

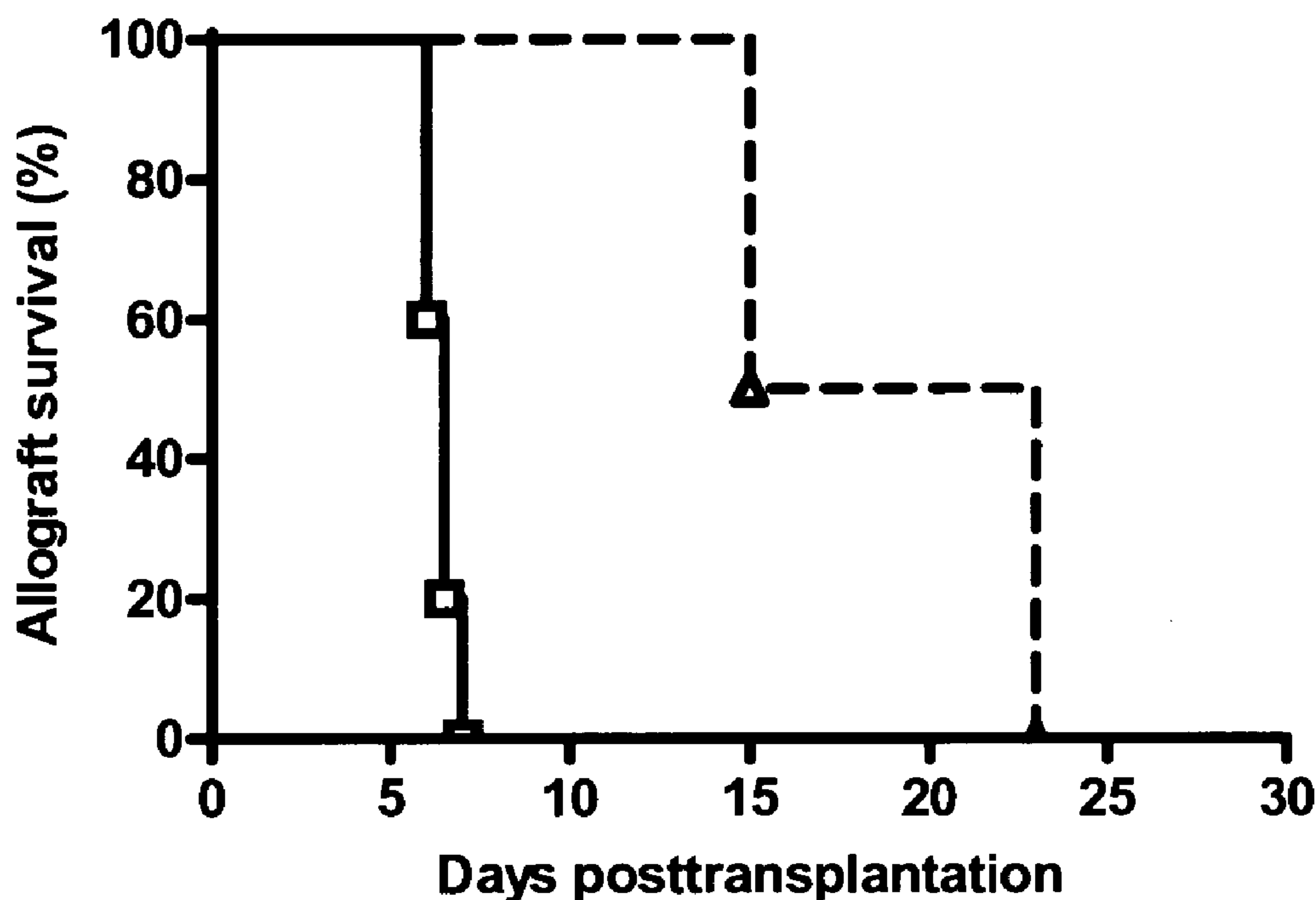


(86) Date de dépôt PCT/PCT Filing Date: 2009/01/13
(87) Date publication PCT/PCT Publication Date: 2009/07/23
(85) Entrée phase nationale/National Entry: 2010/06/28
(86) N° demande PCT/PCT Application No.: EP 2009/000133
(87) N° publication PCT/PCT Publication No.: 2009/090032
(30) Priorité/Priority: 2008/01/15 (US61/011,348)

(51) Cl.Int./Int.Cl. *C07K 16/28* (2006.01),
A61K 39/395 (2006.01)
(71) Demandeur/Applicant:
F. HOFFMANN-LA ROCHE AG, CH
(72) Inventeurs/Inventors:
AUER, JOHANNES, DE;
BERSON, AMY, US;
BRANDT, MICHAEL, DE;
LORA, JOSE M., GB;
RIES, STEFAN, DE;
BORIE, DOMINIQUE CHRISTIAN, US
(74) Agent: GOWLING LAFLEUR HENDERSON LLP

(54) Titre : ANTICORPS AFUCOSYLES CONTRE CCR5 ET LEURS UTILISATIONS
(54) Title: AFUCOSYLATED ANTIBODIES AGAINST CCR5 AND THEIR USE

Fig. 5



(57) Abrégé/Abstract:

An antibody binding to CCR5 and being glycosylated with a sugar chain at Asn297, said antibody being characterized in that the amount of fucose within said sugar chain is 65% or lower has improved properties in anti-inflammatory therapy.

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

(19) World Intellectual Property Organization
International Bureau(43) International Publication Date
23 July 2009 (23.07.2009)

PCT

(10) International Publication Number
WO 2009/090032 A1

(51) International Patent Classification:

C07K 16/28 (2006.01) A61K 39/395 (2006.01)

(21) International Application Number:

PCT/EP2009/000133

(22) International Filing Date: 13 January 2009 (13.01.2009)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

61/011,348 15 January 2008 (15.01.2008) US

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

(72) Inventors; and

(75) Inventors/Applicants (for US only): **AUER, Johannes** [DE/DE]; Angerstrasse 7, 82445 Schwaigen (DE). **BERSON, Amy** [US/US]; 3102 Stelling Drive, Palo Alto, CA 94303 (US). **BRANDT, Michael** [DE/DE]; Faltergatter 29, 82393 Iffeldorf (DE). **LORA, Jose, M.** [ES/GB]; East Heath Road, Flat B, Hampstead, London NW3 1BN (GB). **RIES, Stefan, Ries** [DE/DE]; St. Klara Strasse 9, 82377 Penzberg (DE). **BORIE, Dominique, Christian** [US/US]; 1510 Middlefield Road, Palo Alto, CA 94301 (US).(74) Agent: **BURGER, Alexander**; Roche Diagnostics GmbH, Patent Department (TR-E), Postfach 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, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, 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, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (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, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declaration under Rule 4.17:

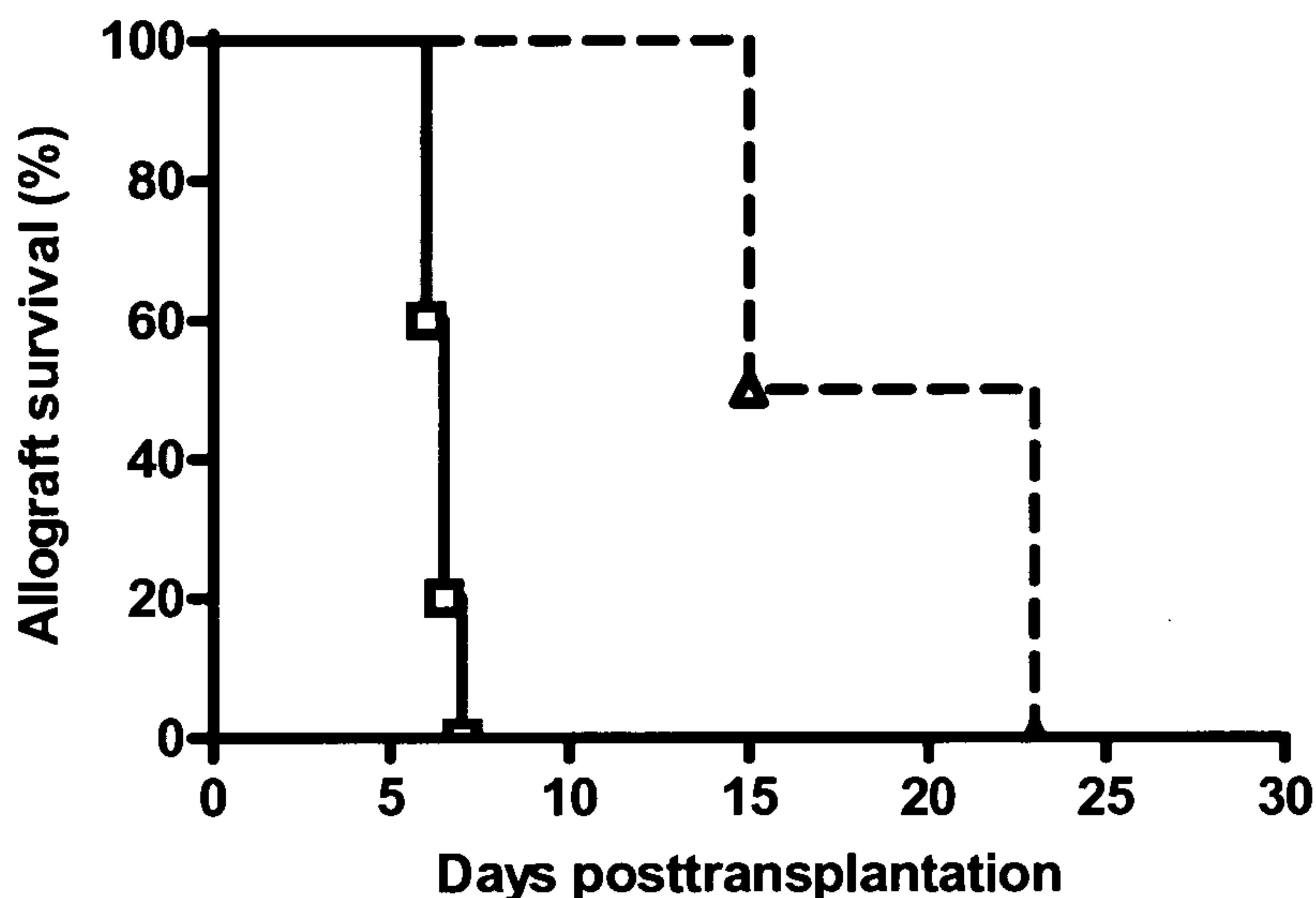
— of inventorship (Rule 4.17(iv))

Published:

— with international search report

[Continued on next page]

(54) Title: AFUCOSYLATED ANTIBODIES AGAINST CCR5 AND THEIR USE

Fig. 5

(57) Abstract: An antibody binding to CCR5 and being glycosylated with a sugar chain at Asn297, said antibody being characterized in that the amount of fucose within said sugar chain is 65 % or lower has improved properties in anti-inflammatory therapy.

WO 2009/090032 A1

WO 2009/090032 A1



— *with (an) indication(s) in relation to deposited biological material furnished under Rule 13bis separately from the description*

— *with sequence listing part of description published separately in electronic form and available upon request from the International Bureau*

Afucosylated antibodies against CCR5 and their use

The present invention relates to afucosylated antibodies against CCR5, methods for
5 their production, pharmaceutical compositions containing said antibodies, and
their use for the treatment of inflammatory conditions, such as acute and chronic
transplant rejection.

Background of the Invention

Chemokines and their receptors are known to participate in allograft rejection by
10 mediating leukocyte trafficking. Panzer, U., et al. (Transplantation 78 (2004) 1341-
50) reported CCR5-positive T cell recruitment in acute human allograft rejection,
and Luckow, B., et al. (Eur. J. Immunol. 34 (2004) 2568-78) observed decreased
intragraft levels of metalloproteinases and arteriosclerosis in CCR5-deficient
animals. Further, Gao, W., et al. (Transplantation 72 (2001) 1199-1205)
15 demonstrated prolonged allograft survival in mice treated with CCR5-specific
monoclonal antibody and in CCR5-deficient mice. Schroeder, C., et al., J. Immunol.
179 (2007) 2289-2299, explored the effects of a CCR5 antagonist in a cynomolgus
monkey cardiac allograft model for investigation of CCR5 modulation during
inflammation and alloimmunity. Moreover, a retrospective study in human
20 transplant recipient cohorts uncovered that CCR5-deficient patients (delta 32)
showed prolonged allograft survival (Fischereder, M., et al., Lancet 357 (2001)
1758-1761).

In experimental models, due to the redundancy of receptor-ligand interaction, the
deficiency or blockade of a single chemokine does not protect the allograft from
25 acute rejection (Fischereder, M., et al., Lancet 357 (2001) 1758-1761; Gao, W., et al.,
Transplantation 72 (2001) 1199-1205; Hancock, W.W., et al., Curr. Opin. Immunol.
12 (2000) 511-516; Hancock, W.W., et al., Curr. Opin. Immunol. 15 (2003) 479-
486).

WO 01/78707 refers to a method of inhibiting graft rejection comprising
30 administering an antagonist of CCR5 function.

Cell-mediated effector functions of monoclonal antibodies can be enhanced by
engineering their oligosaccharide component as described in Umana, P., et al.,
Nature Biotechnol. 17 (1999) 176-180, and US 6,602,684. IgG1 type antibodies, the
most commonly used antibodies in cancer immunotherapy, are glycoproteins that

- 2 -

have a conserved N-linked glycosylation site at Asn297 in each C_H2 domain. The two complex biantennary oligosaccharides attached to Asn297 are buried between the C_H2 domains, forming extensive contacts with the polypeptide backbone, and their presence is essential for the antibody to mediate effector functions such as antibody dependent cellular cytotoxicity (ADCC) (Lifely, M.R., et al., *Glycobiology* 5 (1995) 813-822; Jefferis, R., et al., *Immunol. Rev.* 163 (1998) 59-76; Wright, A. and Morrison, S.L., *Trends Biotechnol.* 15 (1997) 26-32). Umana, P., et al., *Nature Biotechnol.* 17 (1999) 176-180 and WO 99/54342 showed that overexpression in Chinese hamster ovary (CHO) cells of β (1,4)-N-acetylglucosaminyltransferase III ("GnTIII"), a glycosyltransferase catalyzing the formation of bisected oligosaccharides, significantly increases the in vitro ADCC activity of antibodies. Alterations in the composition of the N297 carbohydrate or its elimination affect also binding to Fc γ R and C1q (Umana, P., et al., *Nature Biotechnol.* 17 (1999) 176-180; Davies, J., et al., *Biotechnol. Bioeng.* 74 (2001) 288-294; Mimura, Y., et al., *J. Biol. Chem.* 276 (2001) 45539-45547; Radaev, S., et al., *J. Biol. Chem.* 276 (2001) 16478-16483; Shields, R.L., et al., *J. Biol. Chem.* 276 (2001) 6591-6604; Shields, R.L., et al., *J. Biol. Chem.* 277 (2002) 26733-26740; Simmons, L.C., et al., *J. Immunol. Methods* 263 (2002) 133-147).

Iida, S., et al., *Clin. Cancer Res.* 12 (2006) 2879-2887, show that efficacy of a non-fucosylated anti-CD20 antibody was inhibited by addition of fucosylated anti-CD20 antibodies. The efficacy of a 1:9 mixture (10 μ g/ml) of non-fucosylated and fucosylated anti-CD20 antibodies was inferior to that of a 1,000-fold dilution (0.01 μ g/mL) of non-fucosylated anti-CD20 antibody alone. They conclude that non-fucosylated IgG1, not including fucosylated counterparts, can evade the inhibitory effect of plasma IgG on ADCC through its high Fc γ RIIIa binding. Natsume, A., et al., show in *J. Immunol. Methods* 306 (2005) 93-103, that fucose removal from complex-type oligosaccharide of human IgG1-type antibody results in a great enhancement of antibody-dependent cellular cytotoxicity (ADCC). Satoh, M., et al., *Expert Opin. Biol. Ther.* 6 (2006) 1161-1173, discuss non-fucosylated therapeutic antibodies as next-generation therapeutic antibodies. Satoh concludes that antibodies consisting of only the non-fucosylated human IgG1 form are thought to be ideal. Kanda, Y., et al., *Biotechnol. Bioeng.* 94 (2006) 680-688, compared fucosylated anti-CD20 antibody (96 % fucosylation, CHO/DG44 1H5) with non-fucosylated anti-CD20 antibody. Davies, J., et al., *Biotechnol. Bioeng.* 74 (2001) 288-294, report that for an anti-CD20 antibody increased ADCC correlates with increased binding to Fc γ RIII.

- 3 -

Methods to enhance cell-mediated effector functions of monoclonal antibodies are described e.g. in WO 2005/018572, WO 2006/116260, WO 2006/114700, WO 2004/065540, WO 2005/011735, WO 2005/027966, WO 1997/028267, US 2006/0134709, US 2005/0054048, US 2005/0152894, WO 2003/035835, and
5 WO 2000/061739.

Summary of the Invention

The invention comprises an antibody binding to CCR5, and being glycosylated with a sugar chain at Asn297, said antibody being characterized in that the amount of fucose within said sugar chain is 65 % or lower.

10 The invention comprises an antibody binding to CCR5, and being glycosylated with a sugar chain at Asn297, said antibody being characterized in that the amount of fucose within said sugar chain is in one embodiment between 5 % and 65 %, in another embodiment between 20 % and 40 %.

Antibodies according to the invention comprising such amount of fucose are
15 further termed as afucosylated.

The invention comprises an antibody binding to CCR5, and being glycosylated with a sugar chain at Asn297, said antibody being characterized in showing high binding affinity to the FcγRIII.

In one embodiment the antibody is of human IgG1, or IgG3 type.

20 In another embodiment the amount of NGNA is 1 % or less and/ or the amount of N-terminal alpha-1,3-galactose is 1 % or less within said sugar chain.

In a further embodiment the amount of NGNA is 0.5 % or less, and in still a further embodiment 0.1 % or less, and in another embodiment even not detectable (LCMS).

25 In one embodiment the amount of N-terminal alpha-1,3-galactose within said sugar chain is 0.5 % or less, in a further embodiment 0.1 % or less, and in still a further embodiment even not detectable (LCMS).

In one embodiment the antibody binding to CCR5 is a T-cell epitope depleted antibody, or a monospecific tetravalent antibody, or a multispecific antibody.

- 4 -

The sugar chain shows the characteristics of N-linked glycans attached to Asn297 of an antibody binding to CCR5 recombinantly expressed in a CHO cell.

The invention comprises an afucosylated antibody binding to CCR5, characterized in that said antibody binds to human CCR5, blocks its function so that CCR5+ T-
5 cells in vitro and in vivo are depleted.

Antibodies according to the invention show benefits for patients in need of inhibiting graft rejection.

The antibody is in one embodiment a monoclonal antibody and, in another embodiment, a chimeric antibody (human constant chain), or a humanized
10 antibody, or in still another embodiment a human antibody.

The invention further comprises a pharmaceutical composition containing an antibody according to the invention, optionally together with a buffer and/or an adjuvant useful for the formulation of antibodies for pharmaceutical purposes.

The invention further comprises a pharmaceutical composition comprising an
15 antibody according to the invention.

The invention further provides pharmaceutical compositions comprising an antibody according to the invention and a pharmaceutically acceptable carrier. In one embodiment, the pharmaceutical composition may be included in an article of manufacture or kit. The invention further provides the use of an antibody
20 according to the invention for the manufacture of a pharmaceutical composition for the treatment of graft rejection. The antibody is used in a pharmaceutically effective amount.

The invention further comprises the use of an antibody according to the invention for the manufacture of a pharmaceutical composition for the prevention of allograft
25 rejection and inflammation and immune-mediated diseases. In one embodiment said disease is rheumatoid arthritis (RA) or Chronic Obstructive Pulmonary Disease (COPD), or granulomatous colitis and regional enteritis (Crohn's disease). The antibody is used in a pharmaceutically effective amount.

The invention further comprises a method for the production of a recombinant
30 human antibody according to the invention, characterized by expressing a nucleic acid encoding an antibody binding to CCR5 in a CHO host cell, which fucosylates said antibody in an amount according to the invention, and recovering said

- 5 -

antibody from said cell. The invention further comprises the antibody obtainable by such a recombinant method.

The invention further comprises a CHO cell capable of recombinantly expressing $\beta(1,4)$ -N-acetylglucosaminyltransferase III (GnTIII), optionally also mannosidase II (ManII), and an anti-CCR5 antibody. Such a CHO cell is a CHO cell, transformed with a first DNA sequence encoding a polypeptide having GnTIII activity and optionally a polypeptide having ManII activity, a second DNA sequence encoding at least the variable domain of the heavy chain of an antibody against CCR5, and a third DNA sequence encoding at least the variable domain of the light chain of an antibody against CCR5. In one embodiment the second and third DNA sequences encode the heavy and light chain of an antibody against CCR5 of human IgG1 type.

The invention further comprises a process for the production of an antibody against CCR5 showing the properties according to the invention, comprising the steps of transforming a host cell, preferably a CHO cell, with a first DNA sequence encoding a polypeptide having GnTIII activity, a second DNA sequence encoding at least the variable domain of the heavy chain of an antibody against CCR5, and a third DNA sequence encoding at least the variable domain of the light chain of an antibody against CCR5, cultivating in a fermentation medium said host cell, which expresses, in one embodiment independently, said first, second and third DNA sequences, under conditions that said host cell secretes said antibody to the fermentation medium, and isolating said antibody from the fermentation medium.

The present invention further relates to a method of treating or preventing acute and chronic organ transplant rejection (allograft, xenograft) in a mammal, including a human, comprising administering to said mammal an antibody against CCR5 according to the invention.

Detailed Description of the Invention

The term "CCR5" denotes a human chemokine receptor (see e.g. Swiss Prot P51681 and Mueller, A., and Strange, P.G., Int. J. Biochem. Cell Biol. 36 (2004) 35-38). The term "CCR5 antibody" means an antibody against CCR5, an anti-CCR5 antibody.

The term "antibody" encompasses the various forms of antibodies including but not being limited to whole antibodies, antibody fragments, human antibodies,

- 6 -

humanized antibodies and genetically engineered antibodies as long as the characteristic properties according to the invention are retained.

"Antibody fragments" comprise a portion of a full length antibody, generally at least the antigen binding portion or the variable region thereof. Examples of antibody
5 fragments include diabodies, single-chain antibody molecules, conjugates, e.g. immunotoxins, and multispecific antibodies comprising antibody fragments.

The terms "monoclonal antibody" or "monoclonal antibody composition" as used herein refer to a preparation of antibody molecules of a single amino acid composition. Accordingly, the term "human monoclonal antibody" refers to
10 antibodies displaying a single binding specificity which have variable framework regions and constant regions derived from human germline immunoglobulin sequences.

The term "chimeric antibody" refers to a monoclonal antibody comprising a variable region, i.e. binding region, from one source or species and at least a portion
15 of a constant region derived from a different source or species, usually prepared by recombinant DNA techniques. Chimeric antibodies comprising a murine variable region and a human constant region are especially preferred. Such murine/human chimeric antibodies are the product of expressed immunoglobulin genes comprising DNA segments encoding murine immunoglobulin variable regions and
20 DNA segments encoding human immunoglobulin constant regions. Other forms of "chimeric antibodies" encompassed by the present invention are those in which the class or subclass has been modified or changed from that of the original antibody. Such "chimeric" antibodies are also referred to as "class-switched antibodies." Methods for producing chimeric antibodies involve conventional recombinant
25 DNA techniques and gene transfection techniques now well known in the art. See, e.g., Morrison, S.L., et al., Proc. Natl. Acad. Sci. USA 81 (1984) 6851-6855, US 5,202,238, and US 5,204,244.

The term "humanized antibody" refers to antibodies in which the framework regions (FRs) and/or "complementarity determining regions" (CDRs) have been
30 modified to comprise the CDR of an immunoglobulin of different specificity as compared to that of the parent immunoglobulin. In one embodiment, a murine CDR is grafted into the framework region of a human antibody to prepare a "humanized antibody." See, e.g., Riechmann, L., et al., Nature 332 (1988) 323-327; and Neuberger, M.S., et al., Nature 314 (1985) 268-270. Particularly the CDRs

- 7 -

correspond to those representing sequences recognizing the antigens noted herein for chimeric and bifunctional antibodies. In one embodiment said antigens are epitopes of CCR5.

The term "human antibody", as used herein, is intended to include antibodies
5 having variable and constant regions derived from human germline immunoglobulin sequences. The variable heavy chain region is in one embodiment derived from germline sequence DP-50 (GenBank LO6618) and the variable light chain region is derived from germline sequence L6 (GenBank X01668) or the variable heavy chain region is derived DP-61 (GenBank M99682) and the variable
10 light chain region is derived from germline sequence L15 (GenBank K01323). The constant regions of the antibody are constant regions of human IgG1 type. Such regions can be allotypic and are described by, e.g., Johnson, G. and Wu, T.T., Nucleic Acids Res. 28 (2000) 214-218, and the databases referenced therein.

The term "recombinant human antibody", refers to antibodies having variable and
15 constant regions derived from human germline immunoglobulin sequences in a rearranged form. The recombinant human antibodies according to the invention have been subjected to in vivo somatic hypermutation. Thus, the amino acid sequences of the variable heavy chain regions (VH) and variable light chain regions (VL) of the recombinant antibodies are sequences that, while been derived from
20 and are related to human germline VH and VL sequences, may not naturally exist within the human antibody germline repertoire in vivo.

As used herein, the term "binding" refers to antibody binding to CCR5 with an affinity of about 10^{-13} to 10^{-8} M (K_D), in one embodiment of about 10^{-13} to 10^{-9} M.

The term "nucleic acid molecule", as used herein, is intended to include DNA
25 molecules and RNA molecules. A nucleic acid molecule may be single-stranded or double-stranded, but preferably is double-stranded DNA.

Human constant regions having IgG1 or IgG3 type are described in detail by Kabat, et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991), and by Brüggemann, M., et al., J. Exp. Med. 166 (1987) 1351-1361; Love, T.W., et al., Methods Enzymol.
30 178 (1989) 515-527. Examples are shown in SEQ ID NO: 01 to 03.

Constant regions of human IgG1, IgG2 or IgG3 type are glycosylated at Asn297. "Asn297" according to the invention means amino acid asparagine located at about

- 8 -

position 297 in the Fc region; based on minor sequence variations of antibodies, Asn297 can also be located some amino acids (usually not more than ± 3 amino acids) upstream or downstream of position 297, i.e. between position 294 and position 300.

- 5 The term "variable region" (variable region of a light chain (VL), variable region of a heavy chain (VH)) as used herein denotes each member of the pair of light and heavy chain domains, which is involved directly in binding of the antibody to the antigen. The variable regions of human light and heavy chains have the same general structure, each comprising four "framework regions" (FR), whose
10 sequences are widely conserved, connected by three "hypervariable regions" (or complementarity determining regions, CDRs). The framework regions adopt a β -sheet conformation and the CDRs may form loops connecting the β -sheet structure. The CDRs in each chain are held in their three-dimensional structure by the framework regions and form together with the CDRs from the other chain the
15 antigen binding site. The CDR3 regions of antibody heavy and light chain variable regions are particularly important in the binding specificity/affinity of the antibodies according to the invention and therefore provide a further aspect of the invention.

- The terms "hypervariable region" or "antigen-binding portion of an antibody" when
20 used herein refer to the amino acid residues of an antibody which are responsible for antigen-binding. The hypervariable region comprises amino acid residues from the "complementarity determining regions" or "CDRs". "Framework" or "FR" regions are those variable domain regions other than the hypervariable region residues as herein defined. Therefore, the light and heavy chains of an antibody
25 comprise from N- to C-terminus the domains FR1, CDR1, FR2, CDR2, FR3, CDR3, and FR4. Especially, CDR3 of the heavy chain is the region which contributes most to antigen binding and characterizes the antibody. CDR and FR regions are determined according to the standard definition of Kabat, et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National
30 Institutes of Health, Bethesda, MD. (1991)) and/or are those residues forming a "hypervariable loop".

- The term "epitope" denotes a protein determinant capable of specific binding to an antibody. Epitopes usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and usually have specific three
35 dimensional structural characteristics, as well as specific charge characteristics.

- 9 -

Conformational and non-conformational epitopes are distinguished in that the binding to the former but not the latter is lost in the presence of denaturing agents.

Antibody A (DSM ACC 2683) binds to an epitope including amino acids on the ECL2 domain of CCR5 (Lee, B., et al., J. Biol. Chem. 274 (1999) 9617-9626) which
5 is different from the epitope recognized by antibody 2D7 (2D7 binds to amino acids K171 and E172 of ECL2A but not to ECL2B amino acids 184-189). Epitope binding for antibody A is found to be 20 % for CCR5 mutant K171A or E172A (if glu172 is mutated to ala). 100 % epitope binding is defined for wild-type CCR5. A further embodiment of the invention is therefore an afucosylated antibody binding
10 to CCR5 and binding to the same epitope as antibody A does. Binding inhibition can be detected by an SPR assay using immobilized antibody A and CCR5 at a concentration of 20-50 nM and the antibody to be detected at a concentration of 100 nM. A signal reduction of 50 % or more shows that the antibody competes with antibody A. Epitope binding can also be investigated by using alanine
15 mutation of CCR5 according to the method described by Olson, W.C., et al., J. Virol. 73 (1999) 4145-4155, for epitope mapping. A signal reduction of 75 % or more shows that the mutated amino acid(s) contribute to the epitope of said antibody. Binding to the same epitope is found, if one or more of the amino acids contributing to the epitope of the investigated antibody are identical to one or more
20 amino acids contributing to the epitope of antibody A. In one embodiment the antibody according to the invention binds to the ECL2 domain of CCR5.

Amino acid sequences of preferred CCR5 antibodies according to the invention are described in WO 2006/103100 and WO 2008/037419.

The invention comprises an afucosylated antibody binding to CCR5 characterized
25 in that the variable heavy chain amino acid sequence CDR3 of said antibody is selected from the heavy chain CDR3 sequences SEQ ID NO: 04 or 05.

The antibody is in one embodiment characterized in containing as heavy chain CDRs the CDRs of SEQ ID NO: 06 and as light chain CDRs the CDRs of SEQ ID NO: 07, as heavy chain CDRs the CDRs of SEQ ID NO: 08 and as light chain CDRs
30 the CDRs of SEQ ID NO: 09, as heavy chain CDRs the CDRs of SEQ ID NO: 10 and as light chain CDRs the CDRs of SEQ ID NO: 11, as heavy chain CDRs the CDRs of SEQ ID NO: 12 and as light chain CDRs the CDRs of SEQ ID NO: 13, as heavy chain CDRs the CDRs of SEQ ID NO: 14 and as light chain CDRs the CDRs of SEQ ID NO: 15, as heavy chain CDRs the CDRs of SEQ ID NO: 16 and as light chain

- 10 -

CDRs the CDRs of SEQ ID NO: 19, as heavy chain CDRs the CDRs of SEQ ID NO: 16 and as light chain CDRs the CDRs of SEQ ID NO: 20, as heavy chain CDRs the CDRs of SEQ ID NO: 17 and as light chain CDRs the CDRs of SEQ ID NO: 19, as heavy chain CDRs the CDRs of SEQ ID NO: 17 and as light chain CDRs the CDRs of SEQ ID NO: 20, as heavy chain CDRs the CDRs of SEQ ID NO: 18 and as light chain CDRs the CDRs of SEQ ID NO: 19, or as heavy chain CDRs the CDRs of SEQ ID NO: 18 and as light chain CDRs the CDRs of SEQ ID NO: 20.

The invention in another embodiment comprises an afucosylated antibody binding to CCR5, characterized in that the heavy chain variable domain comprises an amino acid sequence of the formula

Gln-Val-Gln-Leu-X01-X02-Ser-Gly-Pro-Gly-Leu-Val-X03-Pro-Ser-Gln-Ser-Leu-Ser-Ile-Thr-Cys-Thr-Val-Ser-Gly-Phe-Pro-Leu-Gly-Ala-Phe-Gly-Val-His-Trp-Val-Arg-Gln-Ser-Pro-Gly-Lys-Gly-X04-Glu-Trp-Leu-Gly-Val-Ile-Trp-Lys-Gly-Gly-Asn-Thr-Asp-Tyr-Asn-Ala-Ala-Phe-X05-Ser-Arg-Leu-Arg-Ile-Thr-Lys-Asp-Asn-Ser-Lys-Ser-Gln-Val-Phe-Phe-Arg-Met-Asn-Ser-Leu-Gln-Thr-Asp-Asp-Thr-Ala-X06-Tyr-Tyr-Cys-Ala-Lys-Val-Asn-Leu-Ala-Asp-Ala-Met-Asp-Tyr-Trp-Gly-Gln-Gly-Thr-X07-Val-X08-Val-Ser-Ser,

wherein

X01 is Lys or Gln,
 X02 is Gln or Glu,
 X03 is Arg or Lys,
 X04 is Leu or Pro,
 X05 is Met or Lys,
 X06 is Ile or Thr,
 X07 is Ser or Thr,
 X08 is Ile or Thr
 (SEQ ID NO: 14).

In one embodiment this antibody is characterized in that the light chain variable domain of said antibody comprises an amino acid sequence of the formula

Asp-Ile-Gln-Met-Thr-Gln-Ser-Pro-Ala-Ser-Leu-Ser-Ala-Ser-Val-Gly-Glu-Thr-Val-Thr-Ile-Thr-Cys-Arg-Ala-Ser-Gly-Asn-X10-His-Gly-Tyr-Leu-Ala-Trp-X11-Gln-Gln-Lys-X12-Gly-Lys-X13-Pro-X14-Leu-Leu-X15-Tyr-Asn-Thr-Lys-Thr-Leu-Ala-Glu-Gly-Val-Pro-Ser-Arg-Phe-Ser-Gly-Ser-Gly-Ser-Gly-Thr-X16-Phe-X17-X18-X19-Ile-X20-Ser-X21-Gln-Pro-Glu-Asp-Phe-X22-X23-Tyr-Tyr-Cys-Gln-His-His-

- 11 -

Tyr-Asp-Leu-Pro-Arg-Thr-Phe-Gly-Gly-Gly-Thr-Lys-X24-Glu-Ile-Lys,
wherein

X10 is Ile or Ala,

X11 is Phe or Tyr,

5 X12 is Gln or Pro,

X13 is Ser or Ala,

X14 is Gln or Lys,

X15 is Val or Ile,

X16 is Gln or Asp,

10 X17 is Ser or Thr,

X18 is Leu or Ala,

X19 is Lys or Thr,

X20 is Asn or Ser,

X21 is Leu or Ala,

15 X22 is Gly or Ala,

X23 is Asn or Thr,

X24 is Leu or Val

(SEQ ID NO: 15).

20 The antibody according to the invention is in one embodiment characterized in that said antibody binds to CCR5 and comprises a variable heavy or light chain domain selected from the group of variable domains comprising heavy chain variable domains of SEQ ID NO: 14, light chain variable domains of SEQ ID NO: 15, or a CCR5-binding fragment thereof.

25 The antibody according to the invention is in one embodiment characterized in that the constant regions (light and heavy chains) are of human origin. Such constant regions (chains) are well known in the state of the art and e.g. described by Kabat (see e.g. Johnson, G. and Wu, T.T., Nucleic Acids Res. 28 (2000) 214-218). For example, a useful human heavy chain constant region comprises an amino acid
30 sequence independently selected from SEQ ID NO: 01 or 02. For example, a useful human light chain constant region comprises an amino acid sequence of a kappa-light chain constant region of SEQ ID NO: 03. In a further embodiment is the antibody of mouse origin and comprises the antibody variable sequence frame of a mouse antibody according to Kabat (see e.g. Johnson, G. and Wu, T.T., Nucleic
35 Acids Res. 28 (2000) 214-218).

- 12 -

The antibody according to the invention inhibits one or more functions of human CCR5, such as ligand binding to CCR5, signaling activity (e.g. activation of a mammalian G protein, induction of a rapid and transient increase in the concentration of cytosolic free Ca^{2+} , and/or stimulation of a cellular response (e.g. stimulation of chemotaxis, exocytosis or inflammatory mediator release by leukocytes, integrin activation)). The antibodies inhibit binding of RANTES, MIP-1 alpha, and/or MIP-1 beta, to human CCR5 and/or inhibit functions mediated by human CCR5, like leukocyte trafficking, T cell activation, inflammatory mediator release, and/or leukocyte degranulation.

- 10 An antibody according to the invention in one embodiment does not inhibit chemokine binding in a binding assay to CCR1, CCR2, CCR3, CCR4, CCR6, and CXCR4 in an antibody concentration up to 100 $\mu\text{g/ml}$.

Glycosylation of human IgG1 or IgG3 occurs at Asn297 as core fucosylated biantennary complex oligosaccharide, whereby glycosylation is terminated with up to two Gal residues. These structures are designated as G0, G1 (α -1,6- or α -1,3-), or G2 glycan residues, depending from the amount of terminal Gal residues (Raju, T. S., Bioprocess Int., April 2003, 44-53). CHO type glycosylation of antibody Fc parts is e.g. described by Routier, F. H., Glycoconjugate J. 14 (1997) 201-207. Antibodies which are recombinantly expressed in non-glycomodified CHO host cells usually are fucosylated at Asn297 in an amount of at least 85 % (mol-%, molar percentage).

According to the invention the term "amount of fucose" means the amount of fucose within the sugar chain at Asn297, with respect to the sum of all sugar residues attached to Asn297 (e.g. complex, hybrid and high mannose structures) when measured by MALDI-TOF mass spectrometry and calculated as average value (see example 8). The relative amount of fucose is the percentage of fucose-containing structures related to all glycostructures identified in an N-Glycosidase F treated sample (e.g. complex, hybrid- and oligo- and high-mannose structures, resp.) by MALDI-TOF.

The afucosylated anti-CCR5 antibody according to the invention can be expressed in a glycomodified host cell engineered to express at least one nucleic acid encoding a polypeptide having GnTIII activity, optionally also a nucleic acid encoding a polypeptide having ManII activity, in order to fucosylate the Fc region of an antibody according to the invention in an amount according to the invention. In one embodiment, the polypeptide having GnTIII activity is a fusion polypeptide.

- 13 -

Alternatively the α 1,6-fucosyltransferase activity of the host cell can be decreased or eliminated according to US 6,946,292 to generate glycomodified host cells. The amount of antibody fucosylation can be predetermined e.g. either by fermentation conditions or by combination of at least two antibodies with different fucosylation amount.

The anti-CCR5 antibody according to the invention can be produced in a host cell by a method comprising: (a) culturing a host cell engineered to express at least one polynucleotide encoding a polypeptide having GnTIII activity, and optionally a polynucleotide encoding a polypeptide having ManII activity, under conditions which permit the production of said antibody with an amount of fucose (of the oligosaccharide(s) present on the Fc region of said antibody) according to the invention; and (b) isolating said antibody from the cell or the cultivation medium. In one embodiment, the polypeptide having GnTIII activity is a fusion polypeptide, in another embodiment the fusion polypeptide comprises the catalytic domain of GnTIII and the Golgi localization domain of a heterologous Golgi resident polypeptide selected from the group consisting of the localization domain of mannosidase II, the localization domain of β (1,2)-N-acetylglucosaminyltransferase I ("GnTI"), the localization domain of mannosidase I, the localization domain of β (1,2)-N-acetylglucosaminyltransferase II ("GnTII"), and the localization domain of α (1-6) core fucosyltransferase. In one embodiment the Golgi localization domain is obtained from mannosidase II or GnTI.

In a further aspect, the invention is directed to a method for modifying the glycosylation profile of an anti-CCR5 antibody by using the above method. In this aspect, the invention is directed to a method for modifying the glycosylation of an anti-CCR5 antibody by using a fusion polypeptide having GnTIII activity and comprising the Golgi localization domain of a heterologous Golgi resident polypeptide. In one embodiment, the fusion polypeptides of the invention comprise the catalytic domain of GnTIII. In another embodiment, the Golgi localization domain is selected from: the localization domain of mannosidase II, the localization domain of GnTI, the localization domain of mannosidase I, the localization domain of GnTII, or the localization domain of α (1-6) core fucosyltransferase. In one embodiment the Golgi localization domain is obtained from mannosidase II or GnTI.

According to the present invention, these modified oligosaccharides of the anti-CCR5 antibody may be hybrid or complex. In one embodiment the bisected, non-

- 14 -

fucosylated oligosaccharides are hybrid. In another embodiment, the bisected, non-fucosylated oligosaccharides are complex.

As used herein, a "polypeptide having GnTIII activity" refers to polypeptides that are able to catalyze the addition of an N-acetylglucosamine (GlcNAc) residue in β -1-4 linkage to the β -linked mannoside of the trimannosyl core of N-linked oligosaccharides. This includes fusion polypeptides exhibiting enzymatic activity similar to, but not necessarily identical to, an activity of β (1,4)-N-acetylglucosaminyltransferase III, also known as β -1,4-mannosyl-glycoprotein 4-beta-N-acetylglucosaminyl-transferase (EC 2.4.1.144), according to the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB), as determined in a particular biological assay, with or without dose dependency. In the case where dose dependency does exist, it need not be identical to that of GnTIII, but rather substantially similar to the dose-dependence in a given activity as compared to the GnTIII (i.e. the candidate polypeptide will exhibit greater activity or not more than about 25-fold less and, in one embodiment, not more than about tenfold less activity, and, in a further embodiment, not more than about three-fold less activity relative to the GnTIII). As used herein, the term "Golgi localization domain" refers to the amino acid sequence of a Golgi resident polypeptide which is responsible for anchoring the polypeptide in location within the Golgi complex. Generally, localization domains comprise amino terminal "tails" of an enzyme.

The antibodies according to the invention show high binding affinity to the Fc gamma receptor III (Fc γ RIII, CD16a). High binding affinity to Fc γ RIII denotes that binding is enhanced for CD16a/F158 at least 10-fold in relation to the wildtype anti-CCR5 antibody (95 % fucosylation) as reference (see example 5) expressed in CHO host cells, such as CHO DG44 or CHO K1 cells, or/and binding is enhanced for CD16a/V158 at least 20-fold in relation to the wildtype anti-CCR5 antibody measured by Surface Plasmon Resonance (SPR) using immobilized CD16a at an antibody concentration of 100 nM (see example 3). Fc γ RIII binding can be increased by methods according to the state of the art, e.g. by modifying the amino acid sequence of the Fc part or the glycosylation of the Fc part of the antibody.

The term "binding to CCR5" as used herein denotes the binding of the antibody to CCR5 in an in vitro assay, in one embodiment in a binding assay in which the antibody is bound to a surface and binding of CCR5 is measured by Surface Plasmon Resonance (SPR). Binding means a binding affinity (K_D) of 10^{-8} M or less,

- 15 -

in one embodiment of 10^{-13} M to 10^{-9} M. Binding of the antibody to CCR5 or FcγRIII can be investigated by a BIAcore assay (Pharmacia Biosensor AB, Uppsala, Sweden). The affinity of the binding is defined by the terms k_a (rate constant for the association of the antibody from the antibody/antigen complex), k_d (dissociation constant), and K_D (k_d/k_a). The antibodies according to the invention
5 show a K_D of 10^{-8} M or less for the binding to CCR5.

The term "CCR5 expressing cells" refers to such cells which are

- a) naturally expressing CCR5 (such as CD4+ and CD8+ T-cells, as well as monocytes and other immune cells),
 - 10 b) recombinant, engineered mouse L1.2 cells (ATCC HB204) and CHO cells (CHO K1 – ATCC CCL-61, CHO DG44 – Urlaub et al. Cell 33 (1983) 405-412), or other cell lines,
 - c) cells expressing CCR5 after stimulation with cytokines, HIV, sodium butyrate or other stimuli.
- 15 The term "CCR5+" denotes cell expressing and presenting the chemokine receptor CCR5 on its outer cell membrane surface.

The term "antibody-dependent cellular cytotoxicity (ADCC)" refers to lysis of human target cells by an antibody according to the invention in the presence of effector cells. ADCC is measured in one embodiment by the treatment of a
20 preparation of CCR5 expressing cells with an antibody according to the invention in the presence of effector cells such as freshly isolated PBMC or purified effector cells from buffy coats, like monocytes or natural killer (NK) cells or a permanently growing NK cell line.

As used herein, the term "host cell" denotes any kind of cellular system which can
25 be engineered to generate the polypeptides and antigen-binding molecules of the present invention. In one embodiment, the host cell is able to and engineered to allow the production of an antigen binding molecule with modified glycoforms. The host cells have been further manipulated to express increased levels of one or more polypeptides having GnTIII activity. In one embodiment the host cell is a
30 CHO cell.

For the protein expression in the host cell, nucleic acids encoding light and heavy chains or fragments thereof are inserted into expression vectors by standard

- 16 -

methods. Expression is performed in such host cells, and the antibody is recovered from the cells (supernatant or cells after lysis). The general methods for recombinant production of antibodies are well-known in the state of the art and described, for example, in the review articles of Makrides, S.C., *Protein Expr. Purif.* 17 (1999) 183-202; Geisse, S., et al., *Protein Expr. Purif.* 8 (1996) 271-282; Kaufman, R.J., *Mol. Biotechnol.* 16 (2000) 151-160; Werner, R.G., *Drug Res.* 48 (1998) 870-880.

The antibodies may be present in whole cells, in a cell lysate, or in a purified form. Purification is performed in order to eliminate other cellular components or contaminants, e.g. cellular nucleic acids or proteins, by standard techniques, including alkaline/SDS treatment, CsCl banding, column chromatography, agarose gel electrophoresis, and others well known in the art. See e.g. Ausubel, F., et al., ed. *Current Protocols in Molecular Biology*, Greene Publishing and Wiley Interscience, New York (1987).

The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to utilize promoters, enhancers and polyadenylation signals.

A nucleic acid is "operably linked" when it is placed in a functional relationship with another nucleic acid sequence. For example, DNA for a pre-sequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a pre-protein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading frame. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

The monoclonal antibodies are suitably separated from the culture medium by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose chromatography, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography. DNA and RNA encoding the

- 17 -

monoclonal antibody are readily isolated and sequenced using conventional procedures. The hybridoma cells can serve as a source of such DNA and RNA.

Aspects of the invention is a method for the treatment or prevention of a patient suffering from allograft rejection and for the treatment of inflammation and other
5 immune-mediated diseases, characterized by administering to the patient an antibody according to the invention. Likewise are the use of an antibody for the manufacture of a medicament for the treatment or prevention of allograft rejection or for the treatment of inflammation or for the treatment of other immune-mediated diseases aspects of the current invention.

10 Also an embodiment of the invention is a method for the treatment of a patient suffering from graft rejection or graft versus host disease, characterized by administering to the patient an antibody according to the invention. Likewise is the use of an antibody according to the invention for the manufacture of a medicament for the treatment of graft rejection or graft versus host disease an aspect of the
15 current invention.

One aspect of the invention is a pharmaceutical composition comprising an antibody according to the invention. Another aspect of the invention is the use of an antibody according to the invention for the manufacture of a pharmaceutical composition. A further aspect of the invention is a method for the manufacture of a
20 pharmaceutical composition comprising an antibody according to the invention and optionally a pharmaceutically acceptable excipient or carrier. In one embodiment the pharmaceutical composition comprises a combination of an antibody according to the invention and an immunosuppressive agent.

The term "immunosuppressive agent" denotes a compound that when
25 administered to an organism reduces or suppresses the immune response of said organism. Examples of immunosuppressive agents are calcineurin inhibitors, such as Cyclosporin A. Thus, in one embodiment the immunosuppressive agent is a calcineurin inhibitor. In another embodiment is the immunosuppressive agent Cyclosporin A.

30 In another aspect, the present invention provides a composition, e.g. a pharmaceutical composition, containing an antibody of the present invention, formulated together with a pharmaceutical carrier.

- 18 -

As used herein, "pharmaceutical carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like that are physiologically compatible. In one embodiment the carrier is suitable for intravenous, intramuscular, subcutaneous, parenteral, spinal or epidermal administration (e.g. by injection or infusion).

A composition of the present invention can be administered by a variety of methods known in the art. As will be appreciated by the skilled artisan, the route and/or mode of administration will vary depending upon the desired results.

To administer a compound of the invention by certain routes of administration, it may be necessary to coat the compound with, or co-administer the compound with, a material to prevent its inactivation. For example, the compound may be administered to a subject in an appropriate carrier, for example, liposomes, or a diluent. Pharmaceutically acceptable diluents include saline and aqueous buffer solutions.

Pharmaceutical carriers include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. The use of such media and agents for pharmaceutically active substances is known in the art.

The phrases "parenteral administration" and "administered parenterally" as used herein means modes of administration other than enteral and topical administration, usually by injection, and includes, without limitation, intravenous, intramuscular, intraarterial, intrathecal, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticular, subcapsular, subarachnoid, intraspinal, epidural and intrasternal injection and infusion.

The compositions according to the invention may also contain adjuvants such as preservatives, wetting agents, emulsifying agents and dispersing agents. Prevention of presence of microorganisms may be ensured both by sterilization procedures, supra, and by the inclusion of various antibacterial and antifungal agents, for example, paraben, chlorobutanol, phenol, sorbic acid, and the like. It may also be desirable to include isotonic agents, such as sugars, sodium chloride, and the like into the compositions. In addition, prolonged absorption of the injectable pharmaceutical form may be brought about by the inclusion of agents which delay absorption, such as aluminum monostearate and gelatin.

- 19 -

Regardless of the selected route of administration, the compounds of the present invention, which may be used in a suitable hydrated form, and/or the pharmaceutical compositions of the present invention, are formulated into pharmaceutically acceptable dosage forms by conventional methods known to those of skill in the art.

Actual dosage levels of the active ingredients in the pharmaceutical compositions of the present invention may be varied so as to obtain an amount of the active ingredient which is effective to achieve the desired therapeutic response for a particular patient, composition, and mode of administration, without being toxic to the patient. The selected dosage level will depend upon a variety of pharmacokinetic factors including the activity of the particular compositions of the present invention employed, the route of administration, the time of administration, the rate of excretion of the particular compound being employed, the duration of the treatment, other drugs, compounds and/or materials used in combination with the particular compositions employed, the age, sex, weight, condition, general health and prior medical history of the patient being treated, and like factors well known in the medical arts.

The composition must be sterile and fluid to the extent that the composition is deliverable by syringe. In addition to water, the carrier in one embodiment is an isotonic buffered saline solution.

Proper fluidity can be maintained, for example, by use of coating such as lecithin, by maintenance of required particle size in the case of dispersion and by use of surfactants. In many cases, it is advantageous to include isotonic agents, for example, sugars, polyalcohols such as mannitol or sorbitol, and sodium chloride in the composition.

Transplantation is performed according to the state of the art with numerous cell types, tissue types and organ types, e.g. pancreatic islets, corneal, bone marrow, stem cells, skin graft, skeletal muscle, aortic and aortic valves, and organs as heart, lung, kidney, liver, and pancreas.

The invention comprises the use of the antibodies according to the invention for the treatment of a patient suffering from GvHD (graft versus host disease) or HvGD (host versus graft disease) (e.g. after transplantation). The invention comprises also a method for the treatment of a patient suffering from such GvHD and HvGD. Additionally the invention comprises the use of an antibody according

- 20 -

to the invention for the manufacture of a medicament for the treatment of GvHD or HvGD.

The invention also provides the use of an antibody according to the invention in an effective amount for the manufacture of a pharmaceutical agent, preferably
5 together with a pharmaceutically acceptable carrier, for the treatment of a patient suffering from inflammatory mediator release mediated by CCR5.

The term "graft rejection" as used within this application denotes the response of the human immune system to transplanted tissue. If tissue is transplanted from a donor to a host the human leukocyte antigen genes of the donor's tissue are likely
10 to be different from those of the host's tissue. Thus, the host's immune system recognized the transplanted tissue as foreign and effects an immune response called graft rejection. This graft rejection reaction is called "graft versus host disease" (GvHD).

The term "the sugar chains show characteristics of N-linked glycans attached to
15 Asn297 of an antibody binding to CCR5 recombinantly expressed in a CHO cell" denotes that the sugar chain at Asn297 of the antibody according to the invention has the same structure and sugar residue sequence except for the fucose residue as those of an anti-CCR5 antibody expressed in unmodified CHO cells, e.g. as those anti-CCR5 antibodies according to WO 2006/103100.

20 The term "NGNA" as used within this application denotes the sugar residue N-glycolylneuraminic acid.

Thus, the current invention provides a method of treating or preventing acute and chronic organ transplant rejection in a mammal, including a human, characterized in administering to said mammal an antibody according to the invention. Also
25 provided is an antibody according to the invention for the treatment or prevention of acute and chronic organ transplant rejection in a mammal, including a human. Further the invention comprises the use of an antibody according to the invention for the manufacture of a medicament for the treatment of acute and chronic transplant rejection.

30 Five cynomolgus monkeys were chosen as heart allograft recipients. The monkeys were treated with the antibody according to the invention either alone (n=2, monotherapy) or in combination with a therapeutic dose of cyclosporine A (CsA,

- 21 -

n=3, combination therapy). This study showed that the antibody according to the invention is immunosuppressive as

- a prolonged graft survival was observed with monotherapy compared to untreated controls (19 ± 5.7 days versus 6.4 ± 0.4 days; $p=0.034$),
- 5 - suppression of chronic allograft rejection (chronic allograft vasculopathy (CAV)) by combination therapy of the antibody according to the invention and CsA compared to CsA monotherapy (CAV score: 0.2 ± 0.1 versus 1.9 ± 0.4 , $p=0.024$),
- suppression of anti-donor alloantibody production by combination therapy of the antibody according to the invention and CsA compared to CsA monotherapy,
- 10 - prolonged primary graft survival by the combination therapy of the antibody according to the invention and CsA compared to CsA monotherapy with evidence of immunosuppression.

The term “combination therapy” refers to the administration of an anti-CCR5 antibody according to the invention and an immunosuppressive agent as one single
15 formulation or as two separate formulations. The administration can be simultaneous or sequential in either order, wherein in one embodiment there is a time period while both (or all) active agents simultaneously exert their biological activities. Said anti-CCR5 antibody and said immunosuppressive agent are administered either simultaneously or sequentially in a combination therapy (e.g.
20 via an intravenous (i.v.) through a continuous infusion (one for the antibody and eventually one for the immunosuppressive agent)).

It is self-evident that the antibodies are administered to the patient in a “therapeutically effective amount” (or simply “effective amount”), which is the amount of the respective compound or combination that will elicit the biological or
25 medical response of a tissue, system, animal or human that is being sought by the researcher, veterinarian, medical doctor or other clinician.

The amount of said anti-CCR5 antibody and said immunosuppressive agent and the timing of administration will depend on the type (species, gender, age, weight, etc.) and condition of the patient being treated and the severity of the disease or
30 condition being treated. Depending on the type and severity of the disease, about 1 $\mu\text{g/kg}$ to 50 mg/kg (in one embodiment 10-25 mg/kg) of said anti-CCR5 antibody and 1 $\mu\text{g/kg}$ to 50 mg/kg (in one embodiment 5-20 mg/kg) of said

- 22 -

immunosuppressive agent is an initial candidate dosage for administration of both drugs to the patient.

Thus, one aspect of the current invention is the use of an anti-CCR5 antibody for the manufacture of a medicament for the treatment of graft rejection in combination therapy with an immunosuppressive agent. Further the current invention comprises a method for the treatment of graft rejection in a patient by combination therapy with an anti-CCR5 antibody according to the invention and an immunosuppressive agent.

Another aspect of the current invention is the use of an anti-CCR5 antibody for the manufacture of a medicament for the treatment of anti-donor alloantibody production in combination therapy with an immunosuppressive agent. Further the current invention comprises a method for the treatment of anti-donor alloantibody production in a patient by combination therapy with an anti-CCR5 antibody according to the invention and an immunosuppressive agent.

The term "alloantibody" denotes an antibody that is generated by an organism against foreign tissue from a person of the same species.

By targeting CCR5 with an antibody according to the invention acute allograft rejection can be reduced or even prevented as CCR5+ cells are depleted. Therefore, by application of the antibody according to the invention a prolonged allograft survival and a completely prevented chronic rejection (evidenced by CAV incidence and severity) were observed.

Thus, aspects of the current invention are the use of an anti-CCR5 antibody according to the current invention for the prevention of allograft rejection or therapy of alloimmunity in allograft recipients, the use of an anti-CCR5 antibody according to the invention for the manufacture of a medicament for the prevention of allograft rejection or therapy of alloimmunity in allograft recipients, as well as a method for the prevention of allograft rejection or therapy of alloimmunity in allograft recipients by administering an antibody according to the invention to said recipient. In one embodiment is the anti-CCR5 antibody according to the invention administered in combination therapy with an immunosuppressive agent.

In cynomolgus macaques treated with antibody monotherapy at 10 mg/kg every 3-5 days for the first week and then weekly, cardiac allografts survived for 14 and 23 days, significantly longer than in untreated monkeys (MST 6.5 ± 0.4 days; n=5,

- 23 -

p=0.034) (see Figure 5). Acute cellular rejection was confirmed histologically. By using combination therapy of CsA and an anti-CCR5 antibody according to the invention dosed as above, none of three animals treated with combination developed symptomatic rejection through the end of observation at day 85-90.

5 After explant of the grafts it has been found that the grafts were without clinical evidence of rejection.

Thus, it has been found that there was no episode of acute graft rejection detectable during treatment with a combination therapy of an anti-CCR5 antibody according to the invention and CsA and all three grafts were electively explanted with a

10 normal function at day ~90.

In contrast, in four of eight historical animals treated with CsA monotherapy, a first episode of symptomatic acute allograft rejection (graft bradycardia and/or diminished contractility, recipient fever) was detected before 90 days (at 7, 23, 44, and 71 days respectively). In three of these animals rejection was steroid-responsive

15 (three daily steroid boluses with Solu-Medrol®, 10 mg/kg); one graft rejected on day 7 before treatment could be initiated. One animal of the historical control died at day 26 with a septicemia from a central venous line infection. The remaining three historical grafts survived without acute rejection to elective graft explant at day 85-90.

Thus, it has been found that the combination therapy of an anti-CCR5 antibody according to the invention and CsA provides for prolonged graft survival and reduced acute graft rejection. The median survival time (MST) is more than 90 days versus 71 days with CsA monotherapy (p=0.13) and the incidence of acute rejection is 0 of 3 versus 4 of 7 (p=0.2) with combination therapy of an anti-CCR5 antibody

20 according to the invention and CsA compared to CsA alone.

25

Allografts rejecting under monotherapy exhibited typical features of severe acute cellular rejection (Grade 3R, with diffuse inflammatory infiltration with multifocal cardiac myocyte damage and associated edema and hemorrhage) according to the International Society of Heart and Lung Transplantation (ISHLT). In contrast,

30 rejection scores were consistently lower (Grade 0 or 1) in monkeys treated with a combination therapy of an anti-CCR5 antibody according to the invention and CsA versus monotherapy with an anti-CCR5 antibody according to the invention alone or no treatment. Remarkably, whereas all grafts treated with CsA monotherapy exhibited moderate to severe cardiac allograft vasculopathy (CAV severity score 1.9

- 24 -

± 0.4 ; all scores ≥ 1.5 , N=6) at explant, the CAV score associated with combination therapy of an anti-CCR5 antibody according to the invention and CsA was significantly reduced relative to monotherapy with CsA (0.2 ± 0.1 , n=3; p=0.024 versus CsA) (Figure 6).

- 5 Alloantibody elaboration was detected within two weeks in both animals treated with anti-CCR5 antibody monotherapy (10.7 mg/kg). When in one embodiment a higher dose of the anti-CCR5 antibody according to the invention of 18 to 22 mg/kg (21.4 mg/kg) was administered in combination therapy with CsA, two of three animals elaborated only trace/measurable anti-donor IgG antibody with none
10 reaching the positivity threshold of 10 %, and only one of these also exhibited transient low-titer IgM at one month. In contrast, alloantibody production was detected within 90 days in 6/7 (IgM) and 4/7 (IgG) historical control animals treated with monotherapy of CsA. Therefore, a combination therapy with an anti-CCR5 antibody according to the invention attenuates alloantibody elaboration in
15 response to a cardiac allograft in the context of concomitant calcineurin inhibitor therapy. CsA was dosed in one embodiment of the invention at 15 ± 10 mg/kg (i.e. in a dose of from 5 mg/kg to 25 mg/kg) intramuscular daily to achieve therapeutic trough levels of > 400 ng/ml.

Thus, another aspect of the current invention is the use of an anti-CCR5 antibody
20 according to the invention in combination with a calcineurin inhibitor therapy for the attenuation of humoral immunity to alloantigens.

CCR5 depletion as maintenance treatment, thus, may allow for significantly lower doses of CsA to be used.

Comparing histology at the time of graft rejection, which was delayed monotherapy
25 with an anti-CCR5 antibody according to the invention, cellular infiltration of the graft was not prevented by monotherapy alone. Specifically, large numbers of T cells and macrophages enter the graft despite partial CCR5+ cell depletion associated with antibody monotherapy.

Table 1: Summary of the graft outcome and histology for recipients treated with
30 CsA alone or combined with an antibody according to the invention is shown in the following table (Secondary survival indicates the time at which the rejected graft was explanted after a first episode of acute rejection was treated; $>$: indicates a graft explanted while still beating; CAV: cardiac allograft vasculopathy; ISHLT: rejection score according to the International Society of Heart and Lung Transplantation;

- 25 -

- CsA: Cyclosporine A given at 15 ± 10 mg/ml daily under graft explant to achieve trough levels > 400 ng/ml; antibody: treatment with anti-CCR5 antibody according to the invention given at 10 mg/kg on days -1, 5, 8, 14, 21, and 28, or until graft explant (monotherapy) or at 20 mg/kg on days -1, 5, 8, 14, and weekly thereafter until 90 days (combination therapy).

Sample	Primary survival	Secondary survival	Rejection at graft explant		
	[days]	[days]	ISHLT	CAV severity	CAV incidence
Historical control					
Untreated	6		3A	N/A	N/A
Untreated	6		3A	N/A	N/A
Untreated	6.5		3A	N/A	N/A
Untreated	6.5		3B	N/A	N/A
Untreated	7		3B	N/A	N/A
Current study					
antibody at 10 mg/kg/d	14	N/A	3R	0.75	54 %
antibody at 10 mg/kg/d	23	N/A	3R	2.33	98 %
Historical control					
CsA at 15 ± 10 mg/ml/d	7		4	N/A	N/A
CsA at 15 ± 10 mg/ml/d	> 26	N/A			
CsA at 15 ± 10 mg/ml/d	44	> 47		1.5	
CsA at 15 ± 10 mg/ml/d	23	72	2	2.27	97 %
CsA at 15 ± 10 mg/ml/d	71	> 92	4	2.45	100 %
CsA at 15 ± 10 mg/ml/d	> 85	N/A	2/3A	1.55	67 %
CsA at 15 ± 10 mg/ml/d	> 89	N/A	3A	1.5	85 %
CsA at 15 ± 10 mg/ml/d	> 91	N/A	2	2.1	95 %
CAV at 70-90 days				1.9 ± 0.4	89 ± 12 %
Current study					

- 26 -

Sample	Primary survival	Secondary survival	Rejection at graft explant		
CsA at 15 ± 10 mg/ml/d + antibody at 20 mg/kg/d	> 92	N/A	1R	0.26	20 %
CsA at 15 ± 10 mg/ml/d + antibody at 20 mg/kg/d	> 91	N/A	0	0.07	7 %
CsA at 15 ± 10 mg/ml/d + antibody at 20 mg/kg/d	> 91	N/A	0	0.2	10 %
CAV at 90 days				0.2 ± 0.1	12 ± 7 %

Antibody Deposition

Cell line	Deposition No.	Date of Deposit
m<CCR5>Pz03.1C5	DSM ACC 2683	18.08.2004

5 The following examples, figures and sequence listing are provided to aid the understanding of the present invention, the true scope of which is set forth in the appended claims. It is understood that modifications can be made in the procedures set forth without departing from the spirit of the invention.

Figures

10 **Figure 1:** Fc receptor binding on CHO cells, afucosylated antibody (◆), wt antibody (■).

Figure 2: ADCC, afucosylated antibody (■), wt antibody (●) and LALA mutant antibody (▲).

Figure 3: In vivo depletion of CCR5+ cells.

15 **Figure 4:** Monitoring of CCR5 cell expression across CD8+ cells, CD4+ cells and monocytes. Grey: control, unspecific monoclonal antibody; black: treated cynomolgus

- 27 -

Figure 5: Prolonged cardiac allograft survival in cynomolgus monkeys receiving anti-CCR5 antibody according to the invention with

Group	Survival [days]	MST $\pm$ SD	<i>P</i>	% over untreated
not treated	6, 6, 6.5, 6.5, 7	6.4 ± 0.4	-	-
antibody at 10 mg/kg	15, 23	19 ± 5.7	0.034	197%

Figure 6: Combination therapy prevents cardiac allograft vasculopathy in cardiac recipient cynomolgus monkeys with

Group	Graft rejection incidence	CAV incidence	CAV severity
antibody + CsA	0/3	12 ± 7	0.2 ± 0.1
CsA	4/8	89 ± 12	1.9 ± 0.4
<i>P</i>	0.2	0.001	0.024

10 Examples

Material and Methods

Antibodies

As antibodies IgG1 antibody against CCR5 as described in WO 2006/103100 and WO 2008/037419 were used (variable regions see SEQ ID NO: 06 to SEQ ID NO: 13, CDR sequences see SEQ ID NO: 04, 05, and SEQ ID NO: 21 to 35). The antibodies were used as wildtype antibody (WT Ab, 8 % afucosylated), afucosylated antibody (afucosylated Ab) and LALA mutant antibody (see WO 2008/037419) (LALA mutant Ab). The term LALA denotes the amino acid exchange from leucine to alanine at positions 234 and 235 in the constant region of said antibody (L234A, L235A).

Plasmids

The expression system comprises the CMV promoter system (EP 0 323 997) and is described in tables 2 and 3.

- 28 -

Table 2: pETR 3928 (Antibody expression vector)

Element	Length	Description
CCR5 HC	1404	encoding heavy chain of
CCR5 LC	714	encoding light chain of
GS	1122	encoding glutamine-synthetase
SV40E	343	Promoter
hCMV promoter	1142	Promoter
Intron	947	Intron

Table 3: pETR 2896 (Glycosylation vector)

Element	Length	Description
chimeric MPSV promoter (contains enhancer of hCMV promoter)	875	promoter
synthetic intron	1324	intron
GnTIII	1644	encoding N-acetylglucosaminyl-transferase III
ManII	3435	Encoding mannosidase II
pac	600	encoding puromycin acetyltransferase from Streptomyces alboniger
polyA	49	polyadenylation signal

5 Human CCR5 Cell Line

The L1.2 cell line stably expressing human CCR5 receptor (L1.2hCCR5 cells) are used for the human CCR5 chemotaxis assay. L1.2hCCR5 cells are cultured in RPMI 1640 containing 10 % FBS, 10 units/ml Penicillin, 10 µg/ml Streptomycin, 0.1 mM Glutamine, 1 mM Sodium Pyruvate, 55 µM 2-Mercaptoethanol, 250 µg/ml Geneticin (all from Invitrogen). Just prior to the set up of the chemotaxis assay, the cells are spun down and resuspended in Chemotaxis Buffer (Hank's Balanced Salt Solution HBSS (Invitrogen) containing 0.1 % BSA (Sigma) and 10 mM HEPES buffer (Invitrogen Corp., USA)). The cells are used in the chemotaxis assay at a final concentration of 5×10^6 cells/ml.

- 29 -

Cynomolgus CCR5 Cell Line

The L1.2 cell line stably expressing cynomolgus CCR5 receptor (L1.2cynoCCR5 cells) are used for the cynomolgus CCR5 chemotaxis assay. L1.2cynoCCR5 cells are cultured in the same growth media as the L1.2hCCR5 cells. Cells are seeded at a density of 8×10^5 cells/ml in growth media containing 5 mM Sodium Butyrate (Sigma) on the day prior to the assay. Just prior to the set up of the chemotaxis assay, the cells are spun down and resuspended in Chemotaxis Buffer. The cells are used in the chemotaxis assay at a final concentration of 5×10^6 cells/ml.

Preparation of Ligands

CCR5 ligands human MIP1 α , MIP1 β or RANTES (R&D Systems) are diluted in Chemotaxis Buffer and are used at a final concentration of 10 nM. LALA mutant Ab and Isotype control mouse IgG2_A (BD Biosciences, USA) are diluted in Chemotaxis Buffer.

Manufacture of anti-CCR5 antibodies

Plasmid pETR 3928 was transfected in a glutamine prototroph CHO or HEK293 host cell (EP 0 256 055) which was previously transfected with plasmid pETR 2896. The cell line was cultivated as fed batch cultivation for up to 14 days in serum free medium to generate antibody batches with different amounts of fucosylation (samples 1-4, CHO). The antibody was isolated from the supernatant and purified by chromatographic methods.

WT antibody (fucosylation of 92 %) or LALA mutant Ab is recombinantly produced in a HEK 293 or Chinese hamster ovarian (CHO) cell line, CHO-DG44 (Flintoff, W.F., et al., Somat. Cell Genet. 2 (1976) 245-261; Flintoff, W.L., et al., Mol. Cell. Biol. 2 (1982) 275-285; Urlaub, G., et al., Cell 33 (1983) 405-412; Urlaub, G., et al., Somat. Cell Mol. Genet. 12 (1986) 555-566). CHO-DG44 cells were grown in MEM alpha Minus Medium (Gibco No. 22561), 10 % dialyzed FCS (Gibco No. 26400-044) and 2 mmol/L L-Glutamine, 100 μ M Hypoxanthine, 16 μ M Thymidine (HT supplement).

Animals:

Cynomolgus monkeys (*Macaca fascicularis*) (3 to 7 kg) were paired with blood type compatible, mixed lymphocyte reaction (MLR)-mismatched (actual SI range: 6-8).

- 30 -

Animals were housed under conventional conditions in compliance with the Guide for the Care and Use of Laboratory Animals (HHS, NIH Publication 86-23, 1985).

Cardiac transplantation in monkeys and immunosuppression:

All recipient animals underwent heterotopic intraabdominal cardiac allograft transplantation, as described previously (Schroeder. C., et al., J. Immunol. 179 (2007) 2289-2299). Two animals were treated with antibody monotherapy at 10 mg/kg by daily intravenous injections on days -1, 5, 8, 14, 21, and 28. Three additional animals received the antibody at 20 mg/kg on days -1, 5, 8, 14, and weekly thereafter until 90 days, and with additional Cyclosporine A (CsA, generic formulation from Bedford Laboratories, Bedford, OH, USA). CsA was given once daily (intramuscular (IM) at 15 ± 10 mg/kg) from the day of surgery until 90 days to achieve target trough levels (> 400 ng/ml). Animals experiencing acute rejection episodes received a 3-day course of steroids (10 mg/kg boluses, Solu-Medrol®, Pharmacia, Kalamazoo, MI, USA) to attain graft survival of 90 days. Open cardiac biopsies were performed 30 minutes after graft revascularization and on postoperative days 5 (monotherapy only), 14, 28, and 56. Graft function was monitored at least daily by implanted telemetry (Data Sciences International, St. Paul, MN, USA). Clinical acute graft rejection was suspected based on two of three cardinal signs: consistent high body temperature ($> 38.5^{\circ}\text{C}$); a decrease in graft heart rate (to < 120 beats per min (bpm), or a sustained drop of > 40 bpm ($\sim 20\%$) from a stable baseline); or a decrease in graft pulse pressure (systolic minus diastolic) of > 20 mmHg not attributable to technical measurement issues. Graft failure was defined as loss of contraction by telemetry and confirmed by visualization at explant, and was always preceded by signs of acute rejection. Body temperature was measured using the DSI telemetry system daily and recorded as a single morning measurement. Reference animals include historical animals receiving either no treatment (n=5) or CsA dosed to achieve target trough levels > 400 ng/ml (CsA monotherapy, n=8).

CBC and FACS analyses:

Complete blood cell (CBC) assays were performed on freshly collected EDTA-blood using an automated cell counter (Hemavet) using monkey settings. Whole blood collected in EDTA (100 μl), and cells isolated from lymph node (LN) (1×10^6 cells) were analyzed for expression of CCR5 at regular intervals. Briefly, cells were stained for 20 min. at 4°C with PerCP-Cy5.5-conjugated anti-human CD4 mAb

- 31 -

(L200, BD Pharmingen, San Diego, CA, USA), APC-conjugated anti-human CD8 α (3B5, Caltag, Invitrogen, Carlsbad, CA, USA), AlexaFluor488-conjugated anti-human CD14 (M5E2, BD Pharmingen, USA), and PE-conjugated anti-human CD195 (CCR5) (3A9, BD Pharmingen, USA) in FACS wash buffer (PBS (phosphate buffered saline) supplemented with 10 % FCS and 0.2 % sodium azide). Red blood cells were lysed with BD FACSlyse. In some experiments, cells were also stained for CXCR3 with AlexaFluor488-conjugated anti-human CD183 (CXCR3) (1C6, BD Pharmingen, USA) and in two instances graft infiltrating cells (GILs) were isolated by collagenase digestion and Ficoll gradient separation and stained as above. Lymphocyte populations were gated by forward/side scatter analysis to exclude debris. Data analysis and graphic display were conducted using CellQuest or Winlist software. The proportion of CCR5-positive cells among CD4+ or CD8+ lymphocytes in the blood was multiplied by absolute counts of CD4 and CD8 calculated from lymphocytes counts from the differential analysis to obtain absolute counts of CCR5+CD4+ and CCR5+CD8+ cells per μ l of blood.

Detection of anti-donor alloantibody:

Alloantibodies were measured retrospectively by flow cytometry as described previously (Schroeder, C., et al., 2007, see above). Briefly, archived frozen donor splenocytes (0.5×10^6 cells) were incubated with heat-inactivated recipient serum (50 μ l) for 30 min. at 4 °C. After washing, antibody binding was revealed using PE-labeled goat anti-human IgM (Fc γ specific) antibodies (Biosource, Invitrogen, Carlsbad, CA, USA), or biotin-labeled goat anti-monkey IgG (Fc γ specific) antibodies (Nordic, Tilburg, The Netherlands) followed by PE-labeled streptavidin (BD Pharmingen, San Diego, CA, USA). FITC-labeled anti-human CD3 (BD Pharmingen, USA) was added to gate T cells. Data were expressed as the calculated percentage of T cells positive post-transplant after subtraction of pre-transplant levels. Reactivity was defined as an increase of more than 10 %.

Histology:

Tissue was fixed with 10 % formalin and processed routinely for paraffin embedding. Sections of paraffin-embedded tissue were stained with hematoxylin and eosin. Cellular infiltrates were graded for acute rejection by ISHLT criteria. CAV incidence in beating hearts explanted after day 70 was recorded as percent of arteries and arteriolar vessels involved (CAV score ≥ 1) at each time point. CAV severity was scored in these explanted hearts as follows: Grade 0, normal arterial

- 32 -

morphology; Grade 1, activated endothelial cells with enlarged nuclei and/or adherent leukocytes, without luminal narrowing (< 10 %); Grade 2, distinct neointimal thickening, luminal narrowing < 50 %; Grade 3, extensive neointimal proliferation with greater than 50 % luminal occlusion. Scoring was independently performed for each explanted heart by three evaluators blinded with respect to treatment group. The mean CAV score for each biopsy or explant was calculated using the equation: $[(\# \text{grade 0-vessels} \times 0) + (\# \text{grade 1-vessels} \times 1) + (\# \text{grade 2-vessels} \times 2) + (\# \text{grade 3-vessels} \times 3)] / (\text{total number of arterial vessels scored})$. Individual graft mean CAV scores were averaged to calculate the group mean ($\pm$ SD) for each treatment group.

Immunohistochemistry:

Immunohistochemical stains were performed using an automated method as follows. Formalin fixed paraffin embedded (FFPE) tissue sections were deparaffinized and stained on the Ventana ES automated stainer using the ABC method (Ventana Medical Systems, Inc., Tucson, AZ, USA). All reagents placed on the Ventana instrument were purchased from Ventana. Settings were adjusted on mild CC1, conditioner 1, and standard 1. The following primary antibodies were used: CD3 (2GV6, Ventana, USA), CD68 (KP1, DAKO) and CD20 (L26, DAKO, Copenhagen, Denmark). For animals treated with antibody monotherapy and untreated controls, the number of cells was calculated as follows: the area of tissue with low, moderate or strong cellular infiltration was estimated, and then 10 pictures corresponding to representative fields were taken. The number of cells per field was then counted, and the average of cells per field was calculated. If a tissue sample was divided in different blocks, all blocks were processed separately, and the number of cells/field for all blocks was averaged to obtain cell counts for that tissue sample. For animals treated with combination therapy of antibody and CsA and CsA monotherapy controls, cellular infiltration was scored using the following scale: 0, absence of cells; 1, focal staining or weak diffuse; 2, 1-3 nodules or mild diffuse interstitial infiltration; 3, 3-10 nodules or moderate infiltration; 4, >10 nodules or strong infiltration; 5, massive infiltration.

Real-time PCR:

Heart tissue was snap frozen in liquid nitrogen, and stored at -70°C . Total RNA was isolated from cardiac grafts using the RNeasy mini kit from Qiagen (Valencia, CA, USA) for further analyses.

- 33 -

Drug level analysis:

Serum samples were collected at respective study time points and antibody levels were measured. CsA plasma levels were measured by HPLC method.

Statistical analysis:

- 5 Graft survival time was expressed as median survival time (MST) and graphed with use of the Kaplan-Meier method. The log-rank test was used to compare survival time between different groups. Continuous variables were expressed as the mean plus standard deviation unless otherwise indicated and were compared using the Mann-Whitney non parametric test. Nominal variables (i.e. incidence of early
10 rejection) were measured using a contingency table and the Fischer exact test. P-values less than 0.05 were considered statistically significant. All statistical analyses were performed on a personal computer with the statistical package SPSS for Windows XP (Version 11.0, SPSS, Chicago, IL, USA) or GraphPad InStat (version 5.1, GraphPad Software, San Diego, CA, USA).

15 Example 1Binding of anti-CCR5 antibodies to CCR5

- The binding capability of afucosylated antibody (Ab) and WT anti-CCR5 antibody are compared. The murine L1.2hCCR5 cell line was used as target cell line. As secondary antibody: FITC-conjugated AffiniPure F(ab)₂ Fragment goat anti-
20 human IgG Fcγ specific (Jackson ImmunoResearch Lab # 109-096-098) was used. Anti-human CCR5-FITC (Becton-Dickinson, BD 555992) and mouse IgG2a-FITC were used as control antibodies. RPMI 1640 medium + 10 % FCS + 1 % Glutamine + 1 % Sodium Pyruvate + 0.05 mM β-Mercaptoethanol + 0.8 mg/ml G418 was the cell culture medium. To induce hCCR5 expression on the cell surface, 0.2 Mio – 0.5
25 Mio cells/ml were incubated in medium containing 1 mM Sodium Butyrate (Sigma B5887). Cells incubated without sodium butyrate served as negative controls.

Method:

- 0.2 Mio cells/180 μl/well diluted in PBS/0.1 % BSA were plated in a 96-round bottom plate and 20 μl of diluted antibody was added. After 30 min of incubation
30 at 4 °C, cells were washed with PBS/0.1 % BSA and 15 μl/well diluted secondary antibody or controls were added. The cells were incubated for another 30 min at 4 °C followed by two washing steps. Before measuring the cells in the FACSCanto, propidium iodide was added.

- 34 -

Results:

WT Ab and afucosylated Ab showed similar binding on the target cells which was dependent of the antibody concentrations. The EC₅₀ values were calculated for both antibodies using GraphPad Prism 4. The mean values for 100 µg/ml antibody were excluded. The mean values for 100 µg/ml antibody were excluded. EC₅₀ afucosylated Ab: 0.1376; EC₅₀ WT Ab: 0.09407.

Example 2**Cellular Fc Binding**

Fc-binding of afucosylated Ab versus WT Ab was investigated.

10 CHO cell line expressing $\geq 10^4$ CCR5 molecules/cell served as a target cell line. As secondary Ab:antibody FITC-conjugated AffiniPure F(ab)₂ Fragment goat anti-human IgG F(ab)₂ Fragment-specific (Jackson ImmunoResearch Lab # 109-096-097) was used. Anti-human CD16-FITC (Beckman Coulter PN IM0814); mouse IgG1 isotype: mouse IgG1-FITC was used as control. The cell culture medium was
15 IMDM + Glutamax + 25 mM HEPES (Gibco 31980) + 10 % FCS + HT supplements + 6 µM Puromycin. Chinese hamster ovary cells (CHO cells) were cultured in T150 flasks and used for the assay when a density of 13×10^6 cells/flask was reached. Cells were harvested with Trypsin/EDTA.

Cell culture:

20 medium: IMDM + Glutamax + 25 mM HEPES (Gibco 31980) + 10 % FCS + HT supplements + 6 µM Puromycin Chinese hamster ovary cells (CHO cells) were cultured in T150 flasks and used for the assay when a density of 13 Mio cells/flask was reached. Cells were harvested with Trypsin/EDTA.

Method:

25 0.2 Mio cells/180 µl/well diluted in PBS/0.1 % BSA were plated in a 96-round bottom plate and 20 µl of diluted antibody was added. After 30 min of incubation at 4 °C, cells were washed with PBS/0.1 % BSA and 12 µl/well diluted secondary antibody or controls were added. The cells were incubated for another 30 min at 4 °C followed by two washing steps. Cells were fixed with 2 % PFA for 20 min. at
30 4 °C followed by a washing step before measuring them in the FACSCanto (Figure 1).

Example 3**Determination of the affinity of anti-CCR5 antibodies to FcγRIII (CD16a)**

His-CD16a was amine coupled to the surface of a CM5-chip. The measurement was performed on a BIACORE®3000 instrument. The running and dilution buffer was HBS-P. The chip surface was saturated with His-CD16a. Amine coupling groups were saturated. The analyte was added to the buffer flow at a constant concentration of 10 nM, whereas the inhibitor, soluble CD16a was added to the buffer flow at increasing concentrations (0-1000 nM). RU values reflect the affinity between antibody and CD16a.

10 Table 4:

	RU max	IC50 [nM]
WT Ab	48 ± 1	14 ± 1
LALA mutant Ab	no binding	no binding
Ab according to the invention	102 ± 8	9 ± 1
Positive Control	32 ± 1	10 ± 0
Negative Control	no binding	no binding

Example 4**Potential of anti-CCR5 monoclonal antibodies to bind to FcγRIIIa on NK cells**

To determine the ability of the antibodies of the invention to bind to FcγRIIIa (CD16) on Natural Killer (NK) cells, Peripheral Blood Mononuclear Cells (PBMCs) are isolated and incubated with 20 µg/ml of antibody and control antibodies in the presence or absence of 20 µg/ml of a blocking mouse antibody to FcγRIIIa (anti-CD16, clone 3G8, RDI, Flanders, NJ), to verify binding via FcγRIIIa. As negative controls, human IgG2 and IgG4 (The Binding Site), that do not bind FcγRIIIa, are used. Human IgG1 and IgG3 (The Binding Site) are included as positive controls for FcγRIIIa binding. Bound antibodies on NK cells are detected by FACS analysis using a PE-labeled mouse anti-human CD56 (NK-cell surface marker) antibody (BD Biosciences Pharmingen, San Diego, CA) in combination with a FITC-labeled goat F(ab)₂ anti-human IgG (Fc) antibody (Protos

- 36 -

Immunoresearch, Burlingame, CA). Maximum binding ($B_{\max}$) is determined at an antibody concentration of 20 $\mu\text{g/ml}$. Control antibody (human IgG4) shows up to 30 % $B_{\max}$ compared to 100 % $B_{\max}$ for human IgG1. Therefore “no Fc γ RIIIa binding or no ADCC” means at an antibody concentration of 20 $\mu\text{g/ml}$ a $B_{\max}$ value of up to 30 % compared to human IgG1.

Measurement of ADCC and CDC by Chromium⁵¹-release assay

Materials:

Chromium from Amersham, Cat # CJS11; Round bottom polypropylene plates costar, Cat # 3790; Lumaplate-96 (Solid scintillator coated polystyrene plates):
10 From Perkin-Elmer, Part # - 6006633.

Target Cells: CCR5-expressing cells (L1.2 cell line, see example 2).

Effector cells or Complement serum (human PBMCs, isolated NK cells)

Method:

1 x 10⁶ target cells are labeled with 100 μCi of Chromium⁵¹ for 1 hour at 37 °C.
15 Labeled cells are washed four times with the medium and resuspended in 5 ml (concentration 200,000 cells/ml). 50 μl of target cells (at a concentration of 200,000 cells/ml) are plated per well. 50 μl of test antibody is added at different concentration and incubated at 4 °C for 1 hour. Effector cells (at desired ratio) or Complement serum at the desired E:T ratio was added and cells are incubated at
20 37 °C for 4 hours.

Controls:

For Spontaneous lysis control, 50 μl target cells and 100 μl culture media was mixed and measured. Maximum lysis was measured using 50 μl labeled target cells + 50 μl Triton-X-100 (1 % in PBS) + 50 μl media At the end of incubation 50 μl
25 of culture supernatant was added on the Luma plate. Results were read in a top count. Cytotoxicity was calculated using percent cytotoxicity equals sample CPM – spontaneous lysis CPM divided by maximal lysis CPM – spontaneous lysis CPM multiplied by 100.

Results are shown in Figure 2.

- 37 -

Example 5**CCR5 Chemotaxis Assay**

L1.2hCCR5 cells harboring the human or L1.2mCCR5 harboring the cynomolgus CCR5 are cultured in RPMI 1640 containing 10 % Fetal bovine serum, 1x Penicillin/Streptomycin, 1x Glutamine, 1x Sodium Pyruvate, 1x β -Mercaptoethanol, and 250 μ g/ml G418 (all from Invitrogen). Just prior to the set up of the chemotaxis assay, the cells are spun down and resuspended in Chemotaxis Buffer (Hank's Balanced Salt Solution HBSS (Invitrogen) containing 0.1 % BSA and 10mM HEPES). The cells are used in the chemotaxis assay at a final concentration of 5×10^6 cells/ml. CCR5 ligands hMIP1 α , hMIP1 β or hRANTES (R&D Systems) are diluted in Chemotaxis Buffer and are used at a final concentration of 20 nM. Test antibodies or the appropriate isotype control antibodies are diluted in HBSS. Chemotaxis is set up in the 0.5 μ m pore 96-well ChemoTx^R system (Neuroprobe). Each antibody is mixed with one of the CCR5 ligands and 30 μ l of this mixture is placed in the bottom well of the ChemoTx^R system. The filter screen is placed on top of the bottom wells. Each antibody is mixed with the L1.2hCCR5 or L1.2mCCR5 cells and 20 μ l of this mixture is placed on the filter. The plates are then placed in a humidified chamber and incubated at 37 °C and 5 % CO₂ for 3 hours. After incubation, the cells are scraped off the filter and the plates are spun in a table top centrifuge at 2,000 rpm for 10 min. The filter is then removed and the density of the cells that have migrated to the bottom wells is detected using CyQUANT[®] Cell proliferation assay kit (Invitrogen) and the Spectra MAX GeminiXS plate reader (Molecular Devices) according to the manufacturers' instructions. IC₅₀ is calculated using Prism 4 (GraphPad).

L1.2CCR5 cells were seeded at 8×10^5 cells/ml with 2 mM Sodium Butyrate 24 h before assay. Antibodies were diluted in CTX buffer (HBSS, 0.1 % BSA, 10 mM HEPES). Ligands were diluted (MIP1a MIP1b RANTES, 20 nM stock (2x) in CTX buffer). Cells were washed and resuspended in CTX buffer (HBSS, 10 mM HEPES, 0.1 % BSA) at 1×10^7 (2x). In deep well blocks were prepared: antibody + ligand and top suspension antibody + cells. The assay was set up into chemotaxis onto 101-5 ChemoTxR (Neuroprobe) plates, incubated 3 hrs at 37 °C in a humidified chamber. Wash-Scrape cells off top of filter and spin plates 2,000 rpm for 10 min. Filter was removed and 10 ml supernatant from each bottom well was taken. After freezing/thawing 10 ml 2x CyQUANT (Invitrogen) was added to each well and results were read on a fluorescent plate reader.

- 38 -

Results:

Migration of human CCR5 cells was effectively blocked by WT Ab and afucosylated Ab (Table 5).

Table 5:

Chemotaxis IC ₅₀ (Midpoint, [nM])			
Antibody	MIP1a	MIP1b	RANTES
LALA mutant Ab	1.38±0.2	1.50±0.6	5.04±2.9
WT Ab	0.66±0.3	0.44±0.0	1.48±0.2
Afucosylated Ab	0.67±0.1	2.36±1.4	0.55±0.01

5

Example 6In vivo depletion of CCR5⁺ cells

Cynomolgus monkeys received 1 single i.v. infusion of 1 mg/kg or 10 mg/kg afucosylated Ab. Data were shown as % of CCR5⁺ cells in the indicated subsets in blood (CD8⁺ and CD4⁺ T-cells as well as monocytes) (Figure 3).

10

Example 7In vivo depletion Toxicity study

Purpose: To monitor CCR5 cell expression across CD8⁺ cells, CD4⁺ cells and monocytes. Determine if CD8⁺ cells are depleted in animals treated with afucosylated Ab to determine effect of treatment on CCR5 expression in the tissues.

15

- 12 Animals: 4 control, 8 with 21 mg/kg/day
- Dose on Day 1 and Day 15
- Whole blood for staining:
- Pre-dose Day-6, Pre-dose Day 1, 2, 4, 8, 15, 16, 18, 22, 29, 36, 43, 50, 57, 64, 71.
- Stain for CCR5⁺ in CD8⁺, CD4⁺, CD8⁺CD4⁺, Monocytes (by gate)

20

- 39 -

- Stain for CXCR3+ in CD8+ and CD4+
- Depletion confirmation by counting beads
- Spleen, lymph node and bone marrow for staining CCR5 on Day 8, 15, 18, 71.

The results are shown in Figure 4.

5 Example 8

Analysis of glycostructure of antibody

For determination of the relative ratios of fucose- and non-fucose (afucose) containing oligosaccharide structures, released glycans of purified antibody material were analyzed by MALDI-Tof-mass spectrometry. For this, the antibody sample (about 50 µg) was incubated over night at 37 °C with 5 mU N-Glycosidase F (Prozyme# GKE-5010B) in 0.1 M sodium phosphate buffer, pH 6.0, in order to release the oligosaccharide from the protein backbone. Subsequently, the glycan structures released were isolated and desalted using NuTip-Carbon pipette tips (obtained from Glygen: NuTip 1-10 µl, Cat.Nr. #NT1CAR). As a first step, the NuTip-Carbon pipette tips were prepared for binding of the oligosaccharides by washing them with 3 µL 1 M NaOH followed by 20 µL pure water (e.g. HPLC-gradient grade from Baker, #4218), 3 µL 30 % (v/v) acetic acid and again 20 µL pure water. For this, the respective solutions were loaded onto the top of the chromatography material in the NuTip-Carbon pipette tip and pressed through it. Afterwards, the glycan structures corresponding to 10 µg antibody were bound to the material in the NuTip-Carbon pipette tips by pulling up and down the N-Glycosidase F digest described above four to five times. The glycans bound to the material in the NuTip-Carbon pipette tip were washed with 20 µL pure water in the way as described above and were eluted stepwise with 0.5 µL 10 % and 2.0 µL 20 % acetonitrile, respectively. For this step, the elution solutions were filled in a 0.5 mL reaction vials and were pulled up and down four to five times each. For the analysis by MALDI-Tof mass spectrometry, both eluates were combined. For this measurement, 0.4 µL of the combined eluates were mixed on the MALDI target with 1.6 µL SDHB matrix solution (2,5-dihydroxybenzoic acid/2-hydroxy-5-methoxybenzoic acid [Bruker Daltonics #209813] dissolved in 20 % ethanol/5 mM NaCl at 5 mg/ml) and analyzed with a suitably tuned Bruker Ultraflex TOF/TOF instrument. Routinely, 50-300 shots were recorded and summed up to a single experiment. The spectra obtained were evaluated by the flex analysis software (Bruker Daltonics) and masses were determined for the each of

- 40 -

the peaks detected. Subsequently, the peaks were assigned to fucose or afucose (non-fucose) containing glycostructures by comparing the masses calculated and the masses theoretically expected for the respective structures (e.g. complex, hybrid and oligo- or high-mannose, respectively, with and without fucose).

- 5 For determination of the ratio of hybrid structures, the antibody sample was digested with N-Glycosidase F and Endo-Glycosidase H concomitantly. N-glycosidase F releases all N-linked glycan structures (complex, hybrid and oligo- and high mannose structures) from the protein backbone and the Endo-Glycosidase H cleaves all the hybrid type glycans additionally between the two
10 GlcNAc-residues at the reducing end of the glycan. This digest was subsequently treated and analyzed by MALDI-Tof mass spectrometry in the same way as described above for the N-Glycosidase F digested sample. By comparing the pattern from the N-Glycosidase F digest and the combined N-glycosidase F / Endo H digest, the degree of reduction of the signals of a specific glycostructure is used to estimate
15 the relative content of hybrid structures.

The relative amount of each glycostructure was calculated from the ratio of the peak height of an individual glycol structure and the sum of the peak heights of all glycostructures detected. The relative amount of afucose is the percentage of fucose-lacking structures related to all glycostructures identified in the N-
20 Glycosidase F treated sample (e.g. complex, hybrid and oligo- and high-mannose structures, resp.), see Table 6.

Table 6:

Sample	Afucosylation (in %)
No. 1	70.0
No. 2	72.8
No. 3	72.5
No. 4 (HEK293)	57

Example 9

- 25 Anti-CCR5 antibody-based immunosuppressive regimen in a model of heart transplantation in non-human primates (Cynomolgus monkeys).

The efficacy of monoclonal antibody X is demonstrated in depleting CCR5+ cells in Cynomolgus monkeys receiving a heart transplant from a mismatched donor animal. The number of CCR5+ cells is measured in the graft 4-5 days after

- 41 -

transplant. In addition the immunosuppressive effect of monoclonal antibody X is evaluated as monotherapy, as measured by the duration of allograft survival.

Animals:

5

Mismatched adult male Cynomolgus monkeys; approximately 3-6 kg in weight; no pre-existing significant pathology, in particular free of infections such as simian retrovirus.

N=5 transplants in phase I (A+B).

10 [Future phase II studies, to validate observations in phase I, are also described: N=10]

Transplant procedures:

15 All procedures are performed according to IACUC-approved protocol:

Pre-op blood type, MLR (assure ABO compatibility, high-responder=MHC-mismatch). Archive serum, lymphocytes (baseline).

20 Heterotopic heart transplant (one donor, one recipient), tether placement, telemetry implant D0.

Biopsy graft (spleen, lymph node) D4-5, D14, D28-30, D60, explant D90.

Sacrifice D90 or 30 days after earlier graft explant.

Blood draw (cells, serum) on D0 and on day of each biopsy, plus D21, D45, D75, for alloantibody, FACS, RO-level, cells archived for future study.

25 Immunize with vaccines D14, monitor response subsequently

Study design:

30 As monotherapy, anti-CCR5 antibody affords significantly longer graft survival (>10 days) than historical controls (6 days). Animals are treated until rejection or for 90 days (whatever comes first).

35 10 mg/kg CCR5 antibody (intravenous, once every other week) is administered for up to 90 days post transplantation. Graft biopsies and blood samples are taken at days 3-5 and assessed by IHC and FACS, respectively. Subsequently, blood samples are taken at day 14, once per week to day 30, and biweekly thereafter. Biopsies are obtained monthly until experimental termination at graft failure or on Day 90

- 42 -

(whichever first). At termination, graft is assessed for CAV. If graft failure occurs before day 60, the animal is recovered after graft explant for an additional 30 days of immune monitoring if animal condition allows.

5 **Example 10**

Anti-CCR5 antibody-based immunosuppressive regimen in a model of heart transplantation in non-human primates (Cynomolgus monkeys) – Combination therapy

10 In a further experiment the immunosuppressive effect of monoclonal antibody X in combination with one or more other immunosuppressive agents is evaluated, as measured by the incidence and severity of Cardiac Allograft Vasculopathy (CAV).

The animals and transplantation procedures used are described in Example 9.

15 10 mg/kg CCR5 antibody (intravenous, once every other week) is administered for up to 90 days post transplantation. Combination therapy consists of Cyclosporine A (CsA) at doses starting at 12.5 mg/kg (5-20 mg/kg, dosed by levels to achieve “therapeutic” trough CsA >300), as determined by the PI until graft loss. Acute rejection (diagnosed by 2 of 3 cardinal signs: decreased graft heart rate, decreased graft contractility, increased recipient temperature; diagnosis confirmed by biopsy) will be treated with steroids. Graft biopsies and blood samples are taken at days 4-5
20 and 14 and assessed by IHC and FACS, respectively. Subsequently, blood samples are taken at day 14, once per week to day 30, and biweekly thereafter. Biopsies are obtained monthly until experimental termination at graft failure or on Day 90 (whichever first). At termination, graft is assessed for CAV. If graft failure occurs before day 60, the animal is recovered after graft explant for an additional 30 days
25 of immune monitoring if animal condition allows.

Enclosure to letter of November 9, 2009

International Application No. PCT/EP2009/000133

Applicant: F. Hoffmann La Roche AG

Applicant's Ref.: 24712 WO-ASK

Amended Claims

1. Antibody binding to CCR5 and being glycosylated with a sugar chain at Asn297, said antibody being characterized in that the amount of fucose within said sugar chain is 65 % or lower.
2. Antibody according to claim 1, characterized in that the amount of fucose within said sugar chain is between 5 % and 65 %.
3. Antibody according to claim 1 or 2, characterized in that the amount of NGNA is 1 % or less and/or the amount of N-terminal alpha-1,3-galactose is 1 % or less.
4. Antibody according to any one of claims 1 to 3, characterized in that the amount of NGNA is 0.5 % or less.
5. Antibody according to any one of claims 1 to 4, characterized in that the amount of N-terminal alpha-1,3-galactose is 0.5 % or less.
6. Antibody according to any one of claims 1 to 5, characterized in that the antibody is a chimeric, humanized, or human antibody.
7. Antibody according to any one of claims 1 to 6, characterized by an affinity to CCR5 of about 10^{-13} to 10^{-9} M (K_D).
8. Antibody according to any one of claims 1 to 7, characterized by comprising as heavy chain complementary determining regions (CDRs) the CDRs of SEQ ID NO: 06 and as light chain CDRs the CDRs of SEQ ID NO: 07, as heavy chain CDRs the CDRs of SEQ ID NO: 08 and as light chain CDRs the CDRs of SEQ ID NO: 09, as heavy chain CDRs the CDRs of SEQ ID NO: 10 and as light chain CDRs the CDRs of SEQ ID NO: 11, as heavy chain CDRs the CDRs of SEQ ID NO: 12 and as light chain CDRs the CDRs of SEQ ID NO: 13, as heavy chain CDRs the CDRs of SEQ ID NO: 14 and as light chain CDRs the CDRs of SEQ ID NO: 15, as heavy chain CDRs the CDRs of SEQ ID NO: 16 and as light chain CDRs the CDRs of SEQ ID NO: 19, as heavy chain CDRs the CDRs of SEQ ID NO: 16 and as light chain CDRs the CDRs of SEQ ID NO: 20, as heavy chain CDRs the CDRs of SEQ ID NO: 17 and as light chain CDRs the CDRs of SEQ ID NO: 19, as heavy chain CDRs the CDRs of SEQ ID NO: 17 and as light chain CDRs the CDRs of SEQ ID NO: 20, as heavy chain CDRs the CDRs of

AMENDED SHEET

SEQ ID NO: 18 and as light chain CDRs the CDRs of SEQ ID NO: 19, or as heavy chain CDRs the CDRs of SEQ ID NO: 18 and as light chain CDRs the CDRs of SEQ ID NO: 20.

9. The use of an antibody according to any one of claims 1 to 8 for the manufacture of a pharmaceutical composition.
10. A pharmaceutical composition containing an antibody according to claims 1 to 8.
11. Method for the manufacture of a pharmaceutical composition comprising an antibody according to claims 1 to 8.
12. Use of an antibody according to any one of claims 1 to 8 for the manufacture of a medicament for the treatment or prevention of acute and chronic organ transplant rejection in a mammal, including a human.
13. Use of an antibody according to any one of claims 1 to 8 for the manufacture of a medicament for the treatment or prevention of allograft rejection or for the treatment of inflammation or for the treatment of other immune-mediated diseases.
14. Use of an antibody according to any one of claim 1 to 8 for the manufacture of a medicament for the treatment of graft rejection in combination therapy with an immunosuppressive agent.
15. Use according to claim 14, characterized in that said immunosuppressive agent is a calcineurin inhibitor.
16. Use of an antibody according to any one of claims 1 to 8 for the manufacture of a medicament for the treatment of anti-donor alloantibody production in combination therapy with an immunosuppressive agent.
17. Use according to any one of the claims 13 to 16, characterized in that said antibody is administered in a dose of 10 - 25 mg/kg.
18. Use according to any one of the claims 14 to 17, characterized in that said immunosuppressive agent is Cyclosporin A.
19. Use according to claim 18, characterized in that said Cyclosporin A is administered in a dose of 5 - 20 mg/kg.

- 45 -

20. Use of an antibody according to any one of claims 1 to 9 for the manufacture of a medicament for the treatment of anti-donor alloantibody production in combination therapy with an immunosuppressive agent.
21. Use according to any one of the claims 17 to 20, characterized in that said
5 antibody is administered in a dose of 10 - 25 mg/kg.
22. Use according to any one of the claims 18 to 21, characterized in that said immunosuppressive agent is Cyclosporin A.
23. Use according to claim 22, characterized in that said Cyclosporin A is administered in a dose of 5 - 20 mg/kg.

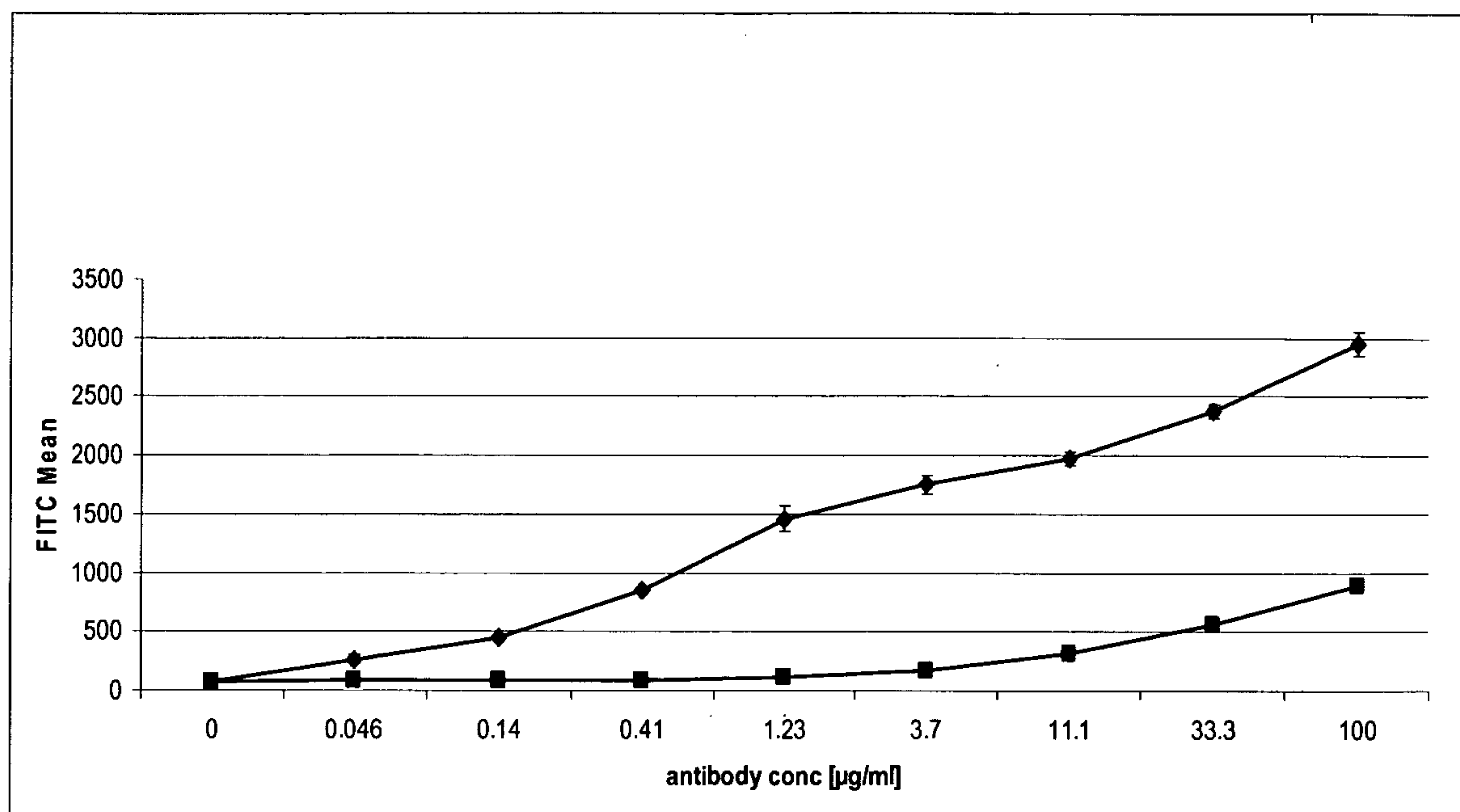
Fig. 1

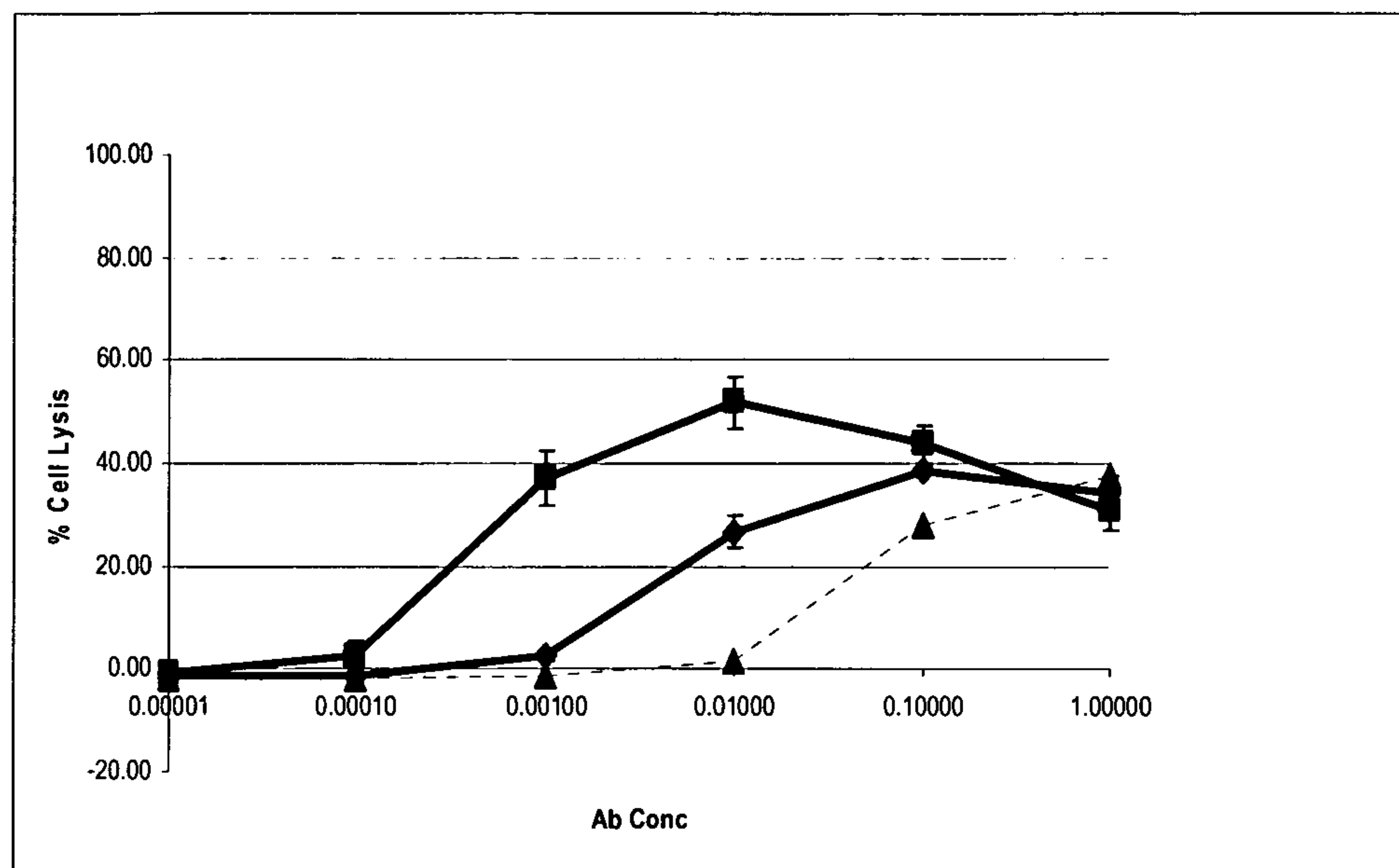
Fig. 2

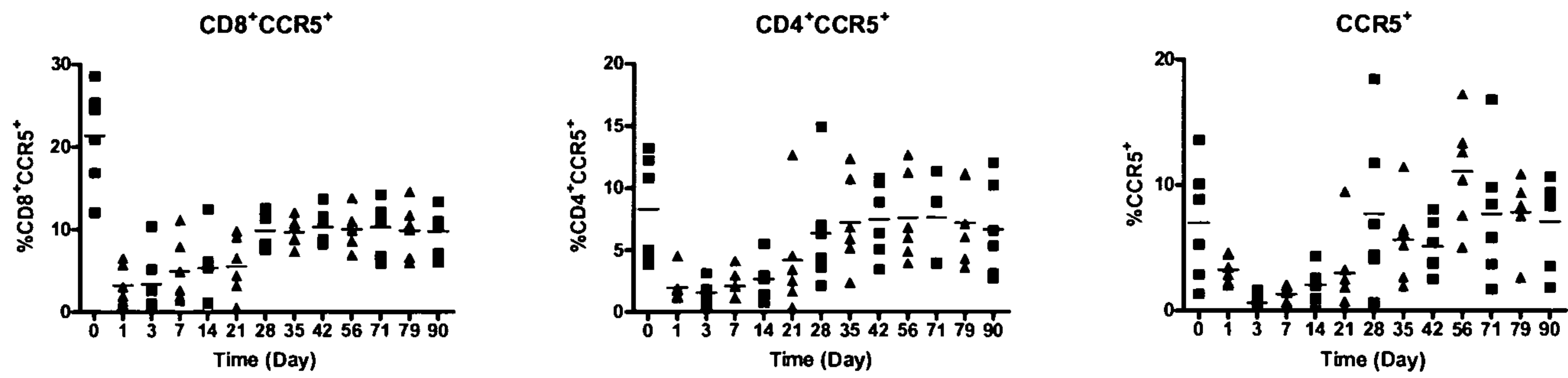
Fig. 3

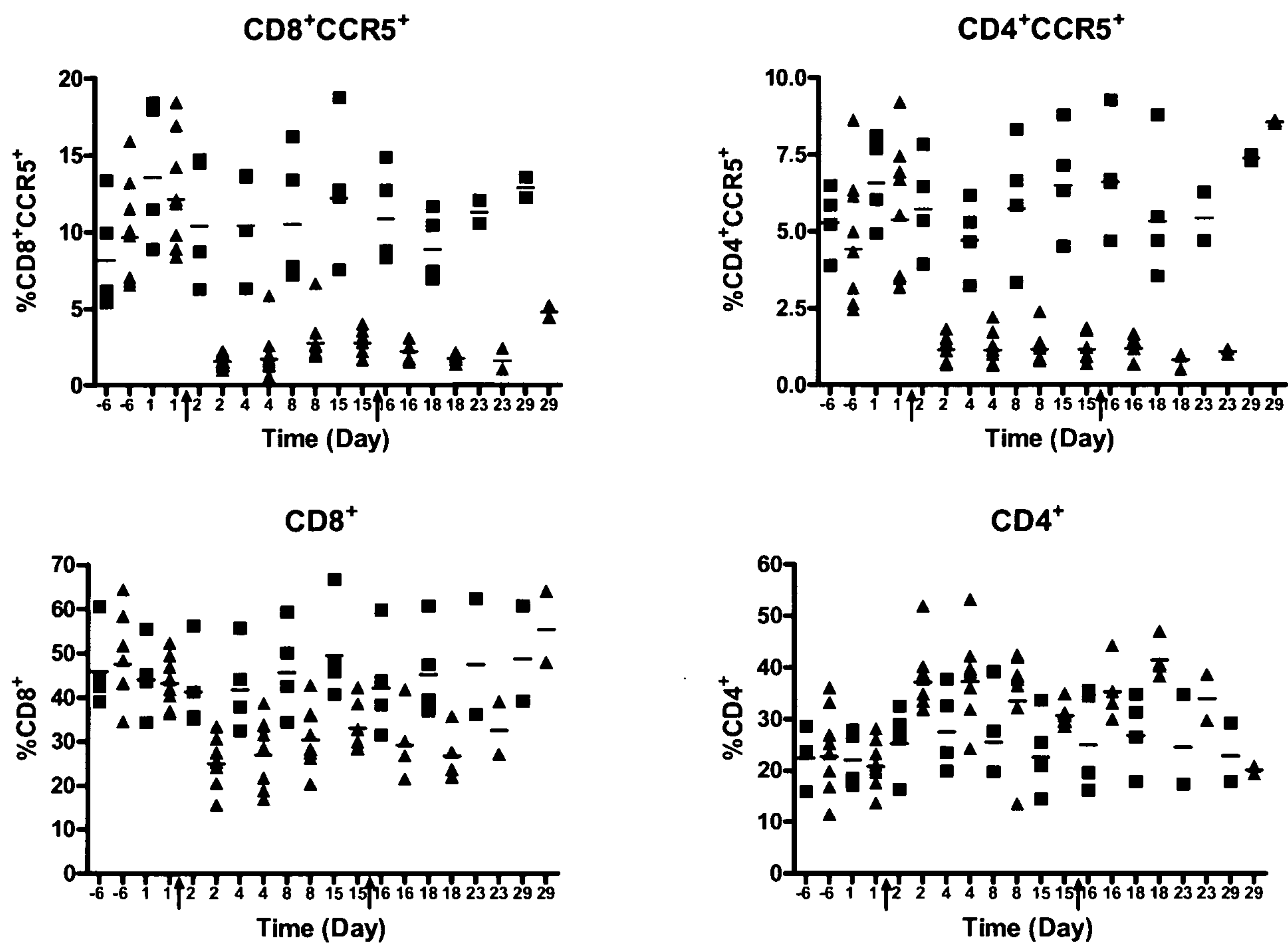
Fig. 4

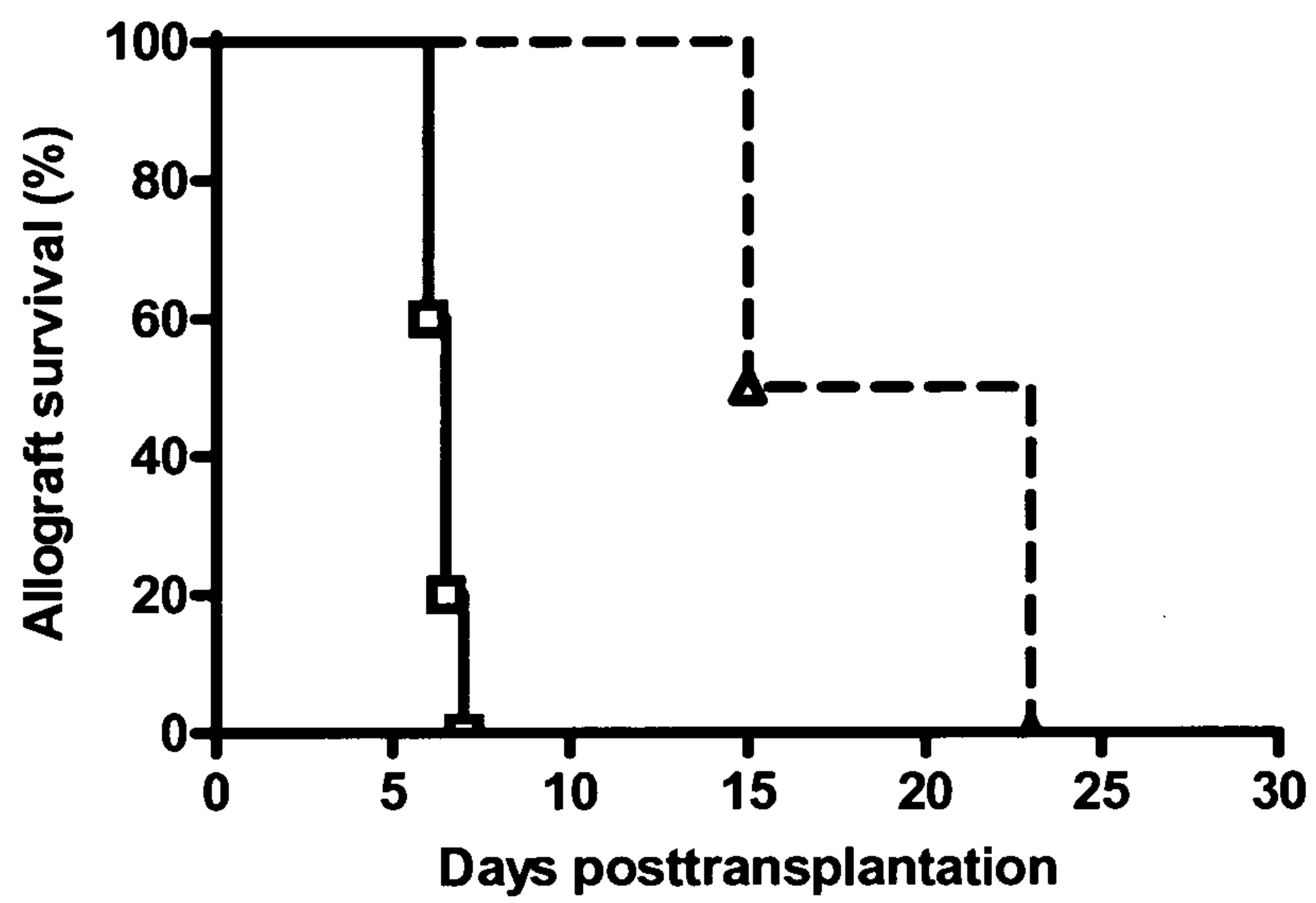
Fig. 5

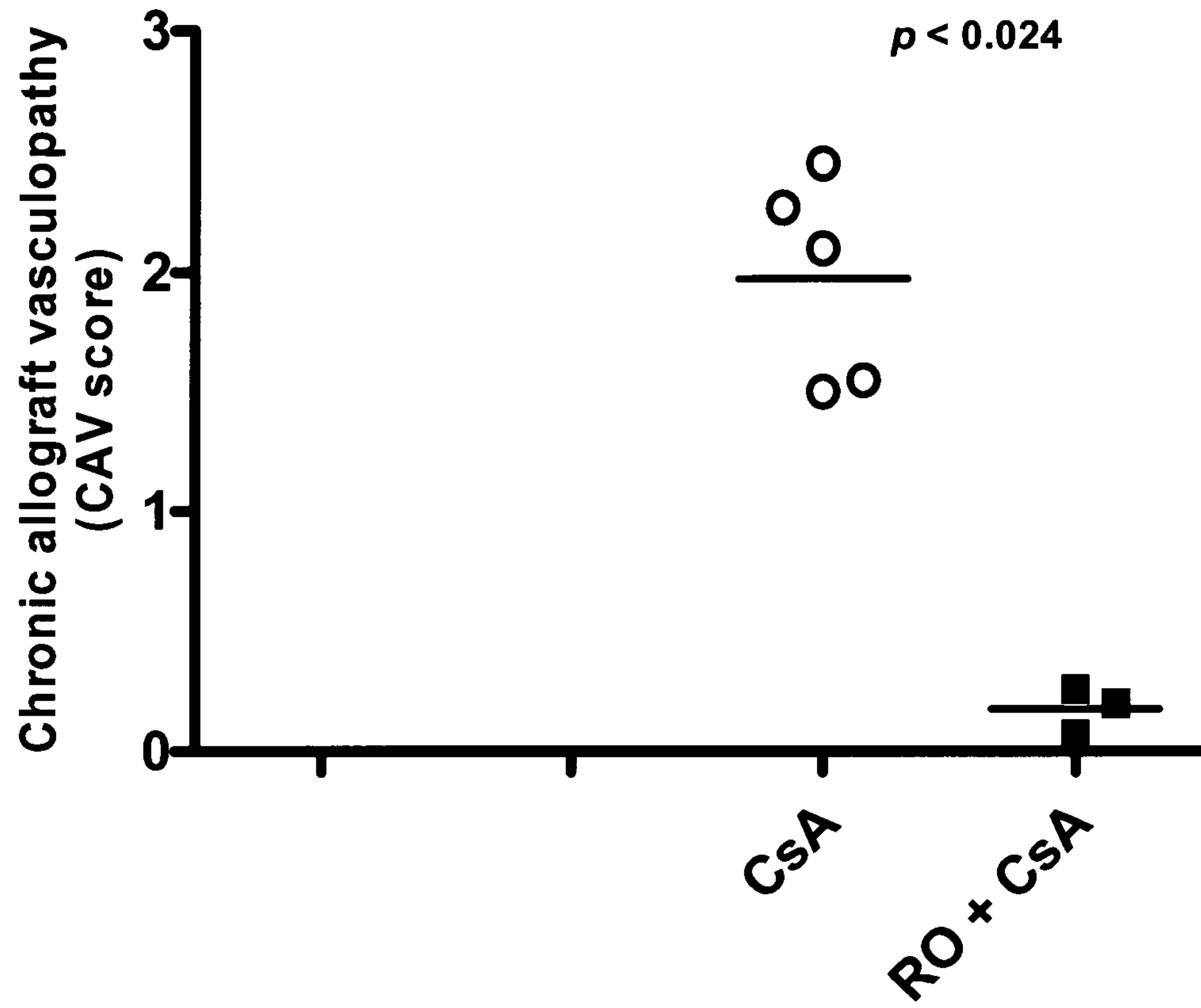
Fig. 6

Fig. 5

