Therapeutic binding molecules

Field of the Invention

The present invention relates to organic compounds, such as to binding molecules against CD45 antigen isoforms, such as for example monoclonal antibodies (mAbs), and use thereof.

Background of the Invention

One approach in the treatment of a variety of diseases is to achieve the elimination or the inactivation of pathogenic leukocytes and the potential for induction of tolerance to inactivate pathological immune responses.

Organ, cell and tissue transplant rejection and the various autoimmune diseases are thought to be primarily the result of T-cell mediated immune response triggered by helper T-cells which are capable of recognizing specific antigens which are captured, processed and presented to the helper T cells by antigen presenting cell (APC) such as macrophages and dendritic cells, in the form of an antigen-MHC complex, i.e. the helper T-cell when recognizing specific antigens is stimulated to produce cytokines such as IL-2 and to express or upregulate some cytokine receptors and other activation molecules and to proliferate. Some of these activated helper T-cells may act directly or indirectly, i.e. assisting effector cytotoxic T-cells or B cells, to destroy cells or tissues expressing the selected antigen. After the termination of the immune response some of the mature clonally selected cells remain as memory helper and memory cytotoxic T-cells, which circulate in the body and rapidly recognize the antigen if appearing again. If the antigen triggering this response is an innocuous environmental antigen the result is allergy, if the antigen is not a foreign antigen, but a self antigen, it can result is autoimmune disease; if the antigen is an antigen from a transplanted organ, the result can be graft rejection.

The immune system has developed to recognize self from non-self. This property enables an organism to survive in an environment exposed to the daily challenges of pathogens. This specificity for non-self and tolerance towards self arises during the development of the T cell repertoire in the thymus through processes of positive and negative selection, which also comprise the recognition and elimination of autoreactive T cells. This type of tolerance is

referred to as central tolerance. However, some of these autoreactive cells escape this selective mechanism and pose a potential hazard for the development of autoimmune diseases. To control the autoreactive T cells that have escaped to the periphery, the immune system has peripheral regulatory mechanisms that provide protection against autoimmunity. These mechanisms are a basis for peripheral tolerance.

Cell surface antigens recognized by specific mAbs are generally designated by a CD (Cluster of Differentiation) number assigned by successive International Leukocyte Typing workshops and the term CD45 applied herein refers to the cell surface leukocyte common antigen CD45; and an mAb to that antigen is designated herein as "anti-CD45".

Antibodies against the leukocyte common antigen (LCA) or CD45 are a major component of anti-lymphocyte globulin (ALG). CD45 belongs to the family of transmembrane tyrosine phosphatases and is both a positive and negative regulator of cell activation, depending upon receptor interaction. The phosphatase activity of CD45 appears to be required for activation of Src-family kinases associated with antigen receptor of B and T lymphocytes (Trowbridge IS et al, Annu Rev Immunol. 1994;12:85-116). Thus, in T cell activation, CD45 is essential for signal 1 and CD45-deficicient cells have profound defects in TCR-mediated activation events.

The CD45 antigen exists in different isoforms comprising a family of transmembrane glycoproteins. Distinct isoforms of CD45 differ in their extracellular domain structure which arise from alternative splicing of 3 variable exons coding for part of the CD45 extracellular region (Streuli MF. et al, J. Exp. Med. 1987; 166:1548-1566). The various isoforms of CD45 have different extra-cellular domains, but have the same transmembrane and cytoplasmic segments having two homologous, highly conserved phosphatase domains of approximately 300 residues. Different isoform combinations are differentially expressed on subpopulations of T and B lymphocytes (Thomas ML. et al, Immunol. Today 1988; 9:320-325). Some monoclonal antibodies recognize an epitope common to all the different isoforms, while other mAbs have a restricted (CD45R) specificity, dependent on which of the alternatively spliced exons (A, B or C) they recognize. For example, monoclonal antibodies recognizing the product of exon A are consequently designated CD45RA, those recognizing the various isoforms containing exon B have been designated CD45RB (Beverley PCL et al, Immunol. Supp. 1988; I:3-5). Antibodies such as UCHL1 selectively bind to the 180 kDa isoform CD45RO (without any of the variable exons A, B or C) which appears to be restricted to a

subset of activated T cells, memory cells and cortical thymocytes and is not detected on B cells (Terry LA et al, Immunol. 1988; 64:331-336).

Description of the Figures

Figure 1 shows that the inhibition of primary MLR by the "candidate mAb" is dose-dependent in the range of 0.001 and 10 µg/ml. "Concentration" is concentration of the "candidate mAb".

Figure 2 shows the plasmid map of the expression vector HCMV-G1 HuA6-VHQ comprising the heavy chain having the nucleotide sequence SEQ ID NO:12 (3921-4274) in the complete expression vector nucleotide sequence SEQ ID NO:15.

Figure 3 shows the plasmid map of the expression vector HCMV-G1 HuA6-VHE comprising the heavy chain having the nucleotide sequence SEQ ID NO:11 (3921-4274) in the complete expression vector nucleotide sequence SEQ ID NO:16.

Figure 4 shows the plasmid map of the expression vector HCMV-K HuAb-VL1 humV1 comprising the light chain having the nucleotide sequence SEQ ID NO:14 (3964-4284) in the complete expression vector nucleotide sequence SEQ ID NO:17.

Figure 5 shows the plasmid map of the expression vector HCMV-K HuAb- VL1 humV2 comprising the light chain having the nucleotide sequence SEQ ID NO:13 (3926-4246) in the complete expression vector nucleotide sequence SEQ ID NO:18.

Figure 6 shows the plasmid map of the expression vector LCVL1SP20 comprising the light chain having the nucleotide sequence SEQ ID NO:33 in the complete expression vector nucleotide sequence SEQ ID NO:36.

Figure 7 shows the plasmid map of the expression vector LCVL2SP20 comprising the light chain having the nucleotide sequence SEQ ID NO:13 in the complete expression vector nucleotide sequence SEQ ID NO:39.

Figure 8 shows the plasmid map of the expression vector HCVHEN73D Sp20 comprising the heavy chain having the nucleotide sequence SEQ ID NO:34 in the complete expression vector nucleotide sequence SEQ ID NO:37.

Figure 9 shows the plasmid map of the expression vector HCVHQN73D Sp20 comprising the heavy chain having the nucleotide sequence SEQ ID NO:35 in the complete expression vector nucleotide sequence SEQ ID NO:38.

Figure 10 shows the plasmid map of the expression vector HCVHESp20 comprising the heavy chain having the nucleotide sequence SEQ ID NO:11 in the complete expression vector nucleotide sequence SEQ ID NO:40.

Figure 11 shows the plasmid map of the expression vector HCVHQSp20 comprising the heavy chain having the nucleotide sequence SEQ ID NO:12 in the complete expression vector nucleotide sequence SEQ ID NO:41.

Figure 12 shows Size Exclusion Chromatography analysis of VHE/humV1, VHE/humV2, VHQ/humV1 and VHQ/humV2, and of VHE/humV1 together with VHE-N73D/humV1.

Figure 13 shows Cation Exchange Chromatography of VHE/humV2, VHE/humV1, VHQ/humV2, VHQ/humV1 and of VHE/humV2 together with VHE-N73D/humV1.

Figure 14 shows Reverse Phase Chromatography of VHE/humV2 and VHE-N73D/humV1.

Description of the Invention

We have now found a binding molecule which comprises a polypeptide sequence which binds to CD45RO and CD45RB, hereinafter also designated as a "CD45RO/RB binding molecule". These binding molecule according to the invention may induce immunosuppression, inhibit primary T cell responses and induce T cell tolerance. Furthermore, the binding molecules of the invention inhibit primary mixed lymphocyte responses (MLR). Cells derived from cultures treated with CD45RO/RB binding molecules preferredly also have impaired proliferative responses in secondary MLR even in the absence of CD45RO/RB binding molecules in the secondary MLR. Such impaired proliferative responses in secondary MLR are an indication of the ability of binding molecules of the invention to induce tolerance. In addition it is found that the CD45RO/RB binding molecules of the invention may induce cell death through apoptosis in human T lymphocytes, may support the differentiation of T cells with a characteristic T regulatory cell (Treg) phenotype; and/or may induce T regulatory cells capable of suppressing naïve T cell activation.

Furthermore, it is found that *in vivo* administration of CD45RO/RB binding molecule to severe combined immunodeficiency (SCID) mice undergoing xeno-GVHD following injection with human PBMC may prolong mice survival, compared to control treated mice, even though circulating human T cells may still be detected in CD45RO/RB binding molecule treated mice. CD45RB/RO binding molecule may also suppress the inflammatory process

that mediates human allograft skin rejection. In addition it is found that CD45RO/RB binding molecule may suppress the inflammatory process that mediates human allograft skin rejection, in particular, may suppress the inflammatory process that mediates human allograft skin rejection *in vivo* in SCID mice transplanted with human skin and engrafted with mononuclear splenocytes. And furthermore, it is found that CD45RB/RO binding molecule may lead to prolonged human islet allograft survival by preventing graft-infiltration and by inhibiting the leukocyte-mediated rejection reaction in vivo.

By "CD45RO/RB binding molecule" is meant any molecule capable of binding specifically to the CD45RB and CD45RO isoforms of the CD45 antigen, either alone or associated with other molecules. The binding reaction may be shown by standard methods (qualitative assay) including for example any kind of binding assay such as direct or indirect immunofluorescence together with fluorescence microscopy or cytofluorimetric (FACS) analysis, enzyme-linked immunosorbent assay (ELISA) or radioimmunoassay in which binding of the molecule to cells expressing a particular CD45 isoform can be visualized. In addition, the binding of this molecule may result in the alteration of the function of the cells expressing these isoforms. For example inhibition of primary or secondary mixed lymphocyte response (MLR) may be determined, such as an in vitro assay or a bioassay for determining the inhibition of primary or secondary MLR in the presence and in the absence of a CD45RO/RB binding molecule and determining the differences in primary MLR inhibition.

Alternatively, the *in vitro* functional modulatory effects can also be determined by measuring the PBMC or T cells or $CD4^+$ T cells proliferation, production of cytokines, change in the expression of cell surface molecules e.g. following cell activation in MLR, or following stimulation with specific antigen such as tetanus toxoid or other antigens, or with polyclonal stimulators such as phytohemagglutinin (PHA) or anti-CD3 and anti-CD28 antibodies or phorbol esters and Ca^{2+} ionophores. The cultures are set up in a similar manner as described for MLR except that instead of allogeneic cells as stimulators soluble antigen or polyclonal stimulators such as those mentioned above are used. T cell proliferation is measured preferably as described above by ^3H-thymidine incorporation.

Cytokine production is measured preferably by sandwich ELISA where a cytokine capture antibody is coated on the surface of a 96-well plate, the supernatants from the cultures are added and incubated for 1 hr at room temperature and a detecting antibody specific for the particular cytokine is then added, following a second-step antibody conjugated to an enzyme

such as Horseradish peroxidase followed by the corresponding substrate and the absorbance is measured in a plate reader. The change in cell surface molecules may be preferably measured by direct or indirect immunofluorescence after staining the target cells with antibodies specific for a particular cell surface molecule. The antibody can be either directly labeled with flourochrome or a fluorescently labeled second step antibody specific for the first antibody can be used, and the cells are analysed with a cytofluorimeter.

The binding molecule of the invention has a binding specificity for both CD45RO and CD45RB ("CD45RB/RO binding molecule").

Preferably the binding molecule binds to CD45RO isoforms with a dissociation constant (Kd) <20nM, preferably with a Kd<15nM or <10nM, more preferably with a Kd<5nM. Preferably the binding molecule binds to CD45RB isoforms with a Kd<50nM, preferably with a Kd<15nM or <10nM, more preferably with a Kd<5nM.

In a further preferred embodiment the binding molecule of the invention binds those CD45 isoforms which

1) include the A and B epitopes but not the C epitope of the CD45 molecule; and/or

2) include the B epitope but not the A and not the C epitope of the CD45 molecule; and/or

3) do not include any of the A, B or C epitopes of the CD45 molecule.

In yet a further preferred embodiment the binding molecule of the invention does not bind CD45 isoforms which include

1) all of the A, B and C epitopes of the CD45 molecule; and/or

2) both the B and C epitopes but not the A epitope of the CD45 molecule.

In further preferred embodiments the binding molecule of the invention further

1) recognises memory and in vivo alloactivated T cells; and/or

2) binds to its target on human T cells, such as for example PEER cells; wherein said binding preferably is with a Kd<15nM, more preferably with a Kd<10nM, most preferably with a Kd<5nM; and/or

3) inhibits in vitro alloreactive T cell function, preferably with an IC_{50} of about less than 100nM, preferably less than 50nM or 30nM, more preferably with an IC_{50} of about 10 or 5nM, most preferably with an IC_{50} of about 0,5nM or even 0,1nM; and/or

4) induces cell death through apoptosis in human T lymphocytes; and/or

5) induces alloantigen-specific T cell tolerance in vitro; and/or

6) prevents lethal xenogeneic graft versus host disease (GvHD) induced in SCID mice by injection of human PBMC when admistiered in an effective amount; and/or

7) binds to T lymphocytes, monocytes, stem cells, natural killer cells and/or granulocytes, but not to platelets or B lymphocytes; and/or

8) supports the differentiation of T cells with a characteristic T regulatory cell (Treg) phenotype; and/or

9) induces T regulatory cells capable of suppressing naïve T cell activation; and/or

10) suppresses the inflammatory process that mediates human allograft skin rejection, in particular, suppresses the inflammatory process that mediates human allograft skin rejection *in vivo* in SCID mice transplanted with human skin and engrafted with mononuclear splenocytes; and/or

11) prolongs human islet allograft survival in a hu-PBL-NOD/SCID mice model.

In a further preferred embodiment the binding molecule of the invention binds to the same epitope as the monoclonal antibody "A6" as described by Aversa et al., Cellular Immunology 158, 314-328 (1994).

Due to the above-described binding properties and biological activities, such binding molecules of the invention are particularly useful in medicine, for therapy and/or prophylaxis. Diseases in which binding molecules of the invention are particularly useful include autoimmune diseases, transplant rejection, dermatitis, inflammatory bowel disease and/or allergies, as will be further set out below.

We have found that a molecule comprising a polypeptide of SEQ ID NO: 1 and a polypeptide of SEQ ID NO: 2 is a CD45RO/RB binding molecule. We also have found the hypervariable regions CDR1', CDR2' and CDR3' in a CD45RO/RB binding molecule of SEQ ID NO:1, CDR1' having the amino acid sequence Arg-Ala-Ser-Gln-Asn-Ile-Gly-Thr-Ser-Ile-Gln (RASQNIGTSIQ) (SEQ ID NO:19), CDR2' having the amino acid sequence Ser-Ser-Ser-Glu-Ser-Ile-Ser (SSSESIS) (SEQ ID NO:20) and CDR3' having the amino acid sequence Gln-Gln-Ser-Asn-Thr-Trp-Pro-Phe-Thr (QQSNTWPFT) (SEQ ID NO:21).

We also have found the hypervariable regions CDR1, CDR2 and CDR3 in a CD45RO/RB binding molecule of SEQ ID NO:2, CDR1 having the amino acid sequence Asn-Tyr-Ile-Ile-His (NYIIH) (SEQ ID NO:22), CDR2 having the amino acid sequence Tyr-Phe-Asn-Pro-Tyr-

Asn-His-Gly-Thr-Lys-Tyr-Asn-Glu-Lys-Phe -Lys-Gly (YFNPYNHGTKYNEKFKG) (SEQ ID NO:23) and CDR3 having the amino acid sequence Ser-Gly-Pro-Tyr-Ala-Trp-Phe-Asp-Thr (SGPYAWFDT) (SEQ ID NO:24).

CDRs are 3 specific complementary determining regions which are also called hypervariable regions which essentially determine the antigen binding characteristics. These CDRs are part of the variable region, e.g. of SEQ ID NO: 1 or SEQ ID NO: 2, respectively, wherein these CDRs alternate with framework regions (FR's) e.g. constant regions. A SEQ ID NO: 1 is part of a light chain, e.g. of SEQ ID NO: 3, and a SEQ ID NO:2 is part of a heavy chain, e.g. of SEQ ID NO: 4, in a chimeric antibody according to the present invention. The CDRs of a heavy chain together with the CDRs of an associated light chain essentially constitute the antigen binding site of a molecule of the present invention. It is known that the contribution made by a light chain variable region to the energetics of binding is small compared to that made by the associated heavy chain variable region and that isolated heavy chain variable regions have an antigen binding activity on their own. Such molecules are commonly referred to as single domain antibodies.

In one aspect the present invention provides a binding molecule comprising at least one antigen binding site, e.g. a CD45RO/RB binding molecule, comprising in sequence the hypervariable regions CDR1, CDR2 and CDR3, said CDR1 having the amino acid sequence Asn-Tyr-Ile-Ile-His (NYIIH) (SEQ ID NO:22), said CDR2 having the amino acid sequence Tyr-Phe-Asn-Pro-Tyr-Asn-His-Gly-Thr-Lys-Tyr-Asn-Glu-Lys-Phe -Lys-Gly (YFNPYNHGTKYNEKFKG) (SEQ ID NO:23) and said CDR3 having the amino acid sequence Ser-Gly-Pro-Tyr-Ala-Trp-Phe-Asp-Thr (SGPYAWFDT) (SEQ ID NO:24); e.g. and direct equivalents thereof.

In another aspect the present invention provides a molecule comprising at least one antigen binding site, e.g. a CD45RO/RB binding molecule, comprising

a) a first domain comprising in sequence the hypervariable regions CDR1, CDR2 and CDR3, said CDR1 having the amino acid sequence Asn-Tyr-Ile-Ile-His (NYIIH) (SEQ ID NO:22), said CDR2 having the amino acid sequence Tyr-Phe-Asn-Pro-Tyr-Asn-His-Gly-Thr-Lys-Tyr-Asn-Glu-Lys-Phe -Lys-Gly (YFNPYNHGTKYNEKFKG) (SEQ ID NO:23) and said CDR3 having the amino acid sequence Ser-Gly-Pro-Tyr-Ala-Trp-Phe-Asp-Thr (SGPYAWFDT) (SEQ ID NO:24); and

b) a second domain comprising in sequence the hypervariable regions CDR1', CDR2' and CDR3', CDR1' having the amino acid sequence Arg-Ala-Ser-Gln-Asn-Ile-Gly-Thr-Ser-Ile-Gln (RASQNIGTSIQ) (SEQ ID NO:19), CDR2' having the amino acid sequence Ser-Ser-Ser-Glu-Ser-Ile-Ser (SSSESIS) (SEQ ID NO:20) and CDR3' having the amino acid sequence Gln-Gln-Ser-Asn-Thr-Trp-Pro-Phe-Thr (QQSNTWPFT) (SEQ ID NO:21),

e.g. and direct equivalents thereof.

In a preferred embodiment the first domain comprising in sequence the hypervariable regions CDR1, CDR2 and CDR3 is an immunoglobulin heavy chain, and the second domain comprising in sequence the hypervariable regions CDR1', CDR2' and CDR3' is an immunoglobulin light chain.

In a further aspect the present invention provides a molecule, e.g. a CD45RO/RB binding molecule, comprising a polypeptide of SEQ ID NO: 1 and/or a polypeptide of SEQ ID NO: 2, preferably comprising in one domain a polypeptide of SEQ ID NO: 1 and in another domain a polypeptide of SEQ ID NO: 2, e.g. a chimeric monoclonal antibody, and in another aspect a molecule, e.g. a CD45RO/RB binding molecule, comprising a polypeptide of SEQ ID NO: 3 and/or a polypeptide of SEQ ID NO: 4, preferably comprising in one domain a polypeptide of SEQ ID NO: 3 and in another domain a polypeptide of SEQ ID NO: 4, e.g. a chimeric monoclonal antibody.

When the antigen binding site comprises both the first and second domains or a polypeptide of SEQ ID NO: 1 or SEQ ID NO:3, respectively, and a polypeptide of SEQ ID NO: 2 or of SEQ ID NO:4, respectively, these may be located on the same polypeptide, or, preferably each domain may be on a different chain, e.g. the first domain being part of an heavy chain, e.g. immunoglobulin heavy chain, or fragment thereof and the second domain being part of a light chain, e.g. an immunoglobulin light chain or fragment thereof.

We have further found that a CD45RO/RB binding molecule according to the present invention is a CD45RO/RB binding molecule in mammalian, e.g. human, body environment. A CD45RO/RB binding molecule according to the present invention can thus be designated as a monoclonal antibody (mAb), wherein the binding activity is determined mainly by the CDR regions as described above, e.g. said CDR regions being associated with other molecules without binding specifity, such as framework, e.g. constant regions, which are substantially of human origin.

In another aspect the present invention provides a CD45RO/RB binding molecule which is not the monoclonal antibody "A6" as described by Aversa et al., Cellular Immunology 158, 314-328 (1994), which is incorporated by reference for the passages characterizing A6.

In another aspect the present invention provides a CD45RO/RB binding molecule according to the present invention which is a chimeric, a humanised or a fully human monoclonal antibody.

Examples of a CD45RO/RB binding molecules include chimeric or humanised antibodies e.g. derived from antibodies as produced by B-cells or hybridomas and or any fragment thereof, e.g. F(ab')2 and Fab fragments, as well as single chain or single domain antibodies. A single chain antibody consists of the variable regions of antibody heavy and light chains covalently bound by a peptide linker, usually consisting of from 10 to 30 amino acids, preferably from 15 to 25 amino acids. Therefore, such a structure does not include the constant part of the heavy and light chains and it is believed that the small peptide spacer should be less antigenic than a whole constant part. By a chimeric antibody is meant an antibody in which the constant regions of heavy and light chains or both are of human origin while the variable domains of both heavy and light chains are of non-human (e.g. murine) origin. By a humanised antibody is meant an antibody in which the hypervariable regions (CDRs) are of non-human (e.g. murine) origin while all or substantially all the other part, e.g. the constant regions and the highly conserved parts of the variable regions are of human origins. A humanised antibody may however retain a few amino acids of the murine sequence in the parts of the variable regions adjacent to the hypervariable regions.

Hypervariable regions, i.e. CDR's according to the present invention may be associated with any kind of framework regions, e.g. constant parts of the light and heavy chains, of human origin. Suitable framework regions are e.g. described in "Sequences of proteins of immunological interest", Kabat, E.A. et al, US department of health and human services, Public health service, National Institute of health. Preferably the constant part of a human heavy chain may be of the IgG1 type, including subtypes, preferably the constant part of a human light chain may be of the κ or λ type, more preferably of the κ type. Preferably, said heavy chain comprises not more than one glycosylation site, most preferably the glycosylation site is a N-glycosylation site, and most preferably the one glycosylation site is located in the constant part of the heavy chain. Most preferably no glycosylation site is present in the variable region, preferably no glycosylation site in the framework region.

A preferred constant part of a heavy chain is a polypeptide of SEQ ID NO: 4 (without the CDR1', CDR2' and CDR3' sequence parts which are specified above) and a preferred constant part of a light chain is a polypeptide of SEQ ID NO: 3 (without the CDR1, CDR2 and CDR3 sequence parts which are specified above).

We also have found a humanised antibody comprising a light chain variable region of amino acid SEQ ID NO:7 or of amino acid SEQ ID NO:8, which comprises CDR1', CDR2' and CDR3' according to the present invention and/or a heavy chain variable region of SEQ:ID NO:9 or of SEQ:ID NO:10, which comprises CDR1, CDR2 and CDR3 according to the present invention. Further another humanised antibody is provided comprising a light chain variable region of amino acid SEQ ID NO:7 or of amino acid SEQ ID NO:8, which comprises CDR1', CDR2' and CDR3' according to the present invention and/or a heavy chain variable region of SEQ:ID NO:31 or of SEQ:ID NO:32, which comprises CDR1, CDR2 and CDR3 according to the present invention.

In another aspect the present invention provides a humanised antibody comprising a polypeptide of SEQ ID NO:9 or of SEQ ID NO:10 and a polypeptide of SEQ ID NO:7 or of SEQ ID NO:8. A still further aspect of the invention provides a binding molecule which is a humanised antibody comprising a polypeptide of SEQ ID NO:31 or of SEQ ID NO:32 and a polypeptide of SEQ ID NO:7 or of SEQ ID NO:8.

In another aspect the present invention provides a humanised antibody comprising

- a polypeptide of SEQ ID NO:9 and a polypeptide of SEQ ID NO:7 (such as VHE/humV2),
- a polypeptide of SEQ ID NO:9 and a polypeptide of SEQ ID NO:8 (such as VHE/humV1),
- a polypeptide of SEQ ID NO:10 and a polypeptide of SEQ ID NO:7 (such as VHQ/humV2),
- a polypeptide of SEQ ID NO:10 and a polypeptide of SEQ ID NO:8 (such as VHQ/humV1),
- a polypeptide of SEQ ID NO:31 and a polypeptide of SEQ ID NO:7 (such as VHEN73D/humV2),
- a polypeptide of SEQ ID NO:31 and a polypeptide of SEQ ID NO:8 (such as VHEN73D/humV1),
- a polypeptide of SEQ ID NO:32 and a polypeptide of SEQ ID NO:7 (such as VHQN73D/humV2), or
- a polypeptide of SEQ ID NO:32 and a polypeptide of SEQ ID NO:8 (such as VHQN73D/humV1).

A polypeptide according to the present invention, e.g. of a herein specified sequence, e.g. of CDR1 (SEQ ID NO:22), CDR2 (SEQ ID NO:23), CDR3 (SEQ ID NO:24), CDR1' (SEQ ID NO:19), CDR2' (SEQ ID NO:20), CDR3' (SEQ ID NO:21), or of a SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:31 or SEQ ID NO:32 includes direct equivalents of said (poly)peptide (sequence); e.g. including a functional derivative of said polypeptide. Said functional derivative may include covalent modifications of a specified sequence, and/or said functional derivative may include amino acid sequence variants of a specified sequence.

"Polypeptide", if not otherwise specified herein, includes any peptide or protein comprising amino acids joined to each other by peptide bonds, having an amino acid sequence starting at the N-terminal extremity and ending at the C-terminal extremity. Preferably the polypeptide of the present invention is a monoclonal antibody, more preferred is a chimeric (V-grafted) or humanised (CDR-grafted) monoclonal antibody. The humanised (CDR-grafted) monoclonal antibody may or may not include further mutations introduced into the framework (FR) sequences of the acceptor antibody. Preferably the humanized or chimeric antibody comprises no more than one glycosylation site. Most preferably said one glycosylation site is a N-glycosylation iste. Most preferably no glycosylation site is present in the variable region, and even more preferably no glycosylation site is present in the variable region of the heavy chain, most preferably no glycosylation site is present in the framework regions (FR's).

A functional derivative of a polypeptide as used herein includes a molecule having a qualitative biological activity in common with a polypeptide to the present invention, i.e. having the ability to bind to CD45RO and CD45RB. A functional derivative includes fragments and peptide analogs of a polpypeptide according to the present invention. Fragments comprise regions within the sequence of a polypeptide according to the present invention, e.g. of a specified sequence. The term "derivative" is used to define amino acid sequence variants, and covalent modifications of a polypeptide according to the present invention. e.g. of a specified sequence. The functional derivatives of a polypeptide according to the present invention, e.g. of a specified sequence, preferably have at least about 65%, more preferably at least about 75%, even more preferably at least about 85%, most preferably at least about 95% overall sequence homology with the amino acid sequence of a polypeptide according to the present invention, e.g. of a specified sequence, and substantially retain the ability to bind to CD45RO and CD45RB. Preferably, the functional

derivative has at least the binding affinity of a binding molecule comprising a polypeptide of SEQ ID NO:1 and/or a polypeptide of SEQ ID NO:2, of a humanised antibody comprising a polypeptide of SEQ ID NO:9 or of SEQ ID NO:10 and/or a polypeptide of SEQ ID NO:7 or of SEQ ID NO:8; or of a humanised antibody comprising a polypeptide of SEQ ID NO:31 or of SEQ ID NO:32 and/or a polypeptide of SEQ ID NO:7 or of SEQ ID NO:8.

The term "covalent modification" includes modifications of a polypeptide according to the present invention, e.g. of a specified sequence; or a fragment thereof with an organic proteinaceous or non-proteinaceous derivatizing agent, fusions to heterologous polypeptide sequences, and post-translational modifications. Covalent modified polypeptides, e.g. of a specified sequence, still have the ability bind to CD45RO and CD45RB by crosslinking. Covalent modifications are traditionally introduced by reacting targeted amino acid residues with an organic derivatizing agent that is capable of reacting with selected sides or terminal residues, or by harnessing mechanisms of post-translational modifications that function in selected recombinant host cells. Certain post-translational modifications are the result of the action of recombinant host cells on the expressed polypeptide. Glutaminyl and asparaginyl residues are frequently post-translationally deamidated to the corresponding glutamyl and aspartyl residues. Alternatively, these residues are deaminated under mildly acidic conditions. Other post-translational modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl, tyrosine or threonyl residues, methylation of the α-amino groups of lysine, arginine, and histidine side chains, see e.g. T. E. Creighton, Proteins: Structure and Molecular Properties, W. H. Freeman & Co., San Francisco, pp. 79-86 (1983). Covalent modifications e.g. include fusion proteins comprising a polypeptide according to the present invention, e.g. of a specified sequence and their amino acid sequence variants, such as immunoadhesins, and N-terminal fusions to heterologous signal sequences.

"Homology" with respect to a native polypeptide and its functional derivative is defined herein as the percentage of amino acid residues in the candidate sequence that are identical with the residues of a corresponding native polypeptide, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent homology, and not considering any conservative substitutions as part of the sequence identity. Neither N- or C-terminal extensions nor insertions shall be construed as reducing identity or homology. Methods and computer programs for the alignment are well known.

"Amino acid(s)" refer to all naturally occurring L-α-amino acids, e.g. and including D-amino acids. The amino acids are identified by either the well known single-letter or three-letter designations.

The term "amino acid sequence variant" refers to molecules with some differences in their amino acid sequences as compared to a polypeptide according to the present invention, e.g. of a specified sequence. Amino acid sequence variants of a polypeptide according to the present invention, e.g. of a specified sequence, still have the ability to bind to CD45RO and CD45RB. Substitutional variants are those that have at least one amino acid residue removed and a different amino acid inserted in its place at the same position in a polypeptide according to the present invention, e.g. of a specified sequence. These substitutions may be single, where only one amino acid in the molecule has been substituted, or they may be multiple, where two or more amino acids have been substituted in the same molecule. Insertional variants are those with one or more amino acids inserted immediately adjacent to an amino acid at a particular position in a polypeptide according to the present invention, e.g. of a specified sequence. Immediately adjacent to an amino acid means connected to either the α-carboxy or α-amino functional group of the amino acid. Deletional variants are those with one or more amino acids in a polypeptide according to the present invention, e.g. of a specified sequence, removed. Ordinarily, deletional variants will have one or two amino acids deleted in a particular region of the molecule.

We also have found the polynucleotide sequences of

- GGCCAGTCAGAACATTGGCACAAGCATACAGTG (SEQ ID NO:25), encoding the amino acid sequence of CDR1;
- TTCTTCTGAGTCTATCTCTGG (SEQ ID NO:26), encoding the amino acid sequence of CDR 2;
- ACAAAGTAATACCTGGCCATTCACGTT (SEQ ID NO:27), encoding the amino acid sequence of CDR 3;
- TTATATTATCCACTG (SEQ ID NO:28), encoding the amino acid sequence of CDR1',
- TTTTAATCCTTACAATCATGGTACTAAGTACAATGAGAAGTTCAAAGGCAG (SEQ ID NO:29), encoding the amino acid sequence of CDR2';
- AGGACCCTATGCCTGGTTTGACACCTG (SEQ ID NO:30), encoding the amino acid sequence of CDR3';
- SEQ ID NO:5 encoding a polypeptide of SEQ ID NO: 1, i.e. the variable region of a light chain of an mAb according to the present invention;

- SEQ ID NO:6 encoding a polypeptide of SEQ ID NO:2, i.e. the variable region of the heavy chain of an mAb according to the present invention;
- SEQ ID NO:11 encoding a polypeptide of SEQ ID NO:9. i.e. a heavy chain variable region including CDR1, CDR2 and CDR3 according to the present invention;
- SEQ ID NO:12 encoding a polypeptide of SEQ ID NO:10, i.e. a heavy chain variable region including CDR1, CDR2 and CDR3 according to the present invention;
- SEQ ID NO:13 encoding a polypeptide of SEQ ID NO:7, i.e. a light chain variable region including CDR1', CDR2' and CDR3' according to the present invention;
- SEQ ID NO:14 encoding a polypeptide of SEQ ID NO:8, i.e. a light chain variable region including CDR1', CDR2' and CDR3' according to the present invention;
- SEQ ID NO:33 encoding a polypeptide of SEQ ID NO:8, i.e. a light chain variable region including CDR1', CDR2' and CDR3' according to the present invention;
- SEQ ID NO:34 encoding a polypeptide of SEQ ID NO:31, i.e. a heavy chain variable region including CDR1, CDR2 and CDR3 according to the present invention; and
- SEQ ID NO:35 encoding a polypeptide of SEQ ID NO:32, i.e. a heavy chain variable region including CDR1, CDR2 and CDR3 according to the present invention;

In another aspect the present invention provides isolated polynucleotides comprising polynucleotides encoding a CD45RO/RB binding molecule, e.g. encoding the amino acid sequence of CDR1, CDR2 and CDR3 according to the present invention and/or, preferably and, polynucletides encoding the amino acid sequence of CDR1', CDR2' and CDR3' according to the present invention; and

Polynucleotides comprising a polynucleotide of SEQ ID NO: 5 and/or, preferably and, a polynucleotide of SEQ ID NO: 6; and

polynucleotides comprising polynucleotides encoding a polypeptide of SEQ ID NO:7 or SEQ ID NO:8 and/or, preferably and, a polypeptide of SEQ ID NO:9 or SEQ ID NO:10; e.g. encoding

- a polypeptide of SEQ ID NO:7 and a polypeptide of SEQ ID NO:9,
- a polypeptide of SEQ ID NO:7 and a polypeptide of SEQ ID NO:10,
- a polypeptide of SEQ ID NO:8 and a polypeptide of SEQ ID NO:9, or
- a polypeptide of SEQ ID NO:8 and a polypeptide of SEQ ID NO:10; and

polynucleotides comprising a polynucleotide of SEQ ID NO:11 or of SEQ ID NO:12 and/or, preferably and, a polynucleotide of SEQ ID NO:13 or a polynucleotide of SEQ ID NO:14, preferably comprising

- a polynucleotide of SEQ ID NO:11 and a polynucleotide of SEQ ID NO:13,
- a polynucleotide of SEQ ID NO:11 and a polynucleotide of SEQ ID NO:14,
- a polynucleotide of SEQ ID NO:12 and a polynucleotide of SEQ ID NO:13, or
- a polynucleotide of SEQ ID NO:12 and a polynucleotide of SEQ ID NO:14; and

polynucleotides comprising polynucleotides encoding a polypeptide of SEQ ID NO:31 or of SEQ ID NO:32 and/or, preferably and, a polypeptide of SEQ ID NO:7 or of SEQ ID NO:8; e.g. encoding

- a polypeptide of SEQ ID NO:31 and a polypeptide of SEQ ID NO:7,
- a polypeptide of SEQ ID NO:31 and a polypeptide of SEQ ID NO:8,
- a polypeptide of SEQ ID NO:32 and a polypeptide of SEQ ID NO:7, or
- a polypeptide of SEQ ID NO:32 and a polypeptide of SEQ ID NO:8; and

polynucleotides comprising a polynucleotide of SEQ ID NO:34 or of SEQ ID NO:35 and/or, preferably and, a polynucleotide of SEQ ID NO:33; SEQ ID NO:14 or 13.

- a polypeptide of SEQ ID NO:34 and a polypeptide of SEQ ID NO:33,
- a polypeptide of SEQ ID NO:34 and a polypeptide of SEQ ID NO:14,
- a polypeptide of SEQ ID NO:34 and a polypeptide of SEQ ID NO:13,
- a polypeptide of SEQ ID NO:35 and a polypeptide of SEQ ID NO:33,
- a polypeptide of SEQ ID NO:35 and a polypeptide of SEQ ID NO:14, or
- a polypeptide of SEQ ID NO:35 and a polypeptide of SEQ ID NO:13.

"Polynucleotide", if not otherwise specified herein, includes any polyribonucleotide or polydeoxyribonucleotide, which may be unmodified RNA or DNA, or modified RNA or DNA, including without limitation single and double stranded RNA, and RNA that is a mixture of single- and double-stranded regions.

A polynucleotide according to the present invention, e.g. a polynucleotide encoding the amino acid sequence CDR1, CDR2, CDR3, CDR1', CDR2', CDR3', or of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:31 or SEQ ID NO:32 respectively, such as a polynucleotide of SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:33, SEQ ID NO:34 or SEQ ID NO:35 respectively, includes allelic variants thereof

and/or their complements; e.g. including a polynucleotide that hybridizes to the nucleotide sequence of SEQ ID NO: 5, SEQ ID NO:6, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:33, SEQ ID NO:34 or SEQ ID NO:35, respectively; e.g. encoding a polypeptide having at least 80% identity to SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:31 or SEQ ID NO:32, respectively, e.g. including a functional derivative of said polypeptide, e.g. said functional derivative having at least 65% homology with SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:31 or SEQ ID NO:32, respectively, e.g. said functional derivative including covalent modifications of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:31 or SEQ ID NO:32, respectively, e.g. said functional derivative including amino acid sequence variants of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:31 or SEQ ID NO:32, respectively; e.g. a SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:33, SEQ ID NO:34 or SEQ ID NO:35, respectively includes a sequence, which as a result of the redundancy (degeneracy) of the genetic code, also encodes a polypeptide of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:31 or SEQ ID NO:32, respectively, or encodes a polypeptide with an amino acid sequence which has at least 80% identity with the amino acid sequence of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:31 or SEQ ID NO:32, respectively. Preferably, the allelic variants or functional derivative has at least the binding affinity of a binding molecule comprising a polypeptide of SEQ ID NO:1 and/or a polypeptide of SEQ ID NO:2, of a humanised antibody comprising a polypeptide of SEQ ID NO:9 or of SEQ ID NO:10 and/or a polypeptide of SEQ ID NO:7 or of SEQ ID NO:8; or of a humanised antibody comprising a polypeptide of SEQ ID NO:31 or of SEQ ID NO:32 and/or a polypeptide of SEQ ID NO:7 or of SEQ ID NO:8.

A CD45RO/RB binding molecule, e.g. which is a chimeric or humanised antibody, may be produced by recombinant DNA techniques. Thus, one or more DNA molecules encoding the CD45RO/RB may be constructed, placed under appropriate control sequences and transferred into a suitable host (organism) for expression by an appropriate vector.

In another aspect the present invention provides a polynucleotide which encodes a single, heavy and/or a light chain of a CD45RO/RB binding molecule according to the present invention; and the use of a polynucleotide according to the present invention for the production of a CD45RO/RB binding molecule according to the present invention by recombinant means.

A CD45RO/RB binding molecule may be obtained according, e.g. analogously, to a method as conventional together with the information provided herein, e.g. with the knowledge of the amino acid sequence of the hypervariable or variable regions and the polynucleotide sequences encoding these regions. A method for constructing a variable domain gene is e.g. described in EP 239 400 and may be briefly summarized as follows: A gene encoding a variable region of a mAb of whatever specificity may be cloned. The DNA segments encoding the framework and hypervariable regions are determined and the DNA segments encoding the hypervariable regions are removed. Double stranded synthetic CDR cassettes are prepared by DNA synthesis according to the CDR and CDR' sequences as specified herein. These cassettes are provided with sticky ends so that they can be ligated at junctions of a desired framework of human origin. Polynucleotides encoding single chain antibodies may also be prepared according to, e.g. analogously, to a method as conventional. A polynucleotide according to the present invention thus prepared may be conveniently transferred into an appropriate expression vector.

Appropriate cell lines may be found according, e.g. analogously, to a method as conventional. Expression vectors, e.g. comprising suitable promotor(s) and genes encoding heavy and light chain constant parts are known e.g. and are commercially available. Appropriate hosts are known or may be found according, e.g. analogously, to a method as conventional and include cell culture or transgenic animals.

In another aspect the present invention provides an expression vector comprising a polynucleotide encoding a CD45RO/RB binding molecule according to the present invention, e.g. of sequence SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40 or SEQ ID NO:41.

In another aspect the present invention provides

- An expression system comprising a polynucleotide according to the present invention wherein said expression system or part thereof is capable of producing a CD45RO/RB

binding molecule according to the present invention, when said expression system or part thereof is present in a compatible host cell;

and

- An isolated host cell which comprises an expression system as defined above.

We have further found that a CD45RO/RB binding molecule according to the present invention inhibit primary alloimmune responses in a dose-dependent fashion as determined by in vitro MLR. The results indicate that the cells which had been alloactivated in the presence of a CD45RO/RB binding molecule according to the present invention are impaired in their responses to alloantigen. This confirms the indication that a CD45RO/RB binding molecule according to the present invention can act directly on the effector alloreactive T cells and modulate their function. In addition, the functional properties of T cells derived from the primary MLR were further studied in restimulation experiments in secondary MLR, using specific stimulator cells or third-party stimulators to assess the specificity of the observed functional effects. We have found that the cells derived from primary MLRs in which a CD45RO/RB binding molecule according to the present invention is present, were impaired in their ability to respond to subsequent optimal stimulation with specific stimulator cells, although there was no antibody added to the secondary cultures. The specificity of the inhibition was demonstrated by the ability of cells treated with a CD45RO/RB binding molecule according to the present invention to respond normally to stimulator cells from unrelated third-party donors. Restimulation experiments using T cells derived from primary MLR cultures thus indicate that the cells which had been alloactivated a CD45RO/RB binding molecule according to the present invention are hyporesponsive, i.e. tolerant, to the original alloantigen. Further biological activities are described in examples 7, and 9 to 13.

Furthermore we have found that cell proliferation in cells pre-treated with a CD45RO/RB binding molecule according to the present invention could be rescued by exogenous IL-2. This indicates that treatment of alloreactive T cells with a CD45RO/RB binding molecule according to the present invention induces a state of tolerance. Indeed, the reduced proliferative responses observed in cells treated with a CD45RO/RB binding molecule according to the present invention, was due to impairement of T cell function, and these cells were able to respond to exogenous IL-2, indicating that these cells are in an anergic, true unresponsive state. The specificity of this response was shown by the ability of cells treated with a CD45RO/RB binding molecule according to the present invention to proliferate normally to unrelated donor cells to the level of the control treated cells.

In addition experiments indicate that the binding of a CD45RO/RB binding molecule according to the present invention to CD45RO and CD45RB may inhibit the memory responses of peripheral blood mononuclear cells (PBMC) from immunized donors to specific recall antigen. Binding of a CD45RO/RB binding molecule according to the present invention to CD45RO and CD45RB thus is also effective in inhibiting memory responses to soluble Ag. The ability of a CD45RO/RB binding molecule according to the present invention to inhibit recall responses to tetanus in PBMC from immunized donors indicate that a CD45RO/RB binding molecule according to the present invention is able to target and modulate the activation of memory T cells. E.g. these data indicate that a CD45RO/RB binding molecule according to the present invention in addition to recognizing alloreactive and activated T cells is able to modulate their function, resulting in induction of T cell anergy. This property may be important in treatment of ongoing immune responses to autoantigens and allergens and possibly to alloantigens as seen in autoimmune diseases, allergy and chronic rejection, and diseases, such as psoriasis, inflammatory bowel disease, where memory responses play a role in the maintenance of disease state. It is believed to be an important feature in a disease situation, such as in autoimmune diseases in which memory responses to autoantigens may play a major role for the disease maintenance.

We have also found that a CD45RO/RB binding molecule according to the present invention may modulate T cell proliferative responses in a mixed lymphocyte response (MLR) *in vivo*, i.e. a CD45RO/RB binding molecule according to the present invention was found to have corresponding inhibitory properties in vivo testing, such as for example, prevention of lethal xenogeneic graft versus host disease (GvHD) or suppression of inflammatory process that mediates human allograft skin rejection in SCID mice models, or prolonging of human islet allograft survival in a hu-PBL-NOD/SCID mice model.

A CD45RO/RB binding molecule according to the present invention may thus have immunosuppressive and tolerogenic properties and may be useful for in vivo and ex-vivo tolerance induction to alloantigens, autoantigens, allergens and bacterial flora antigens, e.g. a CD45RO/RB binding molecule according to the present invention may be useful in the treatment and prophylaxis of diseases e.g. including autoimmune diseases, such as, but not limited to, rheumatoid arthritis, psoriatic arthritis, autoimmune thyroditis, Graves disease, type I and type II diabetes, multiple sclerosis, Crohn's disease (CD), ulcerative colitis (UC), systemic lupus erythematosus, Sjögren syndrome, scleroderma, autoimmune gastritis, glomerulonephritis, transplant rejection, such as, but not limited to, organ and tissue allograft

and xenograft rejection, e.g. for the treatment of recipients of e.g. heart, lung, combined heart-lung, liver, kidney, pancreatic, skin or corneal transplants, graft versus host disease (GVHD), such as following bone marrow transplantation, and/or pancreatic islet cell transplant rejection, and/or also psoriasis, dermatitis such as atopic and contact dermatitis including allergic contact dermatitis, inflammatory bowel disease and/or allergies, including allergic asthma.

In another aspect the present invention provides the use of a CD45RO/RB binding molecule according to the present invention as a pharmaceutical, e.g. in the treatment and prophylaxis of autoimmune diseases, transplant rejection, e.g. pancreatic islet cell transplant rejection or graft versus host disease (GVHD), psoriasis, dermatitis, inflammatory bowel disease and/or allergies.

In another aspect the present invention provides a CD45RO/RB binding molecule according to the present invention for the production of a medicament in the treatment and prophylaxis of diseases associated with autoimmune diseases, transplant rejection, e.g. pancreatic islet cell transplant rejection or graft versus host disease (GVHD), psoriasis, dermatitis, inflammatory bowel disease and/or allergies.

In another aspect the present invention provides a method of treatment and/or prophylaxis of diseases associated with autoimmune diseases, transplant rejection, psoriasis, dermatitis, inflammatory bowel disease and/or allergies comprising administering to a subject in need of such treatment and/or prophylaxis an effective amount of a CD45RO/RB binding molecule according to the present invention, e.g. in the form of a pharmaceutical composition according to the present invention.

One embodiment of the present invention provides a method of treatment and/or prophylaxis of a disease associated with islet cell transplant rejection, e.g. islet cell transplant rejection, comprising administering to a subject in need of such treatment and/or prophylaxis an effective amount of a molecule or a humanised antibody according to the present invention.

In preferred embodiments said CD45RO/RB binding molecule for use as a pharmaceutical, for the production of a medicament or in a method of treatment and/or prophylaxis of a disease associated with autoimmune diseases, transplant rejection, psoriasis, dermatitis, inflammatory bowel disease and/or allergies comprises a polypeptide of SEQ ID NO:31 or of SEQ ID NO:32 and/or a polypeptide of SEQ ID NO:7 or of SEQ ID NO:8. Preferrably the

CD45RO/RB binding molecule comprises a polypeptide of SEQ ID NO:31 and a polypeptide of SEQ ID NO:8.

An "effective amount" of a CD45RO/RB binding molecule is an amount sufficient to effect beneficial or desired results including clinical results such decreasing one or more symptoms resulting from the autoimmune disease, transplant rejection, psoriasis, dermatitis, inflammatory bowel disease and/or allergy, increasing the quality of life of those suffering from, decreasing the dose of other medications required to treat such diseases, enhancing effect of another medication, delaying the progression of the disease, and/or prolonging survival of patients, either directly or indirectly.

An effective amount can be administered in one or more administrations and may or may not be achieved in conjunction with another drug, compound, or pharmaceutical composition. Thus, an "effective amount" may be considered in the context of administering one or more therapeutic agents, and a single agent may be considered to be given in an effective amount if, in conjunction with one or more other agents, a desirable result may be or is achieved.

Further is provided that a CD45RO/RB binding molecule of the invention may be administered as the sole active ingredient or together with other drugs in immunomodulating regimens or other anti-inflammatory agents e.g. for the treatment or prevention of diseases associated with autoimmune diseases, transplant rejection, psoriasis, dermatitis inflammatory bowel disease and/or allergies. For example, a CD45RO/RB binding molecule of the invention may be used in combination with a calcineurin inhibitor, e.g. cyclosporine A, cyclosporine G, FK-506, ABT-281, ASM 981; an mTOR inhibitor, e.g. rapamycin, 40-O-(2-hydroxy)ethyl-rapamycin, CCI779, ABT578, AP23573, AP23464, AP23675, AP23841, TAFA-93, biolimus-7 or bioimus-9; a corticosteroid; cyclophosphamide; azathioprine; methotrexate; a S1P receptor agonist, e.g. FTY 720 or an analogue thereof; leflunomide or analogs thereof; mizoribine; mycophenolic acid; mycophenolate mofetil; 15-deoxyspergualine or analogs thereof; immunosuppressive monoclonal antibodies, e.g., monoclonal antibodies to leukocyte receptors, e.g., MHC, CD2, CDS, CD4, CD11a/CD18, CD7, CD25, CD27, B7, CD40, CD45, CD58, CD137, ICOS, CD150 (SLAM), OX40, 4-1BB or their ligands, e.g. CD154; or other immunomodulatory compounds, e.g. a recombinant binding molecule having at least a portion of the extracellular domain of CTLA4 or a mutant thereof, e.g. an at least extracellular portion of CTLA4 or a mutant thereof joined to a non-CTLA4 protein sequence, e.g. CTLA4Ig (e.g. designated ATCC 68629) or a mutant thereof, e.g. LEA29Y, or

other adhesion molecule inhibitors, e.g. mAbs or low molecular weight inhibitors including LFA-1 antagonists, Selectin antagonists and VLA-4 antagonists.

An effective amount of a CD45RO/RB binding molecule of the invention, alone or in conjunction with another drug, compound, or pharmaceutical composition can be administered by any conventional route, including injection or by gradual infusion over time. The administration may, for example, be oral, intravenous, intraperitoneal, intramuscular, intracavity, subcutaneous, topical or transdermal. By "co-administration" is meant administration of the components of the compositions of the invention together or at substantially the same time, either in the same vehicle or in separate vehicles, so that upon oral administration, for example, both compounds are present simultaneously in the gastrointestinal tract. Preferably, the compounds are administered as a fixed combination.

In another aspect the present invention provides a pharmaceutical composition comprising a CD45RO/RB binding molecule according to the present invention in association with at least one pharmaceutically acceptable carrier or diluent.

The term "pharmaceutically-acceptable carrier or diluent" as used herein means one or more compatible solid or liquid fillers, diluents or encapsulating substances which are suitable for administration into a mammals including humans. The term "carrier" denotes an organic or inorganic ingredient, natural or synthetic, with which the active ingredient is combined to facilitate the application.

The term "pharmaceutically acceptable" means a non-toxic material that does not interfere with the effectiveness of the biological activity of the active ingredients. Such preparations may routinely contain pharmaceutically acceptable concentrations of salts, buffering agents, preservatives, compatible carriers, supplementary immune potentiating agents such as adjuvants and cytokines and optionally other therapeutic agents, such as chemotherapeutic agents.

When used in medicine, the salts should be pharmaceutically acceptable, but non-pharmaceutically acceptable salts may conveniently be used to prepare pharmaceutically-acceptable salts thereof and are not excluded from the scope of the invention.

The pharmaceutical compositions may contain suitable buffering agents, including: acetic acid in a salt; citric acid in a salt; boric acid in a salt; and phosphoric acid in a salt.

The pharmaceutical compositions also may contain, optionally, suitable preservatives, such as: benzalkonium chloride; chlorobutanol; parabens and thimerosal.

The doses of polypeptide or nucleic acid encoding said polypeptide administered to a subject can be chosen in accordance with different parameters, in particular in accordance with the mode of administration used and the state of the subject. Other factors include the desired period of treatment. In the event that a response in a subject is insufficient at the initial doses applied, higher doses (or effectively higher doses by a different, more localized delivery route) may be employed to the extent that patient tolerance permits.

The pharmaceutical compositions may conveniently be presented in unit dosage form and may be prepared by any of the methods well-known in the art of pharmacy. All methods include the step of bringing the active agent into association with a carrier which constitutes one or more accessory ingredients. In general, the compositions are prepared by uniformly and intimately bringing the active compound into association with a liquid carrier, a finely divided solid carrier, or both, and then, if necessary, shaping the product.

Compositions suitable for oral administration may be presented as discrete units, such as capsules, tablets, lozenges, each containing a predetermined amount of the active compound. Other compositions include suspensions in aqueous liquids or non-aqueous liquids such as a syrup, elixir or an emulsion.

Compositions suitable for parenteral administration conveniently comprise a sterile aqueous or non-aqueous preparation of a polypeptide or nucleic acid encoding the polypeptide, which is preferably isotonic with the blood of the recipient. This preparation may be formulated according to known methods using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation also may be a sterile injectable solution or suspension in a non-toxic parenterally-acceptable diluent or solvent, for example, as a solution in 1,3-butane diol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution, and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil may be employed including synthetic mono- or di-glycerides. In addition, fatty acids such as oleic acid may be used in the preparation of injectables. Carrier formulation suitable for oral, subcutaneous, intravenous, intramuscular, etc. administrations can be found in Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, PA.

A pharmaceutical composition may comprise further, e.g. active, ingredients, e.g. other immunomodulatory antibodies such as, but not confined to anti-ICOS, anti-CD154, anti-CD134L or recombinant proteins such as, but not confined to rCTLA-4 (CD152), rOX40 (CD134), or anti-inflammatory agents or immunomodulatory compounds such as, but not confined to cyclosporin A, FTY720, RAD, rapamycin, FK506, 15-deoxyspergualin, steroids; as described above. Such pharmaceutical composition may comprise a CD45RO/RB binding molecule according to the invention and immunomodulating drugs and/or anti-inflammatory agents in separate unit dosage forms, preferably wherein the unit dosage forms are suitable for administration of the component compounds in synergistically effective amounts, together with instruction for use, optionally with further means for facilitating compliance with the administration of the component compounds, e.g. a label or drawings. The compositions of the invention can be administered as a free combination, or can be formulated into a fixed combination. Absolute dosages of the compounds will vary depending on a number of factors, e.g. the individual, the route of administration, the desired duration, the rate of release of the active agent and the nature and severity of the condition to be treated.

Diseases as outlined above to be treated with a CD45RO/RB binding molecule of the present invention alone or in combination with other drugs include, but are not limited to autoimmune diseases, including rheumatoid arthritis, psoriatic arthritis, autoimmune thyroditis, Graves disease, type I and type II diabetes, multiple sclerosis, Crohn's disease (CD), ulcerative colitis (UC), systemic lupus erythematosus, Sjögren syndrome, scleroderma, autoimmune gastritis and glomerulonephritis; transplant rejection, including, but are not limited to, organ and tissue allograft and xenograft rejection, e.g. for the treatment of recipients of e.g. heart, lung, combined heart-lung, liver, kidney, pancreatic, skin or corneal transplants, graft versus host disease (GVHD), such as following bone marrow transplantation, and/or pancreatic islet cell transplant rejection; psoriasis; dermatitis such as atopic and contact dermatitis including allergic contact dermatitis; inflammatory bowel disease and/or allergies, including allergic asthma.

EXAMPLES

The invention will be more fully understood by reference to the following examples. They should not, however, be construed as limiting the scope of the invention. In the following examples all temperatures are in degree Celsius.

The "candidate mAb" or "chimeric antibody" is a CD45RO/RB binding molecule according to the present invention comprising light chain of SEQ ID NO:3 and heavy chain of SEQ ID NO:4.

The "humanised antibody" is a CD45RO/RB binding molecule according to the present invention comprising a polypeptide of SEQ ID NO:8 and polypeptide of SEQ ID NO:9 (VHE/humV1, VHE/VL1 or VHE/VLh), a polypeptide of SEQ ID NO:8 and a polypeptide of SEQ ID NO:10 (VHQ/humV1, VHQ/VL1 or VHQ/VLh); a polypeptide of SEQ ID NO:7 and polypeptide of SEQ ID NO:9 (VHE/humV2, VHE/VL2 or VHE/VLm); a polypeptide of SEQ ID NO:7 and polypeptide of SEQ ID NO:10 (VHQ/humV2, VHQ/VL2 or VHQ/VLm); a polypeptide of SEQ ID NO:8 and polypeptide of SEQ ID NO:31 (VHEN73D/humV1, VHEN73D/VL1 or VHEN73D/VLh); a polypeptide of SEQ ID NO:8 and polypeptide of SEQ ID NO:32 (VHQN73D/humV1, VHQN73D/VL1 or VHQN73D/VLh); a polypeptide of SEQ ID NO:7 and polypeptide of SEQ ID NO:31 (VHEN73D/humV2, VHEN73D/VL2 or VHEN73D/VLm); or a polypeptide of SEQ ID NO:7 and polypeptide of SEQ ID NO:32 (VHQN73D/humV2, VHQN73D/VL2 or VHEN73D /VLm).

The following abbreviations are used:

APC	antigen presenting cell
CEX	cation exchange chromatography
c.p.m.	counts per minute
dhfr	dihydrofolate reductase
EDTA	ethylen dinitrilo tetra acidic acid
ELISA	enzyme linked immuno-sorbant assay
ESI-Q-TOF	electrospray ionization – quadrupole – time of flight
FACS	fluorescence activated cell sorting
Fc	fragment crystallizable
$F(ab')_2$	fragment antigen-binding; bivalent
FITC	fluorescein isothiocyanate
FBS	foetal bovine serum

GVHD	graft-vs-host disease
HCMV	human cytomegalovirus promoter
HPLC	High Performance Liquid Chromatography
IFN-γ	interferon gamma
IgE	immunoglobulin isotype E
IgG	immunoglobulin isotype G
IL-2	interleukin-2
IU	international units
MALDI-TOF	matrix assisted laser desorption ionization – time of flight
MLR	mixed lymphocyte reaction
MLC	mixed lymphocyte culture
MP1	matrix protein 1 from hemophilus influenza
MTX	methotrexate
PBS	phosphate-buffered saline
PBL	peripheral blood leukocytes
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
RP	reverse phase chromatography
SEC	size exclusion chromatography
SCID	severe combined immunodeficiency
T_{reg}	T regulatory cells
xGVHD	xeno-graft-vs-host disease

Example 1: Primary mixed lymphocyte response (MLR)

Cells

Blood samples are obtained from healthy human donors. Peripheral blood mononuclear cells (PBMC) are isolated by centrifugation over Ficoll-Hypaque (Pharmacia LKB) from leukocytes from whole peripheral blood, leukopheresis or buffy coats with known blood type, but unknown HLA type. In some MLR experiments, PBMC are directly used as the stimulator cells after the irradiation at 40 Gy. In the other experiments, T cells were depleted from PBMC by using CD2 or CD3 Dynabeads (Dynal, Oslo, Norway). Beads and contaminating cells are removed by magnetic field. T cell-depleted PBMC are used as simulator cells after the irradiation.

PBMC, $CD3^+$ T cells or $CD4^+$ T cells are used as the responder cells in MLR. Cells are prepared from different donors to stimulator cells. $CD3^+$ T cells are purified by negative selection using anti-CD16 mAb (Zymed, CA), goat anti-mouse IgG Dynabeads, anti-CD14 Dynabeads, CD19 Dynabeads. In addition anti-CD8 Dynabeads are used to purify $CD4^+$ T cells. The cells obtained are analyzed by FACScan or FACSCalibur (Becton Dickinson & Co., CA) and the purity of the cells obtained was >75%. Cells are suspended in RPMI1640 medium, supplemented with 10 % heat-inactivated FBS, penicillin, streptomycin and L-glutamine.

Reagents

The chimeric anti-CD45R0/RB mAb "candidate mAb" and an isotype matched control chimeric antibody is also generated. Mouse (Human) control IgG_1 antibody specific for KLH (keyhole limpet hemocyanin) or recombinant human IL-10 is purchased from BD Pharmingen (San Diego, CA). Anti-human CD154 mAb 5c8 is according to Lederman et al 1992.

Primary Mixed lymphocyte response (MLR)

Aliquots of 1×10^5 PBMC or 5×10^4 of $CD3^+$ or $CD4^+$ cells are mixed with 1×10^5 irradiated PBMC or 5×10^4 T cells-depleted irradiated (50 Gy) PBMC in the each well of 96-well culture plates (Costar, Cambridge, MA) in the presence of the indicated mAb or absence of Ab. In some experiments, $F(ab')_2$ fragment of goat anti-mouse Ig or goat anti-human Ig specific for Fc portion (Jackson ImmunoResearch, West Grove, PA) is added at 10 µg/ml in addition to the candidate mAb To ensure optimal in vitro cross-linking of the target CD45 molecules. The mixed cells are cultured for 4 or 5 days at 37°C in 5% CO_2 and proliferation is determined by pulsing the cells with ^3H-thymidine for the last 16 - 20 hours of culture. Other experiments are similar to those described above, but with the following exceptions: 1) Medium used is EX-VIVO (Bio-Whittaker) containing 10% FBS and 1% human plasma; 2) Anti-mouse total IgG (5 µg/ml) is used as secondary cross-linking step; 3) Irradiation of stimulator cells is 60 Gy.

Primary MLR is performed in the presence of the "candidate mAb" or control chimeric IgG_1 (10 µg/ml) both with a second step reagent, $F(ab')_2$ fragment of goat anti-human Ig specific for Fc portion (10 µg/ml). Percentage inhibition by the "candidate mAb" is calculated in comparison with the cell proliferation in the presence of control IgG_1. Results are shown in TABLE 1 below:

TABLE 1

Inhibition of primary MLR by 10 µg/ml of a candidate mAb according to the present invention

Responder	Stimulator (Irr. PBMC)	% of Inhibition
#211 CD4	#219 CD3	63.51
#220 CD4	#219 CD3 depl.	63.07
#227 CD4	#220 CD3 depl.	65.96
#229 CD4	#219 CD3 depl.	50.76
Average± SD		60.83 ±6.83 *

* Significantly different from control value ($P<0.001$)

A candidate mAb according to the present invention inhibits primary MLR as can be seen from TABLE 1. The average inhibitory effect is 60.83 ± 6.83 % in four different donors-derived $CD4^+$ T cells and statistically significant.

The inhibition of primary MLR by the "candidate mAb" is shown to be dose-dependent in the range of 0.001 and 10 µg/ml of the "candidate mAb" as shown in Figure 1.

The IC_{50} for the inhibition of primary MLR by a "candidate mAb" is determined from the results of three separate MLR experiments using one donor PBMC as responder cells. Thus, responder $CD4^+$ T cells from Donor #229 and #219 and irradiated PBMC depleted of T cells as stimulators are mixed in the presence of a "candidate mAb" or control chimeric Ab with 10 µg/ml of $F(ab')_2$ fragment of goat anti-human Ig. Experiments are repeated 3 times and percentage of proliferation in the presence of a "candidate mAb" is calculated in comparison with the T cell proliferation in the presence of control Ab. IC_{50} value is determined using Origin (V. 6.0®). The cellular activity IC_{50} value is calculated to be 0.87 ± 0.35 nM (0.13 ± 0.052 µg/ml).

Example 2: Secondary MLR

In order to assess whether a "candidate mAb" induces unresponsiveness of $CD4^+$ T cells to specific alloantigens, secondary MLR is performed in the absence of any antibodies after the primary MLC. $CD4^+$ T cells are cultured with irradiated allogeneic stimulator cells (T cells-depleted PBMC) in the presence of the indicated antibody in 96-well culture plates for 10 days (primary MLC). Then, cells are collected, layered on a Ficoll-Hypaque gradient to remove dead cells, washed twice with RPMI, and restimulated with the same stimulator, 3rd party stimulator cells or IL-2 (50 U/ml). The cells are cultured for 3 days and the proliferative response is determined by pulsing the cells with ^3H-thymidine for the last 16 - 20 hours of culture.

Specifically, $CD4^+$ T cells are cultured with irradiated allogeneic stimulator cells (T cells-depleted PBMC taken from other donors) in the presence of 10 μg/ml of the "candidate mAb", control IgG1 chimeric Ab and $F(ab')_2$ fragment of goat anti-human Ig. Primary MLR proliferation is determined on day 5. For secondary MLR, the responder and stimulator cells are cultured for 10 days in the presence of the "candidate mAb", then the cells are harvested, washed twice in RPMI1640 and restimulated with specific stimulator, third-party stimulators or IL-2 (50 U/ml) in the absence of any Ab. Cell proliferation is determined on day 3. Results set out in TABLE 2:

TABLE 2

Responder CD4+ T cells Donor #	% Inhibition of 2ry MLR
#211	49.90*
#220	59.33*
#227	58.68*

* Significantly different from control value (p=<0.001 determined by t-test, SigmaStat V.2.03). # p=<0.046

In order to test whether the impaired proliferation is due to unresponsivess as a consequence of the treatment with a "candidate mAb", the cells derived from primary MLR are cultured in the presence of IL-2 (50 U/ml). Addition of IL-2 results in the rescue of proliferative responses of the T cells which had been treated with a "candidate mAb" in primary MLR, to levels similar to those observed in the presence of IgG_1 control Ab. These data indicate that the impaired secondary response in T cells treated with a "candidate mAb" is due to to functional alteration of the responder T cells which become unresponsive to the specific stimulator cells.

Percentage inhibition is calculated according to the following formula:

$$\frac{\text{c.p.m. with control Ab} - \text{c.p.m. with "candidate mAb}}{\text{c.p.m. with control Ab}} \times 100$$

Statistical analysis is performed using SigmaStat (Vers. 2.03).

The data is analyzed by two-way ANOVA followed by Dunnett method. In all test procedures probabilities <0.05 are considered as significant. In some experiments t-test is used (SigmaStat V.2.03).

Example 3: In vivo survival studies in SCID-mice

Engraftment of hu-PBL in SCID mice

Human peripheral blood mononuclear cells (PBMC) are injected intraperitoneally into SCID mice C.B 17 /GbmsTac-*Prkdc*^{scid} *Lyst*^{bg} mice (Taconic, Germantown, NY) in an amount sufficient to induce a lethal xenogeneic graft-versus-host disease (xGvHD) in >90% of the mice within 4 weeks after cell transfer. Such treated SCID mice are hereinafter designated as hu-PBL-SCID mice

Mab-treatment of hu-PBL-SCID mice

Hu-PBL-SCID mice are treated with a "candidate mAb" or mouse or chimeric isotype matched mAb controls at day 0, immediately after PBMC injection, at day 3, day 7 and at weekly intervals thereafter. Mabs are delivered subcutaneously in 100 µl PBS at a final

concentration of 5 mg/kg body weight. The treatment was stopped when all control mice were dead.

Evaluation of treatment results

The main criterion to assess the efficacy of a "candidate mAb" in this study was the survival of the hu-PBL-SCID mice. The significance of the results is evaluated by the statistical method of survival analysis using the Log-rank test (Mantel method) with the help of the Systat v9.01 software. The method of survival analysis is a non-parametric test, which not only consider whether a particular mouse is still alive but also whether if it was sacrificed for reasons irrelevant to the treatment/disease such as the requirement of perform in vitro analysis with its organs/cells. Biopsies of liver, lung, kidney and spleen are obtained from dead mice for further evaluation. In addition, hu-PBL-SCID mice are weighed at the beginning (before cell transfer) and throughout (every two days) the experiment as an indirect estimation of their health status. Linear regression lines were generated using the body weight versus days post-PBMC transfer values obtained from each mouse and subsequently, their slopes (control versus anti-CD45 treated mice) were compared using the non-parametric Mann-Whitney test.

Results

All hu-PBL-SCID mice treated with mouse mAb controls had infiltrated human leukocytes in the lung, liver and spleen and died (4/4) within ca. 2 to 3 weeks after cell transfer. Death is a likely consequence of xGvHD. Control mAb-treated mice furthermore lost weight in a linear manner, ca. 10% and more within 3 weeks.

All hu-PBL-SCID mice treated with a "candidate mAb" survived (4/4) without any apparent sign of disease more than 4 weeks, even although "candidate mAb"-treatment was stopped after 3 weeks. "Candidate mAb"-treated mice increased weight in a linear manner, up to ca. 5% within 4 weeks.

Example 4: Expression of antibodies of the invention

Expression of humanised antibody comprising a SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10 SEQ ID NO:31 or SEQ ID NO:32

Expression vectors according to the plasmid maps shown in Figures 2 to 11 are constructed, comprising the corresponding nucleotides encoding the amino acid sequence of humanised light chain variable region humV1 (SEQ ID NO:8; Figures 4 and 6), humanised light chain

variable region humV2 (SEQ ID NO:7; Figures 5 and 7), humanised heavy chain variable region VHE (SEQ ID NO:9; Figure 3 and 10), or humanised heavy chain variable region VHQ (SEQ ID NO:10; Figures 2 and 11), humanised heavy chain variable region VHE-N73D (SEQ ID NO:31; Figure 8) or humanised heavy chain variable region VHQ-N73D (SEQ ID NO:32; Figure 9), respectively. These expression vectors have the DNA (nucleotide) sequences SEQ ID NO: 15 and SEQ ID NO: 41 (VHQ), SEQ ID NO: 16 and SEQ ID NO: 40 (VHE), SEQ ID NO: 17 and SEQ ID NO 36 (humV1), SEQ ID NO: 18 and SEQ ID NO: 39 (humV2), or SEQ ID NO 37 (VHE-N73D) and SEQ ID NO 38 (VHQ-N73D).

Construction of humanised antibody heavy and light chain expression vectors for expression in COS cells

Human kappa light chain expression vectors for versions VLh and VLm

In order to construct the final expression vector encoding for the complete humanised light chain of human kappa isotype, DNA fragments encoding the complete light chain variable regions (VLh and VLm) were excised from the VLh and VLm containing PCR-Script cloning vectors (Stratagene) (VLm region) using HindIII and BglII. The gel-purified fragments were then subcloned into the HindIII and BamHI sites of C21-HCMV Kappa expression vector which was created during construction of the humanised anti-IgE antibody TESC-21 (Kolbinger et al 1993) and which originally received from M. Bendig (MRC Collaborative Centre, London, UK) (Maeda et al. 1991). The ligation products were purified by phenol/chloroform extraction, and electroporated into electrocoporation-competent Epicurian Coli® XL1-Blue strain (Cat. N° #200228, Stratagene). After plating on LB/amp agar plates overnight at 37°C, each 12 colonies were picked to prepare plasmid DNA from a 3 ml culture using the BioRobot 9600 (Qiagen). This yielded the light chain expression vectors for the humanised antibody versions VLh and VLm, respectively, as further described in the Figures.

Human gamma-1 heavy chain expression vectors for VHQ

For the construction of the VHQ expression vector, a step-wise approach was taken. First, the complete variable region of VHQ was assembled by PCR according to the methology as described in Kolbinger et al 1993 (Protein Eng. 1993 Nov; 6(8):971-80) and subcloned into the C21-HCMV-gamma-1 expression from which the C21 insert had been removed using the same enzymes. A HindIII/BamHI fragment of PCRScript clone VHQ containing the complete variable region was then subcloned into expression vector C21-HCMV-gamma-1 cleaved

with the same enzymes. This yielded the final expression vector for the humanised antibody version VHQ.

Human gamma-1 heavy chain expression vectors for VHE

The construction of the final VHE expression vector encoding for the complete humanised heavy chain of human gamma-1 isotype was achieved by directly ligating a HindIII and BamHI restricted PCR fragment encoding the variable region into the HindIII and BamHI sites of C21-HCMV gamma-1 expression vector which was created during construction of the humanised anti-IgE antibody TESC-21 (Kolbinger et al. 1993) and which was also originally received from M. Bendig (MRC Collaborative Centre, London, UK) (Maeda et al. 1991).

Transient expression in COS cells

The following transfection protocol is adapted for adherent COS cells in 150 mm cell culture dishes, using SuperFect™ Transfection Reagent (Cat. N°301305, Qiagen). The four different expression vectors described above are used for transient transfection of cells. For expression of humanised antibody, each of two clones containing heavy chain inserts (VHE or VHQ, respectively) are co-transfected into cells with each of the two clones encoding for the light chains (humV1 or humV2, respectively), in total 4 different combinations of heavy and light chain expression vectors (VHE/humV1, VHE/humV2, VHQ/humV1 and VHQ/humV2). Before transfection, the plasmids are linearized with the restriction endonuclease PvuI which cleaves in the region encoding the resistance gene for ampicillin. The day before transfection, 4 x 10^6 COS cells in 30 ml of fresh culture medium are seeded in 150 mm cell culture dishes. Seeding at this cell density generally yielded 80% confluency after 24 hours. On the day of transfection, four different combinations of linearized heavy- and light-chain DNA expression vectors (15 µg each) are diluted in a total volume of 900 µl of fresh medium without serum and antibiotics. 180 µl of SuperFect Transfection Reagent is then mixed thoroughly with the DNA solution. The DNA mixture is incubated for 10 min at room temperature to allow complex formation. While complex formation takes place, the growth medium is removed from COS cell cultures, and cells are washed once with PBS. 9 ml of fresh culture medium (containing 10% FBS and antibiotics) are then added to each reaction tube containing the transfection complexes and well mixed. The final preparation is immediately transferred to each of 4 cultures to be transfected and gently mixed. Cell cultures are then incubated with the DNA complexes for 3 hours at 37°C and 5% CO2. After incubation, the medium containing transfection complexes is removed and replaced with 30

ml of fresh culture medium. At 48 hr post transfection, the culture supernatants are harvested.

Concentration of culture supernatants

For ELISA and FACS analysis, the culture supernatants collected from COS cells transfected with heavy- and light- chain plasmids are concentrated as follows. 10 ml of each supernatant are added to Centriprep YM-50 Centrifugal Filter Devices (Cat. N° 4310, Millipore) as described by the manufacturer. The Centriprep filters are centrifuged for 10 min at 3000 rpm at room temperature. The centrifugation step is then repeated again with the remaining 20 ml of supernatant using only 5 min of centrifugation and supervising the concentration evolution. The intermediate 500 µl of concentrated supernatant is recovered, transferred to new Microcon Centrifugal Filter Devices (Cat. N° 42412, Microcon) and further concentrated following the manufacturer's protocol. The concentrated supernatants are centrifuged four times for 24 min at 3000 rpm at room temperature, one time for 10 min at 6000 rpm and then, three times for 5 min, always supervising the concentration evolution. The final volume of concentrated conditioned medium achieved is 100-120 µl corresponding to a 250 to 300-fold concentration of original culture medium and is stored at 4°C until use. For comparison and control, culture medium from untransfected cells is similarly concentrated, using the same centrifugation protocol described above.

Generation of stable Sp2/0 myeloma transfectants secreting humanised anti-CD45RO/RB antibodies

The mouse myeloma cell line Sp2/0 (ATCC, CRL-1581) is electroporated with the above described CHO-expression vectors encoding heavy (VHE or VHQ) and light (humV1 or humV2) chain of the CD45RO/RB binding humanised antibodies. Four different combinations of heavy and light chain expression vectors (VHE/humV1, VHE/humV2, VHQ/humV1 and VHQ/humV2) are used for transfection according to the following protocol: 20 µg supercoiled DNA of each plasmid is mixed in an electroporation cuvette (0.4 cm gap) with 8 x 10^6 live Sp2/0 cells suspended in DMEM / 10%FCS culture medium. Electroporation settings are 1500 V, 25 µF using a BioRad GenePulser instrument. After electroporation, cells are cultured for 20 h in culture medium (DMEM supplemented with 10% FCS penicillin, streptomycin and L-glutamine). On day two the selection drug G418 (Cat. N° 10131-019, Gibco) is added to a final concentration of 1 mg active drug / ml and the cells are distributed into one 96-well plate, 200 µl each well with approx. 10^5 cells per well. Ten to 15 days later, G418-surviving clones are expanded in G418-containing medium. Secretion of humanised

mAbs from these transfectants is assessed by ELISA, using a coating antibody goat anti-human IgG/Fcγ (Cat. N° 109-005-098, Jackson Labs) and a peroxidase-coupled antibody against human kappa light chain (Cat. N° A-7164, Sigma). Transfectants, which score positive in this assay are selected for a comparison of productivity on a per cell per day basis, again using ELISA (see below). The best clone of each transfectant is selected for immediate subcloning by limiting dilution, using a seeding density of 1 cell per well. Productivity of G418-surviving subclones is again determined as described above. Subclones are expanded in G418-containing selection medium, until the culture volume reaches 150 ml, at which stage the culture is continued without G418 in flasks destined to feed roller bottles.

After the first transfection and selection, stable transfectants grow out of the 96-well plates at a frequency of 20.8 % for VHE/humV1, 11.5 % for VHQ/humV1, 18.8 % for VHE/humV2 and 7.3 % for VHQ/humV2. After two rounds of subcloning the best two producers are clone 1.33.25 (3.87 pg/cell/day) and clone 1.33.26 (3.43 pg/cell/day) for VHE/humV1 and clone 12.1.4 (1.19 pg/cell/day) and clone 12.1.20 (1.05 pg/cell/day) for VHQ/humV1. The stable Sp2/0 transfectants for VHE/humV1 and VHQ/humV1 are subsequently expanded for antibody production and purification.

The antibodies are purified from supernatants of stably transfected SP2/0 myeloma cell lines containing 10% FCS by a combination of affinity chromatography using an immobilized anti-human IgGFc matrix and size-exclusion chromatography. If required, endotoxin is removed using an Acticlean Etox column (Sterogene Bioseparations).

Construction of humanised antibody heavy and light chain expression vectors for expression in Sp2/0 cells

Human kappa light chain expression vectors for versions VLh and VLm:

The humV1 (= VL1) or humV2 (= VL2) cDNA is amplified by PCR from the CHO expression plasmid SEQ ID NO: 17 or SEQ ID NO: 18 respectively, using the primers HuCD45LC-Mlu (5' – AAAACGCGTTGTGACATTCTGCTGACCCAGTCT - 3'; SEQ ID NO: 42) and HuCD45LC-Hind (5' – AAAAAAGCTTGGTCCCCTGGCCGAACGTGAA – 3'; SEQ ID NO: 43). Each 321 bp PCR fragment is digested with *Mlu*I and *Hin*dIII and ligated directly into the light chain expression vector chA6HCk.dhfr which is digested with the same enzymes. The resulting plasmids are named LCVL1Sp20 (SEQ ID NO: 36; Figure 6) and LCVL2Sp20 (SEQ ID NO: 39; Figure 7) respectively. LCVL1Sp20 is then used for the expression in Sp2/0 cells

of the humanised CD45RO/RB-binding molecules VHE/humV1, VHQ/humV1 or VHE-N73D/humV1; LCVL2Sp20 may then used for the expression in Sp2/0 cells of the humanised CD45RO/RB-binding molecules VHE/humV2, VHQ/humV2 or VHE-N73D/humV2.

Human gamma-1 heavy chain expression vectors for VHQ and for VHE

The two humanised V_H cDNA regions are amplified by PCR from the recombinant plasmids HCMV-G1 HuA6-VHE (SEQ ID NO: 16; Figure 3) and HCMV-G1 HuA6-VHQ (SEQ ID NO: 15 Figure 2) respectively, using the PCR primers HuCD45HCEup (5' – CAGGCAGAGGTGCAGCTGGTGGAGTCA – 3'; SEQ ID NO: 44) or HuCD45HCQup (5' – CAGGCACAGGTGCAGCTGGTGGAGTCA – 3'; SEQ ID NO: 45) and HuCD45HClo (5' – AAATCCTTCTAGAACTCACCTGAGGAGAC – 3'; SEQ ID NO: 46). The PCR fragments' 3' ends were digested with *Bst*EII. Each PCR fragment is then cloned into the heavy chain cassette vector HCcassREAL cut with *Bst*EII and the blunt end cutter *Hin*cII. The resulting plasmids are intermediates to the final expression constructs and are named HCcassHVESP20 and HCcassHVQSP20, respectively. This subcloning also leads to a change of the leader sequence which is associated to both of the V_H regions in the original vectors. Whereas the old leader sequence's amino acid sequence is MDWTWRVFCLLAVVAPGAHS (SEQ ID NO: 47), it has been replaced during the subcloning with MAWVWTLPFLMAAAQSVQA (SEQ ID NO: 48).

The intermediates HCcassVHESP20 or HCcassVHQSP20 are digested with ScaI and subsequenctly, the plasmids are digested with *Bam*HI and *Eco*RI. The 2.9 kb fragments containing the Ig heavy chain promoter and the V_H region are purified and ligated with the 8.7 kb fragment of *Bam*HI- and *Eco*RI-digested of a heavy chain expression construct. The resulting plasmids are named HCVHESp20 (SEQ ID NO: 40; Figure 10) and HCVHQSp20 (SEQ ID NO: 41; 11), respectively; and are used for the expression in Sp2/0 cells of the humanised CD45RO/RB-binding molecules VHE/humV1, VHQ/humV1.

Human gamma-1 heavy chain expression vector for VHEN73D

Expression vectors HCVHESp20 (SEQ ID NO: 40; Figure 10) and HCVHQSp20 (SEQ ID NO: 41; Figure 11), are used as templates for site-directed mutagenesis to achieve a N73D mutation (exchange of an asparagine to aspartate in amino acid position 73 of the CD45RO/RB-binding molecule's heavy chain). This mutation eliminate a putative N-glycosylation site. Mutagenesis is performed with the QuikChange® Multi-Site Directed

Mutagenesis kit (Stratagene) and the primer aCD45H-N73D (5' - phospho GCCACACTAACTGCAGACAAATCCATCAGCACAGC – 3'; SEQ ID NO: 49) according to the kit manual. The sequence of the resulting constructs HCVHEN73DSp20 and HCVHQN73DSp20 is confirmed and is disclosed as SEQ NO: 37 and SEQ NO: 38, and Figures 8 and 9, respectively. HCVHEN73DSp20 (SEQ NO: 37) together with the light chain expression construct LCVL1SP20 (SEQ NO: 36) are used for the expression in Sp2/0 cells of the humanised CD45RO/RB-binding molecule VHE-N73D/humV1.

Generation of stable Sp2/0 myeloma transfectants secreting humanised anti-CD45RO/RB antibody VHE-N73D/humV1

The mouse myeloma cell line Sp2/0 Ag14.10 is electroporated with vectors encoding heavy (HCVHEN73DSp20; SEQ ID NO: 37) and light (LCVL1SP20; SEQ ID NO: 36) chain of the humanised CD45RO/RB binding molecule VHEN73D/humV1. For transfection, cells in exponential growth phase with a viability higher than 95% are used. Cells are washed twice with cold TF-buffer (272 mM sucrose, 1 mM $MgCl_2$, 7 mM phosphate buffer pH 7.4) and cell concentration is adjusted to 2 x 10^7 cells/ml in TF-buffer. 0.8 ml cell suspension is mixed with 15 µg each of the heavy and light chain plasmid construct and placed on ice for 10 minutes. Five transfections are done by electroporation using the Biorad Gene Pulser (280V and 25 µF). After electroporation cells are placed on ice for 15 minutes followed by transfer into 50 ml cold culture medium (RPMI-based medium without FCS) and incubated for 2 days at 37 ° C and 5% CO_2.

For selection of transfectants, cells were cultivated in the presence of 1.1 mg/ml G418 (Geneticin, Gibco lot 3069464) for approximately 2 to 3 weeks. The dhfr (dihydrofolate reductase) amplification marker located on the light chain construct allows amplification of the dhfr gene as well as of the transgene by the folic acid analogue methotrexate (MTX). For gene amplification, G418 resistant cells are cultivated in the presence of 200 nM MTX for a period of 2-3 weeks followed by a further increase in the MTX concentration at 1 µM resulting in an amplified heterogeneous cell pool. The antibody concentration is determined by analytical ProteinA HPLC. The best producing pools are selected for cloning by limiting dilution, using a seeding density of 0.3 cell per well to isolate high producer clones.

Example 5: Determination of recombinant human IgG expression by ELISA

Characterization of VHE/humV1, VHE/humV2, VHQ/humV1 and VHQ/humV2

To determine IgG concentrations of recombinant human antibody expressed in the culture supernatants, a sandwich ELISA protocol has been developed and optimized using human IgG as standard. Flat bottom 96-well microtiter plates (Cat. N° 4-39454, Nunc Immunoplate Maxisorp) are coated overnight at 4°C with 100 µl of goat anti-human IgG (whole molecule, Cat. N° I1011, SIGMA) at the final concentration of 0.5 µg/ml in PBS. Wells are then washed 3 times with washing buffer (PBS containing 0.05% Tween 20) and blocked for 1.5 hours at 37°C with blocking buffer (0.5% BSA in PBS). After 3 washing cycles, the antibody samples and the standard human IgG (Cat.No. I4506, SIGMA) are prepared by serial 1.5-fold dilution in blocking buffer. 100 µl of diluted samples or standard are transfered in duplicate to the coated plate and incubated for 1 hour at room temperature. After incubation, the plates are washed 3 times with washing buffer and subsequently incubated for 1 hour with 100 µl of horseradish peroxidase-conjugated goat anti-human IgG kappa-light chain (Cat. N° A-7164, SIGMA) diluted at 1/4000 in blocking buffer. Control wells received 100 µl of blocking buffer or concentrated normal culture medium. After washing, the colorimetric quantification of bound peroxidase in the sample and standard wells is performed, using a TMB Peroxidase EIA Substrate Kit (Cat. N° 172-1067, Bio-Rad) according to the manufacturer's instructions. The peroxidase mixture is added at 100 µl per well and incubated for 30 min at room temperature in the dark. The colorimetric reaction is stopped by addition of 100 µl of 1 M sulfuric acid and the absorbance in each well is read at 450 nm, using an ELISA plate reader (Model 3350-UV, BioRad).

With a correlation coefficient of 0.998 for the IgG standard curve, the following concentrations are determined for the four different culture concentrates (ca. 250-300 fold concentrated) obtained from transfected COS cells:

VHE/humV1 supernatant = 8.26 µg/ml
VHE/humV2 supernatant = 6.27 µg/ml
VHQ/humV1 supernatant = 5.3 µg/ml
VHQ/humV2 supernatant = 5.56 µg/ml

Size Exclusion Chromatography analysis (SEC)

The affinity-purified antibodies VHE/humV1, VHE/humV2, VHQ/humV1 and VHQ/humV2 are analyzed by Size Exclusion Chromatography (SEC) on a TSKgel Super SW3000SWXL in order to determine the protein content, the percentage of small aggregates (oligomeric antibodies) as well as possible by- and degradation products. The chromatograms show for VHE/humV1, VHE/humV2, VHQ/humV1 and VHQ/humV2 that the main peak is split and that the separation is more pronounced for pools with heavy chain E (Figure 12). The results suggest that there are at least two molecules with a retention time typical for an IgG antibody present in each sample.

SDS-PAGE under reducing conditions

Analysis of the humanized CD45RO/RB binding molecules VHE/humV1, VHE/humV2, VHQ/humV1 and VHQ/humV2 by SDS-PAGE under reducing conditions (gradient gel, 4 to 20% Tris-Glycine gel, Novex) shows the presence of an unexpected additional band, migrating slightly above the band corresponding to the heavy chain band in each sample. The difference in mass is estimated to be in the range of 2 to 4 kDa. Western Blot analysis reveals that the upper band is recognized by anti-human (H + L) antibodies suggesting that the additional band is a heavy chain variant.

Cation Exchange Chromatography (CEX)

The charge heterogeneity is evaluated by cation exchange chromatography (CEX) using a PolyCatA column (PolyLC Inc). While the chimeric mAb is essentially free of charge heterogeneity other than the C-terminal lysine variants, all humanized CD45RO/RB binding molecules, i.e. VHE/humV1, VHE/humV2, VHQ/humV1 and VHQ/humV2, are found to be very heterogeneous in charge. A common reason for charge heterogeneity in antibodies is the presence/absence of Lys at the C-terminal end of the antibody heavy chain resulting in three peaks in CEX. The high number of peaks visible on each chromatogram (>10; Figure 13) indicates that at least one additional modification is present in all four CD45RO/RB binding molecules.

SDS-PAGE

In order to evaluate the molecular differences between the two antibody species found in the protein pools of VHE/humV1, VHE/humV2, VHQ/humV1 and VHQ/humV2, VHE/humV2 is chosen to be further analysed. The two SEC peaks of VHE/humV2 are collected in a semi-preparative mode and re-analyzed by SEC to confirm their purity. The fractions collected by SEC (Fraction 1 and Fraction 2) are then analyzed by SDS-PAGE under reducing conditions. The proportion of the unexpected additional band at about 49 kDa is different between the two fractions. Fraction 2 is almost free of this additional higher band.

ESI- Q-TOF Mass Spectrometry

SEC fractions F1, F2 and the protein pool of VHE/humV2 are then further analyzed by ESI-Q-TOF mass spectrometry. In the spectrograms of the protein pool and Fraction 2, the same groups of signals are observed. The first group of signals at about 148'000 Da, is found in the protein pool and the Fraction 2, is not detected in Fraction 1. In Fraction 1, a second group of signals (at about 150'300 Da) is detected in addition to another group of signals at about 152'500 Da. The second group of signals at about 150'320 Da could not be correlated to any usually observed antibody forms. These findings suggests that the two peaks in SEC and the two upper bands in reduced SDS-PAGE correspond each to variants of an expected protein.

Reverse Phase Chromatography

To obtain a less complex pattern in mass spectrometry, the antibody chains are further separately analyzed. The fractions are reduced and alkylated. In order to confirm completeness of the reactions the reduced and alkylated samples are analyzed by reverse phased (RP) chromatography (SORBAX, Poroshell 300SB-C8). After reduction and alkylation, the peak shape is almost the same as after reduction only. A shift in retention time is observed. Similar patterns are obtained for the SEC Fraction 1 and the Fraction 2 reduced and alkylated samples. The VHE/humV2 protein pool sample, SEC Fraction 1 and SEC Fraction 2 (reduced & alkylated) are analyzed with ESI- Q-TOF mass spectrometry. Several mass peaks could be assigned to expected antibody forms. The same major peaks are found in all the three samples (protein pool, F1 and F2) but their intensities in Fraction 2 are very low (Figure 14).

Carbohydrates analysis of heavy chain variants

The reduced and alkylated antibody is injected on the reverse phase (RP) chromatography column and the two peaks corresponding the heavy chain variants are collected. The oligosaccharide profiles of these RP-fractionated antibodies are then determined. Among the anticipated G0 and G1 oligosaccharides residues found in all samples, several other peaks are detected in the chromatogram of the Fraction 1 (traces of those peaks are also detectable in chromatograms of Fraction 2 and protein pool).

Taken together, the results suggest that the larger antibody species contain additional glycosylation, not typically found in SP2/0 expressed monoclonal antibodies, accounting for the mass difference. Since the large, complex glycosylation found by carbohydrate analysis and mass spectrometry would be very unlikely for the conserved glycosylation site in the constant region of the antibody, a search for other possible sites is made in the amino acid sequence of the humanized antibodies. A potential site (N73) for N-glycosylation (N-X-S) is identified in the variable domain of the two heavy chain variants.

Preparative fractionation by CEX

Further analyses is performed using material purified from pool VHE/humV1. To reduce the heterogeneity of the material, the C-terminal -lysine is removed by carboxypeptidase-B treatment. The VHE/humV1 antibody is fractionated by preparative cation exchange chromatography using SP Sepharose (Pharmacia). The column is equilibrated with 25 mM sodium phosphate buffer, pH 6.0 (= buffer A) and bound protein is eluted with 250 mM NaCl in buffer A (= buffer B; gradient from 0-65% B). The purity of the fractions is analysed by using CEX. The fractions collected are re-injected on the SEC column to look for a correlation between the results obtained with the two techniques. The chromatogram indicates that the first group of peaks in CEX correlates with the pre-peak in SEC analysis of the antibody, that the second group of peaks in CEX leads together to the first peak in SEC analysis of the protein pool (or Fraction 1), and that the last peak in CEX elutes as the last peak in SEC (or Fraction 2).

In conclusion, the results described above (oligosaccharide profile for the SEC Fractions 1 and 2, CEX pattern obtained after de-carboxylation, SEC pattern obtained for the fractions collected in CEX, mass spectrometry) strongly suggest the presence of antibody species with complex oligosaccharides causing a) mass heterogeneity, leading to a double peak in

SEC and b) the observed double heavy chain band in reduced SDS-PAGE charge heterogeneity, leading to the CEX pattern.

Analytical characterization of CEX fractions

Since the larger antibody species elute earlier on CEX, showing that they are less positively charged, the presence of sialic acid (a component of complex glycosylation and negatively charged) is measured in the different fractions obtained by preparative CEX. Sialic acid is present in all 4 samples to varying degrees. The sialic acid found is most likely the N-glycolyl-neuraminic acid form (based on the shorter retention time as compared to the N-acetyl-neuraminic acid standard). The very low presence of sialic acid in one CEX fraction in contrast to its very high abundance in another CEX fraction strongly suggest that the negatively charged sugar component contributes to the mass/charge heterogeneity. The CEX fractions are analyzed by ESI-Q-TOF mass spectrometry.

SDS-PAGE

The same fractions are also analyzed by SDS-PAGE under reducing conditions. It can be concluded that the upper heavy chain band corresponds to the portion of antibody with a complex oligosaccharide profile containing sialic acid. The lower heavy chain band corresponds to the expected antibody displaying a conventional oligosaccharide profile.

Separation on Mono-S column of papaïn digested CEX fractions

In order to confirm the usage of the potential glycosylation site in the variable domain of the heavy chain, about 1 mg of each CEX fraction is treated with papaïn to separate the Fab and the Fc part of the antibody. Preparative chromatography is performed on a Mono-S column. Each of the sub-fraction collected is re-injected onto the RP column to identify its content in terms of Fab and/or Fc domains. Each of the sub-fraction collected is than analyzed with ESI- Q-TOF mass spectrometry. The results obtained show that a) the conserved glycosylation site in the Fc part of the heavy chain is occupied by bi-antennary oligosaccharide forms with none (G0), one (G1) or two terminal (G2) galactose units; b) that in contrast, the irregular glycosylation site (N73) carries complex oligosaccharides containing N-glycolyl neuraminic acid.

In conclusion, the asparagine residue N73 in the variable domain of the heavy chain is found to be partially N-glycosylated. These sugar species cause mass heterogeneity as well as charge heterogeneity that are detected by SDS-PAGE, size exclusion chromatography and cation exchange chromatography.

Characterization of VHE-N73D/humV1

In order to eliminate the heterogenicity of the humanized VHE/humV1, VHE/humV2, VHQ/humV1 and VHQ/humV2 antibodies the asparagine residue at position N73 in the variable domain of the heavy chain is substituted by an aspartic acid residue (see example 4). The VHE-N73D/humV1 is then further analysed:

Size Exclusion Chromatography analysis (SEC)

SEC is performed as described above. A clear difference between VHE/humV1 and VHE-N73D /humV1 is observed in SEC. In contrast to the double peak obtained for VHE/humV1, only one peak is obtained for VHE-N73D /humV1 (Figure 12). Approximately 0.2% of aggregates are quantified by SEC.

SDS-PAGE under reducing conditions

VHE/humV2 and VHE-N73D/humV1 are further analysed by SDS-PAGE under reducing conditions as outlined above. Only one band is visible for VHE-N73D/humV1 at the expected heavy chain (HC) position (about 50 kDa). The HC-band observed for VHE-N73D/humV1 corresponds to the lower band of the doublet observed for VHE/humV2. The position of the light chain band is the same for the proteins analyzed.

Cation Exchange Chromatography (CEX)

Cation Exchange Chromatography (CEX) is performed as outlined above. Results obtained for CEX analysis show that the charge heterogeneity of VHE-N73D/humV1 is reduced to the C-terminal Lysine variants (three peaks) as compared to the high charge heterogeneity of VHE/humV2 (>10; Figure 13)

MALDI TOF analyse (mass spectrometry)

The detected mass for the heavy chain and the light chain obtained by MALDI TOF analyse (mass spectrometry) are in close agreement with the expected mass, deduced from the amino acid sequence of VHE-N73D/humV1.

Reverse Phase chromatography (RP)

After reduction with DDT the two humanized antibodies VHE/humV2 and VHE-N73D/humV1 are analyzed by Reverse Phase chromatography (RP). Due to the partial glycosylation on asparagine N73, two "heavy chains" are observed for VHE/humV2 (double peak at about

18.5 min) amongst the peak corresponding to the light chain (at about 17.3 min). For VHE-N73D/humV1, only one peak is observed for the heavy chain (Figure 14).

Example 6: FACS competition analysis (binding affinity)

The human T-cell line PEER is chosen as the target cell for FACS analysis because it expressed the CD45 antigen on its cell surface. To analyze the binding affinity of humanised antibody supernatants, competition experiments using FITC-labeled chimeric antibody as a reference are performed and compared with the inhibition of purified mouse antibody and of chimeric antibody. PEER cell cultures are centrifuged for 10 seconds at 3000 rpm and the medium is removed. Cells are resuspended in FACS buffer (PBS containing 1% FBS and 0.1% sodium azide) and seeded into 96-well round-bottom microtitter plate at a cell density of $1x10^5$ cells per well. The plate is centrifuged and the supernatant is discarded. For blocking studies, 25 µl of concentrated untransfected medium or isotype matched control antibody (negative controls), unlabeled mouse antibody or chimeric antibody (positive controls) as well as concentrated supernatant containing the various combinations of humanised antibody (samples), is first added in each well at the indicated concentrations in the text. After 1 hour of incubation at 4°C, PEER cells are washed with 200 µl of FACS buffer by centrifugation. Cells are subsequently incubated for 1 hour at 4°C with chimeric antibody conjugated with FITC in 25 µl of FACS buffer at the final concentration of 20 µg/ml. Cells are washed and resuspended in 300 µl of FACS buffer containing 2 µg/ml propidium iodide which allows gating of viable cells. The cell preparations are analyzed on a flow cytometer (FACSCalibur, Becton Dickinson).

FACS analyses indicate a dose-dependent blockade of fluorochrome-labeled chimeric antibody by the concentrated humanised antibody culture supernatants. No dose-dependent blockade of chimeric antibody binding is seen with the isotype matched control antibody, indicating that the blocking effect by the different humanised antibody combinations is epitope specific and that epitope specificity appears to be retained after the humanisation process.

Undiluted supernatant from the above mentioned SP2/0 transfectants or chimeric antibody (positive controls) or isotype matched control antibody (negative controls) at 2 µg/ml in culture medium are incubated with $1.5x10^5$ PEER cells in 100 µl for 30 min at 4°C. Then, 100 µl PBS containing FITC-labeled chimeric antibody is added to each sample and incubation at

4°C continues for another 30 minutes. After washing, cells are resuspended in FACS-PBS containing 1 µg/ml 7-Amino-Actinomycin D and analyzed by flow cytometry using a Becton Dickinson FACSCalibur instrument and the CellQuest Pro Software. Gating was on live cells, i.e. 7-Amino-Actinomycin D – negative events.

FACS analyses show that unlabeled humanised CD45RB/RO binding molecules, e.g. VHE/humV1 and VHQ/humV1 but not the isotype matched control antibody compete with FITC-labeled chimeric antibody for binding to the human CD45-positive T cell line PEER.

Specificity of VHE-N73D/humV1

To evaluate whether the modification of the humanised CD45R0/RB binding molecule VHE/humV1, i.e. the exchange of the asparagine to aspartate in amino acid position 73 of the CD45RO/RB-binding molecule's heavy chain, modified the reactivity with the cognate epitope, the reactivity of VHE-N73D/humV1 with a CD45-expressing human T cell line PEER is analysed in a competition binding experiment.

PEER cells are incubated with VHE-N73D/humV1, with its chimeric predecessor or with a non-binding isotype control IgG1 antibody. Unbound antibody is washed off and the cells are subjected to an incubation with fluorochrome fluorescein isothiocyanate (FITC) labeled chimeric anti-CD45R0/RB mAb or FITC-labeled isotype IgG1 control antibody. After washing, the cells are subjected to flow cytometry analysis to quantitate the amount of FITC-labeled antibody bound to the pre-incubated PEER cell: PEER cell cultures are centrifuged for 10 min at 400 times the standard gravity force (g) and the medium is removed. Cells are resuspended in FACS buffer (PBS containing 1% v/v FBS, 0.1 w/v EDTA and 0.1% w/v sodium azide) and seeded into 96-well V-bottom microtiter plates at a cell density of $1x10^5$ cells per well. The plate is centrifuged and the supernatant is discarded. Each sample of cells is incubated for 30 minutes at 4°C in 50 µl of FACS buffer containing 20 µg/ml of either chimeric anti-CD45R0/RB mAb, VHE-N73D/humV1 or IgG1 control Ab. Next, PEER cells are washed twice with 150 µl of FACS buffer by centrifugation. Cells are subsequently incubated for 30 minutes at 4°C in 50 µl of FACS buffer containing 20 µg/ml of either FITC-conjugated chimeric mAb or FITC-conjugated 3G5 control Ab. Finally, cells are washed and resuspended in 200 µl of FACS buffer containing 7-amino actinomycin D (7-AAD) at 1 µg/ml, which allows identification of viable cells during analysis. The cell preparations are analysed on a FACSCalibur flow cytometer (Becton Dickinson).

It is observed that the unlabeled VHE-N73D/humV1 and the unlabeled chimeric anti-CD45R0/RB mAb but not the IgG1 control antibody prevent the FITC-labeled chimeric anti-CD45R0/RB mAb from binding to CD45-expressing PEER cells as determined by a decreased fluorescence signal. In conclusion, it is found that the modification of the VHE/humV1 antibody to VHE-N73D/humV1 does not change the epitope specificity of the humanised anti-CD45R0/RB binding molecule.

Affinity of VHE-N73D/humV1 for human or cynomolgus CD45

To determine the affinity of the VHE-N73D/humV1 antibody for its epitope, the reactivity of VHE-N73D/humV1 with a human CD45-expressing T cell line PEER or the cynomolgus monkey CD45-expressing T cell line HSC-F can be quantitated in a competition binding experiment, similar to the procedure described in Daley et al., 1995; J. Mol. Biol. *253*:243.

Briefly, the staining intensity of PEER or HSC-F cells with FITC-labeled antibody in the presence of various concentrations of unlabeled competitor antibody is used to calculate the unlabeled antibodies' affinities. More precisely, in a first step, the concentration and fluorochrome labeling stochiometry of the FITC-labeled mouse-derived anti-human CD45RB/RO binding molecule A6 (mA6) is measured. Next, the concentration of the target molecule, CD45, on the surface of the PEER or HSC-F cell line is be determined with the help of the above mentioned FITC-conjugated mA6 yes (mouse-derived anti-huCD45) antibody and using fluorescent beads with known molecular labeling degree. With the concentration of cellular CD45 receptors and its FITC-conjugated ligand known, the affinity of FITC-conjugated mA6 to cellular CD45 receptors is determined by a FACS based equilibrium titration procedure. Finally, the affinity of the unlabeled antibody VHE-N73D/humV1 is determined from competition staining reactions with FITC-conjugated mA6 on PEER or HSC-F cells measuring the increase in FITC fluorescence as a function of the total concentration of VHE-N73D/humV1. If the cubic equation which represents the algebraically explicit description of binding equilibria in a mixture of two competing ligands binding to one receptor is implemented into the program Origin 7.5, the software program is used to calculate the dissociation constant (Kd) value for VHE-N73D/humV1 upon re-iterative curve fitting analyses.

In one experiment the dissociation constant of VHE-N73D/humV1 for the human PEER cellular target is calculated as 2.44 ± 0.07 nM – for the cynomolgus HSC-F cell target it is

calculated as 1.67 ± 0.00 nM, assuming divalent antibody binding to the target CD45RO/RB molecules.

Example 7: Biological activities of CD45RB/RO binding molecules

In this study, we have addressed whether CD45RB/RO binding chimeric antibody, when present in cultures of polyclonally activated primary human T cells (i) supports the differentiation of T cells with a characteristic Treg phenotype, (ii) prevents or enhances apoptosis following T cell activation, and (iii) affects expression of subset-specific antigens and receptors after restimulation.

CD45RB/RO binding chimeric antibody enhances cell death in polyclonally activated T cells

Primary T cells (mixture of CD4+ and CD8+ T subsets) were subjected to activation by anti-CD3 plus anti-CD28 mAb (200 ng/ml each) in the presence or absence (=control) of CD45RB/RO binding chimeric antibody. Excess antibodies were removed by washing on day 2. 7-amino-actinomycin D (7-AAD) as a DNA-staining dye taken up by apoptotic and necrotic cells was used to measure cell death following activation. The results show that activation of T cells in the presence of CD45RB/RO binding chimeric antibody increased the fraction of 7-AAD positive cells than two-fold on day 2 after activation. On day 7, the portion of 7-AAD positive cells was again similar in CD45RB/RO binding chimeric antibody-treated and control cultures.

CD45RB/RO binding chimeric antibody but not control mAb treated T cells display a T regulatory cell (Treg) phenotype

Increased expression of CD25 and the negative regulatory protein CTLA-4 (CD152) is a marker of Treg cells. Functional suppression of primary and secondary T cell responses by CD45RB/RO binding chimeric antibody may be due to the induction of Treg cells. To address this issue, T cells were activated by anti-CD3 + CD28 mAbs and cultured in the presence of CD45RB/RO binding chimeric antibody or anti-LPS control mAb. The time course of CTLA-4 and CD25 expression reveals marked differences between controls and CD45RB/RO binding chimeric antibody-treated T cells on days 1 and 3 after secondary stimulation, indicating a Treg phenotype.

Intracellular CTLA-4 expression is sustained in the presence of CD45RB/RO binding chimeric antibody

It has been reported that substantial amounts of CTLA-4 can also be found intracellularly. Therefore, in parallel to surface CTLA-4 staining, intracellular CTLA-4 expression was analyzed. Moderate differences between T cell cultures were seen on day 4 after stimulation. After prolonged culture, however, high levels of intracellular CTLA-4 were sustained only in CD45RB/RO binding chimeric antibody-treated but not in control T cells.

CD45RB/RO binding chimeric antibody-treated T cells become double positive for CD4 and CD8

Following stimulation, T cells induce and upregulate the expression of several surface receptors, such as CD25, CD152 (CTLA-4), CD154 (CD40-Ligand) and others. In contrast, the level of expression of CD4 or CD8 is thought to stay relatively constant. We reproducibly observed a strong increase of both CD4 and CD8 antigens on CD45RB/RO binding chimeric antibody-treated but not on control Ab-treated T cells after activation. The emergence of a CD4/CD8 double-positive T cell population seems to be due to the upregulation of CD4 on the CD8+ subset and conversely, CD8 on the CD4+ subset. This contrasts with a moderately low percentage of double positive T cells in control cultures.

High IL-2 receptor alpha-chain, but very low beta-chain expression by CD45RB/RO binding chimeric antibody-treated T cells

Treg cells are known to be constitutively positive for CD25, the IL-2 receptor alpha-chain. The regulation of other subunits of the trimeric IL-2 receptor on Treg cells is not known. Recently we have compared the expression of the beta-chain of IL-2 receptor, e.g. CD122, on T cells activated and propagated in the presence or absence of CD45RB/RO binding chimeric antibody. The results show that CD45RB/RO binding chimeric antibody-treated T cells have about ten-fold lower CD122 expression as compared to T cells in control cultures. This difference may indicate that Treg cells require factors other than IL-2 to proliferate.

Example 8: Sequences of the invention (CDR sequences of the invention are underlined)

SEQ ID NO:1

Part of the amino acid sequence of chimeric light chain

DILLTQSPAILSVSPGERVSFSCRASQNIGTSIQWYQQRTNGSPRLLIRSSSESISGIPSRFSG
SGSGTDFTLSINSVESEDIADYYCQQSNTWPFTFGSGTKLEIK

SEQ ID NO:2

Part of the amino acid sequence of chimeric heavy chain

EVQLQQSGPELVKPGASVKMSCKASGYTFTNYIIHWVKQEPGQGLEWIGYFNPYNHGTKY
NEKFKGRATLTADKSSNTAYMDLSSLTSEDSAIYYCARSGPYAWFDTWGQGTTVTVSS

SEQ ID NO:3

Amino acid sequence of chimeric light chain

DILLTQSPAILSVSPGERVSFSCRASQNIGTSIQWYQQRTNGSPRLLIRSSSESISGIPSRFSG
SGSGTDFTLSINSVESEDIADYYCQQSNTWPFTFGSGTKLEIKRTVAAPSVFIFPPSDEQLKS
GTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYE
KHKVYACEVTHQGLSSPVTKSFNRGEC

SEQ ID NO:4

Amino acid sequence of chimeric heavy chain

EVQLQQSGPELVKPGASVKMSCKASGYTFTNYIIHWVKQEPGQGLEWIGYFNPYNHGTKY
NEKFKGRATLTADKSSNTAYMDLSSLTSEDSAIYYCARSGPYAWFDTWGQGTTVTVSSAS
TKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLY
SLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPELLGGPSVFL
FPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVV
SVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVS
LTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFS
CSVMHEALHNHYTQKSLSLSPGK

SEQ ID NO:5

Nucleotide sequence encoding a polypeptide of SEQ ID NO:1

GACATTCTGCTGACCCAGTCTCCAGCCATCCTGTCTGTGAGTCCAGGAGAAAGAGTCA
GTTTCTCCTGCAGGGCCAGTCAGAACATTGGCACAAGCATACAGTGGTATCAACAAAGA
ACAAATGGTTCTCCAAGGCTTCTCATAAGGTCTTCTTCTGAGTCTATCTCTGGGATCCCT
TCCAGGTTTAGTGGCAGTGGATCAGGGACAGATTTTACTCTTAGCATCAACAGTGTGGA
GTCTGAAGATATTGCAGATTATTACTGTCAACAAAGTAATACCTGGCCATTCACGTTCGG
CTCGGGGACCAAGCTTGAAATCAAA

SEQ ID NO:6

Nucleotide sequence encoding a polypeptide of SEQ ID NO:2

GAGGTGCAGCTGCAGCAGTCAGGACCTGAACTGGTAAAGCCTGGGGCTTCAGTGAAG
ATGTCCTGCAAGGCCTCTGGATACACATTCACTAATTATATTATCCACTGGGTGAAGCA
GGAGCCTGGTCAGGGCCTTGAATGGATTGGATATTTTAATCCTTACAATCATGGTACTA
AGTACAATGAGAAGTTCAAAGGCAGGGCCACACTAACTGCAGACAAATCCTCCAACACA
GCCTACATGGACCTCAGCAGCCTGACCTCTGAGGACTCTGCGATCTACTACTGTGCAA
GATCAGGACCCTATGCCTGGTTTGACACCTGGGGCCAAGGGACCACGGTCACCGTCTC
CTCA

SEQ ID NO:7

Part of amino acid sequence of humanised light chain designated humV2 (humV2 = VLm)

DILLTQSPAT LSLSPGERAT FSCRASQNIG TSIQWYQQKT NGAPRLLIRS SSESISGIPS
RFSGSGSGTD FTLTISSLEP EDFAVYYCQQ SNTWPFTFGQ GTKLEIK

SEQ ID NO:8

Part of amino acid sequence of humanised light chain designated humV1 (humV1 = VLh)

DILLTQSPAT LSLSPGERAT LSCRASQNIG TSIQWYQQKP GQAPRLLIRS SSESISGIPS
RFSGSGSGTD FTLTISSLEP EDFAVYYCQQ SNTWPFTFGQ GTKLEIK

SEQ ID NO:9

Part of amino acid sequence of humanised heavy chain designated VHE

EVQLVESGAE VKKPGASVKV SCKASGYTFT NYIIHWVKQE PGQGLEWIGY
FNPYNHGTKY NEKFKGRATL TANKSISTAY MELSSLRSED TAVYYCARSG
PYAWFDTWGQ GTTVTVSS

SEQ ID NO:10

Part of amino acid sequence of humanised heavy chain designated VHQ

QVQLVESGAE VKKPGASVKV SCKASGYTFT NYIIHWVKQE PGQGLEWIGY
FNPYNHGTKY NEKFKGRATL TANKSISTAY MELSSLRSED TAVYYCARSG
PYAWFDTWGQ GTTVTVSS

SEQ ID NO:11

Nucleotide sequence encoding amino acid sequence SEQ ID NO:9

GAGGTGCAGCTGGTGGAGTCAGGAGCCGAAGTGAAAAAGCCTGGGGCTTCAGTGAAG
GTGTCCTGCAAGGCCTCTGGATACACATTCACTAATTATATTATCCACTGGGTGAAGCA
GGAGCCTGGTCAGGGCCTTGAATGGATTGGATATTTTAATCCTTACAATCATGGTACTA
AGTACAATGAGAAGTTCAAAGGCAGGGCCACACTAACTGCAAACAAATCCATCAGCACA
GCCTACATGGAGCTCAGCAGCCTGCGCTCTGAGGACACTGCGGTCTACTACTGTGCAA
GATCAGGACCCTATGCCTGGTTTGACACCTGGGGCCAAGGGACCACGGTCACCGTCTC
CTCA

SEQ ID NO:12

Nucleotide sequence encoding amino acid sequence SEQ ID NO:10

CAGGTGCAGCTGGTGGAGTCAGGAGCCGAAGTGAAAAAGCCTGGGGCTTCAGTGAAG
GTGTCCTGCAAGGCCTCTGGATACACATTCACTAATTATATTATCCACTGGGTGAAGCA
GGAGCCTGGTCAGGGCCTTGAATGGATTGGATATTTTAATCCTTACAATCATGGTACTA
AGTACAATGAGAAGTTCAAAGGCAGGGCCACACTAACTGCAAACAAATCCATCAGCACA
GCCTACATGGAGCTCAGCAGCCTGCGCTCTGAGGACACTGCGGTCTACTACTGTGCAA
GATCAGGACCCTATGCCTGGTTTGACACCTGGGGCCAAGGGACCACGGTCACCGTCTC
CTCA

SEQ ID NO:13

Nucleotide sequence encoding amino acid sequence SEQ ID NO:7

GACATTCTGCTGACCCAGTCTCCAGCCACCCTGTCTCTGAGTCCAGGAGAAAGAGCCA
CTTTCTCCTGCAGGGCCAGTCAGAACATTGGCACAAGCATACAGTGGTATCAACAAAAA
ACAAATGGTGCTCCAAGGCTTCTCATAAGGTCTTCTTCTGAGTCTATCTCTGGGATCCC
TTCCAGGTTTAGTGGCAGTGGATCAGGGACAGATTTTACTCTTACCATCAGCAGTCTGG
AGCCTGAAGATTTTGCAGTGTATTACTGTCAACAAAGTAATACCTGGCCATTCACGTTC
GGCCAGGGGACCAAGCTGGAGATCAAA

SEQ ID NO:14

Nucleotide sequence encoding amino acid sequence SEQ ID NO:8

GACATTCTGCTGACCCAGTCTCCAGCCACCCTGTCTCTGAGTCCAGGAGAAAGAGCCA
CTCTCTCCTGCAGGGCCAGTCAGAACATTGGCACAAGCATACAGTGGTATCAACAAAAA
CCAGGTCAGGCTCCAAGGCTTCTCATAAGGTCTTCTTCTGAGTCTATCTCTGGGATCCC

TTCCAGGTTTAGTGGCAGTGGATCAGGGACAGATTTTACTCTTACCATCAGCAGTCTGG
AGCCTGAAGATTTTGCAGTGTATTACTGTCAACAAAGTAATACCTGGCCATTCACGTTC
GGCCAGGGGACCAAGCTGGAGATCAAA

SEQ ID NO:15

Nucleotide sequence of the expression vector HCMV-G1 HuA6-VHQ

(Complete DNA Sequence of a humanised heavy chain expression vector comprising SEQ ID NO:12 (VHQ) from 3921-4274)

```
   1  AGCTTTTTGC AAAAGCCTAG GCCTCCAAAA AAGCCTCCTC ACTACTTCTG
  51  GAATAGCTCA GAGGCCGAGG CGGCCTCGGC CTCTGCATAA ATAAAAAAAA
 101  TTAGTCAGCC ATGGGGCGGA GAATGGGCGG AACTGGGCGG AGTTAGGGGC
 151  GGGATGGGCG GAGTTAGGGG CGGGACTATG GTTGCTGACT AATTGAGATG
 201  CATGCTTTGC ATACTTCTGC CTGCTGGGGA GCCTGGTTGC TGACTAATTG
 251  AGATGCATGC TTTGCATACT TCTGCCTGCT GGGGAGCCTG GGGACTTTCC
 301  ACACCCTAAC TGACACACAT TCCACAGCTG CCTCGCGCGT TTCGGTGATG
 351  ACGGTGAAAA CCTCTGACAC ATGCAGCTCC CGGAGACGGT CACAGCTTGT
 401  CTGTAAGCGG ATGCCGGGAG CAGACAAGCC CGTCAGGGCG CGTCAGCGGG
 451  TGTTGGCGGG TGTCGGGGCG CAGCCATGAC CCAGTCACGT AGCGATAGCG
 501  GAGTGTATAC TGGCTTAACT ATGCGGCATC AGAGCAGATT GTACTGAGAG
 551  TGCACCATAT GCGGTGTGAA ATACCGCACA GATGCGTAAG GAGAAAATAC
 601  CGCATCAGGC GCTCTTCCGC TTCCTCGCTC ACTGACTCGC TGCGCTCGGT
 651  CGTTCGGCTG CGGCGAGCGG TATCAGCTCA CTCAAAGGCG GTAATACGGT
 701  TATCCACAGA ATCAGGGGAT AACGCAGGAA AGAACATGTG AGCAAAAGGC
 751  CAGCAAAAGG CCAGGAACCG TAAAAAGGCC GCGTTGCTGG CGTTTTTCCA
 801  TAGGCTCCGC CCCCCTGACG AGCATCACAA AAATCGACGC TCAAGTCAGA
 851  GGTGGCGAAA CCCGACAGGA CTATAAAGAT ACCAGGCGTT TCCCCCTGGA
 901  AGCTCCCTCG TGCGCTCTCC TGTTCCGACC CTGCCGCTTA CCGGATACCT
 951  GTCCGCCTTT CTCCCTTCGG GAAGCGTGGC GCTTTCTCAT AGCTCACGCT
1001  GTAGGTATCT CAGTTCGGTG TAGGTCGTTC GCTCCAAGCT GGGCTGTGTG
1051  CACGAACCCC CCGTTCAGCC CGACCGCTGC GCCTTATCCG GTAACTATCG
1101  TCTTGAGTCC AACCCGGTAA GACACGACTT ATCGCCACTG GCAGCAGCCA
1151  CTGGTAACAG GATTAGCAGA GCGAGGTATG TAGGCGGTGC TACAGAGTTC
1201  TTGAAGTGGT GGCCTAACTA CGGCTACACT AGAAGGACAG TATTTGGTAT
1251  CTGCGCTCTG CTGAAGCCAG TTACCTTCGG AAAAAGAGTT GGTAGCTCTT
1301  GATCCGGCAA ACAAACCACC GCTGGTAGCG GTGGTTTTTT TGTTTGCAAG
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1351 CAGCAGATTA CGCGCAGAAA AAAAGGATCT CAAGAAGATC CTTTGATCTT
1401 TTCTACGGGG TCTGACGCTC AGTGGAACGA AAACTCACGT TAAGGGATTT
1451 TGGTCATGAG ATTATCAAAA AGGATCTTCA CCTAGATCCT TTTAAATTAA
1501 AAATGAAGTT TTAAATCAAT CTAAAGTATA TATGAGTAAA CTTGGTCTGA
1551 CAGTTACCAA TGCTTAATCA GTGAGGCACC TATCTCAGCG ATCTGTCTAT
1601 TTCGTTCATC CATAGTTGCC TGACTCCCCG TCGTGTAGAT AACTACGATA
1651 CGGGAGGGCT TACCATCTGG CCCCAGTGCT GCAATGATAC CGCGAGACCC
1701 ACGCTCACCG GCTCCAGATT TATCAGCAAT AAACCAGCCA GCCGGAAGGG
1751 CCGAGCGCAG AAGTGGTCCT GCAACTTTAT CCGCCTCCAT CCAGTCTATT
1801 AATTGTTGCC GGGAAGCTAG AGTAAGTAGT TCGCCAGTTA ATAGTTTGCG
1851 CAACGTTGTT GCCATTGCTG CAGGCATCGT GGTGTCACGC TCGTCGTTTG
1901 GTATGGCTTC ATTCAGCTCC GGTTCCCAAC GATCAAGGCG AGTTACATGA
1951 TCCCCCATGT TGTGCAAAAA AGCGGTTAGC TCCTTCGGTC CTCCGATCGT
2001 TGTCAGAAGT AAGTTGGCCG CAGTGTTATC ACTCATGGTT ATGGCAGCAC
2051 TGCATAATTC TCTTACTGTC ATGCCATCCG TAAGATGCTT TTCTGTGACT
2101 GGTGAGTACT CAACCAAGTC ATTCTGAGAA TAGTGTATGC GGCGACCGAG
2151 TTGCTCTTGC CCGGCGTCAA CACGGGATAA TACCGCGCCA CATAGCAGAA
2201 CTTTAAAAGT GCTCATCATT GGAAAACGTT CTTCGGGGCG AAAACTCTCA
2251 AGGATCTTAC CGCTGTTGAG ATCCAGTTCG ATGTAACCCA CTCGTGCACC
2301 CAACTGATCT TCAGCATCTT TTACTTTCAC CAGCGTTTCT GGGTGAGCAA
2351 AAACAGGAAG GCAAAATGCC GCAAAAAAGG GAATAAGGGC GACACGGAAA
2401 TGTTGAATAC TCATACTCTT CCTTTTTCAA TATTATTGAA GCATTTATCA
2451 GGGTTATTGT CTCATGAGCG GATACATATT TGAATGTATT TAGAAAAATA
2501 AACAAATAGG GGTTCCGCGC ACATTTCCCC GAAAAGTGCC ACCTGACGTC
2551 TAAGAAACCA TTATTATCAT GACATTAACC TATAAAAATA GGCGTATCAC
2601 GAGGCCCTTT CGTCTTCAAG AATTCAGCTT GGCTGCAGTG AATAATAAAA
2651 TGTGTGTTTG TCCGAAATAC GCGTTTTGAG ATTTCTGTCG CCGACTAAAT
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SEQ ID NO:16

Nucleotide sequence of the expression vector HCMV-G1 HuA6-VHE

(Complete DNA Sequence of a humanised heavy chain expression vector comprising SEQ ID NO: 11 (VHE) from 3921-4274)

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8251 TCTGTTGTGC CCAGTCATAG CCGAATAGCC TCTCCACCCA AGCGGCCGGA
8301 GAACCTGCGT GCAATCCATC TTGTTCAATC ATGCGAAACG ATCCTCATCC
8351 TGTCTCTTGA TCAGATCTTG ATCCCCTGCG CCATCAGATC CTTGGCGGCA
8401 AGAAAGCCAT CCAGTTTACT TTGCAGGGCT TCCCAACCTT ACCAGAGGGC
8451 GCCCCAGCTG GCAATTCCGG TTCGCTTGCT GTCCATAAAA CCGCCCAGTC
8501 TAGCTATCGC CATGTAAGCC CACTGCAAGC TACCTGCTTT CTCTTTGCGC
8551 TTGCGTTTTC CCTTGTCCAG ATAGCCCAGT AGCTGACATT CATCCGGGGT
8601 CAGCACCGTT TCTGCGGACT GGCTTTCTAC GTGTTCCGCT TCCTTTAGCA
8651 GCCCTTGCGC CCTGAGTGCT TGCGGCAGCG TGAAGCT

SEQ ID NO:17

Nucleotide sequence of the expression vector HCMV-K HuAb-VL1 hum V1 (Complete DNA Sequence of a humanised light chain expression vector comprising SEQ ID NO: 14 (humV1=VLh) from 3964-4284)

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   1  CTAGCTTTTT GCAAAAGCCT AGGCCTCCAA AAAAGCCTCC TCACTACTTC
  51  TGGAATAGCT CAGAGGCCGA GGCGGCCTCG GCCTCTGCAT AAATAAAAAA
 101  AATTAGTCAG CCATGGGGCG GAGAATGGGC GGAACTGGGC GGAGTTAGGG
 151  GCGGGATGGG CGGAGTTAGG GGCGGGACTA TGGTTGCTGA CTAATTGAGA
 201  TGCATGCTTT GCATACTTCT GCCTGCTGGG GAGCCTGGTT GCTGACTAAT
 251  TGAGATGCAT GCTTTGCATA CTTCTGCCTG CTGGGGAGCC TGGGGACTTT
 301  CCACACCCTA ACTGACACAC ATTCCACAGC TGCCTCGCGC GTTTCGGTGA
 351  TGACGGTGAA AACCTCTGAC ACATGCAGCT CCCGGAGACG GTCACAGCTT
 401  GTCTGTAAGC GGATGCCGGG AGCAGACAAG CCCGTCAGGG CGCGTCAGCG
 451  GGTGTTGGCG GGTGTCGGGG CGCAGCCATG ACCCAGTCAC GTAGCGATAG
 501  CGGAGTGTAT ACTGGCTTAA CTATGCGGCA TCAGAGCAGA TTGTACTGAG
 551  AGTGCACCAT ATGCGGTGTG AAATACCGCA CAGATGCGTA AGGAGAAAAT
 601  ACCGCATCAG GCGCTCTTCC GCTTCCTCGC TCACTGACTC GCTGCGCTCG
 651  GTCGTTCGGC TGCGGCGAGC GGTATCAGCT CACTCAAAGG CGGTAATACG
 701  GTTATCCACA GAATCAGGGG ATAACGCAGG AAAGAACATG TGAGCAAAAG
 751  GCCAGCAAAA GGCCAGGAAC CGTAAAAAGG CCGCGTTGCT GGCGTTTTTC
 801  CATAGGCTCC GCCCCCCTGA CGAGCATCAC AAAAATCGAC GCTCAAGTCA
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 901  GAAGCTCCCT CGTGCGCTCT CCTGTTCCGA CCCTGCCGCT TACCGGATAC
 951  CTGTCCGCCT TTCTCCCTTC GGGAAGCGTG GCGCTTTCTC ATAGCTCACG
1001  CTGTAGGTAT CTCAGTTCGG TGTAGGTCGT TCGCTCCAAG CTGGGCTGTG
1051  TGCACGAACC CCCCGTTCAG CCCGACCGCT GCGCCTTATC CGGTAACTAT
1101  CGTCTTGAGT CCAACCCGGT AAGACACGAC TTATCGCCAC TGGCAGCAGC
1151  CACTGGTAAC AGGATTAGCA GAGCGAGGTA TGTAGGCGGT GCTACAGAGT
1201  TCTTGAAGTG GTGGCCTAAC TACGGCTACA CTAGAAGGAC AGTATTTGGT
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1301  TTGATCCGGC AAACAAACCA CCGCTGGTAG CGGTGGTTTT TTTGTTTGCA
1351  AGCAGCAGAT TACGCGCAGA AAAAAAGGAT CTCAAGAAGA TCCTTTGATC
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1451  TTTGGTCATG AGATTATCAA AAAGGATCTT CACCTAGATC CTTTTAAATT
1501  AAAAATGAAG TTTTAAATCA ATCTAAAGTA TATATGAGTA AACTTGGTCT
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1851 CGCAACGTTG TTGCCATTGC TGCAGGCATC GTGGTGTCAC GCTCGTCGTT
1901 TGGTATGGCT TCATTCAGCT CCGGTTCCCA ACGATCAAGG CGAGTTACAT
1951 GATCCCCCAT GTTGTGCAAA AAAGCGGTTA GCTCCTTCGG TCCTCCGATC
2001 GTTGTCAGAA GTAAGTTGGC CGCAGTGTTA TCACTCATGG TTATGGCAGC
2051 ACTGCATAAT TCTCTTACTG TCATGCCATC CGTAAGATGC TTTTCTGTGA
2101 CTGGTGAGTA CTCAACCAAG TCATTCTGAG AATAGTGTAT GCGGCGACCG
2151 AGTTGCTCTT GCCCGGCGTC AACACGGGAT AATACCGCGC CACATAGCAG
2201 AACTTTAAAA GTGCTCATCA TTGGAAAACG TTCTTCGGGG CGAAAACTCT
2251 CAAGGATCTT ACCGCTGTTG AGATCCAGTT CGATGTAACC CACTCGTGCA
2301 CCCAACTGAT CTTCAGCATC TTTTACTTTC ACCAGCGTTT CTGGGTGAGC
2351 AAAAACAGGA AGGCAAAATG CCGCAAAAAA GGGAATAAGG GCGACACGGA
2401 AATGTTGAAT ACTCATACTC TTCCTTTTTC AATATTATTG AAGCATTTAT
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2851 TACGCGATAT CTGGCGATAG CGCTTATATC GTTTACGGGG GATGGCGATA
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3201 ATTAATAGTA ATCAATTACG GGGTCATTAG TTCATAGCCC ATATATGGAG
3251 TTCCGCGTTA CATAACTTAC GGTAAATGGC CCGCCTGGCT GACCGCCCAA
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3601 GTTTGACTCA CGGGGATTTC CAAGTCTCCA CCCCATTGAC GTCAATGGGA
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4951 ACAGGGGAGA GTGTTAGAGG GAGAAGTGCC CCCACCTGCT CCTCAGTTCC
5001 AGCCTGACCC CCTCCCATCC TTTGGCCTCT GACCCTTTTT CCACAGGGGA
5051 CCTACCCCTA TTGCGGTCCT CCAGCTCATC TTTCACCTCA CCCCCCTCCT
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5501 TTCAAAAGAA GAAACCTGCT ATAAAGAGAA TCATTCATTG CAACATGATA
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5601 GTTTAAGTTC ATCATGGTAC TTAGACTTAA TGGAATGTCA TGCCTTATTT
5651 ACATTTTTAA ACAGGTACTG AGGGACTCCT GTCTGCCAAG GGCCGTATTG
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5801 TCTATAACTC AGCAATCCCA CTTCTAGATG ACTGAGTGTC CCCACCCACC
5851 AAAAAACTAT GCAAGAATGT TCAAAGCAGC TTTATTTACA AAAGCCAAAA
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6101 AACCAGGTAA AAATAAAGTT AGAAATTTGG ATGGAAATTA CTCTTAGCTG
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6301 CTTATACCCA GTTAATAGAT AGAAGAGGAA TAAGTAATAG GTCAAGACCA
6351 CGCAGCTGGT AAGTGGGGGG GCCTGGGATC AAATAGCTAC CTGCCTAATC
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6451 AACAAAACAA CAGGCCTGCT ATTTTCCTGG CATCTGTGCC CTGTTTGGCT
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6551 GGGACAGAGG ACAGAATTAA CCTTGCCCAG ACACTGGAAA CCCATGTATG
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6751 CAGGAGTAAC TAACACAGCA TCCCTTCCCT CAAATGACTG ACAATCCCTT
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6901 GAATGTTGAT GAGTATCAAA TCTTTCAAAC TTTGGAGGTT TGAGTAGGGG
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7001 TGGGGGCCAA ATTGAACAAT CAAAGGCAGG CATAATCCAG CTATGAATTC
7051 TAGGATCGAT CCAGACATGA TAAGATACAT TGATGAGTTT GGACAAACCA

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7201 CAATTGCATT CATTTTATGT TTCAGGTTCA GGGGGAGGTG TGGGAGGTTT
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7801 ACACATTATA CACTTAAAAA TTTTATATTT ACCTTAGAGC TTTAAATCTC
7851 TGTAGGTAGT TTGTCCAATT ATGTCACACC ACAGAAGTAA GGTTCCTTCA
7901 CAAAGATCCG GGACCAAAGC GGCCATCGTG CCTCCCCACT CCTGCAGTTC
7951 GGGGGCATGG ATGCGCGGAT AGCCGCTGCT GGTTTCCTGG ATGCCGACGG
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8201 GATGGCAGGT TGGGCGTCGC TTGGTCGGTC ATTTCGAACC CCAGAGTCCC
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8851 CGCGCTGCCT CGTCCTGCAG TTCATTCAGG GCACCGGACA GGTCGGTCTT
8901 GACAAAAAGA ACCGGGCGCC CCTGCGCTGA CAGCCGGAAC ACGGCGGCAT

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8951  CAGAGCAGCC GATTGTCTGT TGTGCCCAGT CATAGCCGAA TAGCCTCTCC
9001  ACCCAAGCGG CCGGAGAACC TGCGTGCAAT CCATCTTGTT CAATCATGCG
9051  AAACGATCCT CATCCTGTCT CTTGATCAGA TCTTGATCCC CTGCGCCATC
9101  AGATCCTTGG CGGCAAGAAA GCCATCCAGT TTACTTTGCA GGGCTTCCCA
9151  ACCTTACCAG AGGGCGCCCC AGCTGGCAAT TCCGGTTCGC TTGCTGTCCA
9201  TAAAACCGCC CAGTCTAGCT ATCGCCATGT AAGCCCACTG CAAGCTACCT
9251  GCTTTCTCTT TGCGCTTGCG TTTTCCCTTG TCCAGATAGC CCAGTAGCTG
9301  ACATTCATCC GGGGTCAGCA CCGTTTCTGC GGACTGGCTT TCTACGTGTT
9351  CCGCTTCCTT TAGCAGCCCT TGCGCCCTGA GTGCTTGCGG CAGCGTGAAG
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SEQ ID NO:18

Nucleotide sequence of the expression vector HCMV-K HuAb-VL1 hum V2 (Complete DNA Sequence of a humanised light chain expression vector comprising SEQ ID NO: 13 (humV2=VLm) from 3926-4246)

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   1  CTAGCTTTTT GCAAAAGCCT AGGCCTCCAA AAAAGCCTCC TCACTACTTC
  51  TGGAATAGCT CAGAGGCCGA GGCGGCCTCG GCCTCTGCAT AAATAAAAAA
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 151  GCGGGATGGG CGGAGTTAGG GGCGGGACTA TGGTTGCTGA CTAATTGAGA
 201  TGCATGCTTT GCATACTTCT GCCTGCTGGG GAGCCTGGTT GCTGACTAAT
 251  TGAGATGCAT GCTTTGCATA CTTCTGCCTG CTGGGGAGCC TGGGGACTTT
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 401  GTCTGTAAGC GGATGCCGGG AGCAGACAAG CCCGTCAGGG CGCGTCAGCG
 451  GGTGTTGGCG GGTGTCGGGG CGCAGCCATG ACCCAGTCAC GTAGCGATAG
 501  CGGAGTGTAT ACTGGCTTAA CTATGCGGCA TCAGAGCAGA TTGTACTGAG
 551  AGTGCACCAT ATGCGGTGTG AAATACCGCA CAGATGCGTA AGGAGAAAAT
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1001  CTGTAGGTAT CTCAGTTCGG TGTAGGTCGT TCGCTCCAAG CTGGGCTGTG
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2851 TACGCGATAT CTGGCGATAG CGCTTATATC GTTTACGGGG GATGGCGATA

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9351 GGCAGCGTGA AG

SEQ ID NO:31:

Part of amino acid sequence of humanised heavy chain designated VHE-N73D

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FNPYNHGTKY NEKFKGRATL TADKSISTAY MELSSLRSED TAVYYCARSG
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SEQ ID NO:32:

Part of amino acid sequence of humanised heavy chain designated VHQ-N73D

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PYAWFDTWGQ GTTVTVSS

SEQ ID NO:33:

Nucleotide sequence encoding amino acid sequence SEQ ID NO: 8

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SEQ ID NO:34:

Nucleotide sequence encoding amino acid sequence SEQ ID NO: 31

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CTCA

SEQ ID NO:35

Nucleotide sequence encoding amino acid sequence SEQ ID NO: 32

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SEQ ID No 36

Nucleotide sequence of expression vector LCVL1Sp20

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7751  GGGACAGATT TTACTCTTAC CATCAGCAGT CTGGAGCCTG AAGATTTTGC
7801  AGTGTATTAC TGTCAACAAA GTAATACCTG GCCATTCACG TTCGGCCAGG
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SEQ ID NO:37

Nucleotide sequence of the expression vector HCVHEN73DSp20

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SEQ ID NO: 38

Nucleotide sequence of the expression vector HCVHQN73DSp20

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SEQ ID NO: 39

Nucleotide sequence of expression vector LCVL2Sp20

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SEQ ID NO: 40

Nucleotide sequence of expression vector HCVHESp20

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SEQ ID NO:41

Nucleotide sequence of expression vector HCVHQSp20

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Example 9: In vitro efficacy of CD45RO/RB binding humanised antibodies

VHE/humV1 and VHQ/humV1

To determine the efficacy of the CD45R0/RB binding humanised antibodies VHE/humV1 and VHQ/humV1 in comparison to the chimeric antibody the ability to induce apoptosis in human T cells and also the ability to inhibit human T cell proliferation is analysed.

Cells and reagents

Peripheral blood mononuclear cells (PBMC) are isolated from leukopheresis samples of healthy human donors with known blood type, but unknown HLA type by centrifugation over Ficoll-Hypaque (Pharmacia LKB). PBMC used as stimulators are first depleted of T and NK cells by using CD3-coated ferromagnetic beads (Miltenyi). Beads and contaminating cells are removed by magnetic field. T cell-depleted PBMC are used as stimulator cells after irradiation (50 Gy). $CD4^+$ T cells are used as responder cells in MLR and are isolated from PBMC with a CD4 T cell negative selection kit (Miltenyi).

The obtained cells are analyzed by FACScan or FACSCalibur (Becton Dickinson & Co., CA) and the purity of the obtained cells is >75%. Cells are suspended in RPMI1640 medium supplemented with 10% heat-inactivated FCS, penicillin, streptomycin and L-glutamine.

Apoptosis assays

Human PBMC of three healthy voluntary donors are cultured in growth medium (RPMI1640+10%FCS) overnight (<16h) in the presence of CD45R0/RB binding chimeric mAb, humanized antibodies (VHE/humV1 and VHQ/humV1) or anti-LPS control mAb. If indicated, a cross-linking reagent, $F(ab')_2$-fragment of goat anti-human IgG (Cat.No. 109-006-098, JacksonLab) is included at a µg/ml concentration being twice as high as the sample's anti-CD45 antibodies concentration. The PBS-concentration in all wells introduced by the antibody reagents is kept constant among all samples, namely at 20% (v/v) for samples without cross-linker or at 40% (v/v) for samples with cross-linker. Earlier experiments demonstrate that the amount of PBS does not affect the readout.

After overnight culture in the presence of the antibodies, the samples are subjected to flow cytometry analyses and stained with the apoptosis marker AnnexinV-FITC (Cat.No. 556419, BD/Pharmingen) and the T cell marker CD2-PE (Cat.No. 556609, BD/Pharmingen). The samples are run in a Becton Dickinson FACSCalibur instrument and the data are analyzed using the CellQuest Pro Software.

From the data collected, curves are fitted using the software Origin v7.0300 The equation used for fitting is

$$y = \frac{A_1 - A_2}{1 + (x/x_0)^p} + A_2 \qquad \text{("Sigmoid-Logistic")}$$

A_1: final value (for fitting sessions set to "shared " and "floating")

A_2: initial value (for fitting sessions set to "shared " and "floating")

p: power

X_0: ED_{50}; IC_{50} (see below).

In the absence of cross-linker, VHE/humV1 is most effective, with an ED_{50} value of 148±71 nM, followed by VHQ/humV1 with 377±219 nM. CD45R0/RB binding chimeric antibody is less effective with an ED_{50} value of 2440±1205 nM.

In the presence of a cross-linking antiserum, the ED_{50} values are shifted dramatically towards higher efficacy by at least two orders of magnitude. In addition, the presence of cross-linker permitted higher levels of apoptosis at very high antibody concentrations, now reaching up to 80 %, whereas the absence of cross-linker only allowed for up to 50% of apoptosis. In the presence of cross-linker, the curves (antibody concentration / % apoptosis) are bi-modal with two plateaus: the first plateau is reached at low antibody concentrations (~ 5nM), where the apoptosis level corresponds to the maximum level obtained in the absence of cross-linker. The second plateau is reached at high antibody concentrations (~ 500 nM) and apoptosis is observed within 70-80% of the T cell population.

Both CD45R0/RB binding humanised mAb are equally effective and better or equal compared to CD45R0/RB binding chimeric mAb with respect to their ability to induce apoptosis in primary human T cells.

Mixed Lymphocyte Reaction assays

One x 10^5 PBMC or 5 x 10^4 of $CD4^+$ cells are mixed with 1 x 10^5 or 5 x 10^4 T cells-depleted irradiated (50 Gy) PBMC in each well of 96-well culture plates in the presence or absence of the different concentrations of mAb.

The mixed cells are cultured for 5 days and proliferation is determined by pulsing the cells with ^{3}H-thymidine for the last 16 – 20 hours of culture. MLR inhibition at each antibody concentration is expressed as percentage inhibition as described in Example 2.

The effect of increasing concentrations of VHE/humV1 and VHQ/humV1 on MLR is evaluated in three responder:stimulator combinations. All antibodies inhibit the MLR in a dose-dependent manner. The IC_{50} values (see above) are similar for the humanized Ab VHE/humV1 (7 ± 7 nM) and VHQ/humV1 (39 ± 54 nM). Both humanised antibodies are more potent in inhibiting MLR than the parental chimeric antibody (IC_{50} of 347 ± 434 nM). As usually seen with MLR experiments, donor variability is high in these experiments.

VHE-N73D/humV1

To address the biologic effect of VHE-N73D/humV1 inducing apoptosis in human peripheral blood mononuclear cells (PBMC), the target PBMC are incubated overnight in the presence of various concentrations of VHE-N73D/humV1 and are subsequently analyzed for binding of the apoptosis marker AnnexinV by flow cytometry analysis: Human PBMC are incubated overnight in 1 ml tissue culture medium containing various concentrations of either VHE-N73D/humV1, or another humanized CD45RO/RB binding molecule variant, VHE/humV1, or chimeric anti-CD45RO/RB mAb or an isotype IgG1 control mAb. In addition, crosslinking $F(ab')_2$ fragments of goat-anti-human Ig Fc at 2x the mass concentration of each mAb is added, mimicking crosslinking of the CD45RO/RB binding molecules by Fc-receptors, which can occur in vivo. On the next day, the cells are washed by centrifugation for 10 min at 400 times the standard gravity force (g) and the medium is removed. Cells are resuspended in FACS buffer (PBS containing 1% v/v FBS, 0.1% w/v EDTA and 0.1% w/v sodium azide) and seeded into 96-well V-bottom microtiter plates at a cell density of $1x10^5$ cells per well. Each sample of cells is incubated for 30 minutes at 4°C in 50 µl of FACS buffer containing 100 µg/ml of normal mouse serum to block unspecific binding sites on cells and with phycoerythrin (PE)-conjugated CD2 to identify human T cells. After washing twice by centrifugation, cells are resuspended in 100 µl calcium-proficient AnnexinV-staining buffer (Vendor BD/Pharmingen kit 556419) containing 1:100 v/v FITC-labeled AnnexinV. Upon incubation for 15 minutes at room temperature in the dark, 7-amino actinomycin D (7-AAD) is added to give a final concentration of 1 µg/ml and analyzed using a FACSCalibur flow cytometer (Becton Dickinson). ED_{50} values for the antibodies' efficacy to induce apoptosis can be calculated from an analysis of the amount of apoptosis (= intensity of the AnnexinV-FITC fluorescence) induced as a function of antibody concentration using the software Origin 7.5 with a Sigmoidal/Logistic curve fitting equation.

Upon such analyses, a biphasic effect of all tested CD45RO/RB binding molecules is observed: at low concentrations of antibody, a low level of less than 30% T cell apoptosis is found. The ED_{50} value for reaching this level of apoptosis is calculated as 0.31 ± 0.13 nM for VHE-N73D/humV1. At higher concentrations of antibody, apoptosis can be induced in up to 70% of the T cells. The ED_{50} value for reaching this higher level of apoptosis is calculated as 352 ± 83 nM for VHE-N73D/humV1. In summary, it is found that VHE-N73D/humV1 also induces apoptosis in human T cells, which can be enhanced by crosslinking

Example 10: Specificity of CD45RB/RO binding molecule

Chimeric CD45RB/RO binding molecule

The CD45 molecule is expressed on all leukocytes. However, different CD45 isoforms are expressed by the various leukocyte subsets. In order to determine the leukocyte subset reactivity of CD45RB/RO binding chimeric antibody molecule immunofluorescent labeling of human leukocytes with subset-specific markers and simultaneous immunofluorescent labeling with a dye-conjugated CD45RB/RO binding chimeric antibody is performed, followed by flow cytometry analysis.

Briefly, specific subsets of a freshly isolated preparation of human peripheral blood mononuclear cells (PBMC), human platelets, human peripheral blood neutrophils or human bone-marrow derived hematopoietic stem cells are identified by incubation with phycoerythrin-coupled antibodies against CD2 (T lymphocytes), CD14 (monocytes), CD19 (B lymphocytes), CD34 (stem cells), CD42a (platelets), CD56 (natural killer cells) or CD66b (granulocytes). Simultaneous binding of a FITC-labeled chimeric CD45RB/RO binding molecule is detected on T lymphocytes, monocytes, stem cells, natural killer cells and granulocytes, but not on platelets or B lymphocytes.

VHE-N73D/humV1

In order to determine the leukocyte subset reactivity of VHE-N73D/humV1 immunofluorescent labeling of human leukocytes with subset-specific markers and simultaneous immunofluorescent labeling with a dye-conjugated VHE-N73D/humV1 is performed, followed by flow cytometry analysis.

Briefly, specific subsets of a freshly isolated preparation of human peripheral blood mononuclear cells (PBMC) or human peripheral blood neutrophils are identified upon incubation with phycoerythrin-coupled antibodies against CD3, CD4, CD8 (T lymphocytes), CD14 (monocytes), CD16 (natural killer cells and monocytes), CD19 (B lymphocytes), or CD66b (granulocytes). Simultaneous binding of a FITC-labeled VHE-N73D/humV1 is detected on T lymphocytes, monocytes, natural killer cells and granulocytes, but not on B lymphocytes. Thus, VHE-N73D/humV1 molecules do not react with human B lymphocytes.

Example 11: In vitro induction of suppressor T cells (T regulatory cells) and of functionally paralyzed T cells

To demonstrate the ability of a CD45RO/RB binding chimeric antibody to induce suppressor T cells, the antibody is included at various concentrations during the generation of CD8+ T cell lines reactive with the antigen matrix protein 1 (MP1) of hemophilus influenza. These lines are generated through repeated co-culture of CD8+ human lymphocytes with CD14+ human monocytes pulsed with the antigen. Later on, CD14+ monocytes can be replaced with a human leukocyte antigen-2 positive cell line as an MP1 antigen-presenting cell (APC). If such MP1-specific CD8+ T cells from a culture including CD45RO/RB binding chimeric antibody are mixed with freshly isolated human CD8+ T cells and this mixture of cells is stimulated with the MP1 antigen on APC, the addition of CD8+ T cells from the culture in the presence of CD45RO/RB binding molecule is able to reduce the IFN-γ production in an antibody-dose-dependent fashion. No CD45RO/RB binding chimeric antibody is present during this IFN-γ assay culture, indicating that the pre-treatment with the CD45RO/RB mAb has induced CD8+ T cells capable of suppressing the activation of freshly isolated T cells. Because of this induction of suppressor T regulatory cells by the CD45RO/RB binding chimeric antibody, the antibody may be useful in diseases, where a dysregulated and/or activated T cell population is thought to contribute to the pathology. Examples of such diseases include autoimmune diseases, transplant rejection, psoriasis, dermatitis, inflammatory bowel disease and allergies.

To demonstrate the ability of a chimeric CD45RO/RB binding molecule to render T cells hyporesponsive (anergic) to further stimulation, i.e. to functionally paralyze T cells, the antibody is included during the generation of CD8+ T cell lines reactive with the antigen matrix protein 1 (MP1) of hemophilus influenza as outlined above. Paralysis is assessed by activating the T cells (exposed prior to CD45RO/RB binding chimeric antibody) with MP1 antigen presented by APC. No CD45RO/RB binding molecule is present in this culture. CD8+ T cells not exposed to CD45RO/RB binding chimeric antibody previously produce IFN-γ upon the mentioned stimulus. In contrast, CD8+ T cells pre-treated with CD45RO/RB binding chimeric antibody show a markedly reduced to inexistent production of this cytokine in response to the antigen-stimulus, demonstrating the CD45RO/RB binding chimeric antibody's ability to functionally paralyze human T cells. Because of this induction of functional T cell hyporesponsiveness by the CD45RO/RB binding molecule, the antibody may be used in diseases, such autoimmune diseases, transplant rejection, psoriasis,

dermatitis, inflammatory bowel disease or allergies, where an activated T cell population is thought to contribute to the pathology.

Example 12: In vivo studies in SCID-hu Skin mice

The therapeutic benefit of the CD45RB/RO binding chimeric antibody in skin inflammation is tested using SCID mice. Human skin from normal individuals is transplanted onto SCID mice (SCID-hu Skin) and the inflammatory process is mimicked by adoptive transferring of mononuclear leukocytes isolated from human individuals unrelated to the human skin donor.

Transplantation of human adult skin in SCID mice (SCID-hu Skin mice)

Two small pieces (1 cm^2) of human adult skin (obtained from the West Hungarian Regional Tissue Bank; WHRTB, Gyor) consisting of the entire epidermis, the papillary dermis and part of the reticular dermis, are transplanted at the right and left upper-back sides of SCID mice C.B 17 /GbmsTac-*Prkdc*^{scid} *Lyst*^{bg} mice (Taconic, Germantown, NY) in replacement of mouse skin. The quality of the grafts is monitored during 5-6 weeks following transplantation and successfully transplanted mice (SCID-hu Skin mice, generally >85%) are selected for in vivo testing of CD45RB/RO binding chimeric antibody.

Engraftment of human mononuclear cells in SCID mice

Mononuclear leukcytes (Spl) are isolated from human adult spleen biopsies (WHRTB, Gyor) after cell suspension (using a cell dissociation sieve equipped with a size 50 mesh) and standard density gradient procedures. Aliquots of ~5 x10^8 Spl are re-suspended in 1.5 ml of RPMI-10% FCS and injected intraperitoneally (i.p.), on experimental day 0, into the SCID-hu Skin mice. These Spl numbers have been found in previous experiments to be sufficient to induce a lethal xeno-GvHD in >90% of the mice within 4-6 weeks after cell transfer.

Antibody treatment of SCID-hu Skin mice

SCID-hu Skin mice, reconstituted with human Spl, are treated with CD45RB/RO binding chimeric antibody or with anti-LPS control mAb at day 0, immediately after mononuclear cell injection, at days 3 and 7 and at weekly intervals thereafter. Antibodies are delivered subcutaneously (s.c.) in 100 µl PBS at a final concentration of 1 mg/kg body weight (b.w.).

Evaluation of anti-CD45 treatment

The efficacy of CD45RB/RO binding chimeric antibody is assessed by the survival of the transplanted mice and by monitoring the rejection of the skin grafts. The significance of the

results is evaluated by the statistical method of survival analysis using the Log-rank test (Mantel method) with the help of Systat v10 software. At the end of the experiment biopsies of human skin grafts and mouse liver, lung, kidney and spleen are obtained from sacrificed mice for histological purposes. All mice are weighed at the beginning (before cell transfer) and throughout the experiment (every two days) as an indirect estimation of their health status. Linear regression lines are generated using the body weight versus days post-PBMC transfer values obtained from each mouse and subsequently, their slopes (control versus anti-CD45 treated mice) are compared using the non parametric Mann-Whitney test.

Results

The human skin grafts are very well tolerated by the SCID mice. Initially, the grafts undergo a period of keratinocyte hyperproliferation resulting in the formation of hyperkeratotic crusts. About 5 weeks after transplantation, the crusts fall off the grafts and reveal a tissue containing all the characteristic structures observed in normal human skin. During this process, the human skin grafts fuse with the adjacent mouse skin and generate a network of freshly grown human vessels that connect the grafts with the underlying mouse tissue. The circulating human Spl transferred into SCID-hu Skin mice (at experimental day 0, approx. 6 weeks after skin transplantation) infiltrate the skin grafts and after recognition of alloantigen molecules expressed on the human skin mount an inflammatory response, that has resemblance with the skin inflammatory process occurring in psoriatic skin, and that in some cases completely destroy the graft.

Treatment of these mice with CD45RB/RO binding chimeric antibody suppresses the inflammatory process and prevents the rejection of the human skin grafts. In contrast, the sample obtained from the control treated mouse shows a massive infiltration with multiple signs of necrosis and a dramatic destruction of the epidermis. This process is easily monitored by eye and documented by simple photography of the mice.

Six out of six SCID-hu Skin mice transferred with allogeneic human Spl and treated with control anti-LPS mAb show a strong inflammatory response clearly visible by eye 23 days after mononuclear cell transfer. All mice show considerable lesions, including erythema, scaling and pronounced pustules. In contrast the skin grafts of all mice treated with CD45RB/RO binding chimeric antibody have a normal appearance. The dramatic differences between the two groups of mice is specifically due to the antibody treatment since the human skin of all mice have an identical look at the beginning of the experiment. This aspect

is not changed until the second week after cell transfer, the time at which the control group started to developed skin lesions. The experiment is terminated at day 34 after mononuclear cell transfer. By that time, one of the control mice is already dead (day 30) and four other are sacrificed (days 27, 27, 27 and 30) due to a strong xeno-GvHD. The pathologic reactions observed in the antibody control treated mice also correlates with a loss of body weight in these animals.

In contrast, the CD45RB/RO binding chimeric antibody treated group displays a healthy status during the whole experimentation time.

Example 13: In vivo activities of an CD45RB/RO binding chimeric antibody in an human islet cell transplantation model

Mice

NOD/SCID female mice (Charles River Laboratories, Calco, Italy) are kept under specific pathogen free conditions. Glucose level in the venous tail blood is quantified using Glucometer Elite system (Bayer, Germany). Diabates is induced in NOD/SCID mice by intravenous injection of 180 mg/kg of streptozotocin (Sigma, St.Luis, MO). A diagnosis of diabetes is made after 2 concecutive glucose measurements higher than 250 mg/dl.

Islet preparations and transplantation

Pancreata are obtained from heart-beating cadaveric multiorgan donors. Islets are isolated according to the method as described in Bertuzzi et al., Diabetes, 1999, 48: 1971-8. The purified islets are cultured in sterile flasks containing 25 ml of M199 medium (Seromed Biochrom, Berlin, Germany) supplemented with 10% FCS, 1% L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin (complete medium). The islets are incubated at 30° in 5% CO_2 and 95% humidified air. Mice are anestetized wirg an intraperitoneal injection of avertin and aliquots of 1500 IE of human islets are transplanted under the kidney capsule of recipient diabetic NOD/SCID mice as described in Davalli A.M. et al., Diabetes, 1996, 45: 1161-7. After transplantation, $50x10^6$ freshly isolated PBMC are injected intraperitoneally to NOD/SCID mice.

Treatment of transplanted mice

Hu-PBL-NOD/SCID transplanted mice are s.c. treated at day 0, +3 and +5 with 1 mg/kg of an CD45RB/RO binding chimeric antibody. Control mice are treated either with saline solution or with IgG purified mAb (Vinci.Biochem, Italy).

Histological analysis

Kidney poles containing human islet graft are snap-frozen in Tissue Tek (Miles Lab., IN) and stores at -70°. 5 µm thick frozen sections are stained with biotinylated mAb against human insulin or human CD3 followed by streptavidin-peroxidase conjugate. Diaminobenzidine (DAKO, Carpenteria, CA) is used as chromogen and hematoxilin as counterstain. Lymphocyte infiltration of the grafts is evaluated on hematoxilin and eosin-stained frozen sections.

Results

Normal NOD/SCID mice transplanted with human islets remain normoglycemic up to 100 days post-transplantation, whereas the mean rejection time of hu-PBL-NOD/SCID mice transplanted with human islets is 35±13 days. Short treatment of hu-PBL-NOD/SCID mice transplanted with human islets with an mAb of the present invention significantly prolongs human islets survival with a survival rate of >70% at day 60 and 50% at day 100 post-transplantation.

Histological analysis of human islet grafts performed 100 day post-transplantational in hu-PBL-NOD/SCID mice show a massive infiltration of $CD3^+$, $CD4^+$ and $CD8^+$ positive T cells in control recipient mice. In contrast, in recipient mice treated with an CD45RB/RO binding chimeric antibody a significantly lower infiltration of human cells is observed. The positive staining for insulin demonstrates the graft function in hu-PBL-NOD/SCID recipient mice treated with an CD45RB/RO binding chimeric antibody compared to control recipient mice. In islet –transplanted and with an CD45RB/RO binding chimeric antibody treated hu-PBL-NOD/SCID mice lower amounts of human IFN-γ are detected in serum compared to control recipient mice.

These data indicate that short term treatment with an CD45RB/RO binding chimeric antibody leads to prolonged human islet allograft survival by preventing graft-infiltration and by inhibiting the leukocyte-mediated rejection reaction in vivo.

Claims

1. A binding molecule comprising at least one antigen binding site, comprising in sequence the hypervariable regions CDR1, CDR2 and CDR3, said CDR1 having the amino acid sequence Asn-Tyr-Ile-Ile-His (NYIIH), said CDR2 having the amino acid sequence Tyr-Phe-Asn-Pro-Tyr-Asn-His-Gly-Thr-Lys-Tyr-Asn-Glu-Lys-Phe -Lys-Gly (YFNPYNHGTKYNEKFKG) and said CDR3 having the amino acid sequence Ser-Gly-Pro-Tyr-Ala-Trp-Phe-Asp-Thr (SGPYAWFDT).

2. A binding molecule according to claim 1 comprising
   a) a first domain comprising in sequence the hypervariable regions CDR1, CDR2 and CDR3, said CDR1 having the amino acid sequence Asn-Tyr-Ile-Ile-His (NYIIH), said CDR2 having the amino acid sequence Tyr-Phe-Asn-Pro-Tyr-Asn-His-Gly-Thr-Lys-Tyr-Asn-Glu-Lys-Phe -Lys-Gly (YFNPYNHGTKYNEKFKG) and said CDR3 having the amino acid sequence Ser-Gly-Pro-Tyr-Ala-Trp-Phe-Asp-Thr (SGPYAWFDT); and
   b) a second domain comprising in sequence the hypervariable regions CDR1', CDR2' and CDR3', CDR1' having the amino acid sequence Arg-Ala-Ser-Gln-Asn-Ile-Gly-Thr-Ser-Ile-Gln (RASQNIGTSIQ), CDR2' having the amino acid sequence Ser-Ser-Ser-Glu-Ser-Ile-Ser (SSSESIS) and CDR3' having the amino acid sequence Gln-Gln-Ser-Asn-Thr-Trp-Pro-Phe-Thr (QQSNTWPFT).

3. A binding molecule according to any one of claims 1 or 2, which is a chimeric or humanised binding molecule.

4. A binding molecule according to any one of claims 1 to 3, wherein the chimeric or humanised binding molecule is a chimeric or humanised monoclonal antibody.

5. A binding molecule according to any one of claims 1 to 4, comprising not more than one glycosylation site.

6. A binding molecule according to claim 5, wherein the glycosylation site is a N-glycosylation site.

7. A binding molecule according to any one of claims 1 to 6, wherein no glycosylation site is present in the variable region.

8. A binding molecule according to any one of claims 1 to 7, comprising a polypeptide of SEQ ID NO:1 and/or a polypeptide of SEQ ID NO:2.

9. A binding molecule according to any one of claims 1 to 7, comprising a polypeptide of SEQ ID NO:3 and/or a polypeptide of SEQ ID NO:4.

10. A binding molecule according to any one of claims 8 or 9 which is a chimeric monoclonal antibody.

11. A binding molecule which is a humanised antibody comprising a polypeptide of SEQ ID NO:9 or of SEQ ID NO:10 and/or a polypeptide of SEQ ID NO:7 or of SEQ ID NO:8.

12. A binding molecule which is a humanised antibody comprising a polypeptide of SEQ ID NO:31 or of SEQ ID NO:32 and/or a polypeptide of SEQ ID NO:7 or of SEQ ID NO:8.

13. A binding molecule which is a humanised antibody comprising
- a polypeptide of SEQ ID NO:9 and a polypeptide of SEQ ID NO:7,
- a polypeptide of SEQ ID NO:9 and a polypeptide of SEQ ID NO:8,
- a polypeptide of SEQ ID NO:10 and a polypeptide of SEQ ID NO:7,
- a polypeptide of SEQ ID NO:10 and a polypeptide of SEQ ID NO:8,
- a polypeptide of SEQ ID NO:31 and a polypeptide of SEQ ID NO:7,
- a polypeptide of SEQ ID NO:31 and a polypeptide of SEQ ID NO:8,
- a polypeptide of SEQ ID NO:32 and a polypeptide of SEQ ID NO:7, or
- a polypeptide of SEQ ID NO:32 and a polypeptide of SEQ ID NO:8.

14. Isolated polynucleotides comprising polynucleotides encoding a binding molecule according to any one of claims 1 to 13.

15. Polynucleotides according to claim 14 encoding the amino acid sequence of CDR1, CDR2 and CDR3 according to claim 2 and/or polynucleotides encoding the amino acid sequence of CDR1', CDR2' and CDR3' according to claim 2.

16. Polynucleotides encoding the amino acid sequence of CDR1', CDR2' and CDR3', according to said CDR1' having the amino acid sequence Arg-Ala-Ser-Gln-Asn-Ile-Gly-Thr-Ser-Ile-Gln (RASQNIGTSIQ), CDR2' having the amino acid sequence Ser-Ser-Ser-Glu-Ser-Ile-Ser (SSSESIS) and CDR3' having the amino acid sequence Gln-Gln-Ser-Asn-Thr-Trp-Pro-Phe-Thr (QQSNTWPFT).

17. Polynucleotides comprising a polynucleotide of SEQ ID NO: 5 and/or a polynucleotide of SEQ ID NO: 6.

18. Polynucletides comprising polynucleotides encoding a polypeptide of SEQ ID NO:7 or SEQ ID NO:8 and/or a polypeptide of SEQ ID NO:9 or SEQ ID NO:10.

19. Polynucleotides comprising a polynucleotide of SEQ ID NO:11 or of SEQ ID NO:12 and/or a polynucleotide of SEQ ID NO:13 or SEQ ID NO:14.

20. Polynucletides comprising polynucleotides encoding a polypeptide of SEQ ID NO:7 or SEQ ID NO:8 and/or a polypeptide of SEQ ID NO:31 or SEQ ID NO:32.

21. Polynucleotides comprising a polynucleotide of SEQ ID NO:34 or of SEQ ID NO:35 and/or a polynucleotide of SEQ ID NO:33; SEQ ID NO:14 or SEQ ID NO:13.

22. An expression vector comprising polynucleotides according to any one of claims 14 to 21.

23. An expression system comprising a polynucleotide according to any one of claims 14 to 21, wherein said expression system or part thereof is capable of producing a polypeptide of any one of claims 1 to 13, when said expression system or part thereof is present in a compatible host cell.

24. An expression system comprising a polynucleotide according to any one of claims 14 to 21, wherein said expression system or part thereof is capable of producing a binding molecule comprising at least one antigen binding site, comprising in sequence the hypervariable regions CDR1, CDR2 and CDR3, said CDR1 having the amino acid

sequence Asn-Tyr-Ile-Ile-His (NYIIH), said CDR2 having the amino acid sequence Tyr-Phe-Asn-Pro-Tyr-Asn-His-Gly-Thr-Lys-Tyr-Asn-Glu-Lys-Phe -Lys-Gly (YFNPYNHGTKYNEKFKG) and said CDR3 having the amino acid sequence Ser-Gly-Pro-Tyr-Ala-Trp-Phe-Asp-Thr (SGPYAWFDT), when said expression system or part thereof is present in a compatible host cell.

25. An isolated host cell which comprises an expression system according to claim 23 or 24.

26. Use of a binding molecule or of a humanised antibody according to any one of claims 1 to 13 as a pharmaceutical.

27. Use according to claim 26 in the treatment and/or prophylaxis of autoimmune diseases, transplant rejection, psoriasis, dermatitis, inflammatory bowel disease and/or allergies.

28. Use according to claim 27 in the treatment and/or prophylaxis of graft versus host disease (GVHD).

29. Use of a binding molecule or of a humanised antibody according to any one of claims 1 to 13 in the preparation of a medicament for treatment of pancreatic islet cell transplant rejection.

30. Use according to any one of claims 26 to 29, wherein the binding molecule or a humanised antibody comprises a polypeptide of SEQ ID NO:31 or of SEQ ID NO:32 and/or a polypeptide of SEQ ID NO:7 or of SEQ ID NO:8.

31. A pharmaceutical composition comprising a molecule or a humanised antibody according to any one of claims 1 to 13 in association with at least one pharmaceutically acceptable carrier or diluent.

32. A method of treatment and/or prophylaxis of diseases associated with autoimmune diseases, transplant rejection, psoriasis, dermatitis, inflammatory bowel disease and/or allergies comprising administering to a subject in need of such treatment and/or prophylaxis an effective amount of a molecule or a humanised antibody according to any one of claims 1 to 13.

33. A method of treatment and/or prophylaxis of psoriasis comprising administering to a subject in need of such treatment and/or prophylaxis an effective amount of a binding molecule or a humanised antibody according to any one of claims 1 to 13.

34. A method of treatment and/or prophylaxis of graft versus host disease (GVHD) comprising administering to a subject in need of such treatment and/or prophylaxis an effective amount of a molecule or a humanised antibody according to any one of claims 1 to 13.

35. A method of treatment and/or prophylaxis of a disease associated with pancreatic islet cell transplant rejection comprising administering to a subject in need of such treatment and/or prophylaxis an effective amount of a binding molecule or a humanised antibody according to any one of claims 1 to 13.

36. The method according to any one of claims 32 to 35, wherein the binding molecule or the humanised antibody comprises a polypeptide of SEQ ID NO:31 or of SEQ ID NO:32 and/or a polypeptide of SEQ ID NO:7 or of SEQ ID NO:8.

37. Use of a binding molecule having a binding specificity for both CD45RO and CD45RB in medicine.

38. The use according to claim 37, wherein the binding molecule is a chimeric, a humanised or a fully human monoclonal antibody.

39. The use according to claim 37 or 38, wherein the binding molecule binds to a CD45RO isoform with a dissociation constant (Kd) <15nM.

40. The use according to any of claims 37 to 38, wherein the binding molecule binds to a CD45RB isoform with a dissociation constant (Kd) <15nM.

41. The use according to any of claims 37 to 40, wherein the binding molecule binds CD45 isoforms which
- include the A and B epitopes, but not the C epitope of the CD45 molecule; and/or

- include the B epitope, but not the A and not the C epitope of the CD45 molecule; and/or
- isoforms which do not include any one of the A, B or C epitopes of the CD45 molecule.

42. The use according to any of claims 37 to 41, wherein the binding molecule does not bind CD45 isoforms which
- include all of the A, B and C epitopes of the CD45 molecule; and/or
- include both the B and C epitopes, but not the A epitope of the CD45 molecule.

43. The use according to any of claims 37 to 42, wherein the binding molecule binds to its target epitope on PEER cells, and wherein said binding is with a Kd<15nM.