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(54) Title: CD20-BINDING POLYPEPTIDE COMPOSITIONS

(57) Abstract: The invention relates to polypeptide compositions that bind to CD20 antigen, especially human CD20 antigen. The polypeptide compositions have the biological activity of known anti-CD20 antibodies, such as 2B8 or Leu16, but show a reduced immunogenicity as compared with the non-modified molecules. The polypeptide compositions include chimeric antibodies, antibody fragments, and fusion proteins of an antibody or antibody fragment with a cytokine.



WO 2005/016969 A3

## CD20-BINDING POLYPEPTIDE COMPOSITIONS

### FIELD OF THE INVENTION

The present invention relates to polypeptide compositions that bind to CD20 antigen.

- 5 More specifically, the invention relates to polypeptide compositions that bind to human CD20 antigen and methods for using the compositions. Such polypeptide compositions include chimeric antibodies, antibody fragments, and fusion proteins of an antibody or antibody fragment with a cytokine.

### 10 BACKGROUND OF THE INVENTION

- There are many instances where the efficacy of a therapeutic protein is limited by an unwanted immune reaction to the therapeutic protein. Several mouse monoclonal antibodies have shown promise as therapies in a number of human disease settings but in certain cases have failed due to the induction of significant degrees of a human anti-murine antibody (HAMA) response (Schroff, R. W. *et al.* (1985) *Cancer Res.* 45: 879-885; Shawler, D.L. *et al.* (1985) *J. Immunol.* 135: 1530-1535). For monoclonal antibodies, a number of techniques have been developed in attempt to reduce the HAMA response (WOA8909622; EPA0239400; EPA0438310; WOA9106667; EPA0699755). These recombinant DNA approaches have generally reduced the
- 20 mouse genetic information in the final antibody construct whilst increasing the human genetic information in the final construct to result in antibody molecules which are generally termed "humanised" antibodies.

- Humanised antibodies, for the most part, are human immunoglobulins (recipient antibody) in which hypervariable region residues of the recipient are replaced by hypervariable region residues from a non-human species (donor antibody) such as mouse, rat, rabbit or primate, and this process is sometimes termed "CDR grafting". Generally additional Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues and in some
- 30 instances other substitutions are made to further restore the antibody function. Typically humanised antibodies are reconstituted into whole antibody molecules comprising two variable domains, in which all or substantially all of the hypervariable loops correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin sequence. The humanised

antibody will also generally comprise at least a portion of a human derived immunoglobulin constant region (Fc) (Jones *et al.* (1986), *Nature* 321: 522-525; Reichmann *et al.* (1988), *Nature* 332: 323-329). Notwithstanding, humanised antibodies have, in several cases, still elicited an immune response in patients (Issacs J.D. (1990) *Sem. Immunol.* 2: 449, 456; Rebello, P.R. *et al.* (1999) *Transplantation* 68: 1417-1420).

Key to the induction of an immune response is the presence within the protein of peptides that can stimulate the activity of T-cells via presentation on MHC class II molecules, so-called "T-cell epitopes". Such T-cell epitopes are commonly defined as any amino acid residue sequence with the ability to bind to MHC Class II molecules. Implicitly, a "T-cell epitope" means an epitope which when bound to MHC molecules can be recognized by a T-cell receptor (TCR), and which can, at least in principle, cause the activation of these T-cells by engaging a TCR to promote a T-cell response.

MHC Class II molecules are a group of highly polymorphic proteins which play a central role in helper T-cell selection and activation. The human leukocyte antigen group DR (HLA-DR) are the predominant isotype of this group of proteins and isotypes HLA-DQ and HLA-DP perform similar functions. In the human population, individuals bear two to four DR alleles, two DQ and two DP alleles. The structure of a number of DR molecules has been solved and these appear as an open-ended peptide binding groove with a number of hydrophobic pockets which engage hydrophobic residues (pocket residues) of the peptide (Brown *et al.* *Nature* (1993) 364: 33; Stern *et al.* (1994) *Nature* 368: 215). Polymorphism identifying the different allotypes of class II molecule contributes to a wide diversity of different binding surfaces for peptides within the peptide binding groove and at the population level ensures maximal flexibility with regard to the ability to recognize foreign proteins and mount an immune response to pathogenic organisms.

An immune response to a therapeutic protein proceeds via the MHC class II peptide presentation pathway. Here exogenous proteins are engulfed and processed for presentation in association with MHC class II molecules of the DR, DQ or DP type. MHC Class II molecules are expressed by professional antigen presenting cells (APCs), such as macrophages and dendritic cells amongst others. Engagement of a

MHC class II peptide complex by a cognate T-cell receptor on the surface of the T-cell, together with the cross-binding of certain other co-receptors such as the CD4 molecule, can induce an activated state within the T-cell. Activation leads to the release of cytokines further activating other lymphocytes such as B cells to produce  
5 antibodies or activating T killer cells as a full cellular immune response.

T-cell epitope identification is the first step to epitope elimination. Identified epitopes may be eliminated by judicious amino acid substitution or other modification strategies. Such an approach is recognized in WO98/52976 and WO00/34317 where  
10 in the latter case computational threading techniques are described as a means to identify polypeptide sequences with the potential to bind a sub-set of human MHC class II DR allotypes and the predicted T-cell epitopes are removed by amino acid substitution within the protein of interest.

15 It would be desirable to identify and to remove, or at least to reduce, T-cell epitopes from a given, in principle therapeutically valuable, but originally immunogenic peptide, polypeptide or protein. One of these therapeutically valuable molecules is a monoclonal antibody with binding specificity for the human B-cell antigen CD20. The preferred monoclonal antibodies of the present invention are modified forms of the  
20 antibody 2B8 and Leu16. Antibody 2B8 is described in U.S patent No. 5,736,137, the disclosure of which is incorporated herein by reference. Antibody Leu16 is described in Wu *et al. Protein Engineering* (2001) 14:1025-1033, the disclosure of which is incorporated herein by reference.

25 CD20 is a non-glycosylated phosphoprotein of 35,000 Daltons, typically designated as the human B lymphocyte restricted differentiation antigen Bp35B. The protein is a highly cell specific surface molecule expressed on pre-B and mature B-cells including greater than 90% of B-cell non-Hodgkin's lymphomas (NHL). Monoclonal antibodies and radioimmunoconjugates targeting CD20 have emerged as new treatments for  
30 NHL. The most significant example includes the parental antibody of the present invention namely monoclonal antibody 2B8 (Reff, M.E. *et al.* (1994) *Blood* 83: 435-445). The variable region domains of 2B8 have been cloned and combined with human constant region domains to produce a chimeric antibody designated C2B8 which is marketed as RITUXAN<sup>TM</sup> in the USA or MABTHERA<sup>®</sup> (rituximab) in

2004264372 02 Jun 2010

- 4 -

Europe. C2B8 is recognized as a valuable therapeutic agent for the treatment of NHL and other B-cell diseases (Maloney, D.G. *et al.* (1997) *J. Clin. Oncol.* 15: 3266-3274; Maloney, D.G. *et al.* (1997) *Blood* 90: 2188-2195).

- 5 An additional example of an anti-CD20 therapeutic is provided by the antibody B1, described in US patent No. 6,090,365. This antibody has similarly achieved registration for use as a NHL therapeutic although in this case the molecule (BEXXAR<sup>TM</sup>) is a <sup>131</sup>I radioimmunoconjugate. The native B1 (non-conjugated) antibody has utility in *ex vivo* purging regimens for autologous bone marrow transplantation therapies for lymphoma and  
10 refractory leukaemia (Freedman, A.S. *et al.* (1990), *J. Clin. Oncol.* 8: 784).

Despite the success of antibodies such as C2B8 (rituximab) and BEXXAR<sup>TM</sup> there is a continued need for anti-CD20 analogues with enhanced properties. There is a particular need for enhancement of the *in vivo* characteristics when administered to the human  
15 subject. In this regard, it is highly desired to provide anti-CD20 antibodies with reduced or absent potential to induce an immune response in the human subject. Such proteins would display an increased circulation time within the human subject and would be of particular benefit in chronic use settings such as is the case for the therapeutic use of anti-CD20 molecules. The present invention provides modified anti-CD20 antibodies that display  
20 enhanced properties *in vivo*.

#### SUMMARY OF THE INVENTION

According to a first aspect of the invention there is provided an anti-CD20 antibody  
25 molecule fused to IL-2 comprising:

- (i) a modified heavy chain variable region polypeptide having the amino acid residue sequence of SEQ ID NO: 9, but which includes at least one amino acid residue substitution in said sequence selected from the group consisting of V<sub>12</sub>K, M<sub>20</sub>V, A<sub>68</sub>T, Q<sub>82</sub>E, T<sub>87</sub>R, S<sub>91</sub>T, D<sub>93</sub>V, and A<sub>114</sub>T,  
30 (ii) a modified light chain variable region polypeptide having the amino acid residue

2004264372 02 Jun 2010

- 4a -

sequence of SEQ ID NO: 11, but which includes at least one amino acid residue substitution in said sequence selected from the group consisting of L<sub>11</sub>I, S<sub>12</sub>T, A<sub>59</sub>S, S<sub>69</sub>T, L<sub>72</sub>M, R<sub>76</sub>S, and V<sub>77</sub>L, and

(iii) a human IgG heavy chain constant region and a human light chain constant region.

5

According to a second aspect of the invention there is provided a pharmaceutical composition comprising an anti-CD20 antibody fusion protein according to the first aspect together with a pharmaceutically acceptable carrier, excipient or diluent.

10 According to a third aspect of the invention there is provided a DNA molecule coding for an anti-CD20 antibody fusion protein according to the first aspect.

According to a fourth aspect of the invention there is provided use of an anti-CD20 antibody fusion protein of the first aspect or a pharmaceutical composition of the second aspect, for the manufacture of a medicament for the treatment of CD20 expressing cancers.

15

According to a fifth aspect of the invention there is provided a pharmaceutical composition comprising the anti-CD20 antibody fusion protein of the first aspect and the monoclonal antibody rituximab.

20

According to a sixth aspect of the invention there is provided an anti-CD20 antibody fusion protein according to the first aspect for use in the treatment of CD20 expressing cancers.

25 According to a seventh aspect of the invention there is provided a method for the treatment of a CD20 expressing cancer in a subject, the method comprising administering to the subject an anti-CD20 antibody of the first aspect or a pharmaceutical composition of the fifth aspect.

30 The present invention provides polypeptide compositions that bind to CD20 antigen, preferably human CD20 antigen. The CD20 compositions comprise one or more anti-

2004264372 02 Jun 2010

- 4b -

CD20 heavy chain and/or light chain variable region polypeptide segments, which can be polypeptide selected from the group consisting of a modified form of the heavy chain variable region (Vh) of anti-CD20 antibody 2B8, a modified form of the light chain variable region (Vk) of anti-CD20 antibody 2B8, a modified form of the heavy chain variable region (Vh) of anti-CD20 antibody Leu16 and a modified form of the light chain variable region (Vk) of anti-CD20 antibody Leu16. The modified Vh and Vk polypeptides differ from the native Vh or Vk regions of 2B8 and Leu16 by one or

more amino acid residue substitution in the native sequence of the antibody Vh and/or Vk region. The amino acid residue substitution(s) in the Vh or Vk polypeptides afford a lower level of immunogenicity to the Cd-20 binding composition of the invention relative to the immunogenicity of the Vh or Vk regions of the native 2B8 and Leu16  
5 antibodies.

A CD20-binding polypeptide composition of the invention comprises at least one polypeptide segment selected from the group consisting of a polypeptide having the amino acid residue sequence of SEQ ID NO: 1 (Vh of 2B8 antibody), but which  
10 includes at least one amino acid residue substitution in SEQ ID NO: 1 selected from the group consisting of V<sub>12</sub>K, A<sub>14</sub>P, M<sub>20</sub>V, L<sub>48</sub>T, A<sub>68</sub>T, Q<sub>82</sub>E, T<sub>87</sub>R, S<sub>91</sub>T, and T<sub>106</sub>W; a polypeptide having the amino acid residue sequence of SEQ ID NO: 4 (Vk of 2B8 antibody), but which includes at least one amino acid residue substitution in SEQ ID NO: 4 selected from the group consisting of L<sub>11</sub>I, S<sub>12</sub>T, S<sub>27</sub>T, V<sub>29</sub>A, G<sub>40</sub>T, V<sub>59</sub>S, S<sub>69</sub>T,  
15 L<sub>72</sub>M, R<sub>76</sub>S, and V<sub>77</sub>L; a polypeptide having the amino acid residue sequence of SEQ ID NO: 9 (Vh of Leu16 antibody), but which includes at least one amino acid residue substitution in SEQ ID NO: 9 selected from the group consisting of V<sub>12</sub>K, M<sub>20</sub>V, A<sub>68</sub>T, Q<sub>82</sub>E, T<sub>87</sub>R, S<sub>91</sub>T, D<sub>93</sub>V, and A<sub>114</sub>T; and a polypeptide having the amino acid residue sequence of SEQ ID NO: 11 (Vk of Leu16 antibody), but which includes at least one  
20 amino acid residue substitution in SEQ ID NO: 11 selected from the group consisting of L<sub>11</sub>I, S<sub>12</sub>T, A<sub>59</sub>S, S<sub>69</sub>T, L<sub>72</sub>M, R<sub>76</sub>S, and V<sub>77</sub>L.

As used herein and in the appended claims, an amino acid residue substitution is designated by listing the single letter code for the native amino acid residue in the  
25 sequence, followed by the position number of that residue (subscripted), followed by the single letter code for the amino acid residue that replaces the native amino acid. Accordingly a substitution L<sub>11</sub>I in SEQ ID NO: 4 means that the Leucine residue (L) at position 11 in SEQ ID NO: 4 (numbered from the N-terminus) is replaced (i.e., substituted) by an isoleucine (I).

30 Preferably the CD20-binding polypeptide compositions of the invention have a binding affinity for human CD20 antigen that is about equal to or stronger than the human CD20 binding affinity of monoclonal antibodies 2B8 or Leu16.

Preferred embodiments of the present invention comprise polypeptide segments such as a heavy chain termed herein VhC comprising the amino acid residue sequence of SEQ ID NO: 2:

QVQLQQPGAELKKPGASVKVSKASGYTFTSYNMHWVKQTPGRGLEWTGAIYPG  
 5 NGDTSYNQKFKGKTTLTADKSSSTAYMELSSLRSEDVAVYYCARSTYYGGDWYF  
 NVWGAGTTVTVSA;

a monoclonal antibody V-region heavy chain termed herein VhD comprising the amino acid residue sequence of SEQ ID NO: 3:

QVQLQQPGAELKKPGASVKVSKASGYTFTSYNMHWVKQTPGRGLEWIGAIYPG  
 10 NGDTSYNQKFKGKTTLTADKSSSTAYMELSSLRSEDVAVYYCARSTYYGGDWYF  
 NVWGAGTTVTVSA;

and a monoclonal antibody V-region heavy chain termed herein VhY comprising the amino acid residue sequence of SEQ ID NO: 10:

EVQLQQSGAELKKPGASVKVSKASGYTFTSYNMHWVKQTPGQGLEWIGAIYPG  
 15 NGDTSYNQKFKGKTTLTADKSSSTAYMELSSLRSEDVAVYYCARSNYYGSSYWFF  
 DVWGTGTTVTVSS.

Other preferred embodiments of the present invention comprise polypeptide segments such as a monoclonal antibody V-region light chain termed herein VkA comprising the amino acid residue sequence of SEQ ID NO: 5:

QIVLSQSPAIITASPGEKVTMTCRASTSASYIHWFQQKPTSSPKPWIYATSNLASGVP  
 20 SRFSGSGSGTYSMTISSLEAEDAATYYCQQWTSNPPTFGGGTKLEIK;

a monoclonal antibody V-region light chain termed herein VkB comprising the amino acid residue sequence of SEQ ID NO: 6:

QIVLSQSPAIITASPGEKVTMTCRASTSVSYIHWFQQKPTSSPKPWIYATSNLASGVP  
 25 SRFSGSGSGTYSMTISSLEAEDAATYYCQQWTSNPPTFGGGTKLEIK;

a monoclonal antibody V-region light chain termed herein VkC comprising the amino acid residue sequence of SEQ ID NO: 7:

QIVLSQSPAIITASPGEKVTMTCRASTSVSYIHWFQQKPGSSPKPWIYATSNLASGV  
 30 PSRFSGSGSGTYSMTISSLEAEDAATYYCQQWTSNPPTFGGGTKLEIK;

a monoclonal antibody V-region light chain termed herein VkD comprising the amino acid residue sequence of SEQ ID NO: 8:

QIVLSQSPAIITASPGEKVTMTCRASSSVSYIHWFQQKPGSSPKPWIYATSNLASGV  
 PSRFSGSGSGTYSMTISSLEAEDAATYYCQQWTSNPPTFGGGTKLEIK;

and a monoclonal antibody V-region light chain termed herein VkZ comprising the amino acid residue sequence of SEQ ID NO: 12:

35

DIVLTQSPALITASPGEKVTMTCRASSSVNYMDWYQKKPGSSPKPWIYATSNLASG  
VPSRFSGSGSGTTYSMTISLSLEAEDAATYYCQWSFNPPTFGGGTKLEIK.

The present invention provides polypeptide compositions having therapeutic potential  
5 for the treatment of CD20 positive B-cell diseases in man. The CD20-binding  
polypeptide compositions of the invention can be in the form of intact antibodies, Fab  
fragments, or fusion proteins comprising whole antibodies and cytokines, fusion  
proteins of antibody fragments and cytokines, or other any other form that binds to  
CD20 antigen.

10 The CD20-binding polypeptide compositions of the invention preferably include a  
combination of a heavy chain (Vh) anti-CD20 antibody variable region and a light  
chain (Vk) anti-CD20 antibody variable region, which together bind to CD20 antigen.  
The Vh/Vk combinations can be configured as intact antibodies, Fab fragments, or  
fusion proteins comprising whole antibodies and cytokines, fusion proteins of antibody  
15 fragments and cytokines, or other any other configuration that binds to CD20 antigen.  
In other preferred embodiments, the V regions are attached to each other through a  
polypeptide backbone.

Preferably, the CD20-binding polypeptide compositions of the invention include Vh  
20 and Vk polypeptides configured as an intact antibody, which includes a light chain  
constant region, and heavy chain CH1, CH2, and CH3 domains. Alternatively, the Vh  
and/or Vk polypeptides can be configured into a Fab fragment or a "minibody" having  
a CH3 domain but lacking a CH2 domain (see e.g., Wu *et al.*, US 5,837,821).  
Alternatively, the Vh and Vk polypeptides can be attached to each other through a  
25 linker to form an "single-chain Fv" antibody molecule. In one set of preferred  
embodiments, human constant regions are combined with the CD-20-binding Vh and  
Vk polypeptides. Such constant regions include those derived from IgA, IgD, IgM,  
IgE, or IgG1, IgG2, IgG3, or IgG4-type immunoglobulins. When a CH2 domain is  
included in the CD20-binding polypeptide composition, it is preferable to include a  
30 human IgG domain, such as an IgG1 CH2 domain. Other preferred embodiments lack  
the N-linked oligosaccharide glycosylation site found in the IgG CH2 domain. The  
absence of the N-linked glycosylation site is preferably engineered by mutation of the  
relevant asparagine, serine or threonine, or adjacent amino acids, or to treat the CH2-  
containing protein with an enzyme such as PNGase F to remove the oligosaccharide.

Preferred combinations of Vh and Vk regions include a polypeptide composition comprising the heavy chain V-region VhC (SEQ ID NO: 2) and a the light chain VkA (SEQ ID NO: 5); a polypeptide composition comprising the heavy chain V-region VhC (SEQ ID NO: 2) and a the light chain V- region VkB (SEQ ID NO: 6); a

5 polypeptide composition comprising the heavy chain V-region VhC (SEQ ID NO: 2) and a the light chain V-region VkC (SEQ ID NO: 7); a polypeptide composition comprising the heavy chain V-region VhC (SEQ ID NO: 2) and a the light chain V- region VkD (SEQ ID NO: 8); a polypeptide composition comprising the heavy chain V-region VhD (SEQ ID NO: 3) and a the light chain V-region VkB (SEQ ID NO: 6); a

10 polypeptide composition comprising the heavy chain V-region VhD (SEQ ID NO: 3) and a the light chain V- region VkD (SEQ ID NO: 8); and a polypeptide composition comprising the heavy chain V-region VhY (SEQ ID NO: 10) and a the light chain V- region VkZ (SEQ ID NO: 12).

One preferred polypeptide composition of the invention includes a V-region heavy

15 chain of antibody 2B8 (SEQ ID NO: 1), which includes one or more amino acid residue substitutions in SEQ ID NO: 1 selected from the group consisting of V<sub>12</sub>K, A<sub>14</sub>P, M<sub>20</sub>V, L<sub>48</sub>T, A<sub>68</sub>T, Q<sub>82</sub>E, T<sub>87</sub>R, S<sub>91</sub>T, and T<sub>106</sub>W.

Another preferred polypeptide composition comprises a V-region light chain of antibody 2B8 (SEQ ID NO: 4) modified to contain one or more amino acid residue

20 substitutions in SEQ ID NO: 4 selected from the group consisting of L<sub>11</sub>I, S<sub>12</sub>T, S<sub>27</sub>T, V<sub>29</sub>A, G<sub>40</sub>T, V<sub>59</sub>S, S<sub>69</sub>T, L<sub>72</sub>M, R<sub>76</sub>S, and V<sub>77</sub>L. Figure 16 illustrates the location of the preferred amino acid residue substitutions in bold face type.

Another preferred CD20-binding polypeptide composition of the invention includes a V-region heavy chain (Vh) of antibody Leu16 (SEQ ID NO: 9) modified to contain

25 one or more substitutions in SEQ ID NO: 9 selected from the group consisting of V<sub>12</sub>K, M<sub>20</sub>V, A<sub>68</sub>T, Q<sub>82</sub>E, T<sub>87</sub>R, S<sub>91</sub>T, D<sub>93</sub>V, and A<sub>114</sub>T.

Yet another preferred polypeptide composition of the invention includes a V-region light chain (Vk) of antibody Leu16 (SEQ ID NO: 11) modified to contain one or more amino acid residue substitutions in SEQ ID NO: 11 selected from the group consisting

30 of L<sub>11</sub>I, S<sub>12</sub>T, A<sub>59</sub>S, S<sub>69</sub>T, L<sub>72</sub>M, R<sub>76</sub>S, and V<sub>77</sub>L. Figure 16 illustrates the location of the preferred amino acid residue substitutions in bold face type.

The CD20-binding polypeptide compositions of the invention also include compositions having one or more anti-CD20 antibody heavy chain V region that

- comprises a polypeptide segment selected from the group consisting of
- SGAELKKPGAS (SEQ ID NO: 15),
- VSCKASGYT (SEQ ID NO: 16),
- LEWTGAIY (SEQ ID NO: 17),
- 5 YNQKFKGKT (SEQ ID NO: 18),
- FKGKTTLTA (SEQ ID NO: 19),
- YMELSSLRS (SEQ ID NO: 20),
- SSLRSEDNAV (SEQ ID NO: 21), and
- DWGTGTTVT (SEQ ID NO: 22).
- 10 Similarly, the CD20-binding polypeptide compositions of the invention also include compositions having one or more anti-CD20 antibody light chain V region that comprises a polypeptide segment selected from the group consisting of
- IITASPGEKV (SEQ ID NO: 23),
- CRASTSASY (SEQ ID NO: 24),
- 15 QQKPTSSP (SEQ ID NO: 25),
- LASGVPSRF (SEQ ID NO: 26),
- FSGSGSGTT (SEQ ID NO: 27), and
- YSMTISSLE (SEQ ID NO: 28).

The V region heavy chains and light chains can be configured, for example, as intact

20 antibodies, antibody fragments, fusion proteins of an antibody with a cytokine, antibody fragment-cytokine fusion proteins, Fab molecules, single-chain Fv molecules, and minibodies. Preferably, the CD20-binding compositions are configured as intact antibodies, more preferably antibodies having human constant regions with and IgG heavy chain constant region isotype, most preferably including an IgG1 heavy chain

25 constant region and having intact effector functions such as antibody-dependent, cell-mediated cytotoxicity.

In another preferred embodiment the CD20-binding composition is configured as antibody-cytokine fusion protein, preferably including human constant regions with

30 the isotype IgG1 and having intact effector functions such as antibody-dependent, cell-mediated cytotoxicity. In such preferred antibody-cytokine fusion proteins, it is useful to include an interleukin such as interleukin-2 (IL-2), IL-4, IL-5, IL-6, IL-7, IL-10, IL-12, IL-13, IL-14, IL-15, IL-16 and IL-18, a hematopoietic factor such as granulocyte-macrophage colony stimulating factor (GM-CSF), granulocyte colony stimulating

factor (G-CSF), erythropoietin, a tumor necrosis factors (TNF) such as TNFalpha, a lymphokine such as lymphotoxin, a regulator of metabolic processes such as leptin, an interferon such as interferon alpha, interferon beta, and interferon gamma, or a chemokine. Preferably, the antibody-cytokine fusion protein of the present invention  
5 displays cytokine biological activity (e.g., stimulation of immune cells, such as T-cells or B-cells).

The polypeptide compositions of the present invention have a number of useful biological properties, including the ability to bind human B-cells; the ability to bind to CD20 antigen; a reduced ability immune response relative to antibodies Leu16, 2B8, or  
10 1H4; and activity against B cell proliferative disorders such as leukemias, lymphomas, rheumatoid arthritis, and other autoimmune diseases. Diagnostic uses for the CD2--binding polypeptide compositions of the present invention are also contemplated, in which composition can be a full length antibody, an antibody fragment (e.g. F(ab')<sub>2</sub>), a radiolabelled antibody, an immobilized antibody, or an antibody conjugated with a  
15 heterologous compound. Such diagnostic method can be used to detect the presence of CD20-presenting cells, to purify or separate CD2--presenting cells, and the like.

The invention also provides pharmaceutical compositions comprising a CD20-binding composition as defined hereinabove together with a pharmaceutically acceptable  
20 carrier, diluent or excipient, as well as method of producing the polypeptide compositions.

## BRIEF DESCRIPTION OF THE DRAWINGS

25 FIGURE 1 shows the native amino acid residue sequence, in single letter code, of the V-region heavy chain protein of the 2B8 antibody and the modified amino acid residue sequences VhC and VhD based on the native sequence.

FIGURE 2 shows the native amino acid residue sequence, in single letter code, of the V-region light chain protein of the 2B8 antibody and modified sequences VkA, VkB,  
30 VhC and VhD based on the native sequence.

FIGURE 3 shows the amino acid residue sequence, in single letter code, of the heavy chain variable regions of Leu16Vh and a modified Leu16Vh region designated VhY;

FIGURE 4 shows the amino acid residue sequence, in a single letter code, of the light chain variable regions of Leu16Vk and a modified Leu16Vk designated VhZ.

FIGURE 5 is a graphical representation of an antibody-dependent cellular cytotoxicity (ADCC) lysis assay described in Example 3; proteins tested were chimeric Leu16 antibody (chLeu16; filled triangles), a fusion protein of chLeu16 and IL2 (chLeu16-IL2; filled squares), LeuVhY/VkZ-IL2 fusion protein (filled squares), chimeric 2B8 antibody (C2B8, filled circles), C2B8-IL2 fusion protein (open squares), and KS-IL2 immunocytokine fusion protein which does not target CD20 (open circles). X-axis: concentration (ng/ml); y-axis: % lysis.

FIGURE 6 illustrates the anti-CD20 binding activity of CD20-binding polypeptide compositions and immunocytokines (ICs). Human Daudi lymphoma cells were incubated with varying concentrations of polypeptide compositions and ICs and relative binding to the cells was assessed by flow cytometry, as described in Example 2.

Figure 6A shows the mean fluorescence intensity (MFI, Y axis), which increases as a function of polypeptide concentration (X axis). Figure 6B shows the fluorescence intensity measured on a Daudi cell sample with no primary antibody present (leftmost peak); an antibody-IL2 fusion directed against EGF receptor, which is not expressed on Daudi cells (left-central peak); and Leu16VhY/VkZ-IL2 (right-most peak). X-axis: fluorescence intensity at 10ug/ml; Y-axis: number of events.

FIGURE 7 illustrates antibody-dependent cell-mediated and complement-dependent cytotoxicity. Figures 7A and 7B represent independent ADCC tests compared to Figure 5. ADCC was determined in a 4-hour assay using  $^{51}\text{Cr}$ -labeled Daudi target cells and human PBMC as effectors (E:T=100). Figure 7A. Antibodies tested were 2B8 (filled circles), chLeu16 (filled diamonds), and Leu16VhY, VkZ (open triangles). Figure 7B. Immunocytokines tested were chLeu16-IL2 (filled squares), and Leu16VhY/VkZ-IL2 (filled triangles), deglycosylated Leu16VhY/VkZ-IL2 (X's), and huKS-IL2 (open circles) as a non-binding control. Y-axis: % lysis, X-axis: concentration (ng/ml).

FIGURE 7C shows CDC activity using  $^{51}\text{Cr}$ -labeled Daudi cells and human plasma as a source of complement, as described in Example 4. Incubation was for 1 hour using the same antibodies and immunocytokines. Antibodies tested were 2B8 (filled circles), chLeu16 (filled diamonds), chLeu16-IL2 (filled squares), and Leu16VhY/VkZ-IL2 (filled triangles), and huKS-IL2 (open circles) as a non-binding control.

FIGURE 8 illustrates the pharmaco-kinetics of epitope-depleted Leu16 immunocytokines in mice, as described in Example 5. A time-concentration analysis was performed following an intravenous (i.v.) dose of each immunocytokine. Serum concentrations were

determined by an ELISA that detects the intact forms for the native (solid squares) and de-glycosylated proteins (open circles). Mice were injected with 25 mg of the indicated immunocytokine and samples were taken by retro-orbital bleeding and assayed by capture with anti-human IgG H&L antisera and detection with an anti-human IL2 antibody.

- 5 Results are normalized to the initial concentration in the serum of each mouse taken immediately after injection. X-axis: hours, Y-axis: % ID remaining

FIGURE 9 shows anti-tumor model evaluations in SCID mice. SCID mice were injected i.v. with  $2 \times 10^6$  CD20+ Daudi lymphoma cells followed by 5 consecutive daily injections of immunocytokines beginning on day 3 (Figure 9A) or day 7 (Figure 9B). The non-targeting control immunocytokine 425-IL2, specific for EGFR, was used at the high dose to illustrate the incomplete activity due to the altered half-life of IL-2. Treatments in Figure 9A were administered on days 3, 4, 5, 6, and 7 and included PBS only (filled diamonds); 425-IL2 (dark, crossed X's); and Leu16VhY/VkZ-IL2 at daily doses of 5 micrograms (mcg) (light Xs), 10 mcg (filled triangles), and 20 mcg (filled squares). In Figure 9A, the latter three doses all showed complete protection of the mice, and the data points are superimposed. Treatments in Figure 9B were administered on days 7, 8, 9, 10, and 11, and included PBS only (filled diamonds); and Leu16VhY/VkZ-IL2 at daily doses of 5 mcg (light Xs); and 20 mcg (filled squares). X-axis: days; Y-axis: % survival.

- 20 FIGURE 10 shows the results of anti-tumor model evaluations (2B8 vs. chLeu16-IL2) in SCID mice; SCID mice were injected with Daudi lymphoma cells and treated with the indicated antibody or immunocytokine beginning 7 days later, as described for Figure 9. Treatments included PBS only (crossed Xs, on days 7-11); rituximab (C2B8; filled circles, 25 mg/kg on days 7, 9 and 11); high dose Leu16VhY/VkZ-IL2 (large squares, 1 mg/kg on days 7-11); low dose Leu16VhY/VkZ-IL2 (small squares, 0.25 mg/kg on days 7-11). (A): healthy mice (B) survival of mice; X-axis: days; Y-axis: % healthy mice (A), % survival of mice (B).

FIGURE 11 shows the results of anti-tumor model evaluations (chLeu16-IL2 vs. deimmunised (DI)-Leu16-IL2) in SCID mice; SCID mice were injected with Daudi lymphoma cells and treated with the indicated antibody or immunocytokine beginning 7 days later, as described for Figure 9. Treatments included PBS only (crossed Xs, on days 7-11); rituximab (filled circles, 25 mg/kg on days 7, 9 and 11); high dose Leu16VhY/VkZ-IL2 (large squares, 1 mg/kg on days 7-11); low dose Leu16VhY/VkZ-IL2 (small squares, 0.25 mg/kg on days 7-11); high dose chLeu16-

IL2 (large triangles, 1 mg/kg on days 7-11); and low dose chLeu16-IL2 (small triangles, 0.25 mg/kg on days 7-11). (A) healthy mice, (B) survival of mice.

X-axis: days; Y-axis: % healthy mice (A), % survival of mice (B).

FIGURE 12 illustrates that loss of ADCC activity has only a partial effect on anti-tumor activity mediated by Leu16VhY/VkZ-IL2 (DI-Leu16-IL2 vs. deglycosyl. DI-Leu16-IL2). Two different doses of native and enzymatically deglycosylated Leu16VhY/VkZ-IL2 were used to treat SCID mice beginning 7 days after injection of Daudi lymphoma cells, as described for Figure 9. Treatments included PBS only (X, on days 7-11); rituximab (filled circles, 25 mg/kg on days 7, 9 and 11); high dose Leu16VhY/VkZ-IL2 (large triangles, 1 mg/kg on days 7-11); low dose Leu16VhY/VkZ-IL2 (small triangles, 0.25 mg/kg on days 7-11); high dose deglycosylated Leu16VhY/VkZ-IL2 (large Xs with thick lines, 1 mg/kg on days 7-11); and low dose de-glycosylated Leu16VhY/VkZ-IL2 (small Xs with thin lines, 0.25 mg/kg on days 7-11). (A) healthy mice, (B) survival of mice. X-axis: days; Y-axis: % healthy mice (A), % survival of mice (B).

FIGURE 13 illustrates that antigen specificity is important for optimal anti-tumor activity. The role of tumor cell targeting was tested by comparing the activity of DI-Leu16-IL2 and another immunocytokine with binding specificity for EGFR, a molecule expressed at only low levels of Daudi lymphoma cells. Treatments included PBS only (X, on days 7-11); rituximab (filled diamonds, 25 mg/kg on days 7, 9 and 11); DI-leu16 antibody (open diamonds, 25 mg/kg on days 7, 9 and 11); medium dose DI-Leu16-IL2 (filled squares, 1 mg/kg on days 7-11); reduced dose DI-Leu16-IL2 (open circle, 1 mg/kg on day 7 and 10); low dose DI-Leu16-IL2 (open squares, 0.25 mg/kg on days 7-11); and medium dose anti-EGFR-IL2 (filled triangle, 1 mg/kg on days 7-11). Results were scored as disease free survival. X-axis: days; Y-axis: % disease free.

FIGURE 14 illustrates that Leu16VhY/VkZ-IL2 is more potent than higher doses of anti-CD20 antibody combined with free IL-2. Anti-tumor activities of medium dose Leu16VhY/VkZ-IL2 (filled squares, 20 mg/mouse on day 7-11) and the corresponding doses of the individual antibody and IL-2 components (open squares, 16.7 mg Leu16VhY, VKZ and 3.3 mg IL-2 by i.v. on days 7-11) were compared to high dose rituximab and subcutaneous (s.c.) IL-2 (open diamonds, 500 mg rituximab on day 7 and 10 mg IL-2 on days 7, 9 and 11), rituximab alone (filled diamond, 500 mg on day 7) or PBS control. X-axis: days; Y-axis: % disease free.

FIGURE 15 illustrates that the presence of normal anti-CD20+ B cells does not diminish the anti-tumor activity of Leu16VhY/VkZ-IL2. The effect of prior implantation of normal human CD20+ B cells was tested in the same SCID/Daudi lymphoma model as described in Methods. Groups of mice were treated with a single high dose of rituximab (diamonds, 25 mg/kg on day 7), Leu16VhY/VkZ-IL2 (squares, 1 mg/kg on days 11-15), the combination of both dosing regimens (triangles, rituximab on day 7 followed by Leu16VhY/VkZ-IL2 on days 11-15) or PBS alone on all dosing days. Half of the groups were injected i.v. with  $4.5 \times 10^6$  PBMC on day 5 (open symbols) or received only PBS (filled symbols). B cell engraftment was confirmed by measuring human antibody levels in the serum of all mice. X-axis: days; Y-axis: % disease free.

FIGURE 16 shows an alignment of the heavy chain and light chain V regions of the anti-CD20 antibodies 2B8, Leu16, and 1H4, as well as the epitope-depleted derivative V regions of 2B8 (VhC, VhD, VkA, VkB, VkC, and VkD, and the epitope-depleted Leu16 V-regions VhY and VkZ.

FIGURE 17 shows a summary of all sequence identity numbers (SEQ ID Nos.: 1 – 43) as used in this application.

## 20 DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

As used herein in the appended claims, the term "polypeptide composition" and grammatical variations thereof refers to single chain polypeptides, as well as multiple chain polypeptides in which the polypeptide chains are chemically bound to each other, such as by one or more disulfide bonds between a cysteine residue in one chain and a cysteine residue on another chain, by an ester bond, an amide bond, or any other suitable linkage.

A CD20-binding polypeptide composition comprises at least one polypeptide segment selected from the group consisting of a modified heavy chain variable region polypeptide (Vh) having the amino acid residue sequence of SEQ ID NO: 1, but which includes at least one amino acid residue substitution in SEQ ID NO: 1 selected from the group consisting of V<sub>12</sub>K, A<sub>14</sub>P, M<sub>20</sub>V, I<sub>48</sub>T, A<sub>68</sub>T, Q<sub>82</sub>E, T<sub>87</sub>R, S<sub>91</sub>T, and T<sub>106</sub>W; a modified light chain variable region polypeptide (Vk) having the amino acid residue sequence of SEQ ID NO: 4, but which includes at least one amino acid residue

substitution in SEQ ID NO: 4 selected from the group consisting of L<sub>11</sub>I, S<sub>12</sub>T, S<sub>27</sub>T, V<sub>29</sub>A, G<sub>40</sub>T, V<sub>59</sub>S, S<sub>69</sub>T, L<sub>72</sub>M, R<sub>76</sub>S, and V<sub>77</sub>L; a modified Vh having the amino acid residue sequence of SEQ ID NO: 9, but which includes at least one amino acid residue substitution in SEQ ID NO: 9 selected from the group consisting of V<sub>12</sub>K, M<sub>20</sub>V, A<sub>68</sub>T, Q<sub>82</sub>E, T<sub>87</sub>R, S<sub>91</sub>T, D<sub>93</sub>V, and A<sub>114</sub>T; and a modified Vk having the amino acid residue sequence of SEQ ID NO: 11, but which includes at least one amino acid residue substitution in SEQ ID NO: 11 selected from the group consisting of L<sub>11</sub>I, S<sub>12</sub>T, A<sub>59</sub>S, S<sub>69</sub>T, L<sub>72</sub>M, R<sub>76</sub>S, and V<sub>77</sub>L.

Preferably, the CD20-binding polypeptide composition includes at least one polypeptide segment having an amino acid residue sequence selected from the group consisting of SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 15 and SEQ ID NO: 28.

15

In a preferred embodiment, the CD20-binding polypeptide composition comprises a modified heavy chain variable region polypeptide and a modified light chain variable region polypeptide. More preferably, the CD20-binding polypeptide composition is in the form of a chimeric antibody and also includes a human heavy chain constant region and a human light chain constant region. Preferably the human heavy chain constant region is an IgG constant region, more preferably an IgG1 constant region. The human light chain constant region preferably is a human kappa light chain constant region. Another preferred embodiment of the CD20-binding polypeptide composition of the invention is a fusion protein comprising a polypeptide composition including modified heavy chain or light chain variable region segment, as described above, fused with a cytokine. Preferably the cytokine is IL2.

Another preferred embodiment of the CD20-binding polypeptide composition of the invention is an antibody molecule, such as a Fab antibody fragment, a single-chain Fv antibody fragment, or a minibody, which includes at least one modified Vh or modified Vk segment, as described above.

A particularly preferred CD20-binding polypeptide composition comprises at least one Vh polypeptide segment having an amino acid residue sequence selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 3 and SEQ ID NO: 10.

- 5 Another particularly preferred CD20-binding polypeptide composition comprises at least one Vk polypeptide segment having an amino acid residue sequence selected from the group consisting of SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, and SEQ ID NO: 12.
- 10 More preferably, the CD20-binding polypeptide composition comprises a Vh polypeptide segment having an amino acid residue sequence selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 3, and SEQ ID NO: 10; and a Vk polypeptide segment having an amino acid residue sequence selected from the group consisting of SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, and SEQ
- 15 ID NO: 12.

Other preferred CD20-binding polypeptide compositions of the present invention comprise combinations of modified Vh and modified Vk segments selected from the group consisting of

- 20 (a) a Vh segment having an amino acid residue sequence of SEQ ID NO: 2 and a Vk segment having an amino acid residue sequence of SEQ ID NO: 5;
- (b) a Vh segment having an amino acid residue sequence of SEQ ID NO: 2 and a Vk segment having an amino acid residue sequence of SEQ ID NO: 6;
- (c) a Vh segment having an amino acid residue sequence of SEQ ID NO: 2 and a Vk
- 25 segment having an amino acid residue sequence of SEQ ID NO: 7;
- (d) a Vh segment having an amino acid residue sequence of SEQ ID NO: 2 and a Vk segment having an amino acid residue sequence of SEQ ID NO: 8;
- (e) a Vh segment having an amino acid residue sequence of SEQ ID NO: 3 and a Vk segment having an amino acid residue sequence of SEQ ID NO: 6;
- 30 (f) a Vh segment having an amino acid residue sequence of SEQ ID NO: 3 and a Vk segment having an amino acid residue sequence of SEQ ID NO: 8; and
- (g) a Vh segment having an amino acid residue sequence of SEQ ID NO: 10 and a Vk segment having an amino acid residue sequence of SEQ ID NO: 12.

Another aspect of the present invention is a pharmaceutical composition comprising a CD20-binding polypeptide composition of the invention, together with a pharmaceutically acceptable carrier, excipient, diluent. The pharmaceutical composition can also include an additional pharmacologically effective drug.

5

The disclosed sequences have been derived from the analysis of the V-region sequences of the mouse monoclonal antibodies 2B8 and Leu16 referred to herein as the "parental" antibodies and disclosed fully in respect of their useful *in vitro* and *in vivo* properties in US patent No. 5,736,137; US patent No. 5,776,456; US patent No. 6,399,061; US patent No. 6,455,043 and foreign equivalents, and in Wu *et al.* (*Protein Engineering* (2001) 14:1025-1033) and references therein.

The sequence analysis so conducted was directed towards identification of the peptides contained within the sequence able to act a MHC class II ligands and therefore function as a T-cell epitope in man. An object of the present invention is to provide an improved version of the parental antibody. Thus knowledge of the positions of potential MHC class II epitopes is an important first step of the engineering process. Epitope identification was conducted computationally, following the scheme outlined in detail in WO 02/069232 and incorporated fully herein by reference. Following this first step, the inventors herein have surprisingly discovered sets of amino acid substitutions that collectively satisfy several different criteria with respect to making desired modifications to the parental antibodies.

The substitution sets within the V-regions of the parental antibody result in a polypeptide sequence with a reduced number of MHC class II ligands and accordingly a reduced propensity for the polypeptides to act as T-cell epitopes. Equally, the said substitution sets result in polypeptide sequences with the retained structural and functional properties of the parental antibody and this is true with respect to the especially desired dual properties of retained and stable expression within a host cell, and of particular importance, a retained ability for the polypeptide to bind the human CD20 antigen and especially bind human B-cells *in vitro*.

30

It is noteworthy that the Vh and Vk amino acid residue sequences of 2B8 and Leu16, as well as the anti-CD20 antibody B1, are quite similar, even though they are of independent origin. Without wishing to be bound by theory, an insight of the invention is as follows. The sequence CEPANPSEKNSPSTQYC (SEQ ID NO: 29) in human

CD20, which is part of the short extracellular peptide of CD20 and which is flanked by cysteines that are likely to form a disulfide bond, corresponds to the sequence CEPSNSSEKNSPSTQYC (SEQ ID NO: 38) in mouse CD20. The mouse NSS sequence is likely to be an N-linked glycosylation site, while the corresponding NPS  
5 sequence in humans is not likely to be an N-linked glycosylation site. This difference between the mouse and human CD20 sequences would reveal, from the vantage point of the mouse immune system, an epitope in the human sequence. Thus, all of the mouse antibodies generated against human CD20 may be selected to recognize a very narrowly defined epitope, and there may be one optimal set of mouse V regions to do  
10 this.

The invention thus provides a set of reduced-immunogenicity consensus V region sequences that are important for recognition of human CD20. For example, in the heavy chain, the sequence segment VSCKASGYT (SEQ ID NO: 16) in the modified heavy chains of the invention is useful in the context of an antibody that binds to  
15 CD20; specifically, this segment is less immunogenic than the corresponding segment, MSCKASGYT (SEQ ID NO: 30), from the 2B8 and Leu16 antibodies. Similarly, the sequences YNQKFKGKT (SEQ ID NO: 18), FKGKTTLTA (SEQ ID NO: 19), and YMELSSLRS (SEQ ID NO: 20) are useful in the context of CD20 antibodies, and are less immunogenic than the corresponding sequences, YNQKFKGKA (SEQ ID NO:  
20 31), FKGKATLTA (SEQ ID NO: 32), and YMQLSSLRS (SEQ ID NO: 33), that are found in the parental 2B8 and Leu16 antibodies.

Considering the light chain, the sequence segment IITASPGGEKV (SEQ ID NO: 23) in the modified light chains of the invention is useful in the context of an antibody that binds to CD20; specifically, this segment is less immunogenic than the corresponding  
25 segment, ILSASPGGEKV (SEQ ID NO: 34), from the parental 2B8 and Leu16 antibodies. Similarly, the sequences LASGVPSRF (SEQ ID NO: 26), FSGSGSGTT (SEQ ID NO: 27), and YSMTISSLE (SEQ ID NO: 28) are useful in the context of CD20 antibodies, and are less immunogenic than the corresponding sequences, LASGVPVARF (SEQ ID NO: 35), FSGSGSGTS (SEQ ID NO: 36), and YSLTISRVE  
30 (SEQ ID NO: 37), that are found in the parental 2B8 and Leu16 antibodies.

Reference to "substantially non-immunogenic" or "reduced immunogenic potential," as used herein and in the appended claims, includes reduced immunogenicity compared to a counterpart or "parent" antibody, i.e. a non-modified murine or chimeric

monoclonal antibody such as 2B8 or Leu16. The term "immunogenicity" includes an ability to provoke, induce or otherwise facilitate a humoral and or T-cell mediated response in a host animal and in particular where the "host animal" is a human.

- 5 The term "antibody molecule" refers to a polypeptide of the immunoglobulin family, including a whole antibody, that is capable of combining, interacting or otherwise associating with an antigen.

The term "antigen" is used herein to refer to a substance that is capable of interacting with the antibody molecule and in the context of the present invention is meant to be

- 10 CD20. The CD20 of the present invention is human CD20 or any CD20 representing an antigen for antibody 2B8. The CD20 may be a soluble CD20 derivative or membrane associated CD20.

The term "immunoglobulin" is used herein to refer to a protein consisting of one or more polypeptides substantially encoded by immunoglobulin genes. The recognized

- 15 immunoglobulin genes include IgG1, IgG2, IgG3, IgG4 constant region genes and in nature multiple immunoglobulin variable region genes. One natural form of immunoglobulin is a tetramer comprising two identical pairs in which each pair has one light chain and one heavy chain. In each pair the heavy and light chain variable regions together provide the binding surface capable of interacting with the antigen.

- 20 The term Vh is used herein to refer to the heavy chain variable region, and the term Vk is used herein to refer to the light chain variable region and in this instance in common with numerous monoclonal antibodies the light chain is a "kappa" (k) type chain.

The V-region includes amino acid residues from a "complementarity determining

- 25 region" or "CDR" (i.e. at about amino acid residues 24-34 (L1), 50-56 (L2) and 89-97 (L3) in the light chain variable domain and at about amino acid residues 31-35 (H1), 50-65 (H2) and 95-102 (H3) in the heavy chain variable domain as defined by Kabat *et al.* (Kabat *et al. Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991)). Alternative
- 30 definitions of the CDRs are also recognized in the art for example according to the scheme of Chothia (Chothia and Lesk (1987) *J. Mol. Biol.* 196: 901-917) in which case the CDRs are found at about amino acid residues 26-32 (L1), 50-52 (L2) and 91-96 (L3) in the light chain, and at about amino acid residues 26-32 (H1), 53-55 (H2) and

96-101 (H3) in the heavy chain. "Framework" or "FR" residues are those V-region residues other than the CDR residues as herein defined.

As used herein, Vh means a polypeptide that is about 110 to 125 amino acid residues  
5 in length, the sequence of which corresponds to any of the specified Vh chains herein which in combination with a Vk are capable of binding human CD20. Similarly, Vk means a polypeptide that is about 95-130 amino acid residues in length, the sequence of which corresponds to any of the specified Vk chains herein which in combination with a Vh are capable of binding human CD20. Full-length immunoglobulin heavy  
10 chains are about 50 kDa molecular weight and are encoded by a Vh gene at the N-terminus and one of the constant region genes at the C-terminus. Similarly, full-length light chains are about 25 kDa molecular weight and are encoded by a V-region gene at the N-terminus and a constant region gene at the C-terminus.

15 In addition to whole a antibody (a tetramer), immunoglobulins may exist in a number of other forms derived by application of recombinant DNA techniques or protein biochemistry. These forms include for example Fv, Fab, Fab' and (Fab)<sub>2</sub> molecules and could all contain any of the Vh or Vk sequences of the present invention. A further example may include a "bi-specific" antibody, that is comprising a Vh/Vk  
20 combination of the present invention in combination with a second Vh/Vk combination with a different antigen specificity.

The term "T-cell epitope," as used herein and in the appended claims, means an amino acid sequence which is able to bind MHC class II, able to stimulate T-cells and / or  
25 also to bind (without necessarily measurably activating) T-cells in complex with MHC class II.

The term "peptide" as used herein and in the appended claims, is a compound that includes two or more amino acid residues. The amino acid residues are linked together  
30 by a peptide bond (defined herein below). There are 20 different common, naturally occurring amino acids involved in the biological production of peptides, and any number of them may be linked in any order to form a peptide chain or ring. The naturally occurring amino acids employed in the biological production of peptides all have the L-configuration. Synthetic peptides can also be prepared employing

conventional synthetic methods, utilizing L-amino acids as well as D-amino acids, or various combinations of amino acids of the two different configurations. Some peptides contain only a few amino acid residues. Short peptides, e.g., having less than ten amino acid residues, are sometimes referred to as "oligopeptides". Other peptides  
5 contain a large number of amino acid residues, e.g. up to 100 or more, and are referred to as "polypeptides".

By convention, a "polypeptide" may be considered as any peptide chain containing three or more amino acid residues, whereas an "oligopeptide" is usually considered as a particular type of "short" polypeptide. Thus, as used herein, it is understood that any  
10 reference to a "polypeptide" herein also includes an oligopeptide. Further, any reference to a "peptide" includes polypeptides, oligopeptides, and proteins. Each different arrangement of amino acid residues forms different polypeptides or proteins. The number of polypeptides—and hence the number of different proteins—that can be formed is practically unlimited.

15 The term "peptide composition" means a composition which at least comprises one polypeptide deriving from an anti-CD20 antibody, preferably 2B8 and Leu16. The polypeptide may exist as single chain (e.g. heavy chain or part thereof, light chain or part thereof, or single-chain Fv) or in a dimeric form. The dimeric form may be a whole antibody, a Fab fragment, a minibody, or any two-chain molecule, wherein the  
20 single chains are linked by a mere chemical bond. The composition may comprise several single chain polypeptides only, which are not linked by a chemical or a cysteine bond.

The invention also relates to an anti-CD20 monoclonal antibody in which substitutions  
25 of at least one amino acid residue have been made at positions within the V-regions of the molecule to result in a substantial reduction in activity of or elimination of one or more potential T-cell epitopes from the protein. It is most preferred to provide modified antibody molecules in which amino acid modification (e.g. a substitution) is conducted within the most immunogenic regions of the parent molecule. The major  
30 preferred embodiments of the present invention comprise modified antibody molecules for which any of the MHC class II ligands are altered such as to eliminate binding or otherwise reduce the numbers of MHC allotypes to which the peptide can bind. For the elimination of T-cell epitopes, amino acid substitutions are made at appropriate points within the peptide sequence predicted to achieve substantial reduction or

elimination of the activity of the T-cell epitope. In practice an appropriate point will preferably equate to an amino acid residue binding within one of the pockets provided within the MHC class II binding groove.

It is most preferred to alter binding within the first pocket of the cleft at the so-called  
5 P1 or P1 anchor position of the peptide. The quality of binding interaction between the P1 anchor residue of the peptide and the first pocket of the MHC class II binding groove is recognized as being a major determinant of overall binding affinity for the whole peptide. An appropriate substitution at this position of the peptide is for a residue less readily accommodated within the pocket, for example, substitution to a  
10 more hydrophilic residue. Amino acid residues in the peptide at positions equating to binding within other pocket regions within the MHC binding cleft are also considered and fall under the scope of the present.

It is understood that single amino acid substitutions within a given potential T cell epitope are the most preferred route by which the epitope may be eliminated.

15 Combinations of substitution within a single epitope may be contemplated, and for example, can be particularly appropriate where individually defined epitopes are in overlap with each other. Moreover, amino acid substitutions either singly within a given epitope, or in combination within a single epitope, may be made at positions not equating to the "pocket residues" with respect to the MHC class II binding groove, but  
20 at any point within the peptide sequence. All such substitutions fall within the scope of the present.

An important and significant feature of the modified antibody molecules of the present invention is that they retain the functional activities of the non-modified parental  
25 antibody. It is therefore particularly desired to produce modified antibodies or modified antibody molecules in which all of the beneficial technical features associated with the therapeutic efficacy of the parental non-modified antibody are exhibited. This is pertinent to the contemplated utility of the invention, namely to provide a composition with therapeutic efficacy in a number of important diseases in man  
30 including especially B-cell lymphoma and other B-cell mediated pathologies. Such a therapeutic is a preferred embodiment of the present invention.

Accordingly, the modified antibody molecule of the present invention exhibits an affinity for its target antigen that is similar to the affinity exhibited by the parental

antibody. The antibody molecule therefore recognizes CD20 positive human B-cells. The therapeutic efficacy of the parental molecule is considered to be mediated by the ability of the antibody to induce antibody-dependent cellular cytotoxicity (ADCC). Critical to this activity is the ability of the antibody constant region (i.e. the Fc domain) to bind human serum complement component C1q. ADCC and C1q binding is mediated by the constant region domain of whole antibody molecules, and the present invention contemplates production of whole antibody molecules comprising a human Fc compatible with ADCC induction. Such constant regions are most preferably IgG1 heavy chains in combination with human kappa light (e.g. Km3) chains.

10

In as far as this invention relates to modified anti-CD20 antibody molecules, compositions containing modified antibodies or fragments of modified antibodies and related compositions are within the scope of the invention. The invention therefore contemplates the use and generation of antibody fragments including for example Fv, Fab, Fab' and F(ab')<sub>2</sub> fragments. Such fragments may be prepared by standard methods (for example; Coligan *et al.* (*Current Protocols in Immunology*, John Wiley & Sons 1991-1997). The present invention also contemplates the various recombinant forms of antibody molecule derived molecular species well known in the art. Such species include stabilised Fv fragments including single chain Fv forms (e.g. scFv) comprising a peptide linker joining the Vh and Vk domains, or an Fv stabilized by inter-chain disulfide linkage (dsFv) and which contain additional cysteine residues engineered to facilitate the conjoining of the Vh and Vk domains. Equally, other compositions are familiar in the art and could include species referred to as "minibodies"; and single variable domain "dAbs". Other species still may incorporate means for increasing the valency of the modified antibody V-region domain, i.e. species having multiple antigen binding sites for example by the engineering of dimerisation domains (e.g. "leucine zippers") or also chemical modification strategies.

In another aspect, the invention relates to fusion proteins in which the anti-CD20 V regions of the invention are coupled to a non-immunoglobulin fusion partner protein, such as an anticancer protein. Examples of such proteins include toxins, such as *Pseudomonas* exotoxin; enzymes, such as bacterial proteases for antibody-dependent prodrug therapy ("ADEPT"), or cytokines. Particularly useful cytokines are wild-type and mutant versions of IL-2, IL-12, fused forms of IL-2 and IL-12, and other

interleukins, interferons, and tumor necrosis factors. Gillies and co-workers (US5,150,650, WO98/25978, WO01/10912, WO02/72605, WO03/48334), Epstein (WO 03/15697), and Halin *et al.* (Cancer Res. (2003) 63:3202-10) have described a number of configurations of anti-cancer antibody V regions and cytokines; these  
5 methods and configurations are incorporated herein by reference. The immunoglobulin constant regions of fusion proteins comprising the V regions of the present invention may be further modified by mutations, such as mutations affecting complement fixation, mutations affecting Fc receptor binding, mutations affecting FcRn binding, and mutations affecting the serum half-life of the fusion protein;  
10 examples of such mutations are described in WO09943713 and WO01/58957, which are incorporated by reference.

In a further aspect, the present invention relates to isolated nucleic acids encoding CD20-binding polypeptide compositions, such as nucleic acids that encode a modified  
15 anti-CD20 antibody molecule, a modified heavy chain variable region polypeptide, a modified light chain variable region polypeptide, anti-CD20 antibody fusion proteins, and the like. The DNA sequence of the polynucleotide encoding the light chain of Leu16 is shown in FIG. 17 (SEQ ID NO: 39). The DNA sequence encoding a preferred epitope-depleted version of the light chain of Leu16 (i.e., including VkZ and  
20 a human constant region) is shown in FIG. 17 as SEQ ID NO: 40. The DNA sequence of the polynucleotide encoding the heavy chain of Leu16 is shown in FIG. 17 (SEQ ID NO: 41). The DNA sequence encoding a preferred epitope-depleted version of the heavy chain of Leu16 (i.e., including VhY and a human constant region) is shown in FIG. 17 as SEQ ID NO: 42. The DNA sequence encoding a preferred epitope-depleted  
25 version of the heavy chain of Leu16 fused to IL-2 (i.e., including VhY, a human constant region, and IL-2 bound to the C-terminus of the constant region) is shown in FIG. 17 as SEQ ID NO: 43.

Yet another aspect the present invention relates to methods for therapeutic treatment of humans using the CD20-binding polypeptide compositions of the invention. The  
30 Examples illustrate how the polypeptide compositions of the invention have been used to treat human cancer cells in mouse models, and the results obtained below are generally illustrative of strategies that would be used to treat human cancers such as B cell lymphoma and other cancers expressing CD20.

The CD20-binding polypeptide compositions of the invention, such as anti-CD20-IL2 fusion proteins, are used as follows: A patient suffering from a CD20-expressing cancer, such as B cell lymphoma, is administered a polypeptide composition of the invention. The preferred route of administration is intravenous or subcutaneous  
5 injection, but intramuscular, intraperitoneal, intradermal, or other routes of injection are also possible. Administration by inhalation, orally, or by suppositories is also possible, as are other routes of administration. Administration is preferably in a four-week cycle of three times per week, followed by no treatment for the next three weeks. Treatments can be more or less frequent depending on the pharmacokinetic behavior of  
10 the fusion protein in a given individual. The preferred dosage for an adult of about 70 kilograms is in the range of about 1 to about 100 milligrams per dose, with a preferred range of about 4 to about 20 milligrams per dose. The most preferred dose is about 10 milligrams for a 70 kg adult treated once per month. Patients are monitored for a response according to standard procedures.

15

## EXAMPLES

The Examples describe certain particular methods for making proteins of the invention, but those skilled in the art of protein expression will recognize that a wide variety of well-known techniques may be used to make proteins of the invention. For example,  
20 the proteins of the invention are preferably made in eukaryotic cells such as mammalian cells, such as NS/0 cells, BHK cells, CHO cells, 293 cells, or PERC6 cells. An protein of the invention may, for example, be purified using, in sequence, some or all of the following steps: Abx Mixed Resin column chromatography, recombinant Protein A chromatography, and Q Sepharose column chromatography, followed by  
25 Pellicon 2 tangential flow diafiltration for buffer exchange into formulation buffer. Virus inactivation and removal steps are interdigitated into these steps. The virus inactivation and removal steps are not necessary for purification *per se*, but are used to satisfy regulatory considerations. In a further aspect the present invention relates to methods for therapeutic treatment of humans using the modified anti-CD20 antibody  
30 molecules. The Examples illustrate how the proteins of the invention have been used to treat human cancer cells in mouse models, and the results obtained below are generally illustrative of strategies that would be used to treat human cancers such as B cell lymphoma and other cancers expressing CD20.

The fusion proteins of the invention are used as follows. A patient suffering from a CD20-expressing cancer, such as B cell lymphoma, is treated. The preferred route of administration is intravenous or subcutaneous injection, but intramuscular, intraperitoneal, intradermal, or other routes of injection are also possible.

- 5 Administration by inhalation, orally, or by suppositories is also possible, as are other routes of administration. Administration is preferably in a four-week cycle of three times per week, followed by no treatment for the next three weeks, but may be more or less frequent depending on the pharmacokinetic behavior of the anti-CD20-IL2 fusion protein in a given individual. Dosing for an adult of about 70 kilograms is in  
10 the range of about 1 to 100 milligrams per dose, with a preferred range of about 4 to 20 milligrams per dose. The most preferred dose is about 10 milligrams for a 70 kg adult treated once per month. Patients are monitored for a response according to standard procedures.

**Example 1. Methods and reagents for expressing DeImmunised Leu16 antibodies.**

- 15 **1A: Cell culture and transfection:** In order to obtain stably transfected clones, plasmid DNA was introduced into the mouse myeloma NS/0 cells by electroporation. About  $5 \times 10^6$  cells were washed once and re-suspended with phosphate buffer solution (PBS). Ten  $\mu\text{g}$  of linearized plasmid DNA was then incubated with the cells in a Gene Pulser cuvette (0.4 cm electrode gap, BioRad) for 10 minutes on ice. Electroporation was  
20 performed using a Gene Pulser (BioRad) with settings at 0.25 V and 500  $\mu\text{F}$ . Cells were allowed to recover for 10 minutes on ice, after which they were re-suspended in growth medium and then plated onto 96-well plates. Stably transfected clones were selected by growth in the presence of 100 nM methotrexate (MTX), which was introduced two days post-transfection. The cells were fed every 3 days for 2 or 3 more  
25 times and MTX-resistant clones appeared in 2 to 3 weeks. Supernatants from clones were assayed by anti-human FC ELISA and analytical high-pressure liquid chromatography (HPLC), to identify high producers (Gillies et al. (1989) J. Immunol. Methods 125:191). High producing clones (yielding about 100  $\mu\text{g}$  of purified protein per ml of cell culture supernatant in medium scale cell cultures) were isolated and  
30 propagated in growth medium containing 100 mM MTX.

**1B: ELISAs:** ELISAs were used to determine the concentrations of protein products in the supernatants of MTX-resistant clones. The anti-huFc ELISA was used to measure

the amount of human Fc-containing proteins. The anti hu-Fc ELISA is described in detail below.

- A. Coating Plates:** ELISA plates were coated with AFFINIPURE™ goat anti-human IgG (H+L) (Jackson Immuno Research) at 5 µg/ml in PBS and 100 (l/well in 96-well plates (Nunc Immuno Plate Maxisorp). Coated plates were covered and incubated at 4°C overnight. Plates were then washed 4 times with 0.05% Tween (Tween 20) in PBS and blocked with 1% bovine serum albumin (BSA)/ 1% goat serum in PBS, 200 µl/well. After incubation with the blocking buffer at 37 °C for 2 hours, the plates were washed 4 times with 0.05% Tween and tapped dry on paper towels.
- B. Incubation with test samples and secondary antibody:** Test samples were diluted to the proper concentrations in sample buffer, which contained 1% BSA/ 1% goat serum/ 0.05% Tween in PBS. A standard curve was prepared with a chimeric antibody (bearing a human Fc), the concentration of which was known. To prepare a standard curve, serial dilutions are made in the sample buffer to give a standard curve ranging from 125 ng/ml to 3.9 ng/ml. The diluted samples and standards were added to the plate, 100 µl/well and the plate incubated at 37 °C for 2 hours.
- After incubation, the plate was washed 8 times with 0.05% Tween in PBS. To each well was then added 100 µl of the secondary antibody, the horse radish peroxidase (HRP)-conjugated anti-human IgG (Jackson Immuno Research), diluted around 1:120,000 in the sample buffer. The exact dilution of the secondary antibody had to be determined separately for each lot of the HRP-conjugated anti-human IgG. After incubation at 37 °C for 2 hours, the plate was then washed 8 times with 0.05% Tween in PBS.
- C. Development:** The ready-made substrate solution (TMB Substrate, BioFX Laboratories, MD) was added to the plate at 100 µl/well and the color allowed to develop at room temperature for 10 minutes. The reaction was stopped by adding 1N HCl, 100 µl/well. The plate was read by a plate reader which was set to a wavelength of 450 nm.
- 1C: Analytical HPLC based method for determining protein concentration:** Protein concentrations were also determined using analytical HPLC. A standard curve for protein concentration over the range 0.78 to 50.0 µg/ml was determined using a pH-based elution protocol with a POROS column (Perceptive Biosystems) on an Agilent 1100 HPLC system, and with the KS-IL2 immunoconjugate as a standard.

**Example 2. Determination of the relative binding affinity of the epitope-depleted Leu-16 antibody-IL2 fusion protein for Daudi tumor cells presenting the CD20 antigen.** The binding of the epitope-depleted Leu16-IL2 fusion protein to Daudi lymphoma cells bearing the CD20 antigen, was compared with the binding of other known anti-CD20 antibodies using flow cytometry analysis. Approximately  $10^6$  Daudi cells were used with various concentrations of antibody in a 100  $\mu$ l volume, for each sample tested. The analysis showed that the modified antibody-IL2 fusion protein with VH Y and VK Z variable regions bound to CD20-expressing cells at least as well as the corresponding chimeric Leu16-IL2 fusion protein. This shows that the mutations introduced into the Leu16 V regions to generate VH Y and VK Z did not interfere with antigen binding. The antibody-IL2 fusion protein with VH Y and VK Z variable regions also compared favorably to RITUXAN (C2B8), as well as the 2B8-IL2 fusion protein containing the murine 2B8 V regions. Data in Table 1 below shows the mean fluorescence intensity, as measured in a flow cytometer, of Daudi cells exposed to different concentrations of antibody or antibody-IL2 fusion protein; this data represents a typical set of experimental results.

Table 1

| Antibody       | Antibody Concentration ( $\mu$ g/ml) |        |        |        |        |        |        |       |
|----------------|--------------------------------------|--------|--------|--------|--------|--------|--------|-------|
|                | 10                                   | 5      | 2.5    | 1.25   | 0.625  | 0.313  | 0.156  | 0.078 |
| 2B8-IL2        | 857.32                               | 720.02 | 512.81 | 369.04 | 229.45 | 147.26 | 85.32  | 52.13 |
| Ab(VhY/VkZ-IL2 | 987.54                               | 848.56 | 677.11 | 539.35 | 336.41 | 219.48 | 124.33 | 74.77 |
| chLeu16-IL2    | 789.20                               | 665.20 | 489.04 | 363.00 | 219.06 | 131.92 | 78.24  | 52.31 |
| 2B8            | 997.18                               | 816.47 | 647.43 | 491.38 | 311.52 | 215.34 | 130.70 | 86.48 |

**Example 3. Antibody-dependent, cell-mediated cytotoxicity (ADCC) driven by anti-CD20-IL2 fusion proteins carrying mutations that reduce immunogenicity.** NS/0 cells that express CD20 were exposed to various concentrations of antibody or antibody-IL2 fusion protein and tested for ADCC lysis according to standard procedures. A typical data set is shown in FIGURE 5. Proteins tested were as follows: chLeu16 is a chimeric antibody consisting of the Leu16 mouse V regions and human IgG1 constant regions; chLeu16-IL2 is a chimeric antibody consisting of the Leu16 mouse V regions and human IgG1 constant regions, with a human IL-2 moiety fused to the C-terminus of the antibody heavy chain; Ab(VhY/VkZ)-IL2 is identical to

chLeu16-IL2 except that the VhHY and VkZ variable domains listed in FIGURES 3 and 4 were present instead of the murine Leu16 V regions; 2B8 is, in this figure, a chimeric antibody consisting of the 2B8 mouse V regions and human IgG1 constant regions; 2B8-IL2 is, in this figure, a chimeric antibody consisting of the 2B8 mouse V regions and human IgG1 constant regions, with a human IL-2 moiety fused to the C-terminus of the antibody heavy chain; and KS-IL2 an anti-EpCAM antibody, with a human IL-2 moiety fused to the C-terminus of the antibody heavy chain. The KS-IL2 protein serves as a negative control. Certain of the antibody-IL2 fusion proteins had mutations of the C-terminal heavy chain amino acids as described by Gillies *et al.* (WO02/66514); these mutations had no effect on the ADCC data.

The data indicate that a fusion protein bearing the VhY and VkZ variable regions was as active as similar molecules carrying CD20-binding murine V regions in stimulating ADCC. IL2 activity was determined by several different cell assays. The results are presented in Table 2 below.

Table 2

|               | T Cell               |                      | T Cell               | + IL2 R <sub>+</sub> |
|---------------|----------------------|----------------------|----------------------|----------------------|
|               | CTLL-2               | HU PBMC              | Kit-225              | TF-1                 |
|               | Avg.ED <sub>50</sub> | Avg.ED <sub>50</sub> | Avg.ED <sub>50</sub> | Avg.ED <sub>50</sub> |
|               | (ng/ml)              | (ng/ml)              | (ng/ml)              | (ng/ml)              |
| KS IL2        | 1.71                 | 2.09                 | 0.08                 | 0.51                 |
| R&D Hu IL2    | 0.65                 | 1.51                 | 0.07                 | 0.71                 |
| ChLeu16-IL2   |                      |                      | 0.06                 | 1.15                 |
| DI-Leu16-IL2  | 1.94                 | 2.03                 | 0.05                 | 1.14                 |
| DI-Leu-16-IL2 | 3.30                 | 3.42                 | 0.09                 | 1.99                 |
| Degly         |                      |                      |                      |                      |

It has also been found that IL-2 based immunocytokines targeting CD20 are highly efficacious in SCID mouse models of well established lymphoma, at least in the absence of normal human B cells expressing the same surface antigen, and that the use of immunocytokines was far more effective than that of the naked antibody in extending the survival of mice with disseminated disease, despite the lack of functional T cells, cells that have been identified in many pre-clinical studies to be the primary effectors of immunocytokine anti-tumor activity.

**Example 4. Complement-dependent cytotoxicity (CDC) driven by anti-CD20-IL2 fusion proteins carrying mutations that reduce immunogenicity.**

To determine CDC activity of antibodies and fusion proteins of the invention, <sup>51</sup>Cr-labeled Daudi cells were incubated for 1 hour with human plasma (diluted 1 in 8) as a  
5 source of complement. Percentage of specific lysis was calculated by subtracting the background radioactivity from the experimental values, dividing by the total releasable radioactivity obtained by detergent lysis, and multiplying by 100.

A comparison of the CDC activities of the chimeric Leu-16 antibody and the corresponding Leu16 antibody with epitope-depleted V regions indicates that the  
10 activities of these two antibodies were essentially identical. A similar comparison of the CDC activities of the chimeric Leu-16 antibody-IL2 fusion and the corresponding Leu16 antibody-IL2 fusion with epitope-depleted V regions indicates that the activities of these two fusion proteins were also essentially identical.

In contrast to ADCC, CDC was somewhat reduced as a consequence of fusing IL-2 to the C-terminus of the H chain (Figure 2B). A similar effect was reported earlier with  
15 an anti-GD2 immunocytokine (Gillies SD, *et al.* Cancer Res. (1999);59:2159-2166.). Typical results are shown in Figure 7C.

**Example 5. Pharmacokinetic profile of an epitope-depleted Leu16-IL2 fusion protein.**

20 In a particularly useful Leu16VhY/VkZ-IL2 construct, FcR binding and ADCC activity were maintained by using a Cg1 heavy chain isotype but have incorporated a modified junction region between the H chain and IL-2 in order to reduce intracellular proteolysis (WO01/58957). Specifically, a Lysine to Alanine change corresponding to the C-terminal amino acid of the antibody heavy chain was used. The resulting protein  
25 had a favorable pharmacokinetic profile following i.v. administration, especially during the distribution phase (Figure 8). The effect of FcR binding on the pharmacokinetic profile was examined by testing the enzymatically de-glycosylated Leu16VhY/VkZ (Lys-Ala)-IL2 in the same experiment. The results indicate that loss of FcR binding improved the pharmacokinetic behavior somewhat. Depending on the  
30 application and the desired frequency of administration, it may be preferable to use a Leu16VhY/VkZ-IL2 molecule with the N-linked glycosylation site in the CH2 domain, which has ADCC activity but a relatively shorter serum half-life, or a Leu16VhY/VkZ-IL2 molecule lacking the N-linked glycosylation site in the CH2 domain, which has no ADCC activity but a relatively longer serum half-life.

**Example 6. Efficacy profile of an epitope-depleted Leu16-IL2 fusion protein.**

SCID mice were injected i.v. with  $5 \times 10^6$  CD20+ Daudi lymphoma cells (day 0) followed by i.v. injection of immunocytokines (5 daily doses of 5 or 20 mg) or control antibody (500 mg every other day for a total of 3 doses) beginning on day 7. A low-targeting control immunocytokine 425-IL2, specific for EGFR (control), was used at the high dose to demonstrate activity due primarily to the altered half-life of IL-2. Results were recorded as general health, e.g. paralysis, which preceded death by 10-14 days, and survival of mice. Figures 9-12 show typical results. Data in Figures 10-12 are from a single, large experiment, but the data are presented in different figures for ease of viewing. In the first anti-tumor experiments Daudi cells were injected i.v. into SCID mice resulting in extensive disseminated disease leading to paralysis of all mice by day 30. Treatment was delayed until day 7 to ensure the tumor cells had fully engrafted. We compared low and moderate doses of both the chimeric and Leu16VhY/VkZ-IL2 immunocytokines to high dose rituximab using 5 daily doses of the immunocytokines and 3 alternate day doses of the antibody. This schedule was chosen due to the much longer circulating half-life of rituximab (several days) compared to the immunocytokines (about 8 hours). Under these conditions, rituximab (25 mg/kg x 3) extended the 50% survival of tumor bearing mice from 39 to 56 days, relative to the PBS control (Figure 4). The low dose chimeric and Leu16VhY/VkZ-IL2 groups (0.25 mg/kg x 5) had similar survival curves (50% survival at 64 days) as the high-dose rituximab. The groups treated with the higher doses of the immunocytokines (1 mg/kg x 5) showed a dramatic increase in survival with no mouse deaths in the Leu16VhY/VkZ-IL2 group at the termination of the experiment (day 110) and only 1 of 8 mice dead in the chLeu16-IL2 group. Thus, the epitope-depleted V regions of the Leu16 antibody were as effective as those of the murine Leu16 antibody, in the context of an IL-2 based immunocytokine, for the treatment of disseminated lymphoma in SCID mice. In the same experiment the contribution of antibody effector function on anti-tumor activity was also tested using the enzymatically de-glycosylated Leu16VhY/VkZ-IL2, which was shown above to have lost ADCC activity (Figure 7B). As shown in Figure 12, a significant portion of the anti-tumor activity was preserved despite the loss of ADCC activity. At later time points, a marked difference between the intact and de-glycosylated forms was observed in the higher dose groups. Therefore, while ADCC appears to play a role in this model, a good deal of the anti-tumor activity in this model can be attributed to targeted delivery of IL-2 to the tumor

alone. To address the importance of actual binding of an epitope-depleted immunocytokine to a target cell, as opposed to an effect of simply extending the serum half-life of IL-2, a control IL-2 immunocytokine targeting EGFR (Cruz *et al.* J Biotechnol. (2002) 26;96:169-183), which is expressed at only very low levels (Figure 5 6B) on this cell line, was tested. It was found that even the higher dose (1 mg/kg) given 5 consecutive days had far less anti-tumor activity in this model than the same dose of the immunocytokines targeting CD20 (Figure 13). The EGFR-targeted immunocytokine also had significantly less activity than the same dose of Leu16VhY/VKkIL2 given only twice, three days apart (days 7 and 10), or at a four- 10 fold lower dose given over 5 days. These results demonstrate the importance of specific tumor cell targeting for anti-tumor activity.

**Anti-tumor activity of separate antibody and IL-2 components.** Clinical trials combining rituximab and IL-2 have shown increased response rates (Friedberg *et al.* Br. J. Haematol. (2002); 117: 828-834). This combination was tested in the same 15 Daudi lymphoma model using two different approaches and compared to treatment with Leu16VhY/VkZ-IL2. In the first case, animals were dosed i.v. for 5 consecutive days with the equivalent molar amounts of Leu16VhY/VkZ antibody and IL-2 contained in 20 mg of Leu16VhY/VkZ-IL2. In the second case, 25 mg/kg of rituximab and 10 mcg of IL-2 were given s.c. every other day for 3 doses. This latter dosing 20 regimen would ensure high levels of antibody as well as a sustained IL-2 activation due to the depot effect of s.c. administration. Results indicated that the two combination protocols resulted in roughly the same degree of anti-tumor activity, with 50% survival of 63 days (Figure 14). Treatment with the equivalent amount of Leu16VhY/VkZ-IL2 immunocytokine, used in the low dose combination group, 25 resulted in long-term survival of all mice. This is particularly noteworthy since the groups treated with the separate antibody and IL-2 are exposed to antibody for a much longer time than Leu16VhY/VkZ-IL2 due to its much longer half-life. Furthermore, the amounts of IL-2 used for comparison were based on mass and not on IL-2 activity units. As shown in Table 2 above, free rIL-2 is approximately 3-fold more active than 30 the equivalent molar amount of IL-2 contained in Leu16VhY/VkZ-IL2 when measured with a mouse cell line expressing the high affinity IL-2R. For mouse immune cells expressing only the intermediate IL-2R, this difference is more than 10-fold in favor of free rIL-2.

**Example 7. Antitumor activity of Leu16VhY, VKZ molecules in mice reconstituted with human immune cells** In the SCID model reconstituted with human B cells, mice were injected i.v. with  $5 \times 10^6$  CD20+ Daudi lymphoma cells on day 0 and  $4.5 \times 10^7$  human PBMC on day 5. One group (n=8) of mice received PBS only; one group received antibody only (500 mg on day 7, 9 and 11); one group received immunocytokine only (20 mg on days 11-15); and one group received the combination of antibody (500 mg on day 7, 9 and 11) and immunocytokine (20 mg on days 11-15). All mice were checked for the presence of human antibodies in their serum by anti-human IgG ELISA on day 21 and 34. Targeting CD20 on B lymphoma cells is complicated by the fact that the antigen is expressed on normal B cells. Thus, therapy involves the targeted depletion of tumor cells in the background of a vast number of normal B cells. Since IL-2 immunocytokine dosing is likely to be limited by the toxicity of the IL-2 component, it is unlikely that the high doses required for normal B cell depletion by the naked antibody could be used for Leu16VhY, VKZ-IL2. Combination treatment is a likely clinical approach in which Leu16VhY, VKZ-IL2 therapy would follow rituximab treatment to first de-bulk both CD20+ tumor cells and normal B cells. In an attempt to create a more realistic tumor model mice were injected with PBMC containing human B cells and then compared monotherapy with rituximab or Leu16VhY/VkZ-IL2 as well as the combination in which the antibody is given as a single dose at day 7, followed by a course of therapy with Leu16VhY/VkZ-IL2 beginning on day 11. A second set of mice were not implanted with human PBMC but received the same treatment regimens. Confirmation that B cells had been implanted was obtained by measuring levels of human IgG in all mice groups. Data in Table 3 show that mice receiving human PBMC all had levels of human IgG of >500 ug/ml demonstrating efficient grafting.

**Table 3.** Human antibody production in SCID mice implanted with human PBMC

| Treatment Group       | Day 21    |           | Day 34    |           |
|-----------------------|-----------|-----------|-----------|-----------|
|                       | - B Cells | + B Cells | - B Cells | + B Cells |
| PBS                   | NT        | >500      | 0         | >500      |
| DI-Leu16-IL2 (d11-15) | NT        | 64.1      | 0.83      | 88.31     |
| Rituximab (d7)        | NT        | 14.32     | 9.12      | 6.18      |
| Combination           | NT        | 9.55      | 6.0       | 5.8       |

Human antibodies were quantitated by anti-IgG ELISA and represent mcg/ml. Antibodies detected in the – B cell group show the contribution of rituximab and Leu16-IL2 (both human IgG) to the total circulating antibody. Antibody levels at day 21 show dramatic decreases in all treatment groups, including monotherapy with Leu16VhY/VkZ-IL2, however the IgG level increased slightly by day 34 indicating continued production by B cells. Treatment with rituximab or the combination, on the other hand, resulted in elimination of human antibody production by day 34. The remaining levels detected in the blood of mice in both rituximab and combination treated groups were clearly rituximab itself, since the same levels were seen in the corresponding groups that were not implanted with PBMC. Results also showed that the anti-tumor activities of all treatment groups were not significantly affected by the presence of CD20+ human B cells (Figure 15). Without wishing to be bound by theory, this may be due in part to the ability of Leu16VhY/VkZ-IL2 alone to eliminate the majority of implanted B cells, as well as tumor cells. The level of activity of Leu16VhY/VkZ-IL2 in this model was markedly reduced compared to earlier experiments due to the delay in initiating treatment (day 11 vs. day 7) however, it should be noted that only a single course of treatment was used to compare different molecules and additional cycles of treatment would be used clinically. We also found that the combination of a single dose of rituximab, followed by a single course of Leu16VhY/VkZ-IL2, had at least additive anti-tumor activity with the majority of mice remaining disease free at the end of the experiment (day 120).

**Example 8. General methods for removing T cell epitopes from anti-CD20 antibodies**  
Haisma *et al.* (Blood 92:184 (1998)) have described another anti-CD20 antibody, termed 1H4, whose V regions are about 95% identical to the V regions of Leu16. Figure 16 shows an alignment of the heavy and light (kappa) V regions of 2B8, Leu16, their epitope-depleted derivatives according to the invention, and 1H4. From the finding that the antibodies 2B8, Leu16, and 1H4 are so similar in sequence, it is an inference of the invention that all of these antibodies recognize the same epitope in CD20. Without wishing to be bound by theory, it is an insight of the invention that CD20 is a highly conserved protein between mouse and humans, and that there is a sequence CEPANPSEKNSPSTQYC (SEQ ID NO: 38) in the extracellular domain of CD20 that corresponds to the sequence CEPSNSSEKNSPSTQYC (SEQ ID NO: 29) in mouse CD20. In human CD20, the sequence NPS is not N-glycosylated, while the corresponding sequence NSS in mouse CD20 is N-glycosylated. According to this theory of the invention, the lack of N-

glycosylation in the human CD20 reveals an antibody epitope that is absent in mouse CD20, so that when mice are immunized with human CD20, this epitope will not be recognized as self by the mouse immune system. Thus, according to the invention, the starting V regions of a monoclonal antibody directed against CD20 are at least 80% identical in sequence, and more often at least 90% identical in sequence, to Leu16 or 2B8 V regions. The antibody 1H4 is one such example. According to the invention, T cell epitopes in an anti-CD20 antibody, for example 1H4, are removed by first aligning the heavy and light chain V regions with Leu16, 2B8, and the corresponding epitope-depleted variants shown in Figure 16, and secondly by introducing mutations in the V regions of the CD20 antibody, such as 1H4, that correspond to the mutations introduced in epitope-depleted versions of Leu16 and/or 2B8. The alignments may be performed by manual alignment or using well-known alignment programs such as BLAST. For example, using the alignments in Figure 16, it is apparent that an anti-CD20 Vh domain with one or more of the following amino acid segments is generated: SGAE LKKPGAS, VSCKASGYT, LEWTGAIY, YNQKFKGKT, FKGKTTLTA, YME LSSLRS, SSLRSED TAV, and DWGTGTTVT i.e., SEQ ID NO: 15-22, respectively. Similarly, using the alignments in Figure 16, it is apparent that an anti-CD20 VL domain with one or more of the following amino acid segments is generated: IITASPG EKV, C RASTSASY, QQKPTSSP, LASGVPSRF, FSGSGSGTT, and YSMTIS SLE, i.e., SEQ ID NO: 23-28, respectively. In particular, a variant of the antibody 1H4 with one or more of the preceding amino acid segments of this paragraph is generated. The utility of the newly introduced mutations may be further verified by checking that T cell epitopes are indeed removed, by using standard methods for detection of T cell epitope activity. For example, methods such as those of Carr *et al.* (US Patent Application 20030153043 and WO02/069232) may be used. Alternatively, peptides corresponding to potential T cell epitopes may be added to immune cells, preferably human immune cells, and tested for the ability to stimulate cell proliferation according to standard techniques. The corresponding V region-containing proteins are configured as whole antibodies, antibody fusion proteins, Fabs, Fab fusion proteins, single-chain Fv proteins, or other standard configurations of antibody V regions. The corresponding V region-containing proteins are then produced according to standard protein expression methods, such as are described in the Examples above.

2004264372 02 Jun 2010

- 35a -

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

5

The reference in this specification to any prior publication (or information derived from it), or to any matter which is known, is not, and should not be taken as an acknowledgment or admission or any form of suggestion that that prior publication (or information derived from it) or known matter forms part of the common general knowledge in the field of

10

endeavour to which this specification relates.

2004264372 02 Jun 2010

- 36 -

**The claims defining the invention are as follows:**

1. An anti-CD20 antibody molecule fused to IL-2 comprising:
  - (i) a modified heavy chain variable region polypeptide having the amino acid residue sequence of SEQ ID NO: 9, but which includes at least one amino acid residue substitution in said sequence selected from the group consisting of V<sub>12</sub>K, M<sub>20</sub>V, A<sub>68</sub>T, Q<sub>82</sub>E, T<sub>87</sub>R, S<sub>91</sub>T, D<sub>93</sub>V, and A<sub>114</sub>T,
  - (ii) a modified light chain variable region polypeptide having the amino acid residue sequence of SEQ ID NO: 11, but which includes at least one amino acid residue substitution in said sequence selected from the group consisting of L<sub>11</sub>I, S<sub>12</sub>T, A<sub>59</sub>S, S<sub>69</sub>T, L<sub>72</sub>M, R<sub>76</sub>S, and V<sub>77</sub>L, and
  - (iii) a human IgG heavy chain constant region and a human light chain constant region.
2. An anti-CD20 antibody fusion protein of claim 1, wherein the IgG constant region is a human IgG1 constant region.
3. An anti-CD20 antibody fusion protein of claim 1 or 2, wherein the human light chain constant region is a human kappa light chain constant region.
4. An anti-CD20 antibody fusion protein according to any one of claims 1 to 3 comprising a heavy chain variable region having the amino acid sequence of SEQ ID NO: 10.
5. An anti-CD20 antibody fusion protein according to any one of claims 1 to 3 comprising a light chain variable region having the amino acid sequence of SEQ ID NO: 12.
6. An anti-CD20 antibody fusion protein according to any one of claims 1 to 3 comprising a heavy chain variable region having the amino acid sequence of SEQ ID NO: 10 and a light chain variable region having the amino acid sequence of SEQ ID NO: 12.

2004264372 02 Jun 2010

- 37 -

7. An anti-CD20 antibody fusion protein according to any one of claims 1 to 6, wherein the C-terminus of the antibody molecule is fused to the N-terminus of IL-2.
8. An anti-CD20 antibody fusion protein according to claim 1 encoded by the DNA sequences of SEQ ID NO: 40 and SEQ ID NO: 43.
9. A pharmaceutical composition comprising an anti-CD20 antibody fusion protein according to any one of claims 1 to 8 together with a pharmaceutically acceptable carrier, excipient or diluent.
10. The pharmaceutical composition of claim 9 further comprising an additional pharmacologically effective drug.
11. A DNA molecule coding for an anti-CD20 antibody fusion protein according to any one of claims 1 to 8.
12. Use of an anti-CD20 antibody fusion protein of any one of claims 1 to 8 or a pharmaceutical composition of claim 9 or 10, for the manufacture of a medicament for the treatment of CD20 expressing cancers.
13. Use of claim 12 wherein said cancer is B-cell lymphoma.
14. A pharmaceutical composition comprising the anti-CD20 antibody fusion protein of claim 8 and the monoclonal antibody rituximab.
15. An anti-CD20 antibody fusion protein according to any of claims 1 to 8 for use in the treatment of CD20 expressing cancers.
16. A method for the treatment of a CD20 expressing cancer in a subject, the method comprising administering to the subject an anti-CD20 antibody of any one of

2004264372 02 Jun 2010

- 38 -

claims 1 to 8 or a pharmaceutical composition of any one of claims 9, 10 or 14.

17. An anti-CD20 antibody molecule fused to IL-2 as claimed in claim 1, and uses thereof, substantially as herein described with reference to the Examples.

**Figure 1****2B8VH:**

QVQLQQPGAELVKAGASVKMSCKASGYTFTSYNMHWVKQTPGGRGLEWIGAIYPGNGDTSYN  
QKFKGKATLTADKSSSTAYMQLSSLTSEDSAVYYCARSTYYGGDTYFNVWGAGTTVTVSA

**VhC:**

QVQLQQPGAELKKPGASVKVSCCKASGYTFTSYNMHWVKQTPGGRGLEWTGAIYPGNGDTSYN  
QKFKGKTTLTADKSSSTAYMELSSLRSEDVAVYYCARSTYYGGDWYFNVWGAGTTVTVSA

**VhD:**

QVQLQQPGAELKKPGASVKVSCCKASGYTFTSYNMHWVKQTPGGRGLEWIGAIYPGNGDTSYN  
QKFKGKTTLTADKSSSTAYMELSSLRSEDVAVYYCARSTYYGGDWYFNVWGAGTTVTVSA

**Figure 2****2B8VL:**

QIVLSQSPAILSASPGEKVTMTCRASSSVSYIHWFQQKPGSSPKPWIYATSNLASGVPVRFSGSGSGTSYSLT  
ISRVEAEDAATYYCQQWTSNPPTFGGGTKLEIK

**VkA:**

QIVLSQSPAIITASPGEKVTMTCRASTSASYIHWFQQKPTSSPKPWIYATSNLASGVPSRF  
SGSGSGTTYSMTISSLEAEDAATYYCQQWTSNPPTFGGGTKLEIK

**VkB:**

QIVLSQSPAIITASPGEKVTMTCRASTSASYIHWFQQKPTSSPKPWIYATSNLASGVPSRF  
SGSGSGTTYSMTISSLEAEDAATYYCQQWTSNPPTFGGGTKLEIK

**VkC:**

QIVLSQSPAIITASPGEKVTMTCRASTSASYIHWFQQKPGSSPKPWIYATSNLASGVPSRF  
SGSGSGTTYSMTISSLEAEDAATYYCQQWTSNPPTFGGGTKLEIK

**VkD:**

QIVLSQSPAIITASPGEKVTMTCRASSSVSYIHWFQQKPGSSPKPWIYATSNLASGVPSRF  
SGSGSGTTYSMTISSLEAEDAATYYCQQWTSNPPTFGGGTKLEIK

### **Figure 3**

#### **Leu-16VH:**

EVQLQQSGAELVKPGASVKMSCKASGYTFTSYNMHWVKQTPGQGLEWIGA  
IYPGNGDTSYNQKFKGKATLTADKSSSTAYMQLSSLTSEDSADYYCARSN  
YYGSSYWFFDVWGAGTTVTVSS

#### **Leu-16VHY:**

EVQLQQSGAELKKPGASVKVSCCKASGYTFTSYNMHWVKQTPGQGLEWIGA  
IYPGNGDTSYNQKFKGKTTLTADKSSSTAYMELSSLRSED<sup>T</sup>AVYYCARSN  
YYGSSYWFFDVWG<sup>T</sup>GT<sup>T</sup>TVTVSS

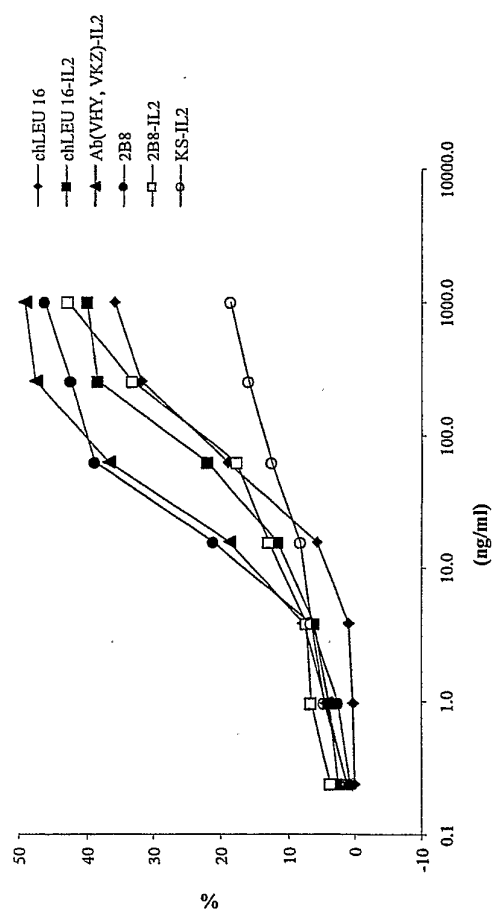
## **Figure 4**

### **Leu-16VL:**

DIVLTQSPAILSASPGEKVTMTCRASSSVNYMDWYQKKPGSSPKPWIYAT  
SNLASGVPARFSGSGSGTYS~~LT~~ISRVEAEDAATYYCQQWSFNPPTFGGG  
TKLEIK

### **Leu-16VLZ:**

DIVLTQSPAIITASPGEKVTMTCRASSSVNYMDWYQKKPGSSPKPWIYAT  
SNLASGVPSRFSGSGSGT~~YS~~MTISS~~LE~~AEDAATYYCQQWSFNPPTFGGG  
TKLEIK

**Figure 5**

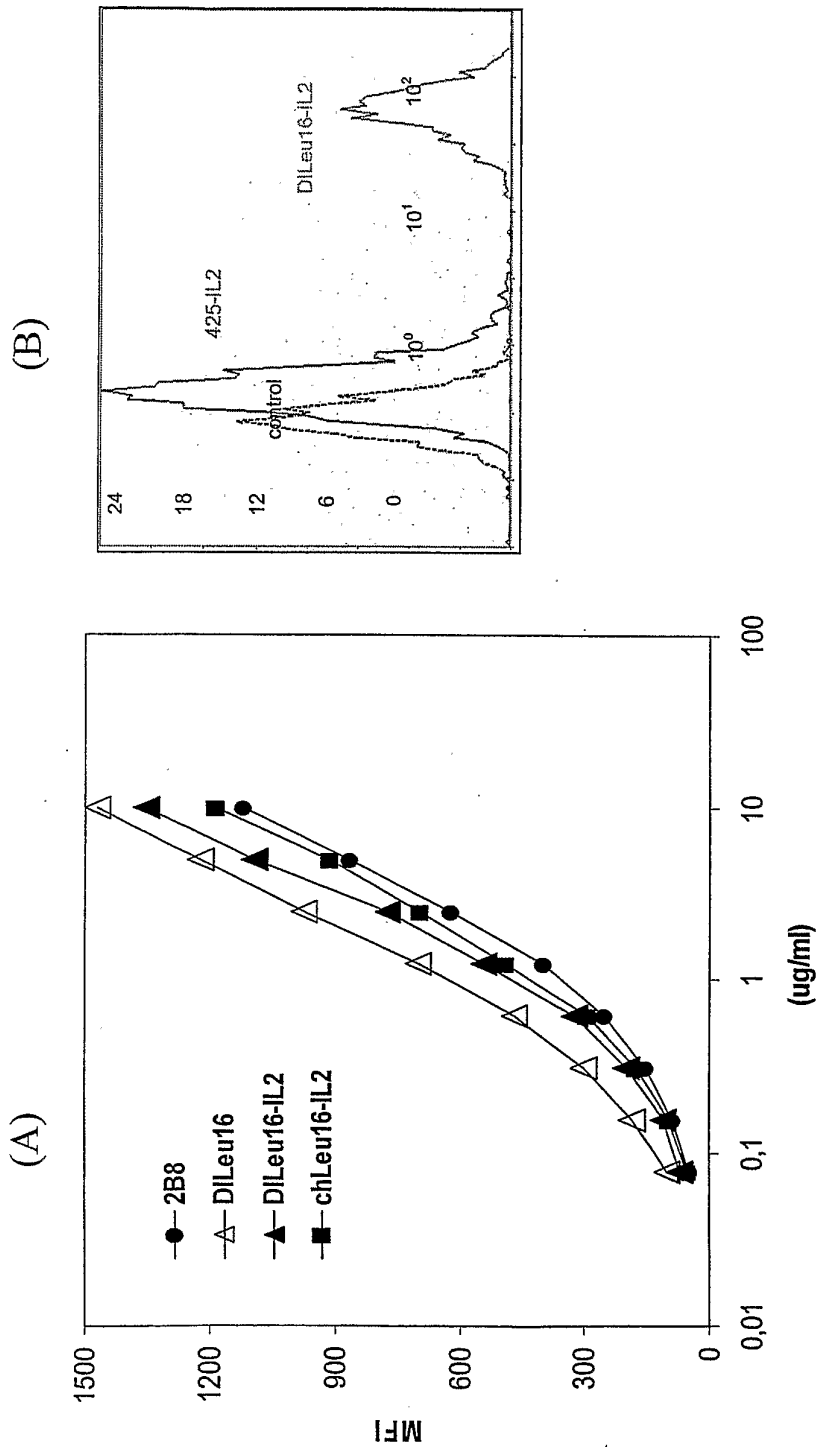


Figure 6.

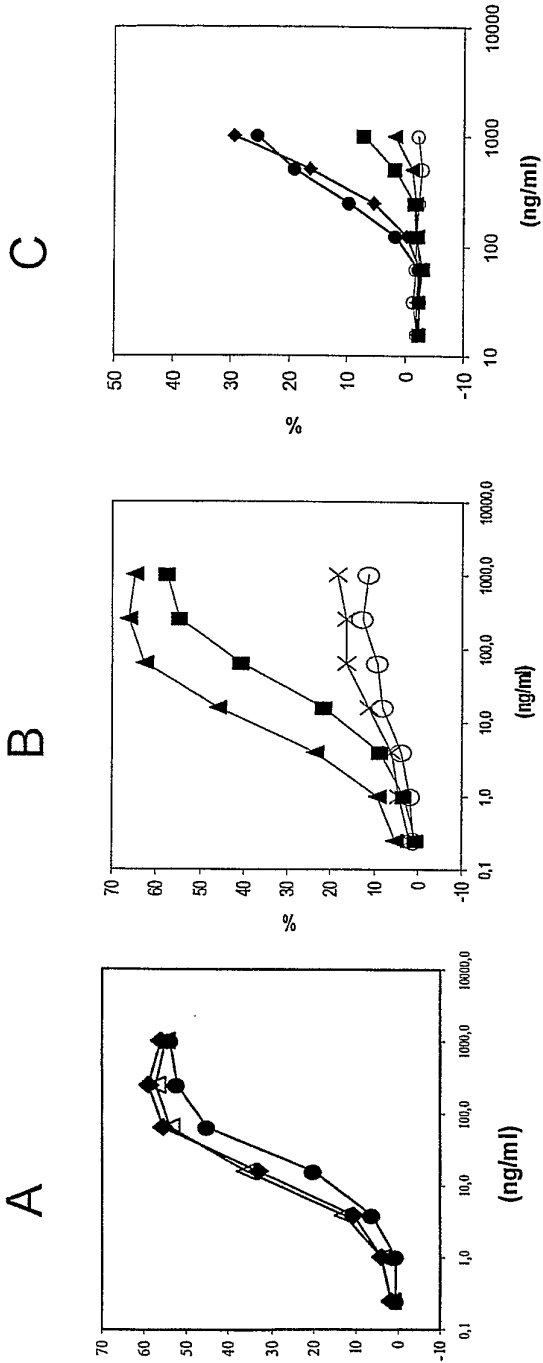
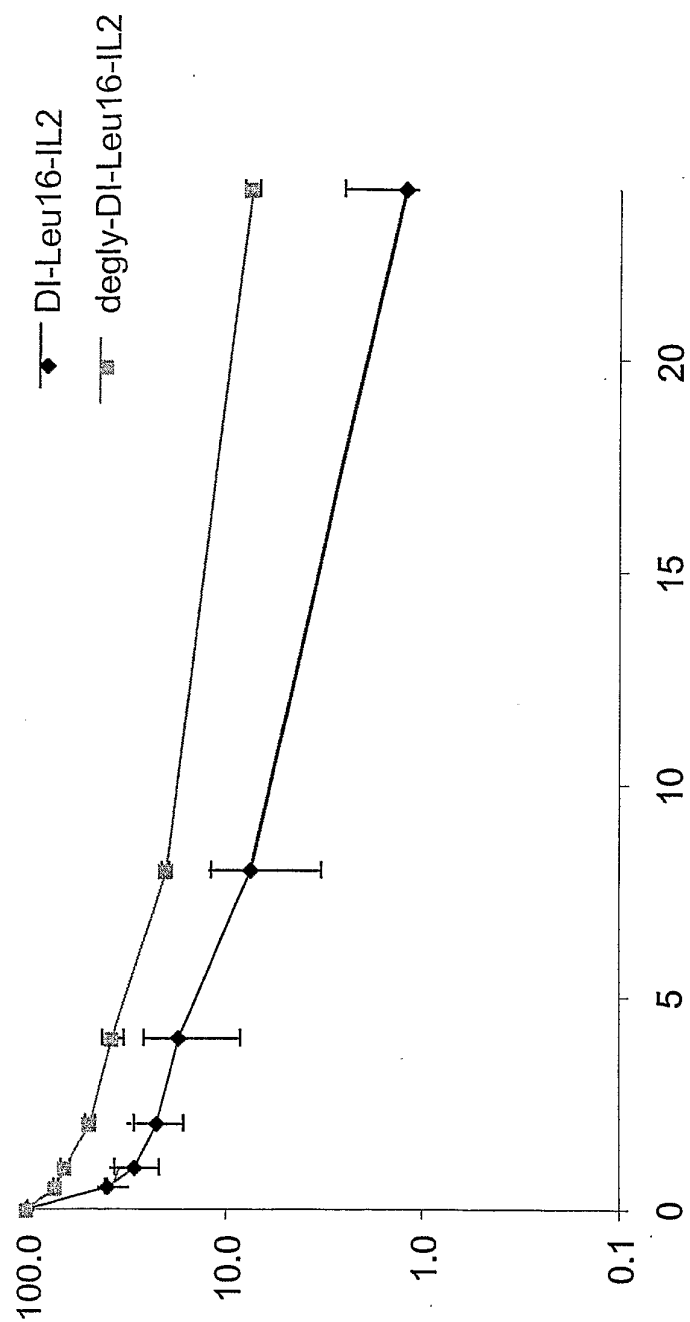
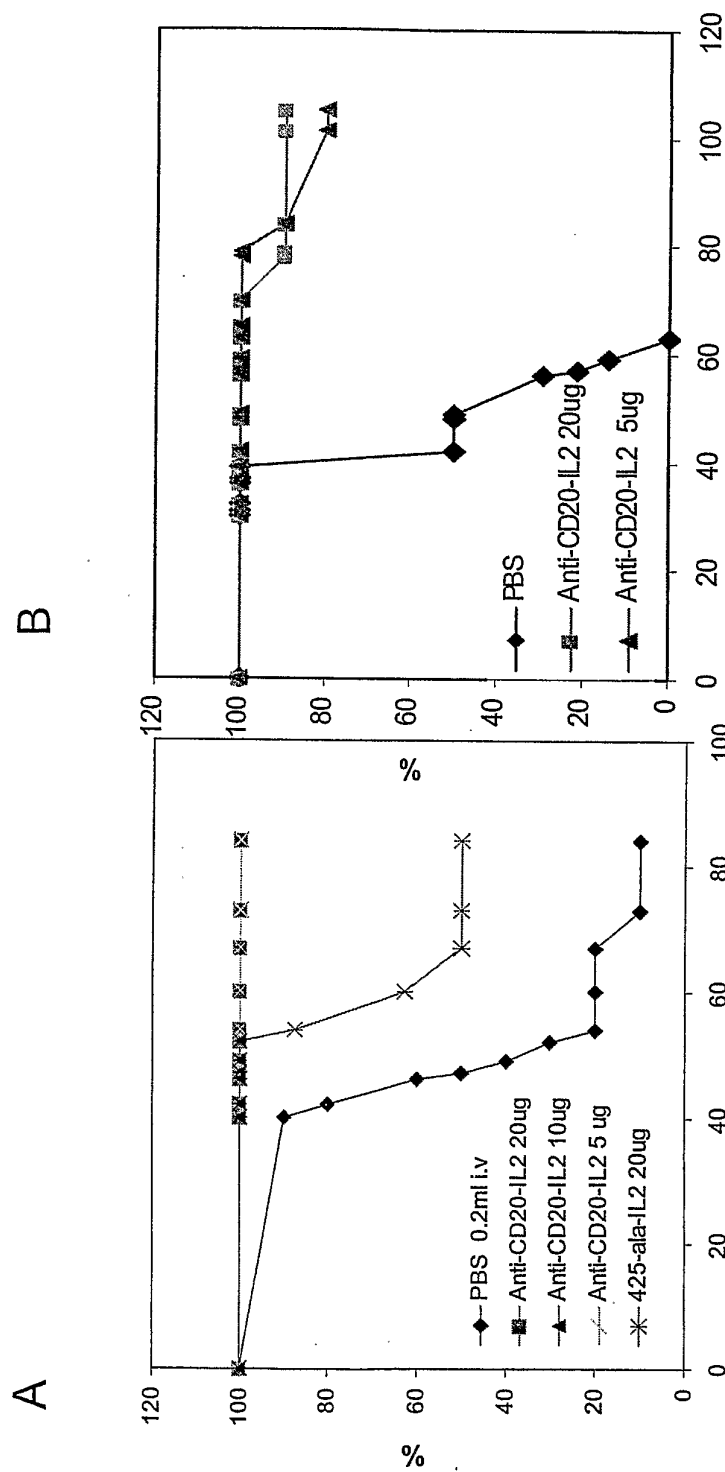
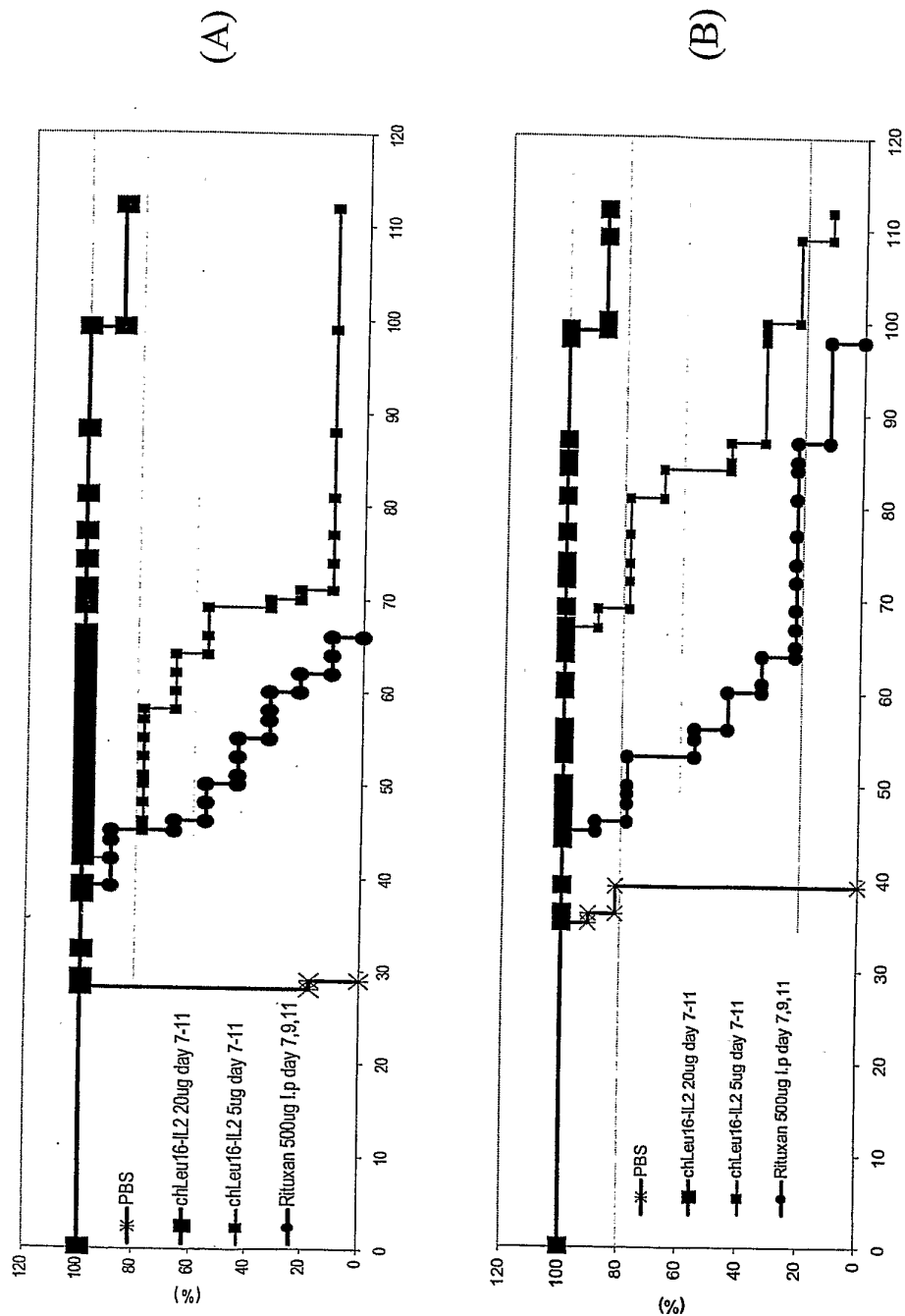


Figure 7.

**Figure 8**





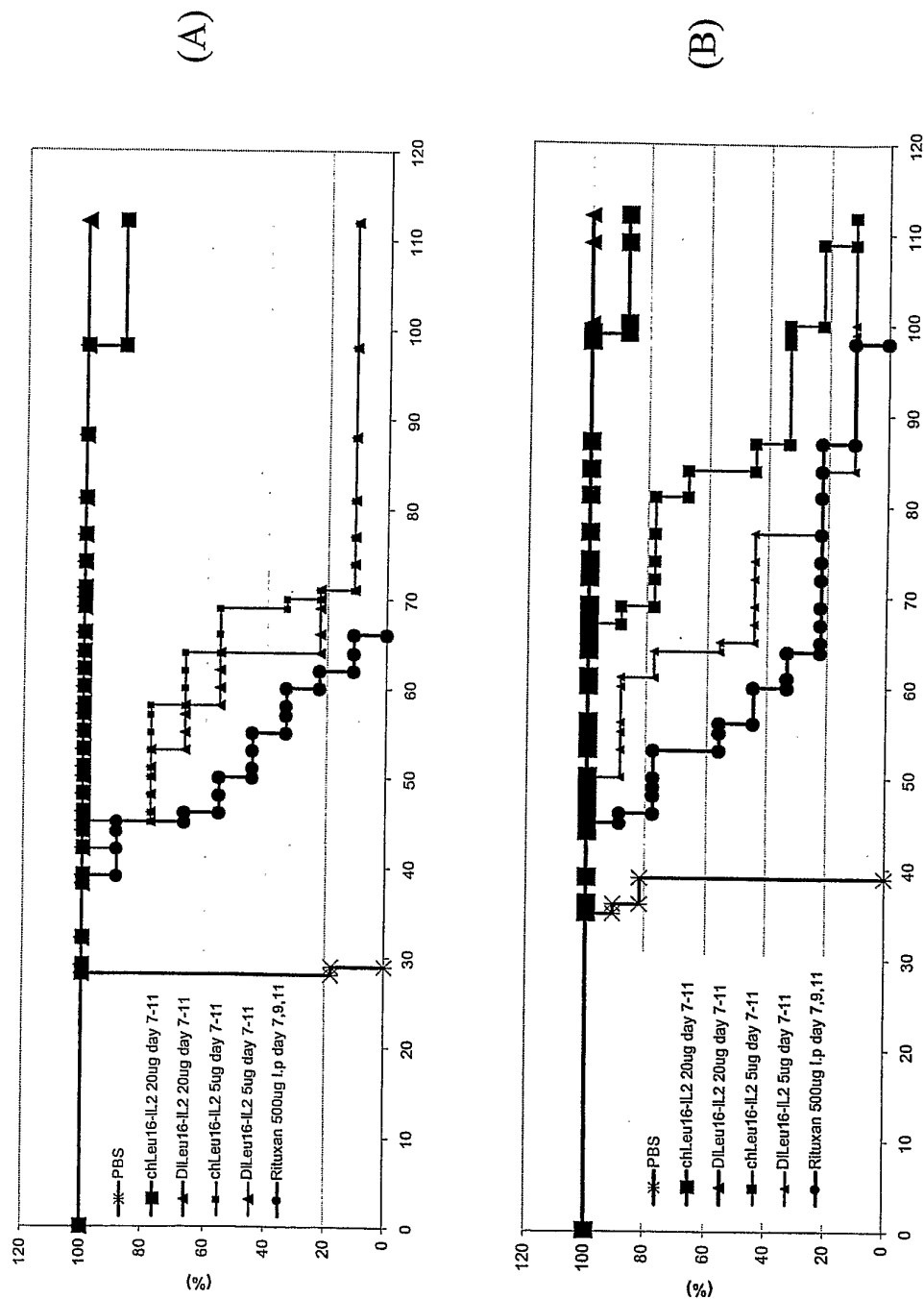
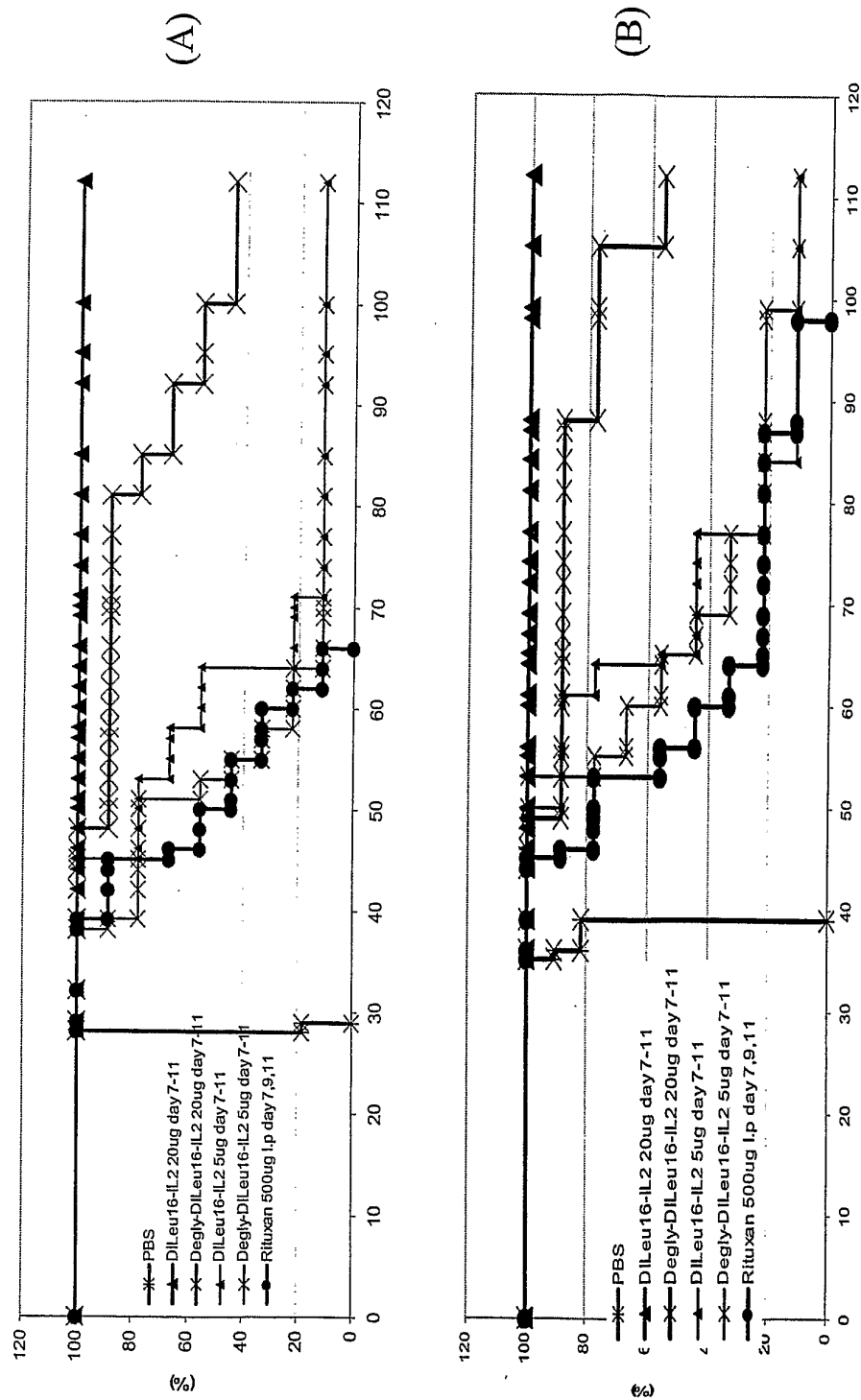
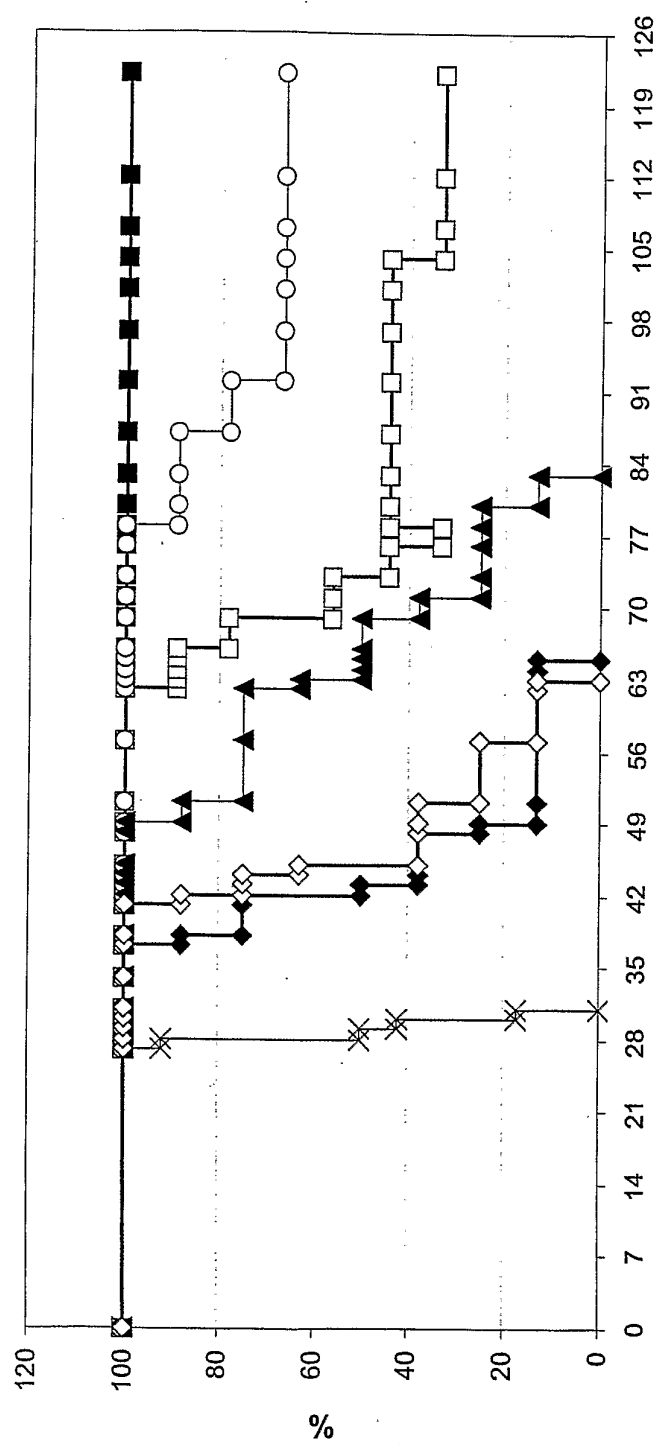


Figure 11.



**Figure 13**

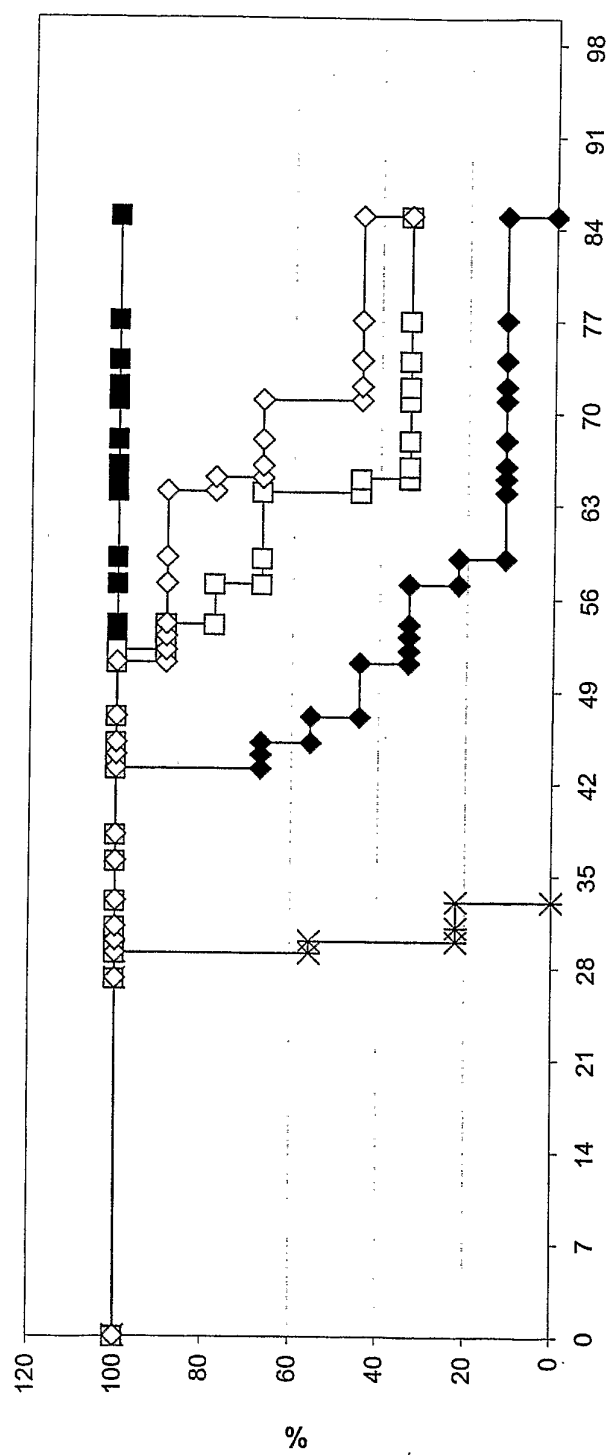
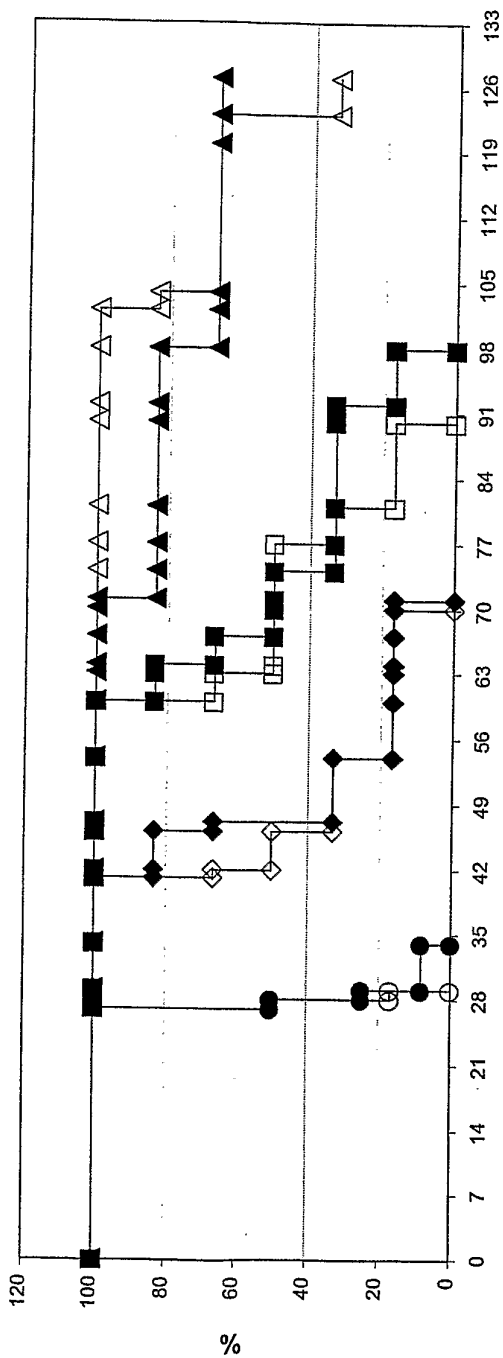
**Figure 14**

Figure 15

**Figure 16**

## Heavy chain sequences of anti-CD20 antibodies

|          |                                                                |  |  |     |  |     |
|----------|----------------------------------------------------------------|--|--|-----|--|-----|
|          | 1                                                              |  |  |     |  | 50  |
| 2B8VH    | QVQLQQPGAELVKAGASVKMSCKASGYTFTSYNMHWVKQTTPGRGLEWIGA            |  |  |     |  |     |
| 2B8VHC   | QVQLQQPGAELKKPGASVKVSCKASGYTFTSYNMHWVKQTTPGRGLEWTGA            |  |  |     |  |     |
| 2B8VHD   | QVQLQQPGAELKKPGASVKVSCKASGYTFTSYNMHWVKQTTPGRGLEWIGA            |  |  |     |  |     |
| Leu16VH  | EVQLQQSGAELVKPGASVKMSCKASGYTFTSYNMHWVKQTTPGQGLEWIGA            |  |  |     |  |     |
| Leu16VHY | EVQLQQSGAELKKPGASVKVSCKASGYTFTSYNMHWVKQTTPGQGLEWIGA            |  |  |     |  |     |
| 1H4 VH   | QVQLQQPGAELVKPGASVKMSCKTSGYTFTSYNVHWVKQTTPGQGLEWIGA            |  |  |     |  |     |
|          | 51                                                             |  |  |     |  | 100 |
| 2B8VH    | IYPNGDTSYNQKFKGKATLTADKSSSTAYMQLSSLTSEDSAVYYCARST              |  |  |     |  |     |
| 2B8VHC   | IYPNGDTSYNQKFKGKTTLTADKSSSTAYMELSSLRSED <sup>T</sup> AVYYCARST |  |  |     |  |     |
| 2B8VHD   | IYPNGDTSYNQKFKGKTTLTADKSSSTAYMELSSLRSED <sup>T</sup> AVYYCARST |  |  |     |  |     |
| Leu16VH  | IYPNGDTSYNQKFKGKATLTADKSSSTAYMQLSSLTSEDSADYYCARSN              |  |  |     |  |     |
| Leu16VHY | IYPNGDTSYNQKFKGKTTLTADKSSSTAYMELSSLRSED <sup>T</sup> AVYYCARSN |  |  |     |  |     |
| 1H4 VH   | IYPNGDTSFNQKFKGKATLTADKSSSTVYMQLSSLTSEDSAVYYCARSN              |  |  |     |  |     |
|          | 101                                                            |  |  | 122 |  |     |
| 2B8VH    | YYGGD--TYFNVWGAGTTTVTVSA                                       |  |  |     |  |     |
| 2B8VHC   | YYGGD--WYFNVWGAGTTTVTVSA                                       |  |  |     |  |     |
| 2B8VHD   | YYGGD--WYFNVWGAGTTTVTVSA                                       |  |  |     |  |     |
| Leu16VH  | YYGSSY-WFFDVWGAGTTTVTVSS                                       |  |  |     |  |     |
| Leu16VHY | YYGSSY-WFFDVWGIGTTTVTVSS                                       |  |  |     |  |     |
| 1H4 VH   | YYGSSYVWFFDVWGAGTTGTGSS                                        |  |  |     |  |     |

## Light chain sequences of anti-CD20 antibodies

|          |                                                                   |  |  |  |  |     |
|----------|-------------------------------------------------------------------|--|--|--|--|-----|
|          | 1                                                                 |  |  |  |  | 50  |
| 2B8VK    | QIVLSQSPAILSASPGEKVTMTCRASSSVSYIHWFOQQKPGSSPKPWIYAT               |  |  |  |  |     |
| 2B8VKA   | QIVLSQSPAIIITASPGEKVTMTCRASTSASYIHWFOQQKTSSSPKPWIYAT              |  |  |  |  |     |
| 2B8VKB   | QIVLSQSPAIIITASPGEKVTMTCRASTSVSYIHWFOQQKTSSSPKPWIYAT              |  |  |  |  |     |
| 2B8VKC   | QIVLSQSPAIIITASPGEKVTMTCRASTSVSYIHWFOQQKPGSSPKPWIYAT              |  |  |  |  |     |
| 2B8VKD   | QIVLSQSPAIIITASPGEKVTMTCRASSSVSYIHWFOQQKPGSSPKPWIYAT              |  |  |  |  |     |
| Leu16VK  | DIVLTQSPAILSASPGEKVTMTCRASSSVNYMDWYQKKPGSSPKPWIYAT                |  |  |  |  |     |
| Leu16VKZ | DIVLTQSPAIIITASPGEKVTMTCRASSSVNYMDWYQKKPGSSPKPWIYAT               |  |  |  |  |     |
| 1H4 VK   | QIVLSQSPTILSASPGEKVTMTCRASSSVSYMDWYQQKPGSSPKPWIYAT                |  |  |  |  |     |
|          | 51                                                                |  |  |  |  | 100 |
| 2B8VL    | SNLASGVPVRFSGSGSGTSYS <del>L</del> TISRVEAEDAATYYCQQWTSNPPTFGGG   |  |  |  |  |     |
| 2B8VKA   | SNLASGVPSRFSGSGSGGT <del>T</del> YSMTISSLEAEDAATYYCQQWTSNPPTFGGG  |  |  |  |  |     |
| 2B8VKB   | SNLASGVPSRFSGSGSGGT <del>T</del> YSMTISSLEAEDAATYYCQQWTSNPPTFGGG  |  |  |  |  |     |
| 2B8VKC   | SNLASGVPSRFSGSGSGGT <del>T</del> YSMTISSLEAEDAATYYCQQWTSNPPTFGGG  |  |  |  |  |     |
| 2B8VKD   | NLASGVPSRFSGSGSGGT <del>T</del> YSMTISSLEAEDAATYYCQQWTSNPPTFGGG   |  |  |  |  |     |
| Leu16VK  | NLASGVPARFSGSGSGTSYS <del>L</del> TISRVEAEDAATYYCQQWSFNPPFTFGGG   |  |  |  |  |     |
| Leu16VKZ | SNLASGVPSRFSGSGSGGT <del>T</del> YSMTISSLEAEDAATYYCQQWSFNPPFTFGGG |  |  |  |  |     |
| 1H4 VK   | SNLASGVPARFSGSGSGTSYS <del>L</del> TISRVEAEDAATYYCQQWISNPPTFGAG   |  |  |  |  |     |
|          | 101                                                               |  |  |  |  |     |
| 2B8VL    | TKLEIK                                                            |  |  |  |  |     |
| 2B8VKA   | TKLEIK                                                            |  |  |  |  |     |
| 2B8VKB   | TKLEIK                                                            |  |  |  |  |     |
| 2B8VKC   | TKLEIK                                                            |  |  |  |  |     |
| 2B8VKD   | TKLEIK                                                            |  |  |  |  |     |
| Leu16VK  | TKLEIK                                                            |  |  |  |  |     |
| Leu16VKZ | TKLEIK                                                            |  |  |  |  |     |
| 1H4 VK   | TKLELK                                                            |  |  |  |  |     |

Figure 17

SEQ ID NO. 1 – Heavy chain V region of the 2B8 antibody

QVQLQQPGAELVKAGASVKMSCKASGYTFTSYNMHWVKQTPGRGLEWIGAIYPGNGDTSYNQKFKG  
KATLTADKSSSTAYMQLSSLTSEDSAVYYCARSTYYGGDTYFNVWGAGTTTVTSA

SEQ ID NO. 2 – Heavy chain V region VhC

QVQLQQPGAELKKPGASVKVSCCKASGYTFTSYNMHWVKQTPGRGLEWTGAIYPGNGDTSYNQKFKG  
KTTLTADKSSSTAYMELSSLRSED TAVYYCARSTYYGGDWYFNVWGAGTTTVTSA

SEQ ID NO. 3 – Heavy chain V region VhD

QVQLQQPGAELKKPGASVKVSCCKASGYTFTSYNMHWVKQTPGRGLEWIGAIYPGNGDTSYNQKFKG  
KTTLTADKSSSTAYMELSSLRSED TAVYYCARSTYYGGDWYFNVWGAGTTTVTSA

SEQ ID NO. 4 – Light chain V region of the 2B8 antibody

QIVLSQSPAILSPGGEKVTMTCRASSSVSYIHWFQQKPGSSPKPWIYATSNLASGVPSRFSGSGS  
GTSYSLTISRVEAEDAATYYCQQWTSNPPTFGGGTKLEIK

SEQ ID NO. 5 – Light chain V region VkA

QIVLSQSPAIITASPGEKVTMTCRASTSVSYIHWFQQKPTSSPKPWIYATSNLASGVPSRFSGSGS  
GTTYSMTISSLEAEDAATYYCQQWTSNPPTFGGGTKLEIK

SEQ ID NO. 6 – Light chain V region VkB

QIVLSQSPAIITASPGEKVTMTCRASTSVSYIHWFQQKPTSSPKPWIYATSNLASGVPSRFSGSGS  
GTTYSMTISSLEAEDAATYYCQQWTSNPPTFGGGTKLEIK

SEQ ID NO. 7 – Light chain V region VcC

QIVLSQSPAIITASPGEKVTMTCRASTSVSYIHWFQQKPGSSPKPWIYATSNLASGVPSRFSGSGS  
GTTYSMTISSLEAEDAATYYCQQWTSNPPTFGGGTKLEIK

SEQ ID NO. 8 – Light chain V region VcD

QIVLSQSPAIITASPGEKVTMTCRASSSVSYIHWFQQKPGSSPKPWIYATSNLASGVPSRFSGSGS  
GTTYSMTISSLEAEDAATYYCQQWTSNPPTFGGGTKLEIK

Figure 17 (contd.)

SEQ ID NO. 9 – Heavy chain V region of the Leu-16 antibody

EVQLQQSGAELVKPGASVKMSCKASGYTFTSYNMHWVKQTPGQGLEWIGA  
IYPGNGDTSYNQKFKGKATLTADKSSSTAYMQLSSLTSEDSADYYCARSN  
YYGSSYWFFDVWGAGTTVTVSS

SEQ ID NO. 10 – Heavy chain V region VhY

EVQLQQSGAELKKPGASVKVSKASGYTFTSYNMHWVKQTPGQGLEWIGA  
IYPGNGDTSYNQKFKGKTTLTADKSSSTAYMELSSLRSED<sup>1</sup>AVYYCARSN  
YYGSSYWFFDVWG<sup>2</sup>TGTTVTVSS

SEQ ID NO. 11 – Light chain V region of the Leu-16 antibody

DIVLTQSPAILSASPGEKVTMTCRASSSVNYMDWYQKKPGSSPKPWIYAT  
SNLASGVPARFSGSGSGTSYSLTISRVEAEDAATYYCQQWSFNPPTFGGG  
TKLEIK

SEQ ID NO. 12 – Light chain V region VLZ

DIVLTQSPAII<sup>1</sup>TASPGEKVTMTCRASSSVNYMDWYQKKPGSSPKPWIYAT  
SNLASGVPS<sup>2</sup>RFSGSGSGT<sup>3</sup>YSMTIS<sup>4</sup>SLEAEDAATYYCQQWSFNPPTFGGG  
TKLEIK

SEQ ID NO. 13 - Heavy chain V region of the 1H4 antibody

1H4 VH QVQLQQPGAELVKPGASVKMSCKTSGYTFTSYNVHWVKQTPGQGLEWIGA  
1H4 VH IYPGNGDTSFNQKFKGKATLTADKSSSTVYMQSSLTSEDSAVYYCARSN  
1H4 VH YYGSSYVWFFDVWGAGTTGTGSS

SEQ ID NO. 14 - Light chain V region of the 1H4 antibody

QIVLSQSPTILSASPGEKVTMTCRASSSVSYMDWYQQKPGSSPKPWIYAT  
SNLASGVPARFSGSGSGTSYSLTISRVEAEDAATYYCQQWISNPPTFGAG  
TKLELK

SEQ ID NO. 15 – A first heavy chain V region segment of an anti-CD20 V region pair

SGAELKKPGAS

Figure 17 (contd.)

SEQ ID NO. 16 – A second heavy chain V region segment of an anti-CD20 V region pair

VSCKASGYT

SEQ ID NO. 17 – A third heavy chain V region segment of an anti-CD20 V region pair

LEWTGAIY

SEQ ID NO. 18 – A fourth heavy chain V region segment of an anti-CD20 V region pair

YNQKFKGKT

SEQ ID NO. 19 – A fifth heavy chain V region segment of an anti-CD20 V region pair

FKGKTTLTA

SEQ ID NO. 20 – A sixth heavy chain V region segment of an anti-CD20 V region pair

YMELSSLRS

SEQ ID NO. 21 – A seventh heavy chain V region segment of an anti-CD20 V region pair

SSLRSED TAV

SEQ ID NO. 22 – A first light chain V region segment of an anti-CD20 V region pair

DWGTGTTVT

SEQ ID NO. 23 – A second light chain V region segment of an anti-CD20 V region pair

IITASPGEKV

SEQ ID NO. 24 – A third light chain V region segment of an anti-CD20 V region pair

CRASTSASY

SEQ ID NO. 25 – A fourth light chain V region segment of an anti-CD20 V region pair

QQKPTSSP

Figure 17 (contd.)

SEQ ID NO. 26 – A fifth light chain V region segment of an anti-CD20 V region pair

LASGVPSRF

SEQ ID NO. 27 – A sixth light chain V region segment of an anti-CD20 V region pair

FSGSGSGTT

SEQ ID NO. 28 – A seventh light chain V region segment of an anti-CD20 V region pair

YSMTISSLE

SEQ ID NO. 29

CEPANPSEKNPSTQYC

SEQ ID NO. 30

MSCKASGYT

SEQ ID NO. 31

YNQKFKGKA

SEQ ID NO. 32

FKGKATLTA

SEQ ID NO. 33

YMLSSLRS

SEQ ID NO. 34

ILSASPGEKV

SEQ ID NO. 35

LASGVPVARF

SEQ ID NO. 36

FSGSGSGTS

Figure 17 (contd.)

SEQ ID NO. 37

YSLTISRVE

SEQ ID NO. 38

CEPSNSSEKNSPSTQYC

SEQ ID NO. 39.

DNA sequence encoding the mature light chain of Leu16 comprising Leu16VK

gacatcggttctgacccagtcctccagcaatcttgtctgcatctccaggggagaagggtcaccatga  
cctgcagagccagctcaagtgtaaattacatggactggtagcagaagaagccagggtcctcccc  
caaaccttgatttatgccacatccaacctggcttctggagtccttgccttctctggcagt  
gggtctgggacctcttactctctcacaatcagcagagtcgaggctgaagatgctgccacttatt  
actgccagcagtgaggcttcaacccacccacgttcggtggtaggaccaagctggagatcaaacg  
taagtggatcccgcaattctaaactctgagggggtaggatgacgtggccattctttgcctaaag  
cattgagtttactgcaaggctcagaaaagcatgcaaagccctcagaatggctgcaaagagctcca  
acaaaacaatttagaactttattaaggaatagggggaagctaggaagaaactcaaacatcaag  
attttaataacgcttcttgggtctccttgctataattatctgggataagcatgctgttttctgtc  
tgtccctaacatgccctgtgattatccgcaaacaacacacccaagggcagaactttgttactta  
aacaccatcctgtttgcttcttctcaggaactgtggctgcaccatctgtcttcatcttcccg  
ccatctgatgagcagttgaaatctggaactgcctctgttggtgtgcctgctgaataacttctatc  
ccagagaggccaaagtacagtggaaggtaggataacgccctccaatcgggtaactcccaggagag  
tgtcacagagcaggacagcaaggacagcacctacagcctcagcagcacctgacgctgagcaaa  
gcagactacgagaaacacaaagtctacgcctgcgaagtaccccatcagggcctgagctcgcccg  
tcacaaagagcttcaacaggggagagtggttag

SEQ ID No 40

DNA sequence encoding an epitope-depleted variant of the mature light chain of Leu16 comprising Leu16 VKZ

Gacattgttctcaccagtcctccagcaatcatcacagcatctccaggggagaagggtcacatga  
cttgcagggccagctcaagtgtaaactacatggactggtagcagaagaagccagggtcctcccc  
caaaccttgatttatgccacatccaacctggcttctggagtccttctcgcttcagtggcagt  
gggtctgggactacttactctatgaccatcagcagcctcagaggctgaagatgctgccacttatt  
actgccagcagtgaggcttcaacccacccacgttcggaggggggaccaagctggaaatcaaacg  
taagtggatcccgcaattctaaactctgagggggtaggatgacgtggccattctttgcctaaag  
cattgagtttactgcaaggctcagaaaagcatgcaaagccctcagaatggctgcaaagagctcca  
acaaaacaatttagaactttattaaggaatagggggaagctaggaagaaactcaaacatcaag  
attttaataacgcttcttgggtctccttgctataattatctgggataagcatgctgttttctgtc  
tgtccctaacatgccctgtgattatccgcaaacaacacacccaagggcagaactttgttactta  
aacaccatcctgtttgcttcttctcaggaactgtggctgcaccatctgtcttcatcttcccg  
ccatctgatgagcagttgaaatctggaactgcctctgttggtgtgcctgctgaataacttctatc  
ccagagaggccaaagtacagtggaaggtaggataacgccctccaatcgggtaactcccaggagag  
tgtcacagagcaggacagcaaggacagcacctacagcctcagcagcacctgacgctgagcaaa

Figure 17 (contd.)

gcagactacgagaaacacaaagtctacgcctgcgaagtcacccatcagggcctgagctcgcccg  
tcacaaagagcttcaacaggggagagtgttag

SEQ ID No 41.

DNA sequence encoding the mature heavy chain of Leu16 comprising Leu16VH

gaggtccagctccagcagctctggagctgagctggtgaagcctggggcttcagtgaaagtgtcct  
gcaaggcttctggatacacattcactagttataaatatgcactgggtaaagcagacacctggaca  
gggcctggaatggattggagctatttatccaggaaatgggtgatacttcctacaatcagaagttc  
aagggcaaggccacactgactgcagacaaatcctccagcacagcctacatgcagctcagcagcc  
tgacatctgaagactctgctgactattactgtgaggaagtaactactacggtagtagctactg  
gttcttcgatgtctggggcgagggaccacggtcacctctcttcaggtaagtaagcttttctg  
gggcaggccaggcctgaccttggcttggggcagggagggggctaaggtagggcaggtagggcgc  
agccaggtagcacaccaatgcccatgagccagacactggacgctgaacctcgcgagacagttaa  
gaacccaggggctctgcgccctgggcccagctctgtcccacaccgcggtcacatggcaccacc  
tctcttgagcctccaccaagggcccatcggtcttccccctggcacccctcctccaagagcacct  
ctgggggcacagcggccctgggctgctggtcaaggactacttccccgaaccggtagcggtgtc  
gtggaactcaggcgccctgaccagcggcgtgcacaccttcccggtgtcctacagtcctcagga  
ctctactccctcagcagcgtggtgacctgacctccagcagcttgggcacccagacctacatct  
gcaacgtgaatcacaagcccagcaacaccaaggtggacaagagagttggtgagaggccagcaca  
gggagggaggggtgtctgctggaagccaggctcagcgtcctgctggacgcatcccggtatgc  
agtcccagtcacagggcagcaaggcagggcccggtctgcctcttccccggaggcctctgcccgc  
ccactcatgctcaggagaggggtcttctggcttttccccaggctctgggcaggcacaggctag  
gtgcccctaaccaggccctgcacacaaagggcaggtgctgggctcagacctgccaagagcca  
tatccgggaggacctgcccctgacctaaagcccaccccaaaggccaaactctccactccctcag  
ctcgacaccttctctcctccagattccagtaactcccaatcttctctctgcagagcccaaat  
cttgtgacaaaactcacacatgcccacctgcccaggtaagccagcccaggcctcgccctccag  
ctcaaggcgggacaggtagccttagagtagcctgcatccagggaaggcccccagccgggtgctga  
cacgtccacctccatctcttctcagcacctgaactcctggggggaccgctcagtccttctcttc  
ccccaaaacccaaggacacctcatgatctcccgacccctgaggtcacatgcgtgggtggg  
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gtcctgcaccaggactggctgaatggcaaggagtacaagtgaaggctctccaacaaagccctcc  
cagcccccatcgagaaaaccatctccaaagccaaagggtgggaccctgggggtgcgagggccaca  
tggacagaggccggctcgggccacctctgacctgagagtgaacgctgtaccaacctctgtccc  
tacagggcagccccgagaaccacaggtgtacacctgcccccatcccgaggagatgaccaag  
aaccaggtagcctgacctgctggtcaaaggcttctatcccagcgacatcgccgtggagtggg  
agagcaatgggcagccggagaaactacaagaccagcctcccggtgctggactccgacggctc  
cttcttctctatagcaagctcacctgggacaagagcaggtggcagcaggggaacgtcttctca  
tgctccgtgatgcatgaggctctgcacaaccactacacgcagaagagcctctcctgtccccgg  
gtaaatga

SEQ ID No 42

DNA sequence encoding an epitope-depleted variant of the mature heavy chain of Leu16  
comprising Leu16 VHY

Gaggtacaactgcagcagctctggggctgagctgaagaagcctggggcctcagtgaaaggtgtcct  
gcaaggcttctggctacacatttaccagttacaatatgcactgggtaaaacagacacctgggtca  
gggcctggaatggattggagctatttatcccggaaatgggtgatacttcctacaatcagaagttc

Figure 17 (contd.)

aaaggcaagacaacattgactgcagacaaatcctccagcacagcctacatggaactcagcagcc  
tgagatctgaggacactgcggtctattactgtgcaagatcgaattactacggcagcagctactg  
gttcttcgatgtctggggcaccgggaccacggtcaccgtctcttcaggtaagctttctggggca  
ggccaggcctgaccttggctttggggcagggaggggggctaagggtgaggcaggtggcgccagcca  
ggtgcacacccaatgcccattgagcccagacactggacgctgaacctcgcggaacagttaagaacc  
cagggggcctctgcgccttggggccagctctgtcccacaccgcggtcacatggcaccacctctct  
tgcagcctccaccaaggggcccatcggtcttccccctggcaccctcctccaagagcacctctggg  
ggcacagcggccctgggctgcctggtcaaggactacttccccgaaccggtgacggtgtcgtgga  
actcaggcgccctgaccagcggcgtgcacaccttcccggtgtcctacagtcctcaggactcta  
ctccctcagcagcgtggtgaccgtgccctccagcagcttgggcacccagacctacatctgcaac  
gtgaatcacaagcccagcaacaccaagggtggacaagagagtgggtgagaggccagcacaggag  
ggagggtgtctgctggaagccaggctcagcgtcctgacctggacgcatcccggtatgcagtc  
cagtcacagggcagcaaggcaggccccgtctgcctcttcaaccggaggcctctgccccccccact  
catgctcaggggagagggtcttctggctttttccccaggctctgggcaggcacaggctaggtgcc  
cctaaccaggccctgcacacaaaggggcaggtgctgggctcagacctgccaagagccatatcc  
gggaggaccctgcccctgacctaaagcccaccccaaaggccaaactctccactccctcagctcgg  
acaccttctctcctcccagattccagtaactcccaatcttctctctgcagagcccaaactctgt  
gacaaaactcacacatgccaccgtgccaggttaagccagcccaggcctcgccctccagctcaa  
ggcgggacaggtgccctagagtgcctgcacccaggacaggccccagccgggtgctgacacgt  
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aaaacccaaggacacctcatgatctcccggaacctctgaggtcacatgcgtgggtggtagctg  
agccacgaagacctgaggtcaagttcaactggtagctggacggcgtggaggtgcataatgcc  
agacaaagccgcgggaggagcagtacaacagcacgtaccgtgtggtcagcgtcctcaccgtcct  
gcaccaggactggctgaatggcaaggagtacaagtgaagggtctccaacaaagccctcccagcc  
cccatcgagaaaaccatctccaagccaaagggtgggacccgtgggggtgcgagggccacatggac  
agaggccggctcggcccacctctgccttgagagtgaacctgtaccaacctctgtccctacag  
ggcagccccgagaaccacaggtgtacacctgcccccatcacgggaggagatgaccaagaacca  
ggtcagcctgacctgcctggtcaaaggcttctatccagcgacatcgccgtggagtgggagagc  
aatgggcagccggagaacaactacaagaccagcctcccggtgctggactccgacgggtccttct  
tctctatagcaagctcaccgtggacaagagcaggtggcagcaggggaacgtcttctcatgctc  
cgtgatgcatgaggctctgcacaacctacacgcagaagagcctctccctgtccccgggtaaa  
tga

## SEQ ID No 43

DNA sequence encoding a fusion protein of an epitope-depleted variant of the mature heavy chain of Leu16 comprising Leu16 VH1 and the cytokine IL2

Gaggtacaactgcagcagctctggggctgagctgaagaagcctggggcctcagtgaagggtgtcct  
gcaaggcttctggctacacatttaccagttacaatatgcactgggtaaaacagacacctggtca  
gggcctggaatggattggagctatcttcccgaaatgggtgatacttcttacaatcagaagttc  
aaaggcaagacaacattgactgcagacaaatcctccagcacagcctacatggaactcagcagcc  
tgagatctgaggacactgcggtctattactgtgcaagatcgaattactacggcagcagctactg  
gttcttcgatgtctggggcaccgggaccacggtcaccgtctcttcaggtaagctttctggggca  
ggccaggcctgaccttggctttggggcagggaggggggctaagggtgaggcaggtggcgccagcca  
ggtgcacacccaatgcccattgagcccagacactggacgctgaacctcgcggaacagttaagaacc  
cagggggcctctgcgccttggggccagctctgtcccacaccgcggtcacatggcaccacctctct  
tgcagcctccaccaaggggcccatcggtcttccccctggcaccctcctccaagagcacctctggg  
ggcacagcggccctgggctgcctggtcaaggactacttccccgaaccggtgacggtgtcgtgga  
actcaggcgccctgaccagcggcgtgcacaccttcccggtgtcctacagtcctcaggactcta  
ctccctcagcagcgtggtgaccgtgccctccagcagcttgggcacccagacctacatctgcaac

Figure 17 (contd.)

gtgaatcacaagcccagcaacaccaaggtggacaagagagtgggtgagagggccagcacagggag  
ggaggggtgtctgctggaagccagggtcagcgctcctgcctggacgcatcccggctatgcagtcc  
cagtccagggcagcaaggcaggccccgtctgcctcttcacccggaggcctctgccgccccact  
catgctcagggagaggggtcttctggctttttccccaggctctgggcaggcacaggctaggtgcc  
cctaaccaggccctgcacacaaaggggcagggtgctgggctcagacctgccaaagagccatatcc  
gggaggaccctgccctgacctaaagcccaccccaaaggccaaactctccactccctcagctcgg  
acaccttctctcctccagattccagtaactcccaatcttctctctgcagagcccaaactcttgt  
gacaaaactcacacatgccaccgtgccaggtaagccagcccaggcctcgccctccagctcaa  
ggcgggacaggtgccctagagttagcctgcatccagggaacaggccccagccgggtgctgacacgt  
ccacctccatctcttccctcagcacctgaactcctggggggaccgtcagctcttctcttcccccc  
aaaacccaaggacaccctcatgatctcccgaccctgaggtcacatgctgggtggtagcgtg  
agccacgaagaccctgagggtcaagttcaactggtagctggacggcgtggagggtgcataatgcc  
agacaaagccgcgggaggagcagtacaacagcacgtaccgtgtgggtcagcgtcctcaccgtcct  
gcaccaggactggctgaatggcaaggagtacaagtgaagggtctccaacaaagccctcccagcc  
cccatcgagaaaaccatctccaaagccaaagggtgggacccgtgggggtgaggggcccacatggac  
agaggccgggtcggccccaccctctgcctgagagtgaaccgtgtaccaacctctgtccctacag  
ggcagccccgagaaccacagggtgtacaccctgcccccatcacgggaggagatgaccaagaacca  
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tcctctatagcaagctcaccgtggacaagagcagggtggcagcaggggaacgtcttctcatgctc  
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gccccaaacttcaagttctacaaagaaaacacagctgcaactggagcatctcctgctggatctcc  
agatgattctgaatggaattaacaactacaagaatcccaaactcaccaggatgctcacattcaa  
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tgctgatgagacagcaaccattgtagaattcctaaacagatggattaccttttgtcaaagcatc  
atctcaacactaacttga