 2B8 murine antibody designated "Y2B8" (US 5,736,137); murine IgG2a "Bl" optionally labeled with 1311 to generate the "131I-B1" antibody (BEXXAR™) (US 5,595,721); murine monoclonal antibody "1F5" (Press et al, Blood 69 (1987) 584-591); "chimeric 2H7" antibody (US 5,677,180); monoclonal antibodies L27, G28-2, 93-1B3, B-Cl or NU-B2 available from the International Leukocyte Typing Workshop (Valentine et al., In: Leukocyte Typing III (McMichael, Ed.) p. 440, Oxford University Press (1987)); and the monoclonal antibody described in US 8,883,980.

The "CD20" antigen is an approx. 35 kDa, non-glycosylated phosphoprotein found on the surface of greater than 90% of B-cells from peripheral blood or lymphoid organs. CD20 is expressed during early pre-B-cell development and remains until plasma cell differentiation. CD20 is present on both normal B-cells as well as malignant B-cells. Other names for CD20 in the literature include "B-lymphocyte-restricted antigen" and "Bp35". The CD20 antigen is described in Clark et al. Proc. Natl. Acad. Sci USA 82 (1985) 1766, for example. See also SEQ ID NO: 05.

An "autoimmune disease" herein is a non-malignant disease or disorder arising from and directed against an individual's own tissues. The term "autoimmune diseases" as used herein specifically excludes malignant or cancerous diseases or conditions, especially excluding B-cell lymphoma, acute lymphoblastic leukemia

(ALL), chronic lymphocytic leukemia (CLL), Hairy cell leukemia and chronic myeloblasts leukemia. Examples of autoimmune diseases or disorders include, but are not limited to, inflammatory responses such as inflammatory skin diseases including psoriasis and dermatitis (e.g. atopic dermatitis); systemic scleroderma and sclerosis; responses associated with inflammatory bowel disease (such as Crohn's disease and ulcerative colitis); respiratory distress syndrome (including adult respiratory distress syndrome; ARDS); dermatitis; meningitis; encephalitis; uveitis; colitis; glomerulonephritis; allergic conditions such as eczema and asthma and other conditions involving infiltration of T-cells and chronic inflammatory responses; atherosclerosis; leukocyte adhesion deficiency; rheumatoid arthritis; systemic lupus erythematosus (SLE); diabetes mellitus (e.g. Type I diabetes mellitus or insulin dependent diabetes mellitus); multiple sclerosis; Reynaud's syndrome; autoimmune thyroiditis; allergic encephalomyelitis; Sjogren's syndrome; juvenile onset diabetes; and immune responses associated with acute and delayed hypersensitivity mediated by cytokines and T-lymphocytes typically found in tuberculosis, sarcoidosis, polymyositis, granulomatosis and vasculitis; pernicious anemia (Addison's disease); diseases involving leukocyte diapedesis; central nervous system (CNS) inflammatory disorder; multiple organ injury syndrome; hemolytic anemia (including, but not limited to cryoglobinemia or Coombs positive anemia); myasthenia gravis; antigen-antibody complex mediated diseases; anti-glomerular basement membrane disease; anti-phospholipid syndrome; allergic neuritis; Graves' disease; Lambert-Eaton myasthenic syndrome; pemphigoid bullous; pemphigus; autoimmune polyendocrinopathies; Reiter's disease; stiff-man syndrome; Bechet disease; giant cell arteritis; immune complex nephritis; IgA nephropathy; IgM polyneuropathies; immune thrombocytopenic purpura (ITP) or autoimmune thrombocytopenia etc.

An "antagonist" is a molecule which, upon binding to a B-cell surface marker, destroys, kills or depletes B-cells in a mammal and/or interferes with one or more B-cell functions, e.g. by reducing or preventing a humoral response elicited by the B-cell. The antagonist is able to deplete B-cells (i.e. reduce circulating B-cell levels) in a mammal treated therewith. Such depletion may be achieved via various mechanisms such antibody-dependent cell-mediated cytotoxicity (ADCC) and/or complement dependent cytotoxicity (CDC), inhibition of B-cell proliferation and/or induction of B-cell death (e.g. via apoptosis). Antagonists include antibodies, synthetic or native sequence peptides and small molecules which bind to the B-cell marker, optionally conjugated with or fused to a cytotoxic agent.

"Growth inhibitory" antagonists are those, which prevent or reduce proliferation of a cell expressing an antigen to which the antagonist binds. For example, the antagonist may prevent or reduce proliferation of B-cells in vitro and/or in vivo.

Antagonists which "induce apoptosis" are those which induce programmed cell death, e.g. of a B-cell, as determined by standard apoptosis assays, such as binding of Annexin V, fragmentation of DNA, cell shrinkage, dilation of endoplasmic reticulum, cell fragmentation, and/or formation of membrane vesicles (called apoptotic bodies).

An antagonist "which binds" an antigen of interest, e.g. a B-cell surface marker, is one capable of binding that antigen with sufficient affinity and/or avidity such that the antagonist is useful as a therapeutic agent for targeting a cell expressing the antigen.

The "central nervous system" or "CNS" refers to the complex of nerve tissues that control bodily function, and includes the brain and spinal cord.

A "blood-brain-barrier receptor" (abbreviated "BBBR" herein) is an extracellular membrane-linked receptor protein expressed on brain endothelial cells which is capable of transporting molecules across the BBB or be used to transport exogenous administrated molecules. Examples of BBBR herein include: transferrin receptor (TfR), insulin receptor, insulin-like growth factor receptor (IGF-R), low density lipoprotein receptors including without limitation low density lipoprotein receptor-related protein 1 (LRP1) and low density lipoprotein receptor-related protein 8 (LRP8), and heparin-binding epidermal growth factor-like growth factor (HB-EGF). One preferred BBBR is transferrin receptor (TfR).

The "transferrin receptor" ("TfR") is a transmembrane glycoprotein (with a molecular weight of about 180,000 Da) composed of two disulphide-bonded sub-units (each of apparent molecular weight of about 90,000 Da) involved in iron uptake in vertebrates. In one embodiment, the TfR as mentioned herein is human TfR comprising the amino acid sequence as in Schneider et al. (Nature 311 (1984) 675-678), for example.

A "multispecific antibody" denotes an antibody having binding specificities for at least two different epitopes on the same antigen or two different antigens. Exemplary multispecific antibodies may bind both a BBBR and a brain antigen. Multispecific antibodies can be prepared as full-length antibodies or antibody fragments (e.g. F(ab')2 bispecific antibodies) or combinations thereof (e.g. full length antibody plus additional scFv or Fab fragments). Engineered antibodies with two, three or more (e.g. four) functional antigen binding sites have also been reported (see, e.g., US 2002/0004587 Al).

An "acceptor human framework" for the purposes herein is a framework comprising the amino acid sequence of a light chain variable domain (VL) framework or a heavy chain variable domain (VH) framework derived from a human immunoglobulin framework or a human consensus framework, as defined below. An acceptor human framework "derived from" a human immunoglobulin framework or a human consensus framework may comprise the same amino acid sequence thereof, or it may contain amino acid sequence changes. In some embodiments, the number of amino acid changes are 10 or less, 9 or less, 8 or less, 7 or less, 6 or less, 5 or less, 4 or less, 3 or less, or 2 or less. In some embodiments, the VL acceptor human framework is identical in sequence to the VL human immunoglobulin framework sequence or human consensus framework sequence.

"Affinity" refers to the strength of the sum total of non-covalent interactions between a single binding site of a molecule (e.g., an antibody) and its binding partner (e.g., an antigen). Unless indicated otherwise, as used herein, "binding affinity" refers to intrinsic binding affinity which reflects a 1:1 interaction between members of a binding pair (e.g., antibody and antigen). The affinity of a molecule X for its partner Y can generally be represented by the dissociation constant (kd). Affinity can be measured by common methods known in the art, such as surface plasmon resonance and including those described herein.

An "affinity matured" antibody refers to an antibody with one or more alterations in one or more hypervariable regions (HVRs), compared to a parent antibody which does not possess such alterations, such alterations resulting in an improvement in the affinity of the antibody for its antigen(s).

The term "antibody" herein is used in the broadest sense and encompasses various antibody structures, including but not limited to monoclonal antibodies, polyclonal antibodies, and multispecific antibodies (e.g., bispecific antibodies) so long as they exhibit the desired antigen-binding activity.

The term "antibody-dependent cellular cytotoxicity (ADCC)" is a function mediated by Fc receptor binding and refers to lysis of target cells by an antibody as reported herein in the presence of effector cells. ADCC is measured in one embodiment by the treatment of a preparation of CD19 expressing erythroid cells (e.g. K562 cells expressing recombinant human CD19) with an antibody as reported herein in the presence of effector cells such as freshly isolated PBMC (peripheral blood mononuclear cells) or purified effector cells from buffy coats, like monocytes or NK (natural killer) cells. Target cells are labeled with 51Cr and subsequently incubated with the antibody. The labeled cells are incubated with effector cells and the supernatant is analyzed for released 51Cr. Controls include the incubation of the target endothelial cells with effector cells but without the antibody. The capacity of the antibody to induce the initial steps mediating ADCC is investigated by measuring their binding to Fey receptors expressing cells, such as cells, recombinantly expressing FcyRI and/or FcyRIIA or NK cells (expressing essentially FcyRIIIA). In one preferred embodiment binding to FcyR on NK cells is measured.

An "antibody fragment" refers to a molecule other than an intact antibody that comprises a portion of an intact antibody that binds the antigen to which the intact antibody binds. Examples of antibody fragments include but are not limited to Fv, Fab, Fab', Fab'-SH, F(ab')2; diabodies; linear antibodies; single-chain antibody molecules (e.g. scFv); and multispecific antibodies formed from antibody fragments.

The term "chimeric" antibody refers to an antibody in which a portion of the heavy and/or light chain is derived from a particular source or species, while the remainder of the heavy and/or light chain is derived from a different source or species.

The "class" of an antibody refers to the type of constant domain or constant region possessed by its heavy chain. There are five major classes of antibodies: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into subclasses (isotypes), e.g., IgGl, IgG2, IgG3, IgG4, IgAl, and IgA2. The heavy chain constant domains that correspond to the different classes of immunoglobulins are called α, δ, ε, γ, and μ, respectively.

The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents a cellular function and/or causes cell death or destruction. Cytotoxic agents include, but are not limited to, radioactive isotopes (e.g., At211, 1131, 1125, Y90, Rel86, Rel88, Sml53, Bi212, P32, Pb212 and radioactive isotopes of Lu); chemotherapeutic agents or drugs (e.g., methotrexate, adriamicin, vinca alkaloids (vincristine, vinblastine, etoposide), doxorubicin, melphalan, mitomycin C, chlorambucil, daunorubicin or other intercalating agents); growth inhibitory agents; enzymes and fragments thereof such as nucleolytic enzymes; antibiotics; toxins such as small molecule toxins or enzymatically active toxins of bacterial, fungal, plant or animal origin, including fragments and/or variants thereof; and the various antitumor or anticancer agents disclosed below.

The term "complement-dependent cytotoxicity (CDC)" refers to lysis of cells induced by the antibody as reported herein in the presence of complement. CDC is measured in one embodiment by the treatment of CD19 expressing human endothelial cells with an antibody as reported herein in the presence of complement. The cells are in one embodiment labeled with calcein. CDC is found if the antibody induces lysis of 20 % or more of the target cells at a concentration of 30 µg/ml. Binding to the complement factor Clq can be measured in an ELISA. In such an assay in principle an ELISA plate is coated with concentration ranges of the antibody, to which purified human Clq or human serum is added. Clq binding is detected by an antibody directed against Clq followed by a peroxidase-labeled conjugate. Detection of binding (maximal binding Bmax) is measured as optical density at 405 nm (OD405) for peroxidase substrate ABTS® (2,2'-azino-di-[3-ethylbenzthiazoline-6-sulfonate (6)]).

"Effector functions" refer to those biological activities attributable to the Fc-region of an antibody, which vary with the antibody class. Examples of antibody effector functions include: Clq binding and complement dependent cytotoxicity (CDC); Fc receptor binding; antibody-dependent cell-mediated cytotoxicity (ADCC); phagocytosis; down regulation of cell surface receptors (e.g. B-cell receptor); and B-cell activation.

Fc receptor binding dependent effector functions can be mediated by the interaction of the Fc-region of an antibody with Fc receptors (FcRs), which are specialized cell surface receptors on hematopoietic cells. Fc receptors belong to the immunoglobulin superfamily, and have been shown to mediate both the removal of antibody-coated pathogens by phagocytosis of immune complexes, and the lysis of erythrocytes and various other cellular targets (e.g. tumor cells) coated with the corresponding antibody, via antibody dependent cell mediated cytotoxicity (ADCC) (see e.g. Van de Winkel, J.G. and Anderson, C.L., J. Leukoc. Biol. 49 (1991) 511-524). FcRs are defined by their specificity for immunoglobulin isotypes: Fc receptors for IgG antibodies are referred to as FcyR. Fc receptor binding is described e.g. in Ravetch, J.V. and Kinet, J.P., Annu. Rev. Immunol. 9 (1991) 457-492; Capel, P.J., et al, Immunomethods 4 (1994) 25-34; de Haas, M., et al, J. Lab. Clin. Med. 126 (1995) 330-341; and Gessner, J.E., et al, Ann. Hematol. 76 (1998) 231-248.

Cross-linking of receptors for the Fc-region of IgG antibodies (FcyR) triggers a wide variety of effector functions including phagocytosis, antibody-dependent cellular cytotoxicity, and release of inflammatory mediators, as well as immune complex clearance and regulation of antibody production. In humans, three classes of FcyR have been characterized, which are:

- FcyRI (CD64) binds monomeric IgG with high affinity and is expressed on macrophages, monocytes, neutrophils and eosinophils. Modification in the Fc-region IgG at least at one of the amino acid residues E233-G236, P238, D265, N297, A327 and P329 (numbering according to EU index of Kabat) reduce binding to FcyRI. IgG2 residues at positions 233-236, substituted into IgGl and IgG4, reduced binding to FcyRI by 10^3-fold and eliminated the human monocyte response to antibody-sensitized red blood cells (Armour, K.L., et al, Eur. J. Immunol. 29 (1999) 2613-2624),

- FcyRII (CD32) binds complexed IgG with medium to low affinity and is widely expressed. This receptor can be divided into two sub-types, FcyRIIA and FcyRIIB. FcyRIIA is found on many cells involved in killing (e.g. macrophages, monocytes, neutrophils) and seems able to activate the killing process. FcyRIIB seems to play a role in inhibitory processes and is found on B-cells, macrophages and on mast cells and eosinophils. On B-cells it seems to function to suppress further immunoglobulin production and isotype switching to, for example, the IgE class. On macrophages, FcyRIIB acts to inhibit phagocytosis as mediated through FcyRIIA. On eosinophils and mast cells the B-form may help to suppress activation of these cells through IgE binding to its separate receptor. Reduced binding for FcyRIIA is found e.g. for antibodies comprising an IgG Fc-region with mutations at least at one of the amino acid residues E233-G236, P238, D265, N297, A327, P329, D270, Q295, A327, R292, and K414 (numbering according to EU index of Kabat),

- FcyRIII (CD16) binds IgG with medium to low affinity and exists as two types. FcyRIIIA is found on NK cells, macrophages, eosinophils and some monocytes and T cells and mediates ADCC. FcyRIIIB is highly expressed on neutrophils.

Reduced binding to FcγRIIIA is found e.g. for antibodies comprising an IgG Fc-region with mutation at least at one of the amino acid residues E233-G236, P238, D265, N297, A327, P329, D270, Q295, A327, S239, E269, E293, Y296, V303, A327, K338 and D376 (numbering according to EU index of Kabat).

Mapping of the binding sites on human IgG1 for Fc receptors, the above mentioned mutation sites and methods for measuring binding to FcγRI and FcγRIIA are described in Shields, R.L., et al., J. Biol. Chem. 276 (2001) 6591-6604.

An "effective amount" of an agent, e.g., a pharmaceutical formulation, refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired therapeutic or prophylactic result.

The term "Fc receptor" as used herein refers to activation receptors characterized by the presence of a cytoplasmatic ITAM sequence associated with the receptor (see e.g. Ravetch, J.V. and Bolland, S., Annu. Rev. Immunol. 19 (2001) 275-290). Such receptors are FcγRI, FcγRIIA and FcγRIIIA. The term "no binding of FcγR" denotes that at an antibody concentration of 10 µg/ml the binding of an antibody as reported herein to NK cells is 10 % or less of the binding found for anti-OX40L antibody LC.001 as reported in WO 2006/029879.

While IgG4 shows reduced FcR binding, antibodies of other IgG subclasses show strong binding. However Pro238, Asp265, Asp270, Asn297 (loss of Fc carbohydrate), Pro329 and 234, 235, 236 and 237 Ile253, Ser254, Lys288, Thr307, Gln311, Asn434, and His435 are residues which provide if altered also reduce FcR binding (Shields, R.L., et al, J. Biol. Chem. 276 (2001) 6591-6604; Lund, J., et al, FASEB J. 9 (1995) 115-119; Morgan, A., et al, Immunology 86 (1995) 319-324; and EP 0 307 434). In one embodiment the antibody as reported herein is of IgG1 or IgG2 subclass and comprises the mutation PVA236, GLPSS331, and/or L234A/L235A. In one embodiment the antibody as reported herein is of IgG4 subclass and comprises the mutation L235E. In one embodiment the antibody further comprises the mutation S228P.

The term "Fc-region" herein is used to define a C-terminal region of an immunoglobulin heavy chain that contains at least a portion of the constant region. The term includes native sequence Fc-regions and variant Fc-regions. In one embodiment, a human IgG heavy chain Fc-region extends from Cys226, or from Pro230, to the carboxyl-terminus of the heavy chain. However, the C-terminal lysine (Lys447) of the Fc-region may or may not be present.

The antibodies as reported herein comprise as Fc-region, in one embodiment an Fc-region derived from human origin. In one embodiment the Fc-region comprises all parts of the human constant region. The Fc-region of an antibody is directly involved in complement activation, Clq binding, C3 activation and Fc receptor binding. While the influence of an antibody on the complement system is dependent on certain conditions, binding to Clq is caused by defined binding sites in the Fc-region. Such binding sites are known in the state of the art and described e.g. by Lukas, T.J., et al, J. Immunol. 127 (1981) 2555-2560; Brunhouse, R. and Cebra, J.J., Mol. Immunol. 16 (1979) 907-917; Burton, D.R., et al, Nature 288 (1980) 338-344; Thommesen, J.E., et al, Mol. Immunol. 37 (2000) 995-1004; Idusogie, E.E., et al, J. Immunol. 164 (2000) 4178-4184; Hezareh, M., et al, J. Virol. 75 (2001) 12161-12168; Morgan, A., et al, Immunology 86 (1995) 319-324; and EP 0 307 434. Such binding sites are e.g. L234, L235, D270, N297, E318, K320, K322, P331 and P329 (numbering according to EU index of Kabat). Antibodies of subclass IgGl, IgG2 and IgG3 usually show complement activation, Clq binding and C3 activation, whereas IgG4 do not activate the complement system, do not bind Clq and do not activate C3.

An "Fc-region of an antibody" is a term well known to the skilled artisan and defined on the basis of papain cleavage of antibodies. In one embodiment the Fc-region is a human Fc-region. In one embodiment the Fc-region is of the human IgG4 subclass comprising the mutations S228P and/or L235E (numbering according to EU index of Kabat). In one embodiment the Fc-region is of the human IgGl subclass comprising the mutations L234A and L235A (numbering according to EU index of Kabat).

"Framework" or "FR" refers to variable domain residues other than hypervariable region (HVR) residues. The FR of a variable domain generally consists of four FR domains: FR1, FR2, FR3, and FR4. Accordingly, the HVR and FR sequences generally appear in the following sequence in VH (or VL): FR1-H1(L1)-FR2-H2(L2)-FR3-H3(L3)-FR4.

The terms "full length antibody", "intact antibody," and "whole antibody" are used herein interchangeably to refer to an antibody having a structure substantially similar to a native antibody structure or having heavy chains that contain an Fc-region as defined herein. A "full length antibody" is an antibody that comprises an antigen-binding variable region as well as a light chain constant domain (CL) and heavy chain constant domains, CHI, CH2 and CH3. The constant domains may be native sequence constant domains (e.g. human native sequence constant domains) or amino acid sequence variants thereof. In more detail a full length antibody comprises two antibody light chains (each comprising a light chain variable domain and a light chain constant domain) and two antibody heavy chains (each comprising a heavy chain variable domain, a hinge region and the heavy chain constant domains CHI, CH2 and CH3). The C-terminal amino acid residues K or GK may be present or not independently of each other in the two antibody heavy chains of a full length antibody.

The terms "host cell", "host cell line", and "host cell culture" are used interchangeably and refer to cells into which exogenous nucleic acid has been introduced, including the progeny of such cells. Host cells include "transformants" and "transformed cells," which include the primary transformed cell and progeny derived therefrom without regard to the number of passages. Progeny may not be completely identical in nucleic acid content to a parent cell, but may contain mutations. Mutant progeny that have the same function or biological activity as screened or selected for in the originally transformed cell are included herein.

A "human consensus framework" is a framework which represents the most commonly occurring amino acid residues in a selection of human immunoglobulin VL or VH framework sequences. Generally, the selection of human immunoglobulin VL or VH sequences is from a subgroup of variable domain sequences. Generally, the subgroup of sequences is a subgroup as in Kabat, E.A. et al, Sequences of Proteins of Immunological Interest, 5th ed., Bethesda MD (1991), NIH Publication 91-3242, Vols. 1-3. In one embodiment, for the VL, the subgroup is subgroup kappa I as in Kabat et al, supra. In one embodiment, for the VH, the subgroup is subgroup III as in Kabat et al, supra.

A "humanized" antibody refers to a chimeric antibody comprising amino acid residues from non-human HVRs and amino acid residues from human FRs. In certain embodiments, a humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the HVRs (e.g., CDRs) correspond to those of a non-human antibody, and all or substantially all of the FRs correspond to those of a human antibody. A humanized antibody optionally may comprise at least a portion of an antibody constant region derived from a human antibody. A "humanized form" of an antibody, e.g., a non-human antibody, refers to an antibody that has undergone humanization.

The term "hypervariable region" or "HVR", as used herein, refers to each of the regions of an antibody variable domain comprising the amino acid residue stretches which are hypervariable in sequence ("complementarity determining regions" or "CDRs") and/or form structurally defined loops ("hypervariable loops"), and/or contain the antigen-contacting residues ("antigen contacts"). Generally, antibodies comprise six HVRs; three in the VH (HI, H2, H3), and three in the VL (LI, L2, L3).

HVRs include

(a) hypervariable loops occurring at amino acid residues 26-32 (LI), 50-52 (L2), 91-96 (L3), 26-32 (HI), 53-55 (H2), and 96-101 (H3) (Chothia, C. and Lesk, A.M., J. Mol. Biol. 196 (1987) 901-917);

(b) CDRs occurring at amino acid residues 24-34 (LI), 50-56 (L2), 89-97 (L3), 31-35b (HI), 50-65 (H2), and 95-102 (H3) (Kabat, E.A. et al, Sequences of Proteins of Immunological Interest, 5th ed. Public Health Service, National Institutes of Health, Bethesda, MD (1991), NIH Publication 91-3242.);

(c) antigen contacts occurring at amino acid residues 27c-36 (LI), 46-55 (L2), 89-96 (L3), 30-35b (HI), 47-58 (H2), and 93-101 (H3) (MacCallum et al, J. Mol. Biol. 262 (1996) 732-745); and

(d) combinations of (a), (b), and/or (c), including amino acid residues 46-56 (L2), 47-56 (L2), 48-56 (L2), 49-56 (L2), 26-35 (HI), 26-35b (HI), 49-65 (H2), 93-102 (H3), and 94-102 (H3).

Unless otherwise indicated, HVR residues and other residues in the variable domain (e.g., FR residues) are numbered herein according to Kabat et al, supra.

An "immunoconjugate" is an antibody conjugated to one or more heterologous molecule(s), including but not limited to a cytotoxic agent.

An "individual" or "subject" is a mammal. Mammals include, but are not limited to, domesticated animals (e.g. cows, sheep, cats, dogs, and horses), primates (e.g., humans and non-human primates such as monkeys), rabbits, and rodents (e.g., mice and rats). In certain embodiments, the individual or subject is a human.

An "isolated" antibody is one, which has been separated from a component of its natural environment. In some embodiments, an antibody is purified to greater than

95% or 99% purity as determined by, for example, electrophoretic (e.g., SDS-PAGE, isoelectric focusing (IEF), capillary electrophoresis) or chromatographic (e.g., ion exchange or reverse phase HPLC). For review of methods for assessment of antibody purity, see, e.g., Flatman, S., et al, J. Chromatogr. B 848 (2007) 79-87.

An "isolated" nucleic acid refers to a nucleic acid molecule that has been separated from a component of its natural environment. An isolated nucleic acid includes a nucleic acid molecule contained in cells that ordinarily contain the nucleic acid molecule, but the nucleic acid molecule is present extrachromosomally or at a chromosomal location that is different from its natural chromosomal location.

"Isolated nucleic acid encoding an anti-human CD20/human transferrin receptor antibody" refers to one or more nucleic acid molecules encoding antibody heavy and light chains (or fragments thereof), including such nucleic acid molecule(s) in a single vector or separate vectors, and such nucleic acid molecule(s) present at one or more locations in a host cell.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical and/or bind the same epitope, except for possible variant antibodies, e.g., containing naturally occurring mutations or arising during production of a monoclonal antibody preparation, such variants generally being present in minor amounts. In contrast to polyclonal antibody preparations, which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody of a monoclonal antibody preparation is directed against a single determinant on an antigen. Thus, the modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by a variety of techniques, including but not limited to the hybridoma method, recombinant DNA methods, phage-display methods, and methods utilizing transgenic animals containing all or part of the human immunoglobulin loci, such methods and other exemplary methods for making monoclonal antibodies being described herein.

A "naked antibody" refers to an antibody that is not conjugated to a heterologous moiety (e.g., a cytotoxic moiety) or radiolabel. The naked antibody may be present in a pharmaceutical formulation.

"Native antibodies" refer to naturally occurring immunoglobulin molecules with varying structures. For example, native IgG antibodies are heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light chains and two identical heavy chains that are disulfide-bonded. From N- to C-terminus, each heavy chain has a variable region (VH), also called a variable heavy domain or a heavy chain variable domain, followed by three constant domains (CHI, CH2, and CH3), whereby between the first and the second constant domain a hinge region is located. Similarly, from N- to C-terminus, each light chain has a variable region (VL), also called a variable light domain or a light chain variable domain, followed by a constant light (CL) domain. The light chain of an antibody may be assigned to one of two types, called kappa (κ) and lambda (λ), based on the amino acid sequence of its constant domain.

The term "package insert" is used to refer to instructions customarily included in commercial packages of therapeutic products, that contain information about the indications, usage, dosage, administration, combination therapy, contraindications and/or warnings concerning the use of such therapeutic products.

"Percent (%) amino acid sequence identity" with respect to a reference polypeptide sequence is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in the reference polypeptide sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for aligning sequences, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared. For purposes herein, however, % amino acid sequence identity values are generated using the sequence comparison computer program ALIGN-2. The ALIGN-2 sequence comparison computer program was authored by Genentech, Inc., and the source code has been filed with user documentation in the U.S. Copyright Office, Washington D.C., 20559, where it is registered under U.S. Copyright Registration No. TXU5 10087. The ALIGN-2 program is publicly available from Genentech, Inc., South San Francisco, California, or may be compiled from the source code. The ALIGN-2 program should be compiled for use on a UNIX operating system, including digital UNIX V4.0D. All sequence comparison parameters are set by the ALIGN-2 program and do not vary.

In situations where ALIGN-2 is employed for amino acid sequence comparisons, the % amino acid sequence identity of a given amino acid sequence A to, with, or against a given amino acid sequence B (which can alternatively be phrased as a given amino acid sequence A that has or comprises a certain % amino acid sequence identity to, with, or against a given amino acid sequence B) is calculated as follows:

100 times the fraction X/Y

where X is the number of amino acid residues scored as identical matches by the sequence alignment program ALIGN-2 in that program's alignment of A and B, and where Y is the total number of amino acid residues in B. It will be appreciated that where the length of amino acid sequence A is not equal to the length of amino acid sequence B, the % amino acid sequence identity of A to B will not equal the % amino acid sequence identity of B to A. Unless specifically stated otherwise, all % amino acid sequence identity values used herein are obtained as described in the immediately preceding paragraph using the ALIGN-2 computer program.

The term "pharmaceutical formulation" refers to a preparation which is in such form as to permit the biological activity of an active ingredient contained therein to be effective, and which contains no additional components which are unacceptably toxic to a subject to which the formulation would be administered.

A "pharmaceutically acceptable carrier" refers to an ingredient in a pharmaceutical formulation, other than an active ingredient, which is nontoxic to a subject. A pharmaceutically acceptable carrier includes, but is not limited to, a buffer, excipient, stabilizer, or preservative.

As used herein, "treatment" (and grammatical variations thereof such as "treat" or "treating") refers to clinical intervention in an attempt to alter the natural course of the individual being treated, and can be performed either for prophylaxis or during the course of clinical pathology. Desirable effects of treatment include, but are not limited to, preventing occurrence or recurrence of disease, alleviation of symptoms, diminishment of any direct or indirect pathological consequences of the disease, preventing metastasis, decreasing the rate of disease progression, amelioration or palliation of the disease state, and remission or improved prognosis. In some embodiments, antibodies as reported herein are used to delay development of a disease or to slow the progression of a disease.

The term "variable region" or "variable domain" refers to the domain of an antibody heavy or light chain that is involved in binding the antibody to antigen. The variable domains of the heavy chain and light chain (VH and VL, respectively) of a native antibody generally have similar structures, with each domain comprising four conserved framework regions (FRs) and three hypervariable regions (HVRs) (see, e.g., Kindt, T.J. et al, Kuby Immunology, 6th ed., W.H. Freeman and Co., N.Y. (2007), page 91). A single VH or VL domain may be sufficient to confer antigen-binding specificity. Furthermore, antibodies that bind a particular antigen may be isolated using a VH or VL domain from an antibody that binds the antigen to screen a library of complementary VL or VH domains, respectively (see, e.g., Portolano, S., et al, J. Immunol. 150 (1993) 880-887; Clackson, T., et al, Nature 352 (1991) 624-628).

The term "vector", as used herein, refers to a nucleic acid molecule capable of propagating another nucleic acid to which it is linked. The term includes the vector as a self-replicating nucleic acid structure as well as the vector incorporated into the genome of a host cell into which it has been introduced. Certain vectors are capable of directing the expression of nucleic acids to which they are operatively linked. Such vectors are referred to herein as "expression vectors".

The term "immunosuppressive agent" as used herein for adjunct therapy refers to substances that act to suppress or mask the immune system of the mammal being treated herein. This would include substances that suppress cytokine production, down-regulate or suppress self-antigen expression, or mask the MHC antigens. Examples of such agents include 2-amino-6-aryl-5-substituted pyrimidines (see US 4,665,077); azathioprine; cyclophosphamide; bromocryptine; danazol; dapsone; glutaraldehyde (which masks the MHC antigens, as described in US 4,120,649); anti-idiotypic antibodies for MHC antigens and MHC fragments; cyclosporin A; steroids such as glucocorticosteroids, e.g., prednisone, methylprednisolone, and dexamethasone; cytokine or cytokine receptor antagonists including anti-interferon-γ, -β, or -a antibodies, anti-tumor necrosis factor-a antibodies, antitumor necrosis factor-β antibodies, anti-interleukin-2 antibodies and anti-IL-2 receptor antibodies; anti-LFA-1 antibodies, including anti-CDl la and anti-CD 18 antibodies; anti-L3T4 antibodies; heterologous anti-lymphocyte globulin; pan-T antibodies, preferably anti-CD3 or anti-CD4/CD4a antibodies; soluble peptide containing a LFA-3 binding domain (WO 90/08187); streptokinase; TGF-β; streptodornase; RNA or DNA from the host; FK506; RS-61443; deoxyspergualin; rapamycin; T-cell receptor (US 5,1 14,721); T-cell receptor fragments (Offner et al, Science 251 (1991) 430-432; WO 90/1 1294; Ianeway, Nature 341 (1989) 482; and WO 91/01 133); and T cell receptor antibodies (EP 0,340,109) such as T10B9.

A "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include alkylating agents such as thiotepa and cyclosphosphamide (CYTOXAN™); alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, trietylenephosphoramide, triethylenethiophosphoramide and trimethylolomelamine; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, ranimustine; antibiotics such as aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, calicheamicin, carabicin, carminomycin, carzinophilin, chromomycins, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin, epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine, 5-FU; androgens such as calusterone, dromostanolone propionate, epitiostanol, mepitiostane, testolactone; anti-adrenals such as aminoglutethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elfornithine;

elliptinium acetate; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidamine; mitoguazone; mitoxantrone; mopidamol; nitracrine; pentostatin; phenamet; pirarubicin; podophyllinic acid; 2-ethylhydrazide; procarbazine; PSK®; razoxane; sizofiran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2"-trichloro triethylamine; urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepa; taxoids, e.g. paclitaxel (TAXOL®, Bristol-Myers Squibb Oncology, Princeton, N.J.) and doxetaxel (TAXOTERE®, Rhone-Poulenc Rorer, Antony, France); chlorambucil; gemcitabine; 6-thioguanine; mercaptopurine; methotrexate; platinum analogs such as cisplatin and carboplatin; vinblastine; platinum; etoposide (VP-16); ifosfamide; mitomycin C; mitoxantrone; vincristine; vinorelbine; navelbine; novantrone; teniposide; daunomycin; aminopterin; xeloda; ibandronate; CPT-11; topoisomerase inhibitor RFS 2000; difluoromethylornithine (DMFO); retinoic acid; esperamicins; capecitabine; and pharmaceutically acceptable salts, acids or derivatives of any of the above. Also included in this definition are anti-hormonal agents that act to regulate or inhibit hormone action on tumors such as anti-estrogens including for example tamoxifen, raloxifene, aromatase inhibiting 4(5)-imidazoles, 4-hydroxytamoxifen, trioxifene, keoxifene, LY117018, onapristone, and toremifene (Fareston); and anti-androgens such as flutamide, nilutamide, bicalutamide, leuprolide, and goserelin; and pharmaceutically acceptable salts, acids or derivatives of any of the above.

TT. COMPOSTTTONS AND METHODS

In one aspect, the invention is based, in part, on the finding that the bispecific anti-human CD20/human transferrin receptor antibody as reported herein has improved properties. In certain embodiments, bispecific anti-human CD20/human transferrin receptor antibodies are provided. Antibodies as reported herein are useful, e.g., for the diagnosis or treatment of Parkinson's disease or multiple sclerosis.

A. Exemplary bispecific anti-human CD20/human transferrin receptor Antibodies

In one aspect, the invention provides isolated bispecific antibodies that bind to human CD20 and human transferrin receptor. The antibodies are bispecific antibodies consisting of a (full length) core antibody and a fused Fab fragment in which certain domains are crosswise exchanged. Thus, the resulting bispecific antibody is asymmetric. Therefore, the bispecific antibodies are produced using the heterodimerization technology called knobs-into-holes using a first heavy chain with the so-called knob mutations (HCknob) and a second heavy chain with the so-called hole mutations (HChole).

Antibody 0039, which is also an aspect of the current invention, is composed of four polypeptides that have the amino acid sequence of SEQ ID NO: 06 to 09.

Antibody 0039 is a bispecific antibody comprising

a) one full length antibody comprising two pairs each of a full length antibody light chain and a full length antibody heavy chain, wherein the binding sites formed by each of the pairs of the full length heavy chain and the full length light chain specifically bind to a first antigen, and

b) one additional Fab fragment, wherein the additional Fab fragment is fused to the C-terminus of one heavy chain of the full length antibody, wherein the binding site of the additional Fab fragment specifically binds to a second antigen,

wherein each of the full length antibody light chains comprises in the constant light chain domain (CL) at position 123 the amino acid residue arginine (instead of the wild-type glutamic acid residue; E123R mutation) and at position 124 the amino acid residue lysine (instead of the wild-type glutamine residue; Q124K mutation) (numbering according to Kabat),

wherein each of the full length antibody heavy chains comprises in the first constant heavy chain domain (CHI) at position 147 an glutamic acid residue (instead of the wild-type lysine residue; K147E mutation) and at position 213 an glutamic acid residue (instead of the wild-type lysine amino acid residue; K213E mutation) (numbering according to Kabat EU index),

wherein the additional Fab fragment specifically binding to the second antigen comprises a domain crossover such that the light chain variable domain (VL) and the heavy chain variable domain (VH) are replaced by each other, and

wherein the first antigen is human CD20 and the second antigen is human transferrin receptor.

Antibody 0041, which is also an aspect of the current invention, is composed of four polypeptides that have the amino acid sequence of SEQ ID NO: 01 to 03 and SEQ ID NO: 10.

Antibody 0041 is a bispecific antibody comprising

a) one full length antibody comprising two pairs each of a full length antibody light chain and a full length antibody heavy chain, wherein the binding sites formed by each of the pairs of the full length heavy chain and the full length light chain specifically bind to a first antigen, and

b) one additional Fab fragment, wherein the additional Fab fragment is fused to the C-terminus of one heavy chain of the full length antibody, wherein the binding site of the additional Fab fragment specifically binds to a second antigen,

wherein each of the full length antibody light chains comprises in the constant light chain domain (CL) at position 123 the amino acid residue arginine (instead of the wild-type glutamic acid residue; E123R mutation) and at position 124 the amino acid residue lysine (instead of the wild-type glutamine residue; Q124K mutation) (numbering according to Kabat),

wherein each of the full length antibody heavy chains comprises in the first constant heavy chain domain (CHI) at position 147 an glutamic acid residue (instead of the wild-type lysine residue; K147E mutation) and at position 213 an glutamic acid residue (instead of the wild-type lysine amino acid residue; K213E mutation) (numbering according to Kabat EU index),

wherein the additional Fab fragment specifically binding to the second antigen comprises a domain crossover such that the constant light chain domain (CL) and the constant heavy chain domain 1 (CHI) are replaced by each other, and

wherein the first antigen is human CD20 and the second antigen is human transferrin receptor.

Antibody 0040, which is also an aspect of the current invention, is composed of three polypeptides that have the amino acid sequence of SEQ ID NO: 11 to 13 and SEQ ID NO: 22.

Antibody 0040 is a bispecific antibody comprising

a) one full length antibody comprising two pairs each of a full length antibody light chain and a full length antibody heavy chain, wherein the binding sites formed by each of the pairs of the full length heavy chain and the full length light chain specifically bind to a first antigen, and

b) one additional Fab fragment, wherein the additional Fab fragment is fused to the C-terminus of one heavy chain of the full length antibody, wherein the binding site of the additional Fab fragment specifically binds to a second antigen,

wherein the additional Fab fragment specifically binding to the second antigen comprises a domain crossover such that the constant light chain domain (CL) and the constant heavy chain domain 1 (CHI) are replaced by each other, and

wherein the first antigen is human CD20 and the second antigen is human transferrin receptor.

Antibody 0042, which is also an aspect of the current invention, is composed of four polypeptides that have the amino acid sequence of SEQ ID NO: 14 to 17.

Antibody 0042 is a bispecific antibody comprising

a) a first light chain and a first heavy chain derived from a first antibody which specifically binds to a first antigen; and

b) a second light chain and a second heavy chain derived from a second antibody which specifically binds to a second antigen, wherein

in the second light chain the constant domain CL is replaced by the constant domain CHI of the second heavy chain; and

in the second heavy chain the constant domain CHI is replaced by the constant domain CL of the second light chain; and

i) wherein in the constant domain CL of the first light chain the amino acids at position 124 and 123 (numbering according to Kabat) are substituted independently from each other by an amino acid selected from K, R and H; and wherein in the constant domain CHI of the first heavy chain the amino acids at position 147 and 213 (numbering according to EU index of Kabat) are substituted independently from each other by an amino acid selected from E or D; or

ii) wherein in the constant domain CL of the second heavy chain the amino acids at position 124 and 123 (numbering according to Kabat) are substituted independently from each other by an amino acid selected from K, R and H; and wherein in the constant domain CHI of the second light chain the amino acids at position 147 and 213 (numbering according to EU index of Kabat) are substituted independently from each other by an amino acid selected from E or D.

The bispecific antibodies as reported herein have been obtained by transient expression in CHO cells. The yields are shown in the following Table.

antibody	**0039**	**0040**	**0041**	**0042**
c [µg/ml]	8.4	25.3	32	22.5
amount [mg]	29.4	88.6	112.0	78.8

Different allocation/combination of the respective polypeptides on different expression plasmids and different ratios of the resulting plasmids have been used for the recombinant production of the bispecific antibodies. The results obtained in HEK 293 cells are presented in the following Table.

antibody	**molar plasmid ratio**		**relative peak area (non-reduced) [%]**		
			½ mAb hole	**hole-hole chain dimer**	**antibody monomer**
0039	1:3	LC+HC-hole : CrossLC+HCknob	9	10	80
0039	1:4		6	6	88
0039	1:3:2	LC+HC-hole : CrossLC+HCknob : CrossLC	17	9	74
0039	1:4:2		10	5	85
0039	1:1:3	LC : LC+HC-hole : CrossLC+HCknob	6	6	88
0039	1:1:4		3	4	93
0040	1:3	LC+HC-hole : CrossLC+HCknob	10	18	72
0040	1:4		6	7	87
0040	1:3:2	LC+HC-hole : CrossLC+HCknob : CrossLC	5	7	89
0040	1:4:2		3	5	92

antibody	molar plasmid ratio		relative peak area (non-reduced) [%]		
0040	1:1:3	LC : LC+HC-hole : CrossLC+HCknob	16	48	35
0040	1:1:4		6	23	69
0041	1:3	LC+HC-hole : CrossLC+HCknob	3	5	92
0041	1:4		1	2	97
0041	1:3:2	LC+HC-hole : CrossLC+HCknob : CrossLC	-	2	98
0041	1:4:2		-	1	99
0041	1:1:3	LC : LC+HC-hole : CrossLC+HCknob	-	3	97
0041	1:1:4		-	2	97
0042	1:2	LC+HC-hole : CrossLC+HCknob	-	1	99

The bispecific antibodies have been produced in CHO cells in small scale and the by-product distribution has been analyzed after a first purification step using a protein A affinity chromatography and after the second purification step using a preparative size-exclusion chromatography. The results are presented in the following Table.

antibody	molar plasmid ratio	harvest 3 liter fermentation after protein A product monomer (CE-SDS not red./ yield)	by-product distribution (CE-SDS not red.)			
			LC	HC hole	½ mAb hole	hole-hole dimer + $^{3}/_{4}$ mAb
0039	1:3:2	55% 26 mg	16	6	11	12
0040	1:3:2	44% 74.5 mg	23	8	7	17
0041	1:3:2	82% > 80 mg	11	0	0	7
0042	1:2	83% 68.4 mg	9	0	0	8

antibody	plasmid ratio	harvest 3 liter fermentation after protein A and preparative SEC product monomer (CE-SDS not red./ yield)	by-product distribution (CE-SDS not red.)			
			LC	HC hole	½ mAb hole	hole-hole dimer + $^3/_4$ mAb
0039	1:3:2	8.2 mg 73%	10	0	2	15
0040	1:3:2	29.7 mg 77%	10	0	0	14
0041	1:3:2	> 44 mg 79%	8	0	0	13
0042	1:2	43.6 mg > 90%	3	0	0	3

antibody	plasmid ratio	harvest 3l after protein A purification monomer SEC	by-products SEC [%]		end product SEC	by-products SEC [%]	
			HMW	LMW		HMW	LMW
0039	1:3:2	90%	2	7	95%	0	5
0040	1:3:2	89%	5	6	96.5%	1	2.5
0041	1:3:2	94%	6	0	97.5%	0.5	2
0042	1:2	95%	2	3	97%	1	2

The bispecific antibodies have been produced in different cell lines. The results are shown in the following Table.

antibody	**micro purification with protein A product monomer (CE-SDS)**		**harvest 3l after protein A purification monomer (yield / CE-SDS not red.)**	**end product (preparative protein A and preparative SEC purification) (yield / CE-SDS not red.)**
	CHO-K1	**HEK293**	**CHO**	**CHO**
0039	80%	93%	7.4 mg/l 55 %	8.2 mg 73 %
0040	83%	92%	21.3 mg/l 44 %	29.7 mg 77 %
0041	85%	99%	> 21 mg/l 82 %	> 44 mg 79 %
0042	91%	99%	19 mg/l 83 %	43.8 mg 94 %

The aggregation temperature for antibodies 0039, 0040, 0041 and 0042 was determined to be approx. 54-56°C, approx. 50-53°C, approx. 51-53°C, and approx. 55-57°C, respectively.

Overall antibody 0041 showed improved properties and is therefore the preferred aspect of the invention. The improved properties lie, amongst others, in the improved side-product profile.

One aspect as reported herein is a bispecific antibody comprising

a) one full length antibody comprising two pairs each of a full length antibody light chain and a full length antibody heavy chain, wherein the binding sites formed by each of the pairs of the full length heavy chain and the full length light chain specifically bind to a first antigen, and

b) one additional Fab fragment, wherein the additional Fab fragment is fused to the C-terminus of one heavy chain of the full length antibody, wherein the binding site of the additional Fab fragment specifically binds to a second antigen,

wherein each of the full length antibody light chains comprises in the constant light chain domain (CL) at position 123 the amino acid residue arginine (instead of the wild-type glutamic acid residue; E123R mutation) and at position 124 the amino acid residue lysine (instead of the wild-type glutamine residue; Q124K mutation) (numbering according to Kabat),

wherein each of the full length antibody heavy chains comprises in the first constant heavy chain domain (CHI) at position 147 an glutamic acid residue (instead of the wild-type lysine residue; K147E mutation) and at position 213 an glutamic acid residue (instead of the wild-type lysine amino acid residue; K213E mutation) (numbering according to Kabat EU index),

wherein the additional Fab fragment specifically binding to the second antigen comprises a domain crossover such that the constant light chain domain (CL) and the constant heavy chain domain 1 (CHI) are replaced by each other, and

wherein the first antigen is human CD20 and the second antigen is human transferrin receptor.

One aspect as reported herein is a bispecific antibody comprising

a) one full length antibody comprising two pairs each of a full length antibody light chain and a full length antibody heavy chain, wherein the binding sites formed by each of the pairs of the full length heavy chain and the full length light chain specifically bind to a first antigen, and

b) one additional Fab fragment, wherein the additional Fab fragment is fused to the C-terminus of one heavy chain of the full length antibody, wherein the binding site of the additional Fab fragment specifically binds to a second antigen,

wherein each of the full length antibody light chains comprises in the constant light chain domain (CL) at position 123 the amino acid residue arginine (instead of the wild-type glutamic acid residue; E123R mutation) and at position 124 the amino acid residue lysine (instead of the wild-type glutamine residue; Q124K mutation) (numbering according to Kabat),

wherein each of the full length antibody heavy chains comprises in the first constant heavy chain domain (CHI) at position 147 an glutamic acid residue (instead of the wild-type lysine residue; K147E mutation) and at position 213 an glutamic acid residue (instead of the wild-type lysine amino acid residue; K213E mutation) (numbering according to Kabat EU index), wherein the additional Fab fragment specifically binding to the second antigen comprises a domain crossover such that the constant light chain domain (CL) and the constant heavy chain domain 1 (CHI) are replaced by each other,

wherein the first antigen is human CD20 and the second antigen is human transferrin receptor,

wherein the human CD20 binding site comprises a heavy chain variable domain (VH) sequence having at least 90%, 91%, 92%, 93%, 94%, 95%, 96%o, 97%o, 98%o, 99%o, or 100% sequence identity to the amino acid sequence of SEQ ID NO: 18 and a light chain variable domain (VL) sequence having at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO: 19, and

wherein the human transferrin receptor binding site comprises a heavy chain variable domain (VH) sequence having at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO: 20 and a light chain variable domain (VL) sequence having at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%o, or 100%o sequence identity to the amino acid sequence of SEQ ID NO: 21.

In certain embodiments, a VH sequence having at least 90%>, 91%>, 92%, 93%>, 94%, 95%, 96%, 97%, 98%, or 99% identity contains substitutions (e.g., conservative substitutions), insertions, or deletions relative to the reference sequence, but a binding site comprising that sequence retains the ability to bind to its antigen. In certain embodiments, a total of 1 to 10 amino acids have been substituted, inserted and/or deleted in SEQ ID NO: 18 or 20. In certain embodiments, substitutions, insertions, or deletions occur in regions outside the HVRs (i.e., in the FRs).

In certain embodiments, a VL sequence having at least 90%>, 91%>, 92%, 93%>, 94%, 95%, 96%, 97%, 98%, or 99% identity contains substitutions (e.g., conservative substitutions), insertions, or deletions relative to the reference sequence, but a binding site comprising that sequence retains the ability to bind to its antigen. In certain embodiments, a total of 1 to 10 amino acids have been substituted, inserted and/or deleted in SEQ ID NO: 19 or 21. In certain embodiments, substitutions, insertions, or deletions occur in regions outside the HVRs (i.e., in the FRs).

In one embodiment, the human CD20 binding site comprises the VH sequence as in SEQ ID NO: 18, including post-translational modifications of that sequence and the VL sequence as in SEQ ID NO: 19, including post-translational modifications of that sequence.

In one embodiment, the human transferrin receptor binding site comprises the VH sequence as in SEQ ID NO: 20, including post-translational modifications of that sequence and the VL sequence as in SEQ ID NO: 21, including post-translational modifications of that sequence.

In one embodiment the bispecific antibody comprises

i) a light chain that has a sequence identity to SEQ ID NO: 01 of at least 70 %, at least 80 %, at least 90 %, or 95 % or more,

ii) a heavy chain that has a sequence identity to SEQ ID NO: 02 of at least 70 %, at least 80 %, at least 90 %, or 95 % or more,

iii) a light chain that has a sequence identity to SEQ ID NO: 03 of at least 70 %, at least 80 %, at least 90 %, or 95 % or more, and

iv) a heavy chain Fab fragment that has a sequence identity to SEQ ID NO: 04 of at least 70 %, at least 80 %, at least 90 %, or 95 % or more,

wherein

SEQ ID NO: 01 has the amino acid sequence
DIVMTQTPLSLPVTPGEPASISCRSSKSLLHSNGITYLYWYLQKPGQSP
QLLIYQMSNLVSGVPDRFSGSGSGTDFTLKISRVEAEDVGVYYCAQN
LELPYTFGGGTKVEIKRTVAAPSVFIFPPSDRKLKSGTASVVCLLNNF
YPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKAD
YEKHKVYACEVTHQGLSSPVTKSFNRGEC,

SEQ ID NO: 02 has the amino acid sequence
QVQLVQSGAEVKKPGSSVKVSCKASGYAFSYSWINWVRQAPGQGLE
WMGRIFPGDGDTDYNGKFKGRVTITADKSTSTAYMELSSLRSEDTAV
YYCARNVFDGYWLVYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSG

GTAALGCLVEDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSS
VVTVPSSSLGTQTYICNVNHKPSNTKVDEKVEPKSCDKTHTCPPCPAP
EAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYV
DGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVS
NKALGAPIEKTISKAKGQPREPQVCTLPPSRDELTKNQVSLSCAVKGF
YPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLVSKLTVDKSRWQQ
GNVFSCSVMHEALHNHYTQKSLSLSPG,

SEQ ID NO: 03 has the amino acid sequence AIQLTQSPSSLSASVGDRVTITCRASQSISSYLAWYQQKPGKAPKLLIY
RASTLASGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQNYASSNV
DNTFGGGTKVEIKSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYF
PEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTY
ICNVNHKPSNTKVDKKVEPKSC, and

SEQ ID NO: 04 has the amino acid sequence QSMQESGPGLVKPSQTLSLTCTVSGFSLSSYAMSWIRQHPGKGLEWI
GYIWSGGSTDYASWAKSRVTISKTSTTVSLKLSSVTAADTAVYYCAR
RYGTSYPDYGDASGFDPWGQGTLVTVSSASVAAPSVFIFPPSDEQLKS
GTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTY
SLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC.

One aspect as reported herein is a bispecific antibody comprising

a) one full length antibody comprising two pairs each of a full length antibody light chain and a full length antibody heavy chain, wherein the binding sites formed by each of the pairs of the full length heavy chain and the full length light chain specifically bind to a first antigen, and

b) one additional Fab fragment, wherein the additional Fab fragment is fused to the C-terminus of one heavy chain of the full length antibody, wherein the binding site of the additional Fab fragment specifically binds to a second antigen,

wherein each of the full length antibody light chains comprises in the constant light chain domain (CL) at position 123 the amino acid residue arginine (instead of the wild-type glutamic acid residue; E123R mutation) and at position 124 the amino acid residue lysine (instead of the wild-type glutamine residue; Q124K mutation) (numbering according to Kabat), wherein each of the full length antibody heavy chains comprises in the first constant heavy chain domain (CHI) at position 147 an glutamic acid residue (instead of the wild-type lysine residue; K147E mutation) and at position 213 an glutamic acid residue (instead of the wild-type lysine amino acid residue; K213E mutation) (numbering according to Kabat EU index),

wherein the additional Fab fragment specifically binding to the second antigen comprises a domain crossover such that the constant light chain domain (CL) and the constant heavy chain domain 1 (CHI) are replaced by each other,

wherein the first antigen is human CD20 and the second antigen is human transferrin receptor,

wherein the human CD20 binding site comprises a heavy chain variable domain (VH) that has the amino acid sequence of SEQ ID NO: 18 and a light chain variable domain (VL) that has the amino acid sequence of SEQ ID NO: 19, and

wherein the human transferrin receptor binding site comprises a heavy chain variable domain (VH) that has the amino acid sequence of SEQ ID NO: 20 and a light chain variable domain (VL) that has the amino acid sequence of SEQ ID NO: 21.

One aspect as reported herein is a bispecific antibody comprising

a) one full length antibody comprising two pairs each of a full length antibody light chain and a full length antibody heavy chain, wherein the binding sites formed by each of the pairs of the full length heavy chain and the full length light chain specifically bind to a first antigen, wherein the full length antibody comprises an Fc-region that is formed by the Fc-region polypeptides, each comprising the CHI, CH2 and CH3 domain, of the two full length heavy chains and

b) one additional Fab fragment, wherein the additional Fab fragment is fused to the C-terminus of one heavy chain of the full length antibody, wherein the binding site of the additional Fab fragment specifically binds to a second antigen, wherein each of the full length antibody light chains comprises in the constant light chain domain (CL) at position 123 the amino acid residue arginine (instead of the wild-type glutamic acid residue; E123R mutation) and at position 124 the amino acid residue lysine (instead of the wild-type glutamine residue; Q124K mutation) (numbering according to Kabat),

wherein each of the full length antibody heavy chains comprises in the first constant heavy chain domain (CHI) at position 147 an glutamic acid residue (instead of the wild-type lysine residue; K147E mutation) and at position 213 an glutamic acid residue (instead of the wild-type lysine amino acid residue; K213E mutation) (numbering according to Kabat EU index),

wherein the additional Fab fragment specifically binding to the second antigen comprises a domain crossover such that the constant light chain domain (CL) and the constant heavy chain domain 1 (CHI) are replaced by each other,

wherein the first antigen is human CD20 and the second antigen is human transferrin receptor,

wherein the human CD20 binding site comprises a heavy chain variable domain (VH) that has the amino acid sequence of SEQ ID NO: 18 and a light chain variable domain (VL) that has the amino acid sequence of SEQ ID NO: 19,

wherein the human transferrin receptor binding site comprises a heavy chain variable domain (VH) that has the amino acid sequence of SEQ ID NO: 20 and a light chain variable domain (VL) that has the amino acid sequence of SEQ ID NO: 21, and

wherein the Fc-region polypeptides are

a) of the human subclass IgGl ,

b) of the human subclass IgG4,

c) of the human subclass IgGl with the mutations L234A, L235A and P329G,

d) of the human subclass IgG4 with the mutations S228P, L235E and P329G,

e) of the human subclass IgGl with the mutations L234A, L235A and P329G in both Fc-region polypeptides and the mutations T366W and S354C in one Fc-region polypeptide and the mutations T366S, L368A, Y407V and Y349C in the respective other Fc-region polypeptide,

f) of the human subclass IgG4 with the mutations S228P and P329G in both Fc-region polypeptides and the mutations T366W and S354C in one Fc-region polypeptide and the mutations T366S, L368A, Y407V and Y349C in the respective other Fc-region polypeptide,

g) of the human subclass IgGl with the mutations L234A, L235A, P329G, 1253A, H310A and H435A in both Fc-region polypeptides and the mutations T366W and S354C in one Fc-region polypeptide and the mutations T366S, L368A, Y407V and Y349C in the respective other Fc-region polypeptide, or

h) of the human subclass IgGl with the mutations L234A, L235A, P329G, M252Y, S254T and T256E in both Fc-region polypeptides and the mutations T366W and S354C in one Fc-region polypeptide and the mutations T366S, L368A, Y407V and Y349C in the respective other Fc-region polypeptide, or

i) a full length antibody of the human subclass IgGl with the mutations L234A, L235A, P329G, H310A, H433A and Y436A in both heavy chains and the mutations i) T366W, and ii) S354C or Y349C, in one heavy chain and the mutations i) T366S, L368A, and Y407V, and ii) Y349C or S354C, in the respective other heavy chain.

In a further aspect, a bispecific anti-human CD20/human transferrin receptor antibody according to any of the above embodiments may incorporate any of the features, singly or in combination, as described in Sections 1-3 below:

1. Chimeric and Humanized Antibodies

In certain embodiments, an antibody provided herein is a chimeric antibody. Certain chimeric antibodies are described, e.g., in US 4,816,567; and Morrison, S.L. et al., Proc. Natl. Acad. Sci. USA 81 (1984) 6851-6855). In one example, a chimeric antibody comprises a non-human variable region (e.g., a variable region derived from a mouse, rat, hamster, rabbit, or non-human primate, such as a monkey) and a human constant region. In a further example, a chimeric antibody is a "class switched" antibody in which the class or subclass has been changed from that of the parent antibody. Chimeric antibodies include antigen-binding fragments thereof.

In certain embodiments, a chimeric antibody is a humanized antibody. Typically, a non-human antibody is humanized to reduce immunogenicity to humans, while retaining the specificity and affinity of the parental non-human antibody. Generally, a humanized antibody comprises one or more variable domains in which HVRs, e.g., CDRs, (or portions thereof) are derived from a non-human antibody, and FRs (or portions thereof) are derived from human antibody sequences. A humanized antibody optionally will also comprise at least a portion of a human constant region. In some embodiments, some FR residues in a humanized antibody are substituted with corresponding residues from a non-human antibody (e.g., the antibody from which the HVR residues are derived), e.g., to restore or improve antibody specificity or affinity.

Humanized antibodies and methods of making them are reviewed, e.g., in Almagro, J.C. and Fransson, J., Front. Biosci. 13 (2008) 1619-1633, and are further described, e.g., in Riechmann, I. et al, Nature 332 (1988) 323-329; Queen, C. et al, Proc. Natl. Acad. Sci. USA 86 (1989) 10029-10033; US 5, 821,337, US 7,527,791, US 6,982,321, and US 7,087,409; Kashmiri, S.V. et al, Methods 36 (2005) 25-34 (describing specificity determining region (SDR) grafting); Padlan, E.A., Mol. Immunol. 28 (1991) 489-498 (describing "resurfacing"); Dall'Acqua, W.F. et al, Methods 36 (2005) 43-60 (describing "FR shuffling"); and Osbourn, J. et al, Methods 36 (2005) 61-68 and Klimka, A. et al, Br. J. Cancer 83 (2000) 252-260 (describing the "guided selection" approach to FR shuffling).

Human framework regions that may be used for humanization include but are not limited to: framework regions selected using the "best-fit" method (see, e.g., Sims, M.J. et al., J. Immunol. 151 (1993) 2296-2308; framework regions derived from the consensus sequence of human antibodies of a particular subgroup of light or heavy chain variable regions (see, e.g., Carter, P. et al, Proc. Natl. Acad. Sci. USA 89 (1992) 4285-4289; and Presta, L.G. et al, J. Immunol. 151 (1993) 2623-2632); human mature (somatically mutated) framework regions or human germline framework regions (see, e.g., Almagro, J.C. and Fransson, J., Front. Biosci. 13

(2008) 1619-1633); and framework regions derived from screening FR libraries (see, e.g., Baca, M. et al, J. Biol. Chem. 272 (1997) 10678-10684 and Rosok, M.J. et al, J. Biol. Chem. 271 (19969 2261 1-22618).

2. Human Antibodies

In certain embodiments, an antibody provided herein is a human antibody. Human antibodies can be produced using various techniques known in the art. Human antibodies are described generally in van Dijk, M.A. and van de Winkel, J.G., Curr. Opin. Pharmacol. 5 (2001) 368-374 and Lonberg, N., Curr. Opin. Immunol. 20 (2008) 450-459.

Human antibodies may be prepared by administering an immunogen to a transgenic animal that has been modified to produce intact human antibodies or intact antibodies with human variable regions in response to antigenic challenge. Such animals typically contain all or a portion of the human immunoglobulin loci, which replace the endogenous immunoglobulin loci, or which are present extrachromosomally or integrated randomly into the animal's chromosomes. In such transgenic mice, the endogenous immunoglobulin loci have generally been inactivated. For review of methods for obtaining human antibodies from transgenic animals, see Lonberg, N., Nat. Biotech. 23 (2005) 1117-1 125. See also, e.g., US 6,075,181 and US 6,150,584 describing XENOMOUSE™ technology; US 5,770,429 describing HUMAB® technology; US 7,041,870 describing K-M MOUSE® technology, and US 2007/0061900, describing VELOCIMOUSE® technology). Human variable regions from intact antibodies generated by such animals may be further modified, e.g., by combining with a different human constant region.

Human antibodies can also be made by hybridoma-based methods. Human myeloma and mouse-human heteromyeloma cell lines for the production of human monoclonal antibodies have been described. (See, e.g., Kozbor, D., J. Immunol. 133 (1984) 3001-3005; Brodeur, B.R. et al, Monoclonal Antibody Production Techniques and Applications, Marcel Dekker, Inc., New York (1987), pp. 51-63; and Boerner, P. et al., J. Immunol. 147 (1991) 86-95) Human antibodies generated via human B-cell hybridoma technology arc also described in Li, J. et al., Proc. Natl. Acad. Sci. USA 103 (2006) 3557-3562. Additional methods include those described, for example, in US 7,189,826 (describing production of monoclonal human IgM antibodies from hybridoma cell lines) and Ni, J., Xiandai Mianyixue

26 (2006) 265-268 (describing human-human hybridomas). Human hybridoma technology (Trioma technology) is also described in Vollmers, H.P. and Brandlein, S., Histology and Histopathology 20 (2005) 927-937 and Vollmers, H.P. and Brandlein, S., Methods and Findings in Experimental and Clinical Pharmacology 27 (2005) 185-191.

Human antibodies may also be generated by isolating Fv clone variable domain sequences selected from human-derived phage display libraries. Such variable domain sequences may then be combined with a desired human constant domain.

3. Antibody Variants

In certain embodiments, amino acid sequence variants of the bispecific antibodies as provided herein are contemplated. For example, it may be desirable to improve the binding affinity and/or other biological properties of the antibody. Amino acid sequence variants of an antibody may be prepared by introducing appropriate modifications into the nucleotide sequence encoding the antibody, or by peptide synthesis. Such modifications include, for example, deletions from, and/or insertions into and/or substitutions of residues within the amino acid sequences of the antibody. Any combination of deletion, insertion, and substitution can be made to arrive at the final construct, provided that the final construct possesses the desired characteristics, e.g., antigen-binding.

a) Substitution, Insertion, and Deletion Variants

In certain embodiments, antibody variants having one or more amino acid substitutions are provided. Sites of interest for substitutional mutagenesis include the HVRs and FRs. Conservative substitutions are shown in the Table below under the heading of "preferred substitutions". More substantial changes are provided in Table 1 under the heading of "exemplary substitutions," and as further described below in reference to amino acid side chain classes. Amino acid substitutions may be introduced into an antibody of interest and the products screened for a desired activity, e.g., retained/improved antigen binding, decreased immunogenicity, or improved ADCC or CDC.

TABLE

Original Residue	**Exemplary Substitutions**	**Preferred Substitutions**
Ala (A)	Val; Leu; Ile	Val
Arg (R)	Lys; Gln; Asn	Lys
Asn (N)	Gln; His; Asp, Lys; Arg	Gln
Asp (D)	Glu; Asn	Glu
Cys (C)	Ser; Ala	Ser
Gln (Q)	Asn; Glu	Asn
Glu (E)	Asp; Gln	Asp
Gly (G)	Ala	Ala
His (H)	Asn; Gln; Lys; Arg	Arg
Ile (I)	Leu; Val; Met; Ala; Phe; Norleucine	Leu
Leu (L)	Norleucine; Ile; Val; Met; Ala; Phe	Ile
Lys (K)	Arg; Gln; Asn	Arg
Met (M)	Leu; Phe; Ile	Leu
Phe (F)	Trp; Leu; Val; Ile; Ala; Tyr	Tyr
Pro (P)	Ala	Ala
Ser (S)	Thr	Thr
Thr (T)	Val; Ser	Ser
Trp (W)	Tyr; Phe	Tyr
Tyr (Y)	Trp; Phe; Thr; Ser	Phe
Val (V)	Ile; Leu; Met; Phe; Ala; Norleucine	Leu

Amino acids may be grouped according to common side-chain properties:

(1) hydrophobic: Norleucine, Met, Ala, Val, Leu, He;

(2) neutral hydrophilic: Cys, Ser, Thr, Asn, Gin;

(3) acidic: Asp, Glu;

(4) basic: His, Lys, Arg;

(5) residues that influence chain orientation: Gly, Pro;

(6) aromatic: Trp, Tyr, Phe.

Non-conservative substitutions will entail exchanging a member of one of these classes for another class.

One type of substitutional variant involves substituting one or more hypervariable region residues of a parent antibody *(e.g.* a humanized or human antibody). Generally, the resulting variant(s) selected for further study will have modifications (e.g., improvements) in certain biological properties (e.g., increased affinity, reduced immunogenicity) relative to the parent antibody and/or will have substantially retained certain biological properties of the parent antibody. An exemplary substitutional variant is an affinity matured antibody, which may be conveniently generated, e.g., using phage display-based affinity maturation techniques such as those described herein. Briefly, one or more HVR residues are mutated and the variant antibodies displayed on phage and screened for a particular biological activity (e.g. binding affinity).

Alterations (e.g., substitutions) may be made in HVRs, e.g., to improve antibody affinity. Such alterations may be made in HVR "hotspots," i.e., residues encoded by codons that undergo mutation at high frequency during the somatic maturation process (see, e.g., Chowdhury, P.S., Methods Mol. Biol. 207 (2008) 179-196), and/or residues that contact antigen, with the resulting variant VH or VL being tested for binding affinity. Affinity maturation by constructing and reselecting from secondary libraries has been described, e.g., in Hoogenboom, H.R. et al. in Methods in Molecular Biology 178 (2002) 1-37. In some embodiments of affinity maturation, diversity is introduced into the variable genes chosen for maturation by any of a variety of methods (e.g., error-prone PCR, chain shuffling, or oligonucleotide-directed mutagenesis). A secondary library is then created. The library is then screened to identify any antibody variants with the desired affinity. Another method to introduce diversity involves HVR-directed approaches, in which several HVR residues (e.g., 4-6 residues at a time) are randomized. HVR residues involved in antigen binding may be specifically identified, e.g., using alanine scanning mutagenesis or modeling. CDR-H3 and CDR-L3 in particular are often targeted.

In certain embodiments, substitutions, insertions, or deletions may occur within one or more HVRs so long as such alterations do not substantially reduce the ability of the antibody to bind antigen. For example, conservative alterations (e.g., conservative substitutions as provided herein) that do not substantially reduce binding affinity may be made in HVRs. Such alterations may, for example, be outside of antigen contacting residues in the HVRs. In certain embodiments of the variant VH and VL sequences provided above, each HVR either is unaltered, or contains no more than one, two or three amino acid substitutions.

A useful method for identification of residues or regions of an antibody that may be targeted for mutagenesis is called "alanine scanning mutagenesis" as described by Cunningham, B.C. and Wells, J.A., Science 244 (1989) 1081-1085. In this method, a residue or group of target residues (e.g., charged residues such as arg, asp, his, lys, and glu) are identified and replaced by a neutral or negatively charged amino acid (e.g., alanine or polyalanine) to determine whether the interaction of the antibody with antigen is affected. Further substitutions may be introduced at the amino acid locations demonstrating functional sensitivity to the initial substitutions. Alternatively, or additionally, a crystal structure of an antigen-antibody complex to identify contact points between the antibody and antigen. Such contact residues and neighboring residues may be targeted or eliminated as candidates for substitution. Variants may be screened to determine whether they contain the desired properties.

Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Examples of terminal insertions include an antibody with an N-terminal methionyl residue. Other insertional variants of the antibody molecule include the fusion to the N- or C-terminus of the antibody to an enzyme (e.g. for ADEPT) or a polypeptide which increases the serum half-life of the antibody.

b) Glycosylation variants

In certain embodiments, a bispecific antibody as provided herein is altered to increase or decrease the extent to which the antibody is glycosylated. Addition or deletion of glycosylation sites to an antibody may be conveniently accomplished by altering the amino acid sequence such that one or more glycosylation sites is created or removed.

Where the antibody comprises an Fc-region, the carbohydrate attached thereto may be altered. Native antibodies produced by mammalian cells typically comprise a branched, biantennary oligosaccharide that is generally attached by an N-linkage to Asn297 of the CH2 domain of the Fc-region. See, e.g., Wright, A. and Morrison, S.L., TIBTECH 15 (1997) 26-32. The oligosaccharide may include various carbohydrates, e.g., mannose, N-acetyl glucosamine (GlcNAc), galactose, and sialic acid, as well as a fucose attached to a GlcNAc in the "stem" of the biantennary oligosaccharide structure. In some embodiments, modifications of the oligosaccharide in an antibody as reported herein may be made in order to create antibody variants with certain improved properties.

In one embodiment, antibody variants are provided having a carbohydrate structure that lacks fucose attached (directly or indirectly) to an Fc-region. For example, the amount of fucose in such antibody may be from 1% to 80%, from 1% to 65%, from 5%> to 65%o or from 20%> to 40%>. The amount of fucose is determined by calculating the average amount of fucose within the sugar chain at Asn297, relative to the sum of all glycostructures attached to Asn 297 (e. g. complex, hybrid and high mannose structures) as measured by MALDI-TOF mass spectrometry, as described in WO 2008/077546, for example. Asn297 refers to the asparagine residue located at about position 297 in the Fc-region (EU numbering of Fc-region residues); however, Asn297 may also be located about ± 3 amino acids upstream or downstream of position 297, i.e., between positions 294 and 300, due to minor sequence variations in antibodies. Such fucosylation variants may have improved ADCC function. See, e.g., US 2003/0157108; US 2004/0093621. Examples of publications related to "defucosylated" or "fucose-deficient" antibody variants include: US 2003/0157108; WO 2000/61739; WO 2001/29246; US 2003/01 15614; US 2002/0164328; US 2004/0093621; US 2004/0132140; US 2004/01 10704; US 2004/01 10282; US 2004/0109865; WO 2003/0851 19; WO 2003/084570; WO 2005/035586; WO 2005/035778; WO 2005/053742; WO 2002/031 140; Okazaki, A. et al, J. Mol. Biol. 336 (2004) 1239-1249; Yamane-Ohnuki, N. et al, Biotech. Bioeng. 87 (2004) 614-622. Examples of cell lines capable of producing defucosylated antibodies include Led 3 CHO cells deficient in protein fucosylation (Ripka, J. et al, Arch. Biochem. Biophys. 249 (1986) 533-545; US 2003/0157108; and WO 2004/056312, especially at Example 11), and knockout cell lines, such as alpha- 1,6-fucosyltransferase gene, *FUT8,* knockout CHO cells (see, e.g., Yamane-Ohnuki, N. et al, Biotech. Bioeng. 87 (2004) 614-622; Kanda, Y. et al, Biotechnol. Bioeng. 94 (2006) 680-688; and WO 2003/085107).

Antibodies variants are further provided with bisected oligosaccharides, e.g., in which a biantennary oligosaccharide attached to the Fc-region of the antibody is bisected by GlcNAc. Such antibody variants may have reduced fucosylation and/or improved ADCC function. Examples of such antibody variants are described, e.g., in WO 2003/01 1878; US 6,602,684; and US 2005/0123546. Antibody variants with at least one galactose residue in the oligosaccharide attached to the Fc-region are also provided. Such antibody variants may have improved CDC function. Such antibody variants are described, e.g., in WO 1997/30087; WO 1998/58964; and WO 1999/22764.

c) Fc-region variants

In certain embodiments, one or more amino acid modifications may be introduced into the Fc-region of a bispecific antibody as provided herein, thereby generating an Fc-region variant. The Fc-region variant may comprise a human Fc-region sequence *(e.g.*, a human IgGl, IgG2, IgG3 or IgG4 Fc-region) comprising an amino acid modification *(e.g.* a substitution) at one or more amino acid positions.

In certain embodiments, herein is contemplated an antibody variant that possesses some but not all effector functions, which make it a desirable candidate for applications in which the half-life of the antibody *in vivo* is important yet certain effector functions (such as complement and ADCC) are unnecessary or deleterious. *In vitro* and/or *in vivo* cytotoxicity assays can be conducted to confirm the reduction/depletion of CDC and/or ADCC activities. For example, Fc receptor (FcR) binding assays can be conducted to ensure that the antibody lacks FcyR binding (hence likely lacking ADCC activity), but retains FcRn binding ability. The primary cells for mediating ADCC, NK cells, express Fc(RIII only, whereas monocytes express FcyRI, FcyRII and FcyRIII. FcR expression on hematopoietic cells is summarized in Table 3 on page 464 of Ravetch, J.V. and Kinet, J.P., Annu. Rev. Immunol. 9 (1991) 457-492. Non-limiting examples of *in vitro* assays to assess ADCC activity of a molecule of interest is described in US 5,500,362 (see, e.g. Hellstrom, I. et al, Proc. Natl. Acad. Sci. USA 83 (1986) 7059-7063; and Hellstrom, I. et al, Proc. Natl. Acad. Sci. USA 82 (1985) 1499-1502); US 5,821,337 (see Bruggemann, M. et al, J. Exp. Med. 166 (1987) 1351-1361). Alternatively, non-radioactive assays methods may be employed (see, for example, ACTI™ non-radioactive cytotoxicity assay for flow cytometry (CellTechnology, Inc. Mountain View, CA; and CytoTox 96® non-radioactive cytotoxicity assay (Promega, Madison, WI). Useful effector cells for such assays include peripheral blood mononuclear cells (PBMC) and Natural Killer (NK) cells. Alternatively, or additionally, ADCC activity of the molecule of interest may be assessed *in vivo*, *e.g.*, in an animal model such as that disclosed in Clynes, R. et al, Proc. Natl. Acad. Sci. USA 95 (1998) 652-656. Clq binding assays may also be carried out to confirm that the antibody is unable to bind Clq and hence lacks CDC activity. See, e.g., Clq and C3c binding ELISA in WO 2006/029879 and WO 2005/100402. To assess complement activation, a CDC assay may be performed (see, for example, Gazzano-Santoro, H. et al, J. Immunol. Methods 202 (1996) 163-171; Cragg, M.S. et al, Blood 101 (2003) 1045-1052; and Cragg, M.S. and M.J. Glennie, Blood 103 (2004) 2738-2743). FcRn binding and *in vivo* clearance/half-life determinations can also be performed using methods known in the art (see, e.g., Petkova, S.B. et al, Int. Immunol. 18 (2006: 1759-1769).

Antibodies with reduced effector function include those with substitution of one or more of Fc-region residues 238, 265, 269, 270, 297, 327 and 329 (US 6,737,056). Such Fc mutants include Fc mutants with substitutions at two or more of amino acid positions 265, 269, 270, 297 and 327, including the so-called "DANA" Fc mutant with substitution of residues 265 and 297 to alanine (US 7,332,581).

Certain antibody variants with improved or diminished binding to FcRs are described. (See, e.g., US 6,737,056; WO 2004/056312, and Shields, R.L. et al, J. Biol. Chem. 276 (2001) 6591-6604)

In certain embodiments, an antibody variant comprises an Fc-region with one or more amino acid substitutions, which improve ADCC, e.g., substitutions at positions 298, 333, and/or 334 of the Fc-region (EU numbering of residues).

In some embodiments, alterations are made in the Fc-region that result in altered *(i.e.,* either improved or diminished) Clq binding and/or Complement Dependent Cytotoxicity (CDC), e.g., as described in US 6,194,551, WO 99/51642, and Idusogie, E.E. et al, J. Immunol. 164 (2000) 4178-4184.

Antibodies with increased half-lives and improved binding to the neonatal Fc receptor (FcRn), which is responsible for the transfer of maternal IgGs to the fetus (Guyer, R.L. et al, J. Immunol. 117 (1976) 587-593, and Kim, J.K. et al, J. Immunol. 24 (1994) 2429-2434), are described in US 2005/0014934. Those antibodies comprise an Fc-region with one or more substitutions therein which improve binding of the Fc-region to FcRn. Such Fc variants include those with substitutions at one or more of Fc-region residues: 238, 256, 265, 272, 286, 303, 305, 307, 311, 312, 317, 340, 356, 360, 362, 376, 378, 380, 382, 413, 424 or 434, e.g., substitution of Fc-region residue 434 (US 7,371,826).

See also Duncan, A.R. and Winter, G., Nature 322 (1988) 738-740; US 5,648,260; US 5,624,821; and WO 94/29351 concerning other examples of Fc-region variants.

d) Cysteine engineered antibody variants

In certain embodiments, it may be desirable to create cysteine engineered antibodies, e.g., "thioMAbs," in which one or more residues of an antibody are substituted with cysteine residues. In particular embodiments, the substituted residues occur at accessible sites of the antibody. By substituting those residues with cysteine, reactive thiol groups are thereby positioned at accessible sites of the antibody and may be used to conjugate the antibody to other moieties, such as drug moieties or linker-drug moieties, to create an immunoconjugate, as described further herein. In certain embodiments, any one or more of the following residues may be substituted with cysteine: V205 (Kabat numbering) of the light chain; A118 (EU numbering) of the heavy chain; and S400 (EU numbering) of the heavy chain Fc-region. Cysteine engineered antibodies may be generated as described, e.g., in US 7,521,541.

e) Antibody Derivatives

In certain embodiments, a bispecific antibody as provided herein may be further modified to contain additional non-proteinaceous moieties that are known in the art and readily available. The moieties suitable for derivatization of the antibody include but are not limited to water soluble polymers. Non-limiting examples of water soluble polymers include, but are not limited to, polyethylene glycol (PEG), copolymers of ethylene glycol/propylene glycol, carboxymethylcellulose, dextran, polyvinyl alcohol, polyvinyl pyrrolidone, poly-1, 3-dioxolane, poly-l,3,6-trioxane, ethylene/maleic anhydride copolymer, polyaminoacids (either homopolymers or random copolymers), and dextran or poly(n-vinyl pyrrolidone)polyethylene glycol, propropylene glycol homopolymers, prolypropylene oxide/ethylene oxide co-polymers, polyoxyethylated polyols (e.g., glycerol), polyvinyl alcohol, and mixtures thereof. Polyethylene glycol propionaldehyde may have advantages in manufacturing due to its stability in water. The polymer may be of any molecular weight, and may be branched or unbranched. The number of polymers attached to the antibody may vary, and if more than one polymer is attached, they can be the same or different molecules. In general, the number and/or type of polymers used for derivatization can be determined based on considerations including, but not limited to, the particular properties or functions of the antibody to be improved, whether the antibody derivative will be used in a therapy under defined conditions, etc.

In another embodiment, conjugates of an antibody and non-proteinaceous moiety that may be selectively heated by exposure to radiation are provided. In one embodiment, the non-proteinaceous moiety is a carbon nanotube (Kam, N.W. et al., Proc. Natl. Acad. Sci. USA 102 (2005) 11600-1 1605). The radiation may be of any wavelength, and includes, but is not limited to, wavelengths that do not harm ordinary cells, but which heat the non-proteinaceous moiety to a temperature at which cells proximal to the antibody-non-proteinaceous moiety are killed.

B. Recombinant Methods and Compositions

Antibodies may be produced using recombinant methods and compositions, e.g., as described in US 4,816,567. In one embodiment, isolated nucleic acid encoding a bispecific anti-human CD20/human transferrin receptor antibody described herein is provided. In a further embodiment, one or more vectors (e.g., expression vectors) comprising such nucleic acid are provided. In a further embodiment, a host cell comprising such nucleic acid is provided. In one embodiment, the host cell is eukaryotic, e.g. a Chinese Hamster Ovary (CHO) cell or lymphoid cell (e.g., Y0, NS0, Sp20 cell). In one embodiment, a method of making a bispecific anti-human CD20/human transferrin receptor antibody is provided, wherein the method comprises culturing a host cell comprising a nucleic acid encoding the antibody, as provided above, under conditions suitable for expression of the antibody, and optionally recovering the antibody from the host cell (or host cell culture medium).

For recombinant production of a bispecific anti-human CD20/human transferrin receptor antibody, nucleic acid encoding an antibody, e.g., as described above, is isolated and inserted into one or more vectors for further cloning and/or expression in a host cell. Such nucleic acid may be readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the antibody).

Suitable host cells for cloning or expression of antibody-encoding vectors include prokaryotic or eukaryotic cells described herein. For example, antibodies may be produced in bacteria, in particular when glycosylation and Fc effector function are not needed. For expression of antibody fragments and polypeptides in bacteria, see, e.g., US 5,648,237, US 5,789,199, and US 5,840,523. (See also Charlton, K.A., In: Methods in Molecular Biology, Vol. 248, Lo, B.K.C. (ed.), Humana Press, Totowa, NJ (2003), pp. 245-254, describing expression of antibody fragments in *E. coli.)*

After expression, the antibody may be isolated from the bacterial cell paste in a soluble fraction and can be further purified.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for antibody-encoding vectors, including fungi and yeast strains whose glycosylation pathways have been "humanized," resulting in the production of an antibody with a partially or fully human glycosylation pattern. See Gerngross, T.U., Nat. Biotech. 22 (2004) 1409-1414; and Li, H. et al, Nat. Biotech. 24 (2006) 210-215.

Suitable host cells for the expression of glycosylated antibody are also derived from multicellular organisms (invertebrates and vertebrates). Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains have been identified which may be used in conjunction with insect cells, particularly for transfection of *Spodoptera frugiperda* cells.

Plant cell cultures can also be utilized as hosts. See, e.g., US 5,959,177, US 6,040,498, US 6,420,548, US 7,125,978, and US 6,417,429 (describing PLANTIBODIES™ technology for producing antibodies in transgenic plants).

Vertebrate cells may also be used as hosts. For example, mammalian cell lines that are adapted to grow in suspension may be useful. Other examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7); human embryonic kidney line (293 or 293 cells as described, e.g., in Graham, F.L. et al, J. Gen Virol. 36 (1977) 59-74); baby hamster kidney cells (BHK); mouse Sertoli cells (TM4 cells as described, e.g., in Mather, J.P., Biol. Reprod. 23 (1980) 243-252); monkey kidney cells (CV1); African green monkey kidney cells (VERO-76); human cervical carcinoma cells (HELA); canine kidney cells (MDCK; buffalo rat liver cells (BRL 3A); human lung cells (W138); human liver cells (Hep G2); mouse mammary tumor (MMT 060562); TRI cells, as described, e.g., in Mather, J.P. et al, Annals N.Y. Acad. Sci. 383 (1982) 44-68; MRC 5 cells; and FS4 cells. Other useful mammalian host cell lines include Chinese hamster ovary (CHO) cells, including $DHFR^-$ CHO cells (Urlaub, G. et al, Proc. Natl. Acad. Sci. USA 77 (1980) 4216-4220); and myeloma cell lines such as Y0, NSO and Sp2/0. For a review of certain mammalian host cell lines suitable for antibody production, see, e.g., Yazaki, P. and Wu, A.M., Methods in Molecular Biology, Vol. 248, Lo, B.K.C. (ed.), Humana Press, Totowa, NJ (2004), pp. 255-268.

C. Methods and Compositions for Diagnostics and Detection

In certain embodiments, any of the bispecific anti-human CD20/human transferrin receptor antibodies provided herein are useful for detecting the presence of CD20 in a biological sample. The term "detecting" as used herein encompasses quantitative or qualitative detection. In certain embodiments, a biological sample comprises a cell or tissue.

In one embodiment, a bispecific anti-human CD20/human transferrin receptor antibody for use in a method of diagnosis or detection is provided. In a further aspect, a method of detecting the presence of CD20 in a biological sample is provided. In certain embodiments, the method comprises contacting the biological sample with a bispecific anti-human CD20/human transferrin receptor antibody as described herein under conditions permissive for binding of the bispecific anti-human CD20/human transferrin receptor antibody to CD20, and detecting whether a complex is formed between the bispecific anti-human CD20/human transferrin receptor antibody and CD20. Such method may be an *in vitro* or *in vivo* method.

In certain embodiments, labeled bispecific anti-human CD20/human transferrin receptor antibodies are provided. Labels include, but are not limited to, labels or moieties that are detected directly (such as fluorescent, chromophoric, electron-dense, chemiluminescent, and radioactive labels), as well as moieties, such as enzymes or ligands, that are detected indirectly, e.g., through an enzymatic reaction or molecular interaction. Exemplary labels include, but are not limited to, the radioisotopes ^{32}P, ^{14}C, ^{125}I, ^{3}H, and ^{131}I, fluorophores such as rare earth chelates or fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, luceriferases, e.g., firefly luciferase and bacterial luciferase (US 4,737,456), luciferin, 2,3-dihydrophthalazinediones, horseradish peroxidase (HRP), alkaline phosphatase, β-galactosidase, glucoamylase, lysozyme, saccharide oxidases, e.g., glucose oxidase, galactose oxidase, and glucose-6-phosphate dehydrogenase, heterocyclic oxidases such as uricase and xanthine oxidase, coupled with an enzyme that employs hydrogen peroxide to oxidize a dye precursor such as HRP, lactoperoxidase, or microperoxidase, biotin/avidin, spin labels, bacteriophage labels, stable free radicals, and the like.

D. Immunoconjugates

The invention also provides immunoconjugates comprising an anti-human CD20/human transferrin receptor antibody as reported herein conjugated to one or more cytotoxic agents, such as chemotherapeutic agents or drugs, growth inhibitory agents, toxins (e.g., protein toxins, enzymatically active toxins of bacterial, fungal, plant, or animal origin, or fragments thereof), or radioactive isotopes.

In one embodiment, an immunoconjugate is an antibody-drug conjugate (ADC) in which a bispecific antibody is conjugated to one or more drugs, including but not limited to a maytansinoid (see US 5,208,020, US 5,416,064 and EP 0 425 235 Bl); an auristatin such as monomethyl auristatin drug moieties DE and DF (MMAE and MMAF) (see US 5,635,483, US 5,780,588, and US 7,498,298); a dolastatin; a calicheamicin or derivative thereof (see US 5,712,374, US 5,714,586, US 5,739,1 16, US 5,767,285, US 5,770,701, US 5,770,710, US 5,773,001, and US 5,877,296; Hinman, L.M. et al, Cancer Res. 53 (1993) 3336-3342; and Lode, H.N. et al, Cancer Res. 58 (1998) 2925-2928); an anthracycline such as daunomycin or doxorubicin (see Kratz, F. et al., Curr. Med. Chem. 13 (2006) 477-523; Jeffrey, S.C. et al, Bioorg. Med. Chem. Lett. 16 (2006) 358-362; Torgov, M.Y. et al, Bioconjug. Chem. 16 (2005) 717-721; Nagy, A. et al, Proc. Natl. Acad. Sci. USA 97 (2000) 829-834; Dubowchik, G.M. et al, Bioorg. & Med. Chem. Letters 12 (2002) 1529-1532; King, H.D. et al, J. Med. Chem. 45 (20029 4336-4343; and US 6,630,579); methotrexate; vindesine; a taxane such as docetaxel, paclitaxel, larotaxel, tesetaxel, and ortataxel; a trichothecene; and CC1065.

In another embodiment, an immunoconjugate comprises a bispecific antibody as described herein conjugated to an enzymatically active toxin or fragment thereof, including but not limited to 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. See, for example, WO 93/21232 published Oct. 28, 1993.

In another embodiment, an immunoconjugate comprises a bispecific antibody as described herein conjugated to a radioactive atom to form a radioconjugate. A variety of radioactive isotopes are available for the production of radioconjugates. Examples include At^{211}, I^{131}, I^{125}, Y^{90}, Re^{186}, Re^{188}, Sm^{153}, Bi^{212}, P^{32}, Pb^{212} and radioactive isotopes of Lu. When the radioconjugate is used for detection, it may comprise a radioactive atom for scintigraphic studies, for example TC^{99m} or I^{123}, or a spin label for nuclear magnetic resonance (NMR) imaging (also known as magnetic resonance imaging, MRI), such as iodine-123 again, iodine-131, indium-I l l, fluorine-19, carbon-13, nitrogen-15, oxygen-17, gadolinium, manganese or iron.

Conjugates of a bispecific antibody and cytotoxic agent may be made using a variety of bifunctional coupling agents such as N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP), succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HC1), active esters (such as disuccinimidyl suberate), aldehydes (such as glutaraldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as toluene 2,6-diisocyanate), and bis-active fluorine compounds (such as l,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta, E.S. et al, Science 238 (1987) 1098-1 104. Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triamine pentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody. See WO 94/1 1026. The linker may be a "cleavable linker" facilitating release of a cytotoxic drug in the cell. For example, an acid-labile linker, peptidase-sensitive linker, photolabile linker, dimethyl linker or disulfide-containing linker (Chari, R.V. et al, Cancer Res. 52 (1992) 127-131; US 5,208,020) may be used.

The immunoconjugates or ADCs herein expressly contemplate, but are not limited to such conjugates prepared with cross-linker reagents including, but not limited to, BMPS, EMCS, GMBS, HBVS, LC-SMCC, MBS, MPBH, SBAP, SIA, SIAB, SMCC, SMPB, SMPH, sulfo-EMCS, sulfo-GMBS, sulfo-KMUS, sulfo-MBS, sulfo-SIAB, sulfo-SMCC, and sulfo-SMPB, and SVSB (succinimidyl-(4-vinylsulfone)benzoate) which are commercially available (e.g., from Pierce Biotechnology, Inc., Rockford, IL., U.S.A).

Conjugates of a bispecific antibody as reported herein and one or more small molecule toxins, such as a calicheamicin, a maytansine (US 5,208,020), a trichothene, and CC1065 are also contemplated herein. In one embodiment of the invention, the bispecific antibody is conjugated to one or more maytansine molecules (e.g. about 1 to about 10 maytansine molecules per antagonist molecule). Maytansine may, for example, be converted to May-SS-Me which may be reduced to May-SH3 and reacted with modified antagonist (Chari et al. Cancer Research 52: 127-131 (1992)) to generate a maytansinoid-antibody conjugate.

Alternatively, the bispecific antibody as reported herein is conjugated to one or more calicheamicin molecules. The calicheamicin family of antibiotics are capable of producing double-stranded DNA breaks at sub-picomolar concentrations. Structural analogues of calicheamicin which may be used include, but are not limited to, γï I, a2 I, a3 I, N-acetyl-γI I, PSAG and θI 1 (Hinman et al. Cancer Research 53: 3336-3342 (1993) and Lode et al. Cancer Research 58: 2925-2928 (1998)).

The present invention further contemplates a bispecific antibody as reported herein conjugated with a compound with nucleolytic activity (e.g. a ribonuclease or a DNA endonuclease such as a deoxyribonuclease; DNase).

Alternatively, a fusion protein comprising the bispecific antibody as reported herein and cytotoxic agent may be made, e.g. by recombinant techniques or peptide synthesis.

E. **Pharmaceutical Formulations**

Pharmaceutical formulations of a bispecific anti-human CD20/human transferrin receptor antibody as described herein are prepared by mixing such antibody having the desired degree of purity with one or more optional pharmaceutically acceptable carriers (Remington's Pharmaceutical Sciences, 16th edition, Osol, A. (ed.) (1980)), in the form of lyophilized formulations or aqueous solutions. Pharmaceutically acceptable carriers are generally nontoxic to recipients at the dosages and concentrations employed, and include, but are not limited to: buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyl dimethylbenzyl 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 dextrins; 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 polyethylene glycol (PEG). Exemplary pharmaceutically acceptable carriers herein further include interstitial drug dispersion agents such as soluble neutral-active hyaluronidase glycoproteins (sHASEGP), for example, human soluble PH-20 hyaluronidase glycoproteins, such as rhuPH20 (HYLENEX®, Baxter International, Inc.). Certain exemplary sHASEGPs and methods of use, including rhuPH20, are described in US 2005/0260186 and US 2006/0104968. In one aspect, a sHASEGP is combined with one or more additional glycosaminoglycanases such as chondroitinases.

Exemplary lyophilized antibody formulations are described in US 6,267,958. Aqueous antibody formulations include those described in US 6,171,586 and WO 2006/044908, the latter formulations including a histidine-acetate buffer. Exemplary anti-CD20 antibody formulations are described in W098/56418, expressly incorporated herein by reference.

Lyophilized formulations adapted for subcutaneous administration are described in WO 97/04801. Such lyophilized formulations may be reconstituted with a suitable diluent to a high protein concentration and the reconstituted formulation may be administered subcutaneously to the mammal to be treated herein.

The formulation herein may also contain more than one active ingredients as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. Such active ingredients are suitably present in combination in amounts that are effective for the purpose intended.

Active ingredients may be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methyl methacrylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences, 16th edition, Osol, A. (ed.) (1980).

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semi-permeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, *e.g.* films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinyl alcohol)), polylactides (US 3,773,919), copolymers of L-glutamic acid and γ ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid.

The formulations to be used for *in vivo* administration are generally sterile. Sterility may be readily accomplished, e.g., by filtration through sterile filtration membranes. In one embodiment the formulation is isotonic.

F. Therapeutic Methods and Compositions

Any of the bispecific anti-human CD20/human transferrin receptor antibodies provided herein may be used in therapeutic methods.

In one aspect, a bispecific anti-human CD20/human transferrin receptor antibody for use as a medicament is provided. In further aspects, a bispecific anti-human CD20/human transferrin receptor antibody for use in preventing and/or treating a B-cell proliferative disease is provided. In certain embodiments, a bispecific anti-human CD20/human transferrin receptor antibody for use in a method of treatment is provided. In certain embodiments, herein is provided a bispecific anti-human CD20/human transferrin receptor antibody for use in a method of treating an individual having a B-cell proliferative disease comprising administering to the individual an effective amount of the bispecific anti-human CD20/human transferrin receptor antibody. In one such embodiment, the method further comprises administering to the individual an effective amount of at least one additional therapeutic agent, such as listed below. In further embodiments, herein is provided a bispecific anti-human CD20/human transferrin receptor antibody for use in depleting brain sequestered B-cells expressing CD20. In certain embodiments, herein is provided a bispecific anti-human CD20/human transferrin receptor antibody for use in a method of depleting brain sequestered B-cells expressing CD20 in an individual comprising administering to the individual an effective of the bispecific anti-human CD20/human transferrin receptor antibody to deplete brain sequestered B-cells expressing CD20. An "individual" according to any of the above embodiments is preferably a human.

In a further aspect, herein is provided the use of a bispecific anti-human CD20/human transferrin receptor antibody in the manufacture or preparation of a medicament. In one embodiment, the medicament is for treatment of a B-cell proliferative disease. In a further embodiment, the medicament is for use in a method of treating a B-cell proliferative disease comprising administering to an individual having a disease associated with B-cell proliferative an effective amount of the medicament. In one such embodiment, the method further comprises administering to the individual an effective amount of at least one additional therapeutic agent, such as listed below. In a further embodiment, the medicament is for the depletion of brain sequestered B-cells expressing CD20. In a further embodiment, the medicament is for use in a method of depleting brain sequestered B-cells expressing CD20 in an individual comprising administering to the individual an amount effective of the medicament to deplete brain sequestered B-cells expressing CD20. An "individual" according to any of the above embodiments may be a human.

In a further aspect, herein is provided a method for treating a B-cell proliferative disease. In one embodiment, the method comprises administering to an individual having a B-cell proliferative disease an effective amount of a bispecific anti-human CD20/human transferrin receptor antibody. In one such embodiment, the method further comprises administering to the individual an effective amount of at least one additional therapeutic agent, such as given below. An "individual" according to any of the above embodiments may be a human.

In a further aspect, herein is provided a method for depleting circulating B-cells expressing CD20 in an individual. In one embodiment, the method comprises administering to the individual an effective amount of a bispecific anti-human CD20/human transferrin receptor antibody to deplete circulating B-cells expressing CD20. In one embodiment, an "individual" is a human.

In a further aspect, herein are provided pharmaceutical formulations comprising any of the bispecific anti-human CD20/human transferrin receptor antibodies provided herein, e.g., for use in any of the above therapeutic methods. In one embodiment, a pharmaceutical formulation comprises any of the bispecific anti-human CD20/human transferrin receptor antibodies provided herein and a pharmaceutically acceptable carrier. In another embodiment, a pharmaceutical formulation comprises any of the bispecific anti-human CD20/human transferrin receptor antibodies provided herein and at least one additional therapeutic agent, e.g., as given below.

Antibodies as reported herein can be used either alone or in combination with other agents in a therapy. For instance, an antibody as reported herein may be co-administered with at least one additional therapeutic agent. In certain embodiments, an additional therapeutic agent is a therapeutic agent effective to treat the same or a different B-cell proliferative disease as the bispecific antibody as reported herein is being employed to treat.

Such combination therapies noted above encompass combined administration (where two or more therapeutic agents are included in the same or separate formulations), and separate administration, in which case, administration of the antibody as reported herein can occur prior to, simultaneously, and/or following, administration of the additional therapeutic agent or agents. In one embodiment, administration of the bispecific anti-human CD20/human transferrin receptor antibody and administration of an additional therapeutic agent occur within about one month, or within about one, two or three weeks, or within about one, two, three, four, five, or six days, of each other. Antibodies as reported herein can also be used in combination with other interventional therapies such as, but not limited to, radiation therapy, behavioral therapy, or other therapies known in the art and appropriate for the neurological disorder to be treated or prevented.

An antibody as reported herein (and any additional therapeutic agent) can be administered by any suitable means, including parenteral, intrapulmonary, and intranasal, and, if desired for local treatment, intralesional administration. Parenteral infusions include intramuscular, intravenous, intraarterial, intraperitoneal, or subcutaneous administration. Dosing can be by any suitable route, e.g. by injections, such as intravenous or subcutaneous injections, depending in part on whether the administration is brief or chronic. Various dosing schedules including but not limited to single or multiple administrations over various time-points, bolus administration, and pulse infusion are contemplated herein.

In addition, the bispecific antibody may suitably be administered by pulse infusion, e.g., with declining doses of the bispecific antibody. The dosing can be given by injections, intravenous or subcutaneous injections, depending in part on whether the administration is brief or chronic.

Antibodies as reported herein would be formulated, dosed, and administered in a fashion consistent with good medical practice. Factors for consideration in this context include the particular disorder being treated, the particular mammal being treated, the clinical condition of the individual patient, the cause of the disorder, the site of delivery of the agent, the method of administration, the scheduling of administration, and other factors known to medical practitioners. The antibody need not be, but is optionally formulated with one or more agents currently used to prevent or treat the disorder in question. The effective amount of such other agents depends on the amount of antibody present in the formulation, the type of disorder or treatment, and other factors discussed above. These are generally used in the same dosages and with administration routes as described herein, or about from 1 to 99% of the dosages described herein, or in any dosage and by any route that is empirically/clinically determined to be appropriate.

Lipid-based methods of transporting the fusion construct or a compound across the BBB include, but are not limited to, encapsulating the fusion construct or a compound in liposomes that are coupled to monovalent binding entity that bind to receptors on the vascular endothelium of the BBB (see e.g., US 2002/0025313), and coating the monovalent binding entity in low-density lipoprotein particles (see e.g., US 2004/0204354) or apolipoprotein E (see e.g., US 2004/0131692).

For the prevention or treatment of disease, the appropriate dosage of an antibody as reported herein (when used alone or in combination with one or more other additional therapeutic agents) will depend on the type of disease to be treated, the type of antibody, the severity and course of the disease, whether the antibody is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the antibody, and the discretion of the attending physician. The antibody is suitably administered to the patient at one time or over a series of treatments. Depending on the type and severity of the disease, about 1 μg/kg to 15 mg/kg (e.g. 0.5mg/kg - 10 mg/kg) of antibody can be an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. One typical daily dosage might range from about 1 μg/kg to 100 mg/kg or more, depending on the factors mentioned above. For repeated administrations over several days or longer, depending on the condition, the treatment would generally be sustained until a desired suppression of disease symptoms occurs. One exemplary dosage of the antibody would be in the range from about 0.05 mg/kg to about 10 mg/kg. Thus, one or more doses of about 0.5 mg/kg, 2.0 mg/kg, 4.0 mg/kg or 10 mg/kg (or any combination thereof) may be administered to the patient. Such doses may be administered intermittently, e.g. every week or every three weeks *(e.g.* such that the patient receives from about two to about twenty, or *e.g.* about six doses of the antibody). An initial higher loading dose, followed by one or more lower doses may be administered. However, other dosage regimens may be useful. The progress of this therapy is easily monitored by conventional techniques and assays.

It is understood that any of the above formulations or therapeutic methods may be carried out using an immunoconjugate as reported herein in place of or in addition to a bispecific anti-human CD20/human transferrin receptor antibody.

The composition comprising a bispecific antibody as reported herein which binds to a B-cell surface antigen will be formulated, dosed, and administered in a fashion consistent with good medical practice. Factors for consideration in this context include the particular disease or disorder being treated, the particular mammal being treated, the clinical condition of the individual patient, the cause of the disease or disorder, the site of delivery of the agent, the method of administration, the scheduling of administration, and other factors known to medical practitioners. The therapeutically effective amount of the antagonist to be administered will be governed by such considerations.

Beside the treatment of B-cell proliferative diseases the bispecific antibody as reported herein can be used in the treatment of autoimmune diseases. Examples of autoimmune diseases or disorders include, but are not limited to, immune-mediated thrombocytopenias, such as acute idiopathic thrombocytopenic purpurea and chronic idiopathic thrombocytopenic purpurea, dermatomyositis, Sydenham's chorea, lupus nephritis, rheumatic fever, polyglandular syndromes, Henoch-Schonlein purpura, post-streptococcal nephritis, erythema nodosum, Takayasu's arteritis, Addison's disease, erythema multiforme, polyarteritis nodosa, ankylosing spondylitis, Goodpasture's syndrome, thromboangitis ubiterans, primary biliary cirrhosis, Hashimoto's thyroiditis, thyrotoxicosis, chronic active hepatitis, polymyositis/dermatomyositis, polychondritis, pamphigus vulgaris, Wegener's granulomatosis, membranous nephropathy, amyotrophic lateral sclerosis, tabes dorsalis, polymyaglia, pernicious anemia, rapidly progressive glomerulonephritis and fibrosing alveolitis, inflammatory responses such as inflammatory skin diseases including psoriasis and dermatitis (e.g. atopic dermatitis); systemic scleroderma and sclerosis; responses associated with inflammatory bowel disease (such as Crohn's disease andulcerative colitis); respiratory distress syndrome (including adult respiratory distress syndrome; ARDS); dermatitis; meningitis; encephalitis; uveitis; colitis; glomerulonephritis; allergic conditions such as eczema and asthma and other conditions involving infiltration of T cells and chronic inflammatory responses; atherosclerosis; leukocyte adhesion deficiency; rheumatoid arthritis; systemic lupus erythematosus (SLE); diabetes mellitus (e.g. Type 1 diabetes mellitus or insulin dependent diabetes mellitus); multiple sclerosis; Reynaud's syndrome; autoimmune thyroiditis; allergic encephalomyelitis; Sjogren's syndrome; juvenile onset diabetes; and immune responses associated with acute and delayed hypersensitivity mediated by cytokines and T-lymphocytes typically found in tuberculosis, sarcoidosis, polymyositis, granulomatosis and vasculitis; pernicious amenia (Addison's disease); diseases involving leukocyte diapedesis; central nervous system (CNS) inflammatory disorder; multiple organ injury syndrome; hemolytic anemia (including, but not limited to cryoglobinemia or Coombs positive anemia); myasthenia gravis; antigen-antibody complex mediated diseases; anti-glomerular basement membrane disease; anti-phospholipid syndrome; allergic neuritis; Graves' disease; Lambert-Eaton myasthenic syndrome; pemphigoid bullous; pemphigus; autoimmune polyendocrinopathies; Reiter's disease; stiff-man syndrome; Bechet disease; giant cell arteritis; immune complex nephritis; IgA nephropathy; IgM polyneuropathies; immune thrombocytopenic purpura (ITP) or autoimmune thrombocytopenia etc. In this aspect of the invention, the ABMs of the invention are used to deplete the blood of normal B-cells for an extended period.

As noted above, however, these suggested amounts of bispecific antibody are subject to a great deal of therapeutic discretion. The key factor in selecting an appropriate dose and scheduling is the result obtained, as indicated above. For example, relatively higher doses may be needed initially for the treatment of ongoing and acute diseases. To obtain the most efficacious results, depending on the disease or disorder, the antagonist is administered as close to the first sign, diagnosis, appearance, or occurrence of the disease or disorder as possible or during remissions of the disease or disorder.

Aside from administration of isolated bispecific antibodies as reported herein to the patient the present application contemplates administration of the bispecific antibody by gene therapy. Such administration of nucleic acid encoding the bispecific antibody is encompassed by the expression "administering a therapeutically effective amount of a bispecific antibody". See, for example, WO96/07321 concerning the use of gene therapy to generate intracellular antibodies.

There are two major approaches to getting the nucleic acid (optionally contained in a vector) into the patient's cells; in vivo and ex vivo. For in vivo delivery the nucleic acid is injected directly into the patient, usually at the site where the bispecific antibody is required. For ex vivo treatment, the patient's cells are removed, the nucleic acid is introduced into these isolated cells and the modified cells are administered to the patient either directly or, for example, encapsulated within porous membranes which are implanted into the patient (see, e.g. US 4,892,538 and US 5,283,187). There are a variety of techniques available for introducing nucleic acids into viable cells. The techniques vary depending upon whether the nucleic acid is transferred into cultured cells in vitro, or in vivo in the cells of the intended host. Techniques suitable for the transfer of nucleic acid into mammalian cells in vitro include the use of liposomes, electroporation, microinjection, cell fusion, DEAE-dextran, the calcium phosphate precipitation method, etc. A commonly used vector for ex vivo delivery of the gene is a retrovirus.

The currently preferred in vivo nucleic acid transfer techniques include transfection with viral vectors (such as adenovirus, Herpes simplex I virus, or adeno-associated virus) and lipid-based systems (useful lipids for lipid-mediated transfer of the gene are DOTMA, DOPE and DC-Choi, for example). In some situations it is desirable to provide the nucleic acid source with an agent that targets the target cells, such as an antibody specific for a cell surface membrane protein or the target cell, a ligand for a receptor on the target cell, etc. Where liposomes are employed, proteins which bind to a cell surface membrane protein associated with endocytosis may be used for targeting and/or to facilitate uptake, e.g. capsid proteins or fragments thereof tropic for a particular cell type, antibodies for proteins which undergo internalization in cycling, and proteins that target intracellular localization and enhance intracellular half-life. The technique of receptor-mediated endocytosis is described, for example, by Wu et al, J. Biol. Chem. 262:4429-4432 (1987); and Wagner et al, Proc. Natl. Acad. Sci. USA 87:3410-3414 (1990). For review of the currently known gene marking and gene therapy protocols see Anderson et al., Science 256:808-813 (1992). See also WO 93/25673 and the references cited therein.

General techniques for conjugating additional therapeutic agents to antibodies are well known (see, e.g., Arnon et al., "Monoclonal Antibodies for Immunotargeting of Drugs in Cancer Therapy", in Monoclonal Antibodies and Cancer Therapy, Reisfeld et al. (eds.), pp. 243-56 (Alan R. Liss, Inc. 1985); Hellstrom et al, "Antibodies For Drug Delivery", in Controlled Drug Delivery (2nd Ed.), Robinson et al. (eds.), pp. 623-53 (Marcel Dekker, Inc. 1987); Thorpe, "Antibody Carriers Of Cytotoxic Agents In Cancer Therapy: A Review", in Monoclonal Antibodies '84: Biological And Clinical Applications, Pinchera et al. (eds.), pp. 475-506 (1985); and Thorpe et al., "The Preparation And Cytotoxic Properties Of Antibody-Toxin Conjugates", Immunol. Rev., 62 (1982) 119-58).

III. Articles of Manufacture

In another aspect as reported herein, an article of manufacture containing materials useful for the treatment, prevention and/or diagnosis of the disorders described above is provided. The article of manufacture comprises a container and a label or package insert on or associated with the container. Suitable containers include, for example, bottles, vials, syringes, IV solution bags, etc. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition, which is by itself or combined with another composition effective for treating, preventing and/or diagnosing the condition and may have a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). At least one active agent in the composition is an antibody as reported herein. The label or package insert indicates that the composition is used for treating the condition of choice. Moreover, the article of manufacture may comprise (a) a first container with a composition contained therein, wherein the composition comprises an antibody as reported herein; and (b) a second container with a composition contained therein, wherein the composition comprises a further cytotoxic or otherwise therapeutic agent. The article of manufacture in this embodiment may further comprise a package insert indicating that the compositions can be used to treat a particular condition. Alternatively, or additionally, the article of manufacture may further comprise a second (or third) container comprising a pharmaceutically-acceptable buffer, such as bacteriostatic water for injection (BWFI), phosphate-buffered saline, Ringer's solution and dextrose solution. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, and syringes.

It is understood that any of the above articles of manufacture may include an immunoconjugate as reported herein in place of or in addition to a bispecific antibody as reported herein.

IV. EXAMPLES

The following are examples of methods and compositions of the invention. It is understood that various other embodiments may be practiced, given the general description provided above.

Materials & general methods

General information regarding the nucleotide sequences of human immunoglobulins light and heavy chains is given in: Kabat, E.A., et al, Sequences of Proteins of Immunological Interest, 5th ed., Public Health Service, National Institutes of Health, Bethesda, MD (1991). Amino acids of antibody chains are numbered and referred to according to numbering according to Kabat (Kabat, E.A., et al, Sequences of Proteins of Immunological Interest, 5th ed., Public Health Service, National Institutes of Health, Bethesda, MD (1991)).

Recombinant DNA techniques

Standard methods were used to manipulate DNA as described in Sambrook, J. et al., Molecular Cloning: A laboratory manual; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989. The molecular biological reagents were used according to the manufacturer's instructions.

Gene synthesis

Desired gene segments were prepared from oligonucleotides made by chemical synthesis. The long gene segments, which were flanked by singular restriction endonuclease cleavage sites, were assembled by annealing and ligating oligonucleotides including PCR amplification and subsequently cloned via the indicated restriction sites. The DNA sequences of the subcloned gene fragments were confirmed by DNA sequencing. Gene synthesis fragments were ordered according to given specifications at Geneart (Regensburg, Germany).

DNA sequence determination

DNA sequences were determined by double strand sequencing performed at MediGenomix GmbH (Martinsried, Germany) or SequiServe GmbH (Vaterstetten, Germany).

DNA and protein sequence analysis and sequence data management

The GCG's (Genetics Computer Group, Madison, Wisconsin) software package version 10.2 and Infomax's Vector NTl Advance suite version 8.0 was used for sequence creation, mapping, analysis, annotation and illustration.

Expression vectors

For the expression of the described bispecific antibodies, expression plasmids for transient expression (e.g. in HEK293 cells) based either on a cDNA organization with or without a CMV-intron A promoter or on a genomic organization with a CMV promoter can be applied.

Beside the antibody expression cassette the vectors contain:

- an origin of replication which allows replication of this plasmid in E. coli, and
- a β-lactamase gene which confers ampicillin resistance in E. coli.

The transcription unit of the antibody gene is composed of the following elements:

- unique restriction site(s) at the 5' end
- the immediate early enhancer and promoter from the human cytomegalovirus ,
- the intron A sequence in the case of cDNA organization,
- a 5'-untranslated region derived from a human antibody gene,
- an immunoglobulin heavy chain signal sequence,
- the respective antibody chain encoding nucleic acid either as cDNA or with genomic exon-intron organization,
- a 3' untranslated region with a polyadenylation signal sequence,
- a terminator sequence, and
- unique restriction site(s) at the 3' end.

The fusion genes encoding the antibody chains are generated by PCR and/or gene synthesis and assembled by known recombinant methods and techniques by connection of the according nucleic acid segments e.g. using unique restriction sites in the respective vectors. The subcloned nucleic acid sequences are verified by DNA sequencing. For transient transfections larger quantities of the plasmids are prepared by plasmid preparation from transformed E. coli cultures (Nucleobond AX, Macherey-Nagel).

For all constructs knob-into-hole heterodimerization technology was used with a typical knob (T366W) substitution in the first CH3 domain and the corresponding hole substitutions (T366S, L368A and Y407V) in the second CH3 domain (as well as two additional introduced cysteine residues S354C/Y349'C) (contained in the respective corresponding heavy chain (HC) sequences depicted above).

Cell culture techniques

Standard cell culture techniques as described in Current Protocols in Cell Biology (2000), Bonifacino, J.S., Dasso, M., Harford, J.B., Lippincott-Schwartz, J. and Yamada, K.M. (eds.), John Wiley & Sons, Inc., are used.

Transient transfections in HEK293 system

The bispecific antibodies are produced by transient expression. Therefore a transfection with the respective plasmids using the HEK293 system (Invitrogen) according to the manufacturer's instruction is done. Briefly, HEK293 cells (Invitrogen) growing in suspension either in a shake flask or in a stirred fermenter in serum-free FreeStyle™ 293 expression medium (Invitrogen) are transfected with a mix of the respective expression plasmids and 293fectin™ or fectin (Invitrogen). For 2 L shake flask (Corning) HEK293 cells are seeded at a density of $1.0*10^6$ cells/mL in 600 mL and incubated at 120 rpm, 8% $C0_2$. On the next day the cells are transfected at a cell density of approx. $1.5*10^6$ cells/mL with approx. 42 mL of a mixture of A) 20 mL Opti-MEM medium (Invitrogen) comprising 600 µg total plasmid DNA (1 µg/mL) and B) 20 ml Opti-MEM medium supplemented with 1.2 mL 293 fectin or fectin (2 µl/mL). According to the glucose consumption glucose solution is added during the course of the fermentation. The supernatant containing the secreted antibody is harvested after 5-10 days and antibodies are either directly purified from the supernatant or the supernatant is frozen and stored.

Protein determination

The protein concentration of purified antibodies and derivatives was determined by determining the optical density (OD) at 280 nm, using the molar extinction coefficient calculated on the basis of the amino acid sequence according to Pace, et al, Protein Science 4 (1995) 241 1-1423.

Antibody concentration determination in supematants

The concentration of antibodies and derivatives in cell culture supematants was estimated by immunoprecipitation with protein A agarose-beads (Roche Diagnostics GmbH, Mannheim, Germany). Therefore, 60 μL protein A Agarose beads were washed three times in TBS-NP40 (50 mM Tris buffer, pH 7.5, supplemented with 150 mM NaCl and 1% Nonidet-P40). Subsequently, 1-15 mL cell culture supernatant was applied to the protein A Agarose beads pre-equilibrated in TBS-NP40. After incubation for at 1 hour at room temperature the beads were washed on an Ultrafree-MC-filter column (Amicon) once with 0.5 mL TBS-NP40, twice with 0.5 mL 2x phosphate buffered saline (2xPBS, Roche Diagnostics GmbH, Mannheim, Germany) and briefly four times with 0.5 mL 100 mM Na-citrate buffer (pH 5.0). Bound antibody was eluted by addition of 35 μΐ NuPAGE® LDS sample buffer (Invitrogen). Half of the sample was combined with NuPAGE® sample reducing agent or left unreduced, respectively, and heated for 10 min at 70 °C. Consequently, 5-30 μΐ were applied to a 4-12% NuPAGE® Bis-Tris SDS-PAGE gel (Invitrogen) (with MOPS buffer for non-reduced SDS-PAGE and MES buffer with NuPAGE® antioxidant running buffer additive (Invitrogen) for reduced SDS-PAGE) and stained with Coomassie Blue.

The concentration of the antibodies in cell culture supematants was quantitatively measured by affinity HPLC chromatography. Briefly, cell culture supematants containing antibodies that bind to protein A were applied to an Applied Biosystems Poros A/20 column in 200 mM KH_2P0_4, 100 mM sodium citrate, pH 7.4 and eluted with 200 mM NaCl, 100 mM citric acid, pH 2.5 on an Agilent HPLC 1100 system. The eluted antibody was quantified by UV absorbance and integration of peak areas. A purified standard IgGl antibody served as a standard.

Alternatively, the concentration of antibodies and derivatives in cell culture supematants was measured by Sandwich-IgG-ELISA. Briefly, StreptaWell High Bind Streptavidin A-96 well microtiter plates (Roche Diagnostics GmbH, Mannheim, Germany) were coated with 100 μL/weII biotinylated anti-human IgG capture molecule F(ab')2<h-Fcy> BI (Dianova) at 0.1 μg/mL for 1 hour at room temperature or alternatively overnight at 4°C and subsequently washed three times with 200 μL/well PBS, 0.05% Tween (PBST, Sigma). Thereafter, 100 μL/weII of a dilution series in PBS (Sigma) of the respective antibody containing cell culture supematants was added to the wells and incubated for 1-2 hour on a shaker at room temperature. The wells were washed three times with 200 μL/weII PBST and bound antibody was detected with 100 μL F(ab')2<hFcy>POD (Dianova) at 0.1 μg/mL as the detection antibody by incubation for 1-2 hours on a shaker at room temperature. Unbound detection antibody was removed by washing three times with 200 μL/well PBST. The bound detection antibody was detected by addition of 100 μL ABTS/well followed by incubation. Determination of absorbance was performed on a Tecan Fluor Spectrometer at a measurement wavelength of 405 nm (reference wavelength 492 nm).

<u>Preparative antibody purification</u>

Antibodies were purified from filtered cell culture supernatants referring to standard protocols. In brief, antibodies were applied to a protein A Sepharose column (GE healthcare) and washed with PBS. Elution of antibodies was achieved at pH 2.8 followed by immediate neutralization. Aggregated protein was separated from monomeric antibodies by size exclusion chromatography (Superdex 200, GE Healthcare) in PBS or in 20 mM Histidine buffer comprising 150 mM NaCl (pH 6.0). Monomeric antibody fractions were pooled, concentrated (if required) using e.g., a MILLIPORE Amicon Ultra (30 MWCO) centrifugal concentrator, frozen and stored at -20°C or -80°C. Part of the samples were provided for subsequent protein analytics and analytical characterization e.g. by SDS-PAGE, size exclusion chromatography (SEC) or mass spectrometry.

<u>SDS-PAGE</u>

The NuPAGE® Pre-Cast gel system (Invitrogen) was used according to the manufacturer's instruction. In particular, 10% or 4-12% NuPAGE® Novex® Bis-TRIS Pre-Cast gels (pH 6.4) and a NuPAGE® MES (reduced gels, with NuPAGE® antioxidant running buffer additive) or MOPS (non-reduced gels) running buffer was used.

<u>CE-SDS</u>

Purity and antibody integrity were analyzed by CE-SDS using microfluidic Labchip technology (PerkinElmer, USA). Therefore, 5 μL of antibody solution was prepared for CE-SDS analysis using the HT Protein Express Reagent Kit according manufacturer's instructions and analyzed on LabChip GXII system using a HT Protein Express Chip. Data were analyzed using LabChip GX Software.

Analytical size exclusion chromatography

Size exclusion chromatography (SEC) for the determination of the aggregation and oligomeric state of antibodies was performed by HPLC chromatography. Briefly, protein A purified antibodies were applied to a Tosoh TSKgel G3000SW column in 300 mM NaCl, 50 mM KH_2PO_4/K_2HPO_4 buffer (pH 7.5) on an Dionex Ultimate® system (Thermo Fischer Scientific), or to a Superdex 200 column (GE Healthcare) in 2 x PBS on a Dionex HPLC-System. The eluted antibody was quantified by UV absorbance and integration of peak areas. BioRad Gel Filtration Standard 151-1901 served as a standard.

Mass spectrometry

This section describes the characterization of the bispecific antibodies with emphasis on their correct assembly. The expected primary structures were analyzed by electrospray ionization mass spectrometry (ESI-MS) of the deglycosylated intact antibody and in special cases of the deglycosylated/limited LysC digested antibody.

The antibodies were deglycosylated with N-Glycosidase F in a phosphate or Tris buffer at 37°C for up to 17 h at a protein concentration of 1 mg/ml. The limited LysC (Roche Diagnostics GmbH, Mannheim, Germany) digestions were performed with 100 µg deglycosylated antibody in a Tris buffer (pH 8) at room temperature for 120 hours, or at 37°C for 40 min, respectively. Prior to mass spectrometry the samples were desalted via HPLC on a Sephadex G25 column (GE Healthcare). The total mass was determined via ESI-MS on a maXis 4G UHR-QTOF MS system (Bruker Daltonik) equipped with a TriVersa NanoMate source (Advion).

Chemical degradation test

Samples were split into three aliquots and re-buffered into 20 mM His/His*HCl, 140 mM NaCl, pH 6.0 or into PBS, respectively, and stored at 40 °C (His/NaCl) or 37 °C (PBS). A control sample was stored at -80 °C.

After incubation ended, samples were analyzed for relative active concentration (BIAcore), aggregation (SEC) and fragmentation (capillary electrophoresis or SDS-PAGE) and compared with the untreated control.

Thermal stability

Samples were prepared at a concentration of 1 mg/mL in 20 mM Histidine/Histidine chloride, 140 mM NaCl, pH 6.0, transferred into an optical 384-well plate by centrifugation through a 0.4 μιη filter plate and covered with paraffin oil. The hydrodynamic radius was measured repeatedly by dynamic light scattering on a DynaPro Plate Reader (Wyatt) while the samples were heated with a rate of 0.05 °C/min from 25 °C to 80 °C.

Alternatively, samples were transferred into a 10 μL micro-cuvette array and static light scattering data as well as fluorescence data upon excitation with a 266 nm laser were recorded with an OptimlOOO instrument (Avacta Inc.), while they were heated at a rate of 0.1 °C/min from 25 °C to 90 °C.

The aggregation onset temperature is defined as the temperature at which the hydrodynamic radius (DLS) or the scattered light intensity (OptimlOOO) starts to increase.

Alternatively, samples were transferred in a 9 μL multi-cuvette array. The multi-cuvette array was heated from 35 °C to 90 °C at a constant rate of 0.1 °C/minute in an OptimlOOO instrument (Avacta Analytical Inc.). The instrument continuously records the intensity of scattered light of a 266 nm laser with a data point approximately every 0.5 °C. Light scattering intensities were plotted against the temperature. The aggregation onset temperature (T_agg) is defined as the temperature at which the scattered light intensity begins to increase.

The melting temperature is defined as the inflection point in fluorescence intensity vs. wavelength graph.

Example 1

Expression and purification

The bispecific antibodies were produced as described above in the general materials and methods section.

The bispecific antibodies were purified from the supernatant by a combination of protein A affinity chromatography and size exclusion chromatography. The obtained products were characterized for identity by mass spectrometry and analytical properties such as purity by CE-SDS, monomer content and stability.

The expected primary structures were analyzed by electrospray ionization mass spectrometry (ESI-MS) of the deglycosylated intact antibody and deglycosylated/plasmin digested or alternatively deglycosylated/limited LysC digested antibody as described in the general methods section.

Additional analytical methods (e.g. thermal stability, mass spectrometry and functional assessment) were only applied after protein A and SEC purification.

Example 2

Determination of binding to transferrin receptor in vitro

Binding of the bispecific antibodies to murine transferrin receptor is tested by FACS analysis on mouse X63.AG8-563 myeloma cells. If the Aβ antibody shows a certain tendency to non-specifically bind to Ag8 cells, specific binding can be quantified by co-incubation with a 20fold excess of anti-mouse-TfR antibody. Cells are harvested by centrifugation, washed once with PBS and $5x10^4$ cells incubated with a 1.5 pM to 10 nM dilution series of the polypeptide fusions with or without addition of 200 nM anti-mouse TfR antibody in 100 µL RPMI/10% FCS for 1.5 h on ice. After 2 washes with RPMI/10% FCS, cells are incubated with goat-anti-human IgG coupled to Phycoerythrin (Jackson Immunoresearch) at a dilution of 1:600 in RPMI/19% FCS for 1.5 h on ice. Cells are again washed, resuspended in RPMI/10% FCS and Phycoerythrin fluorescence measured on a FACS-Array instrument (Becton-Dickinson).

Example 3

Surface plasmon resonance-based binding assay for human TfR-antibody interaction

The binding experiment were carried out on a BIAcore B 4000 (GE Healthcare) equipped with CI sensor chip (GE Healthcare, cat.no. BR1 005-35) pre-treated with anti-human Fab antibody (GE Healthcare, cat.no 28-9583-25) using a standard amine coupling chemistry procedure accordingly to the vendor's manual.

For kinetic measurements the sample antibody was immobilized applying a contact time of 60 seconds and a flow rate of 10 µL/ηιη in phosphate buffer saline pH 7.4, 0.05 % Tween 20 at 25 °C. Recombinant His6-tagged human transferrin receptor (R&D systems, cat.no 2474-TR-050) was applied in increasing concentrations and the signal monitored over the time. An average time span of 150 seconds of association time and 600 seconds of dissociation time at 30µL/ηiη flow rate was recorded. Data were fit using a 1:1 binding model (Langmuir isotherm).

Patent Claims

1. A bispecific antibody comprising

   a) one antibody comprising two pairs each of a antibody light chain and a antibody heavy chain, wherein the binding sites formed by each of the pairs of the heavy chain and the light chain specifically bind to a first antigen, and

   b) one additional Fab fragment, wherein the additional Fab fragment is fused to the C-terminus of one of the heavy chains of the antibody, wherein the binding site of the additional Fab fragment specifically binds to a second antigen,

   wherein each of the antibody light chains comprises in the constant light chain domain (CL) at position 123 the amino acid residue arginine (instead of the wild-type glutamic acid residue; E123R mutation) and at position 124 the amino acid residue lysine (instead of the wild-type glutamine residue; Q124K mutation) (numbering according to Kabat),

   wherein each of the antibody heavy chains comprises in the first constant heavy chain domain (CHI) at position 147 an glutamic acid residue (instead of the wild-type lysine residue; K147E mutation) and at position 213 an glutamic acid residue (instead of the wild-type lysine amino acid residue; K213E mutation) (numbering according to Kabat EU index),

   wherein the additional Fab fragment specifically binding to the second antigen comprises a domain crossover such that the constant light chain domain (CL) and the constant heavy chain domain 1 (CHI) are replaced by each other, and

   wherein the first antigen is human CD20 protein and the second antigen is human transferrin receptor.

2. The antibody according to claim 1, wherein the additional Fab fragment is fused to the C-terminus of the heavy chain by a peptidic linker.

3. The antibody according to any one of claims 1 and 2, wherein

a) the heavy chain that is fused to the additional Fab fragments has as C-terminal heavy chain amino acid residues the tripeptide LSP wherein the proline thereof is directly fused to the first amino acid residue of the additional Fab fragment or of the peptidic linker via a peptide bond, and

b) the heavy chain that is not fused to the additional Fab fragments has as C-terminal heavy chain amino acid residues the tripeptide LSP, or SPG, or PGK.

4. The antibody according to anyone of claims 1 to 3, wherein the antibody is

a) a full length antibody of the human subclass IgGl, or

b) a full length antibody of the human subclass IgG4, or

c) a full length antibody of the human subclass IgGl with the mutations L234A, L235A and P329G,

d) a full length antibody of the human subclass IgG4 with the mutations S228P, L235E and P329G,

e) a full length antibody of the human subclass IgGl with the mutations L234A, L235A and P329G in both heavy chains and the mutations T366W and S354C in one heavy chain and the mutations T366S, L368A, Y407V and Y349C in the respective other heavy chain,

f) a full length antibody of the human subclass IgG4 with the mutations S228P and P329G in both heavy chains and the mutations T366W and S354C in one heavy chain and the mutations T366S, L368A, Y407V and Y349C in the respective other heavy chain,

g) a full length antibody of the human subclass IgGl with the mutations L234A, L235A, P329G, I253A, H310A and H435A in both heavy chains and the mutations T366W and S354C in one heavy chain and the mutations T366S, L368A, Y407V and Y349C in the respective other heavy chain,

h) a full length antibody of the human subclass IgGl with the mutations L234A, L235A, P329G, M252Y, S254T and T256E in both heavy chains and the mutations T366W and S354C in one heavy chain and the mutations T366S, L368A, Y407V and Y349C in the respective other heavy chain, or

i) a full length antibody of the human subclass IgGl with the mutations L234A, L235A, P329G, H310A, H433A and Y436A in both heavy chains and the mutations i) T366W, and ii) S354C or Y349C, in one heavy chain and the mutations i) T366S, L368A, and Y407V, and ii) Y349C or S354C, in the respective other heavy chain.

5. The antibody according to any one of claims 1 to 4, wherein the bispecific antibody comprises

i) a light chain that has a sequence identity to SEQ ID NO: 01 of 70 % or more,

ii) a heavy chain that has a sequence identity to SEQ ID NO: 02 of 70 % or more,

iii) a light chain that has a sequence identity to SEQ ID NO: 03 of 70 % or more, and

iv) a heavy chain Fab fragment that has a sequence identity to SEQ ID NO: 04 of70 % or more.

6. The antibody according to any one of claims 1 to 4, wherein the bispecific antibody comprises a light chain that has the amino acid sequence of SEQ ID NO: 01, a heavy chain that has the amino acid sequence of SEQ ID NO: 02, a light chain that has the amino acid sequence of SEQ ID NO: 03, and an antibody Fab fragment comprising the amino acid sequences of SEQ ID NO: 04.

7. The antibody according to any one of claims 1 to 6, wherein the antibody is monoclonal.

8. A pharmaceutical formulation comprising the antibody according to any one of claims 1 to 7 and a pharmaceutically acceptable carrier.

9. The antibody according to any one of claims 1 to 7 for use as a medicament.

10. The antibody according to any one of claims 1 to 7 for the treatment of multiple sclerosis.

11. Use of the antibody according to any one of claims 1 to 7 in the manufacture of a medicament.

12. The use of claim 11, wherein the medicament is for treatment of multiple sclerosis.

13. The use of claim 11, wherein the medicament is for depleting brain sequestered B-cells expressing CD20.

14. A method of treating an individual having multiple sclerosis comprising administering to the individual an effective amount of the antibody according to any one of claims 1 to 7.

15. A method of depleting brain sequestered B-cells expressing CD20 in an individual comprising administering to the individual an effective amount of the antibody according to any one of claims 1 to 7 to deplete brain sequestered B-cells expressing CD20.

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/073413

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 2014/033074 A1 (HOFFMANN LA ROCHE [CH]; HOFFMANN LA ROCHE [US]) 6 March 2014 (2014-03-06) page 30, line 31 - page 32, line 20; claims 7,20 -----	1-15
X	WO 2012/075037 A1 (GENENTECH INC [US]; HOFFMANN LA ROCHE [CH]; DENNIS MARK [US]; WATTS RY) 7 June 2012 (2012-06-07) claims 12,21,27 -----	1-15
X	WO 2014/189973 A2 (GENENTECH INC [US]; HOFFMANN LA ROCHE [CH]) 27 November 2014 (2014-11-27) page 14, line 31 - page 15, line 10 page 39, line 6 - line 14; claims 1,20 page 35, line 25 - page 36, line 5 ----- -/--	1-15

[X] Further documents are listed in the continuation of Box C.

[X] 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
12 December 2016	09/01/2017

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Marinoni J-C

1

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2015/101588 A1 (HOFFMANN LA ROCHE [CH]; HOFFMANN LA ROCHE [US]) 9 July 2015 (2015-07-09) page 3, line 28 - page 4, line 2 page 19, line 24 - line 33 page 3, line 13 - line 17 page 4, line 22 - line 26 page 5, line 17 - line 23 claims 1,6, 10, 13, 14 -----	1-15
Y	WO 2010/033587 A2 (GENENTECH INC [US]; SMITH CRAIG [US]; CHIN PETER S [US]) 25 March 2010 (2010-03-25) the whole document -----	1-15
Y	WO 2012/096924 A1 (GLAXO GROUP LTD [GB]; DEROSIER FREDERICK J [US]; FREEDMAN IMMANUEL [US]) 19 July 2012 (2012-07-19) the whole document -----	1-15
Y	HAWKER KATHLEEN ET AL: "Rituximab in Patients with Primary Progressive Multiple Sclerosis Results of a Randomized Double-Blind Placebo-Controlled Multicenter Trial", ANNALS OF NEUROLOGY, JOHN WILEY AND SONS, BOSTON, US, vol. 66, no. 4, 1 October 2009 (2009-10-01), pages 460-471, XP002567460, ISSN: 0364-5134, DOI: 10.1002/ANA.21867 [retrieved on 2009-09-09] the whole document -----	1-15
A	WO 2014/082179 A1 (ZYMEWORKS INC [CA]) 5 June 2014 (2014-06-05) -----	1-15
A	H-CHRISTIAN VON BÜDINGEN ET AL: "B cells in multiple sclerosis: connecting the dots", CURRENT OPINION IN IMMUNOLOGY, vol. 23, no. 6, 7 October 2011 (2011-10-07), pages 713-720, XP028336245, ISSN: 0952-7915, DOI: 10.1016/J.COI.2011.09.003 [retrieved on 2011-09-13] abstract -----	1-15

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2016/073413

Patent document cited in search report		Publication date	Patent family member(s)			Publication date
WO 2014033074	A1	06-03-2014	AU	2013307406	A1	29-01-2015
			CA	2879496	A1	06-03-2014
			CN	104520329	A	15-04-2015
			EP	2890712	A1	08-07-2015
			HK	1205521	A1	18-12-2015
			JP	2015528452	A	28-09-2015
			KR	20150039798	A	13-04-2015
			RU	2015111093	A	20-10-2016
			SG	11201500583P	A	29-04-2015
			US	2015322149	A1	12-11-2015
			WO	2014033074	A1	06-03-2014
WO 2012075037	A1	07-06-2012	AR	084020	A1	17-04-2013
			AU	2011336716	A1	09-05-2013
			CA	2818173	A1	07-06-2012
			CL	2013001535	A1	07-03-2014
			CN	103443125	A	11-12-2013
			CO	6721052	A2	31-07-2013
			CR	20130255	A	18-09-2013
			EA	201390785	A1	29-11-2013
			EC	SP13012724	A	30-08-2013
			EP	2646470	A1	09-10-2013
			JP	2014500886	A	16-01-2014
			KR	20130137654	A	17-12-2013
			MA	34749	B1	03-12-2013
			PE	09812014	A1	20-08-2014
			SG	190900	A1	31-07-2013
			TW	201305200	A	01-02-2013
			US	2012171120	A1	05-07-2012
			US	2015329636	A1	19-11-2015
			WO	2012075037	A1	07-06-2012
WO 2014189973	A2	27-11-2014	AR	096364	A1	23-12-2015
			AU	2014268726	A1	19-11-2015
			CA	2908743	A1	27-11-2014
			CL	2015003406	A1	15-07-2016
			CN	105358578	A	24-02-2016
			CR	20150618	A	08-02-2016
			EA	201592213	A1	29-04-2016
			EP	2999716	A2	30-03-2016
			HK	1215584	A1	02-09-2016
			JP	2016526878	A	08-09-2016
			KR	20160011198	A	29-01-2016
			PH	12015502565	A1	29-02-2016
			SG	11201509566R	A	30-12-2015
			TW	201500373	A	01-01-2015
			US	2015110791	A1	23-04-2015
			WO	2014189973	A2	27-11-2014
WO 2015101588	A1	09-07-2015	CA	2932547	A1	09-07-2015
			CN	105873947	A	17-08-2016
			EP	3092251	A1	16-11-2016
			WO	2015101588	A1	09-07-2015
WO 2010033587	A2	25-03-2010	AR	073295	A1	28-10-2010
			AU	2009293363	A1	25-03-2010
			BR	PI0918604	A2	01-12-2015
			CA	2737074	A1	25-03-2010

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
		CN 102215868 A	12-10-2011
		CN 104826107 A	12-08-2015
		EA 201170465 Al	31-10-2011
		EP 2331132 A2	15-06-2011
		EP 3095463 A2	23-11-2016
		HK 1210596 Al	29-04-2016
		JP 2012502919 A	02-02-2012
		JP 2015007057 A	15-01-2015
		JP 2016147856 A	18-08-2016
		SG 10201406297T A	30-12-2014
		TW 201014605 A	16-04-2010
		TW 201438738 A	16-10-2014
		US 2010158903 Al	24-06-2010
		US 2012225070 Al	06-09-2012
		US 2014363431 Al	11-12-2014
		US 2015307618 Al	29-10-2015
		WO 2010033587 A2	25-03-2010
		ZA 201101854 B	30-05-2012
WO 2012096924 Al	19-07-2012	EP 2663331 Al	20-11-2013
		JP 2014506258 A	13-03-2014
		US 2013315901 Al	28-11-2013
		US 2016090421 Al	31-03-2016
		WO 2012096924 Al	19-07-2012
WO 2014082179 Al	05-06-2014	AU 2013351888 Al	04-06-2015
		CA 2893562 Al	05-06-2014
		CN 105026430 A	04-11-2015
		EP 2925785 Al	07-10-2015
		JP 2016508117 A	17-03-2016
		KR 20150103008 A	09-09-2015
		US 2015307594 Al	29-10-2015
		WO 2014082179 Al	05-06-2014