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(54) Title: MONOCLONAL ANTIBODIES AGAINST CD30 LACKING IN FUCOSYL AND XYLOSYL RESIDUES

(57) Abstract: The invention pertains to anti-CD30 antibodies that lack fucosyl and xylosyl residues. The antibodies of the invention exhibit increased antibody-dependent cellular cytotoxicity (ADCC) activity, including the ability to lyse CD30-expressing cell lines that are not lysed by the fucosylated and xylosylated form of the antibodies. The invention also provides host cells that express the anti-CD30 antibodies that lack fucosyl and xylosyl residues, wherein the host cells are deficient for a fucosyltransferase and a xylosyltransferase. Methods of using the antibodies to inhibit the growth of CD30+ cells, such as tumor cells, are also provided.
MONOCLONAL ANTIBODIES AGAINST CD30 LACKING IN FUCOSYL AND XYLOSYL RESIDUES

Related Applications
This application claims priority to U.S. Provisional Application No.: 60/759298, filed on January 17, 2006, U.S. Provisional Application No.: 60/790373, filed on April 7, 2006, U.S. Provisional Application No.: 60/791178, filed on April 11, 2006, U.S. Provisional Application No.: 60/812702, filed on June 9, 2006, U.S. Provisional Application No.: 60/836998, filed on August 11, 2006, and U.S. Provisional Application No.: 60/837202, filed on August 11, 2006. This application also corresponds to [Alston & Bird LLP attorney docket No.: 040989/322372] and [Alston & Bird LLP attorney docket No.: 040989/322364], filed on even date herewith. The entire contents of each of the aforementioned applications are hereby expressly incorporated herein by this reference.

Background of the Invention
The CD30 cell surface molecule is a member of the tumor necrosis factor receptor (TNF-R) superfamily. This family of molecules has variable homology among its members and includes nerve growth factor receptor (NGFR), CD120(a), CD120(b), CD27, CD40 and CD95. These molecules are typically characterized by the presence of multiple cysteine-rich repeats in the extracytoplasmic region (de Bruin, P.C., et al. Leukemia 9:1620-1627 (1995)). Members of this family are considered crucial for regulating proliferation and differentiation of lymphocytes.

CD30 is a type I transmembrane glycoprotein with six (human) or three (murine and rat) cysteine-rich repeats with a central hinge sequence. CD30 exists as a 120 kDa membrane molecule which develops from an intercellular precursor protein of 90 kDa. It is shed from the cell surface as a soluble protein (sCD30) of approximately 90 kDa. Shedding of sCD30 occurs as an active process of viable CD30 cells and is not merely caused by the release from dying or dead cells. cDNAs encoding the CD30 protein have been cloned from expression libraries of the HTLV-I human T-cell line HUT-102 by immunoscreening with monoclonal antibodies Ki-I and Ber-H2 (Schwab, U., et al. Nature 299:65 (1982)). The mouse and rat CD30 cDNA has been found to encode 498 and 493 amino acids, respectively. Human CD30 cDNA encodes an additional 90 amino acids, partially duplicated from one of the cysteine rich domains. The CD30 gene has been mapped to Ip36 in humans and 5q36.2 in rats.
CD30 is preferentially expressed by activated lymphoid cells. Specifically, stimulation of CD30 in lymphoid cells has been shown to induce pleiotropic biological effects, including proliferation, activation, differentiation and cell death, depending on cell type, stage of differentiation and presence of other stimuli (Grass, HJ. et al, Blood 83:2045-2056 (1994)). CD30 was originally identified by the monoclonal antibody Ki-I, which is reactive with antigens expressed on Hodgkin and Reed-Sternberg cells of Hodgkin's disease (Schwab et al, Nature 299:65 (1982)). Accordingly, CD30 is widely used as a clinical marker for Hodgkin's lymphoma and related hematological malignancies (Froese et al., J. Immunol. 139:2081 (1987); Carde et al., Eur. J. Cancer 26:474 (1990)).

CD30 was subsequently shown to be expressed on a subset of non-Hodgkin's lymphomas (NHL), including Burkitt's lymphoma, anaplastic large-cell lymphomas (ALCL), cutaneous T-cell lymphomas, nodular small cleaved-cell lymphomas, lymphocytic lymphomas, peripheral T-cell lymphomas, Lennert's lymphomas, immunoblastic lymphomas, T-cell leukemia/lymphomas (ATLL), adult T-cell leukemia (T-ALL), and entroblastic/centrocytic (cb/cc) follicular lymphomas (Stein et al., Blood 66:848 (1985); Miettinen, Arch. Pathol. Lab. Med. 116:1 197 (1992); Piris et al., Histopathology 17:211 (1990); Burns et al, Am. J. Clin. Pathol. 93:327(1990); and Eckert et al, Am. J. Dermatopathol. 11:345 (1989)), as well as several virally-transformed lines such as human T-Cell Lymphotrophic Virus I or II transformed T-cells, and Epstein-Barr Virus transformed B-cells (Stein et al, Blood 66:848 (1985); Andreesen et al, Blood 63:1299 (1984)). In addition, CD30 expression has been documented in embryonal carcinomas, nonembryonal carcinomas, malignant melanomas, mesenchymal tumors, and myeloid cell lines and macrophages at late stages of differentiation (Schwarting et al, Blood 74:1678 (1989); Pallesen et al, Am J. Pathol. 133:446 (1988); Mechtersheimer et al, Cancer 66:1732 (1990); Andreesen et al, Am. J. Pathol. 134:187 (1989)).

Since the percentage of CD30-positive cells in normal individuals is quite small, the expression of CD30 in tumor cells renders it an important target for antibody mediated therapy to specifically target therapeutic agents against CD30-positive neoplastic cells (Chaiarle, R., et al Clin. Immunol 90(2):157-164 (1999)). Antibody mediated therapy has been shown to increase cytotoxicity of CD30-positive cells by both complement activation and antibody dependent cellular cytotoxicity (ADCC) (Pohl C, et al Int J Cancer 54:418 (1993)). However, while the results obtained to date clearly establish CD30 as a useful target for immunotherapy, they also show that currently available murine antibodies do not constitute ideal therapeutic agents. Passive antibody therapy has not been effective in vitro

A number of plant species have been targeted for use in "molecular farming" of mammalian proteins of pharmaceutical interest. These plant expression systems provide for low cost production of biologically active mammalian proteins and are readily amenable to rapid and economical scale-up (Ma et al. (2003) Nat. Rev. Genet. 4:794-805; Raskin et al. (2002) Trends Biotechnol. 20:522-531). The differences in glycosylation patterns between plants and mammals offer a challenge to the feasibility of plant expression systems to produce high quality recombinant mammalian proteins for pharmaceutical use. Methods are needed to alter the glycosylation pattern in plant expressed proteins, specifically to inhibit plant-specific glycosylation of the eukaryotic core structure, to advantageously produce recombinant mammalian proteins with a humanized glycosylation pattern.

Accordingly, the need exists for improved therapeutic antibodies against CD30 which are more effective for treating and/or preventing diseases mediated by CD30.

Summary of the Invention

The present invention provides isolated human monoclonal antibodies which bind to human CD30, as well as derivatives (e.g., immunoconjugates and bispecific molecules)- and other therapeutic compositions containing such antibodies, alone or in combination with additional therapeutic agents. Also provided are methods for treating a variety of diseases involving CD30 expression using the antibodies and compositions of the invention.

In one aspect, the invention pertains to an isolated defucosylated and dxylosylated monoclonal antibody, or an antigen-binding portion thereof, wherein the antibody binds to human CD30 with a $K_D$ of $10^x$ M or less, more preferably $1x10^{-9}$ M or less, more preferably $5x10^{-9}$ or less or even more preferably $1x10^{-9}$ or less.

In another aspect, the invention pertains to an isolated glycoprotein composition comprising an anti-CD30 antibody composition comprising a substantially homogeneous N-glycosylation profile, wherein at least 90% of the iV-glycans species present in said profile are GlcNAc2Man3GlcNAc2 (GO), said profile comprising a trace amount of precursor N-
glycan species, wherein said precursor N-glycan species is selected from the group consisting of Man3GlcNAc2, GlcNaclMan3GlcNAc2 wherein GlcNacl is attached to the 1,3 mannose arm (MGn), GlcNacl Man3GlcNAc2 wherein GlcNacl is attached to the 1,6 mannose arm (GnM), and any combination thereof.

The defucosylated and dexylosylated antibodies of the present invention bind to CD30 and inhibit the growth of cells expressing CD30 by enhancing antibody dependent cellular cytotoxicity (ADCC) in the presence of human effector cells (e.g., monocytes or mononuclear cells), as compared to the fucosylated form of the antibody. In one embodiment, the defucosylated and dexylosylated antibody mediates increased ADCC of cells expressing CD30 in the presence of human effector cells but not in the presence of mouse effector cells.

Preferably, the defucosylated and dexylosylated antibody of the invention is a monoclonal antibody. In one aspect, the invention pertains to a humanized or chimeric monoclonal antibody. Preferably, the humanized or chimeric antibody is prepared from a mouse anti-CD30 antibody selected from the group consisting of: AC1O, HeFi-I, Ber-H2, Ki-I, Ki-4, HRS-3, Irac, HRS-4, M44, M67, Ber-H8. In another aspect, the invention pertains to a human monoclonal antibody.

In one embodiment of the invention, the human monoclonal antibody comprises:
(a) a heavy chain variable region comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 1, 2 and 3; and
(b) a light chain variable region comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 4, 5 and 6.

wherein the antibody binds CD30 and lacks fucosyl residues.
A preferred combination comprises:
(a) a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 1; and
(b) a light chain variable region comprising the amino acid sequence of SEQ ID NO: 4.

Another preferred combination comprises:
(a) a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 2; and
(b) a light chain variable region comprising the amino acid sequence of SEQ ID NO: 5.

Another preferred combination comprises:
(a) a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 3; and
(b) a light chain variable region comprising the amino acid sequence of SEQ ID NO: 6.

In another aspect, the invention provides a defucosylated and dexylosylated anti-CD30 antibody comprising:

- a heavy chain variable region that comprises CDR1, CDR2, and CDR3 sequences; and
- a light chain variable region that comprises CDR1, CDR2, and CDR3 sequences, wherein:
  (a) the heavy chain variable region CDR1 sequence comprises an amino acid sequence selected from the group consisting of amino acid sequences of SEQ ID NOs: 7, 8, and 9;
  (b) the heavy chain variable region CDR2 sequence comprises an amino acid sequence selected from the group consisting of amino acid sequences of SEQ ID NOs: 10, 11, and 12;
  (c) the heavy chain variable region CDR3 sequence comprises an amino acid sequence selected from the group consisting of amino acid sequences of SEQ ID NOs: 13, 14, and 15;
  (d) the light chain variable region CDR1 sequence comprises an amino acid sequence selected from the group consisting of amino acid sequences of SEQ ID NOs: 16, 17, and 18;
  (e) the light chain variable region CDR2 sequence comprises an amino acid sequence selected from the group consisting of amino acid sequences of SEQ ID NOs: 19, 20, and 21; and
  (f) the light chain variable region CDR3 sequence comprises an amino acid sequence selected from the group consisting of amino acid sequences of SEQ ID NOs: 22, 23, and 24; wherein the antibody binds CD30 and lacks fucosyl residues.

A preferred combination comprises:

- (a) a human heavy chain variable region CDR1 comprising SEQ ID NO:7;
- (b) a human heavy chain variable region CDR2 comprising SEQ ID NO:10;
- (c) a human heavy chain variable region CDR3 comprising SEQ ID NO:13;
- (d) a human light chain variable region CDR1 comprising SEQ ID NO:16;
- (e) a human light chain variable region CDR2 comprising SEQ ID NO:19; and
- (f) a human light chain variable region CDR3 comprising SEQ ID NO:22.

Another preferred combination comprises:

- (a) a human heavy chain variable region CDR1 comprising SEQ ID NO:8;
- (b) a human heavy chain variable region CDR2 comprising SEQ ID NO:11;
- (c) a human heavy chain variable region CDR3 comprising SEQ ID NO:14;
- (d) a human light chain variable region CDR1 comprising SEQ ID NO:17;
(e) a human light chain variable region CDR2 comprising SEQ ID NO:20; and
(f) a human light chain variable region CDR3 comprising SEQ ID NO:23.

Yet another preferred combination comprises:

(a) a human heavy chain variable region CDR1 comprising SEQ ID NO:9;
(b) a human heavy chain variable region CDR2 comprising SEQ ID NO:12;
(c) a human heavy chain variable region CDR3 comprising SEQ ID NO:15;
(d) a human light chain variable region CDR1 comprising SEQ ID NO:18;
(e) a human light chain variable region CDR2 comprising SEQ ID NO:21; and
(f) a human light chain variable region CDR3 comprising SEQ ID NO:24.

In another aspect, the invention provides a defucosylated and dexylosylated human anti-CD30 antibody which comprises a heavy chain variable region that is a product of or derived from a human V\textsubscript{H} 4-34 or V\textsubscript{H} 3-07 gene. The invention also provides a defucosylated and dexylosylated human anti-CD30 antibody which comprises a light chain variable region that is a product of or derived from a human V\textsubscript{L} L15, A27 or L6 gene. The invention still further provides a defucosylated and dexylosylated human anti-CD30 antibody which comprises a heavy chain variable region that is a product of or derived from a human V\textsubscript{H} 4-34 or V\textsubscript{H} 3-07 gene and a light chain variable region that is a product of or derived from a human V\textsubscript{L} L15, A27 or L6 gene.

In another aspect of the invention, antibodies, or antigen-binding portions thereof, are provided that compete for binding to CD30 with any of the aforementioned antibodies.

The antibodies of the invention can be, for example, full-length antibodies, for example of an IgGl, IgG2 or IgG4 isotype. Alternatively, the antibodies can be antibody fragments, such as Fab, Fab' or Fab'2 fragments, or single chain antibodies.

The invention also provides an immunoconjugate comprising an antibody of the invention, or antigen-binding portion thereof, linked to a therapeutic agent, such as a cytotoxin or a radioactive isotope. The invention also provides a bispecific molecule comprising an antibody, or antigen-binding portion thereof, of the invention, linked to a second functional moiety having a different binding specificity than said antibody, or antigen binding portion thereof.

Compositions comprising an antibody, or antigen-binding portion thereof, or immunoconjugate or bispecific molecule of the invention and a pharmaceutically acceptable carrier are also provided.
Nucleic acid molecules encoding the antibodies, or antigen-binding portions thereof, of the invention are also encompassed by the invention, as well as expression vectors comprising such nucleic acids and host cells comprising such expression vectors.

In another aspect, the invention pertains to a host cell comprising immunoglobulin heavy and light chain genes encoding an anti-CD30 antibody, wherein said host cell lacks both a fucosyltransferase and a xylosyltransferase or has its endogenous fucosyltransferase and xylosyltransferase function inhibited such that the anti-CD30 antibody expressed by said host cell lacks fucosyl and xylosyl residues. Preferably, the immunoglobulin heavy and light chain genes are human immunoglobulin heavy and light chain genes. Preferably, the fucosyltransferase which is missing or inhibited is FUT8 or FucT. Preferably, the xylosyltransferase which is missing or inhibited is XyIT. Preferably, the host cell is a CHO cell or a plant host cell.

In another aspect, the invention provides a method of inhibiting growth of CD30+ cells. The method involves contacting the cells with a defucosylated and defucosylated anti-CD30 antibody under conditions sufficient to induce antibody-dependent cellular cytotoxicity (ADCC) of said cells. The cells can be, for example, tumor cells. Preferably, the anti-CD30 antibody is a human antibody.

The invention also provides a method of inhibiting growth of tumor cells expressing CD30 in a subject. The method involves administering to the subject a defucosylated and defucosylated anti-CD30 antibody in an amount effective to inhibit growth of tumor cells expressing CD30 in the subject. Preferably, the anti-CD30 antibody is a human antibody.

In preferred embodiments, the tumor cells are Hodgkin’s Disease (HD) tumor cells or anaplastic large-cell lymphoma (ALCL) tumor cells.

Other features and advantages of the instant invention will be apparent from the following detailed description and examples which should not be construed as limiting. The contents of all references, Genbank entries, patents and published patent applications cited throughout this application are expressly incorporated herein by reference.

**Brief Description of the Drawings**

Figure 1A shows the nucleotide sequence (SEQ ID NO: 30) and amino acid sequence (SEQ ID NO: 1) of the heavy chain variable region of the 5Fl 1 human monoclonal antibody. The CDR1 (SEQ ID NO: 7), CDR2 (SEQ ID NO: 10) and CDR3 (SEQ ID NO: 13) regions are delineated and the V, D and J germline derivations are indicated.
Figure 1B shows the nucleotide sequence (SEQ ID NO: 33) and amino acid sequence (SEQ ID NO: 4) of the light chain variable region of the 5Fl 1 human monoclonal antibody. The CDR1 (SEQ ID NO: 16), CDR2 (SEQ ID NO: 19) and CDR3 (SEQ ID NO: 22) regions are delineated and the V and J germline derivations are indicated.

Figure 2A shows the nucleotide sequence (SEQ ID NO: 31) and amino acid sequence (SEQ ID NO: 2) of the heavy chain variable region of the 17Gl human monoclonal antibody. The CDR1 (SEQ ID NO: 8), CDR2 (SEQ ID NO: 11) and CDR3 (SEQ ID NO: 14) regions are delineated and the V and J germline derivations are indicated.

Figure 2B shows the nucleotide sequence (SEQ ID NO: 34) and amino acid sequence (SEQ ID NO: 5) of the light chain variable region of the 17Gl human monoclonal antibody. The CDR1 (SEQ ID NO: 17), CDR2 (SEQ ID NO: 20) and CDR3 (SEQ ID NO: 23) regions are delineated and the V and J germline derivations are indicated.

Figure 3A shows the nucleotide sequence (SEQ ID NO: 32) and amino acid sequence (SEQ ID NO: 3) of the heavy chain variable region of the 2H9 human monoclonal antibody. The CDR1 (SEQ ID NO: 9), CDR2 (SEQ ID NO: 12) and CDR3 (SEQ ID NO: 15) regions are delineated and the V, D, and J germline derivations are indicated.

Figure 3B shows the nucleotide sequence (SEQ ID NO: 35) and amino acid sequence (SEQ ID NO: 6) of the light chain variable region of the 2H9 human monoclonal antibody. The CDR1 (SEQ ID NO: 18), CDR2 (SEQ ID NO: 21) and CDR3 (SEQ ID NO: 24) regions are delineated and the V and J germline derivations are indicated.

Figure 4 is a graph showing the cytotoxic cell killing activity of the defucosylated and defucosylated forms of 5Fl 1 on the L540 human Hodgkin's lymphoma cell line, as compared to an isotype-matched control antibody (1D4).

Figure 5 is a graph showing the cytotoxic cell killing activity of the defucosylated and defucosylated forms of 5Fl 1 on the L428 human Hodgkin's lymphoma cell line, as compared to an isotype-matched control antibody (1D4).

Figure 6 is a graph showing the cytotoxic cell killing activity of the defucosylated and defucosylated forms of 5Fl 1 on the L1236 human Hodgkin's lymphoma cell line, as compared to an isotype-matched control antibody (1D4).

Figure 7 is a graph showing the cytotoxic cell killing activity of the defucosylated and defucosylated forms of 5Fl 1 on the Karpas human T cell lymphoma cell line, as compared to an isotype-matched control antibody (1D4).
Figures 8A-8B show the amino acid sequences of the human germlines V_H 4-34, V_H 3-07, V_K L15, V_K A27, and V_K L6 (SEQ ID NOs:25-29, respectively), the CDRs are delineated.

Figure 9 is a graph showing blockade of ADCC activity with an anti-CD16 antibody.

Figure 10 is a graph showing the cytotoxic cell killing activity of the fucosylated and defucosylated forms of 5Fl 1 in the presence of mouse (left panel) or human (right panel) effector cells.

Figure 11 is a graph showing an ADCC assay using cynomolgus blood.

Figure 12 shows glycosyltransferase activity in *Lemma* wild-type and 5Fl 1 LEX^0^ RNAi lines. Microsomal membranes from wild-type (WT) and 5Fl 1 LEX^0^ RNAi (line numbers are indicated) plants were incubated in the presence of a reaction buffer containing GDP-Fuc, UDP-Xyl and GnGn-dabsyl-peptide acceptor. Mass peaks corresponding to fucosylated (white bars) or xylosylated (black bars) products synthesized by microsomes from each line were measured by positive reflectron mode MALDI-TOF MS and normalized, in percent, to the WT positive control. Boiled wildtype membranes (BWT) indicate background ion counts.

Figure 13 shows SDS-PAGE of plant extracts and protein A or hydroxyapatite purified samples from 5Fl 1 LEX^0^ under non-reducing (Figure 13A) and reducing (Figure 13B) conditions, respectively. MAb purified from a CHO cell line (5Fl 1 CHO) was used as a positive control. Mark12 molecular weight markers were included on the gels. Gels were stained with Colloidal Blue.

Figure 14 shows the spectra obtained from negative, reflectron mode MALDI-TOF mass spectrometric analysis of 2-AA labeled JV-glycans released from 5Fl 1 mAbs expressed in CHO (5Fl 1 CHO), wild-type *Lemma* (5Fl 1 LEX), or *Lemma* transformed with the XylT/FucT RNAi construct (5Fl 1 LEX^0^). Significant peaks are identified by the corresponding mass ([M-H]^+^). The * indicates the location of matrix artefacts.

Figure 15 shows the spectra obtained from NP-HPLC-QTOF MS analysis of 2-AA labeled TV-glycans released from 5Fl 1 mAbs expressed in CHO (5Fl 1 CHO), wild-type *Lemma* (5Fl 1 LEX), *oxLemma* transformed with the XylT/FucT RNAi construct (5Fl 1 LEX^0^). 2-AA labeled N-glycans were separated by normal phase chromatography and detected by fluorescence. The most abundant peaks from each sample (labeled a-i) were characterized by on-line negative mode QTOF MS and their corresponding QTOF mass spectra ([M-2H]^2^) are shown.
Figure 16 shows *in vitro* activity of 5Fl1 mAbs as measured by flow cytometric analysis of 5Fl1 CHO, LEX, or glyco-optimized LEX\(^{\text{opt}}\) mAb binding to CD30 expressed on L540 cells. L540 cells were incubated with increasing concentrations of the indicated antibody as outlined in Example 6 herein below. Geo Mean Fluorescence Intensity (GMFI) is plotted against the various concentrations of mAb used. ■: 5Fl1 CHO; ▲: 5Fl1 LEX;T: 5Fl1 LEX\(^{\text{opt}}\).

Figure 17 shows equilibrium binding of glyco-optimized and wild-type mAb to two different human FcR\(\gamma\)IIIa allotypes (Val\(^{158}\) or Phe\(^{158}\)). The binding signal as a function of FcR\(\gamma\)IIIa was fit to a one-site binding model. ■: 5Fl1 CHO; ▲: 5Fl1 LEX;T: 5Fl1 LEX\(^{\text{opt}}\).

Figure 18 shows ADCC activity of 5Fl1 mAb derived from CHO, LEX (wild-type *Lemna* glycosylation), or LEX\(^{\text{opt}}\) (RNAi transgenic *Lemna*). Human effector cells from a FcR\(\gamma\)IIIaPhe\(^{158}\) homozygote donor and a FcR\(\gamma\)IIIaPhe/Val\(^{158}\) heterozygote donor were incubated with BATDA-labeled L540 cells at an effector:target ratio of 50:1 in the presence of increasing concentrations of the indicated antibodies. Specific percent lysis at each mAb concentration is plotted. Human mAb1 not recognizing antigen on L540 cells was used as an isotype control in all experiments. EC50 values (\(\mu\)g/mL), binding constants and maximal percent lysis were calculated using GraphPad Prism 3.0 software. ■: 5Fl1 CHO; ▲: 5Fl1 LEX;T: 5FU LEX\(^{\text{opt}}\).

Figure 19 shows intact mass analysis of the 5Fl1 LEX mAb compositions produced in wild-type *L. minor* comprising the MDXAOI construct. When XyIT and FucT expression are not suppressed in *L. minor*, the recombinantly produced 5Fl1 LEX mAb composition comprises at least 7 different glycoforms, with the GOX\(\text{F}^3\) glycoform being the predominate species present. Note the absence of a peak representing the GO glycoform.

Figure 20 shows glycan mass analysis of the heavy chain of the 5Fl1 LEX mAb produced in wild-type *L. minor* comprising the MDXAOI construct. When XyIT and FucT expression are not suppressed in *L. minor*, the predominate JV-glycan species present is GOX\(\text{F}^3\), with additional major peaks reflecting the GOX species. Note the minor presence of the GO glycan species.

Figure 21 shows intact mass analysis of the 5Fl1 LEX\(^{\text{opt}}\) mAb compositions produced in transgenic *L. minor* comprising the MDXAO4 construct. When XyIT and FucT expression are suppressed in *L. minor*, the intact mAb composition contains only GOJV-glycans. In addition, the composition is substantially homogeneous for the GO glycoform (peak 2), wherein both glycosylation sites are occupied by the GOiV-glycan species, with two
minor peaks reflecting trace amounts of precursor glycoforms (peak 1, showing mAb having an Fc region wherein the C\textsubscript{H}2 domain of one heavy chain has a GO glycan species attached to Asn 297, and the C\textsubscript{H}2 domain of the other heavy chain is unglycosylated; and peak 3, showing mAb having an Fc region wherein the Asn 297 glycosylation site on each of the C\textsubscript{H}2 domains has a GO glycan species attached, with a third GO glycan species attached to an additional glycosylation site within the mAb structure).

Figure 22 shows glycan mass analysis of the heavy chain of the 5FL1 LEX\textsuperscript{Opt} mAb produced in transgenic \textit{L. minor} comprising the MDXA04 construct. When XyIT and FucT expression are suppressed in \textit{L. minor}, the only readily detectable iV-glycan species attached to the Asn 297 glycosylation sites of the C\textsubscript{H}2 domains of the heavy chains is GO.

**Detailed Description of the Invention**

The present invention provides antibody compositions that bind specifically to CD30 and/or CD30 expressing cells with high affinity. In one embodiment, the antibodies of the invention lack fucosyl residues on the antibody carbohydrate chains. In another embodiment, the antibodies of the invention lack xylosyl residues on the antibody carbohydrate. In yet another embodiment, the antibodies of the invention lack both fucosyl and xylosyl residues on the antibody carbohydrate. In yet another embodiment, the antibodies of the invention comprise a substantially homogeneous G0N-glycosylation profile. Furthermore, the antibodies exhibit enhanced antibody directed cellular cytotoxic (ADCC) killing of CD30+ cells. In one embodiment, the antibodies of the present invention are fully human antibodies and are particularly useful for the therapeutic treatment in humans of disorders associated with CD30 expressing cells. Methods of using anti-CD30 antibodies for therapeutic treatment (e.g., to treat and/or prevent diseases associated with expression of CD30) are also encompassed by the invention.

In order that the present invention may be more readily understood, certain terms will be defined as follows. Additional definitions are set forth throughout the detailed description.

The terms "CD30" and "CD30 antigen" are used interchangeably herein, and include any variants, isoforms and species homologs of human CD30 which are naturally expressed by cells. The complete amino acid sequence of human CD30 protein has the Genbank accession number NP_001234. The complete cDNA sequence encoding the human CD30 protein has the Genbank accession number NM_001243.
As used herein, the terms "antibody that lacks fucosyl residues", "defucosylated antibody," and "nonfucosylated antibody" are used interchangeably and are intended to refer to an antibody in which the carbohydrate portion of the antibody does not contain a fucosyl residue or from which the fucosyl residue has been removed. An antibody that lacks fucosyl residues can be generated, for example, by expression of the antibody in a cell or expression system that minimizes or does not attach fucosyl residues to the antibody carbohydrate chain, or by chemical modification of the antibody to remove fucosyl residues from the carbohydrate chain (e.g., treatment of the antibody with a fucosidase). As such, the terms "lacks fucosyl residues" and "defucosylated" are not intended to be limited by the mechanism by which the antibody with altered carbohydrate structure is prepared.

As used herein, the terms "antibody that lacks xylosyl residues," "dexylosylated antibody," and "nonxylosylated antibody" are used interchangeably and are intended to refer to an antibody in which the carbohydrate portion of the antibody does not contain a xylosyl residue or from which the xylosyl residue has been removed. An antibody that lacks xylosyl residues can be generated, for example, by expression of the antibody in a cell or expression system that minimizes or does not attach xylosyl residues to the antibody carbohydrate chain, or by chemical modification of the antibody to remove xylosyl residues from the carbohydrate chain (e.g., treatment of the antibody with a xylosidase). As such, the terms "lacks xylosyl residues" and "dexylosylated" are not intended to be limited by the mechanism by which the antibody with altered carbohydrate structure is prepared.

As used herein, the term "antibody expressing fucosyl residues" and "fucosylated antibody" are used interchangeably and are intended to refer to an antibody in which the carbohydrate portion of the antibody contains fucosyl.

As used herein, the term "antibody expressing xylosyl residues" and "xylosylated antibody" are used interchangeably and are intended to refer to an antibody in which the carbohydrate portion of the antibody contains xylosyl.

For purposes of the present invention, the terms "N-glycan," "N-linked glycan," and "glycan" are used interchangeably and refer to an N-linked oligosaccharide, e.g., one that is or was attached by an N-acetylglucosamine (GlcNAc) residue linked to the amide nitrogen of an asparagine residue in a protein. The predominant sugars found on glycoproteins are glucose, galactose, mannose, fucosyl, N-acetylglucosamine (GaINAc), N-acetylgalactosamine (GaINAc), and sialic acid (e.g., N-acetyl-neuraminic acid (NeuAc)). The processing of the sugar groups occurs cotranslationally in the lumen of the ER and continues in the Golgi apparatus for N-linked glycoproteins.
The N-glycans attached to glycoproteins differ with respect to the number of branches (antennae) comprising peripheral sugars (e.g., GlcNAc, galactose, fucosyl, and sialic acid) that are added to the trimannose core structure. N-glycans are commonly classified according to their branched constituents (e.g., complex, high mannose, or hybrid). A "complex" type N-glycan typically has at least one GlcNAc attached to the 1,3 mannose arm and at least one GlcNAc attached to the 1,6 mannose arm of a "trimannose" core. Where one GlcNAc is attached to each mannose arm, the species of N-linked glycan is denoted herein as "GlcNAc2Man3GlcNAc2" or "GnGn." Where only one GlcNAc is attached, the N-glycan species is denoted herein as "GlcNAc1Man3GlcNAc2," wherein the GlcNAc is attached to either the 1,3 mannose arm (denoted "NGn" herein) or the 1,6 mannose arm (denoted "GnM" herein) (see Figure 30). Complex N-glycans may also have galactose ("Gal") or N-acetylgalactosamine ("GalNAc") sugar residues that are optionally modified with sialic acid or derivatives (e.g., "NeuAc," where "Neu" refers to neuraminic acid and "Ac" refers to acetyl). Where a galactose sugar residue is attached to each GlcNAc on each mannose arm, the species of N-linked glycan is denoted herein as "Gal2GlcNAc2Man3GlcNAc2." Complex N-glycans may also have intrachain substitutions comprising "bisecting" GlcNAc and core fucosyl ("Fuc"). Complex N-glycans may also have multiple antennae on the "trimannose core," often referred to as "multiple antennary glycans." A "high mannose" type N-glycan has five or more mannose residues. A "hybrid" N-glycan has at least one GlcNAc on the terminal of the 1,3 mannose arm of the trimannose core and zero or more mannoses on the 1,6 mannose arm of the trimannose core.

The terms "GO glycan" and "GO glycan structure" and "GO glycan species" are used interchangeably and are intended to mean the complex N-linked glycan having the GlcNAc2Man3GlcNAc2 structure, wherein no terminal sialic acids (NeuAcs) or terminal galactose (Gal) sugar residues are present. If a GO glycan comprises a fucosyl ("Fuc") residue attached to the trimannose core structure, it is referred to herein as a "GOF3 glycan" (having the plant-specific α1,3-linked fucosyl residue) or "GOF6 glycan" (having the mammalian α1,6-linked fucosyl residue). hi plants, a GO glycan comprising the plant-specific β1,2-linked xylosyl residue attached to the trimannose core structure is referred to herein as a "GOX glycan," and a GO glycan comprising both the plant-specific β1,2-linked xylosyl residue and plant-specific α1,3-linked fucosyl residue attached to the trimannose core structure is referred to herein as a "GOFXF3 glycan."
The terms "substantially homogeneous," "substantially uniform," "substantially a single glycoform," and "substantial homogeneity" in the context of a glycosylation profile for a glycoprotein composition or glycoprotein product are used interchangeably and are intended to mean a glycosylation profile wherein at least 80%, at least 85%, at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or at least 99% of the total N-glycan species within the profile are represented by one desired N-glycan species, with a trace amount of precursor N-glycan species appearing in the profile. By "trace amount" is intended that any given precursor N-glycan species that is present in the glycosylation profile is present at less than 5%, preferably less than 4%, less than 3%, less than 2%, less than 1%, and even less than 0.5% or even less than 0.1% of the total amount of N-glycan species appearing in the profile. By "precursor" N-glycan species is intended an N-glycan species that is incompletely processed. Examples of precursor N-glycan species present in trace amounts in the glycoprotein compositions or glycoprotein products of the invention, and thus appearing in the glycosylation profiles thereof, are the Man3GlcNAc2, MGn (GlcNaclMan3GlcNAc2 wherein GlcNacl is attached to the 1,3 mannose arm), and GnM (GlcNaclMan3GlcNAc2 wherein GlcNacl is attached to the 1,6 mannose arm) precursor N-glycan species described above.

Thus, for example, where the desired N-glycan species within a glycoprotein product or composition is GO, a substantially homogeneous glycosylation profile for that product or composition would be one wherein at least 80%, 80%, at least 85%, at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or at least 99% of the total amount of N-glycan species appearing in the glycosylation profile for the product or composition is represented by the GO glycan species, with a trace amount of precursor N-glycan species appearing in the glycosylation profile. For such a composition, a representative precursor N-glycan species appearing in its glycosylation profile would be the Man3GlcNAc2, MGn (GlcNaclMan3GlcNAc2 wherein GlcNacl is attached to the 1,3 mannose arm), and GnM (GlcNaclMan3GlcNAc2 wherein GlcNacl is attached to the 1,6 mannose arm) precursor N-glycan species described above.

The term "glyco-optimized" refers to an antibody having a particular N-glycan structure that produces certain desirable properties, including but not limited to, enhanced antibody-dependent cell-mediated cytotoxicity (ADCC) and effector cell receptor binding activity when compared to CHO-expressed antibodies.

The term "duckweed" refers to members of the family Lemnaceae. This family currently is divided into five genera and 38 species of duckweed as follows: genus Lemna (L.

The term "immune response" refers to the action of, for example, lymphocytes, antigen-presenting cells, phagocytic cells, granulocytes, and soluble macromolecules produced by the above cells or the liver (including antibodies, cytokines, and complement) that results in selective damage to, destruction of, or elimination from the human body of invading pathogens, cells or tissues infected with pathogens, cancerous cells, or, in cases of autoimmunity or pathological inflammation, normal human cells or tissues.

A "signal transduction pathway" refers to the biochemical relationship between various of signal transduction molecules that play a role in the transmission of a signal from one portion of a cell to another portion of a cell. As used herein, the phrase "cell surface receptor" includes, for example, molecules and complexes of molecules capable of receiving a signal and the transmission of such a signal across the plasma membrane of a cell. An example of a "cell surface receptor" of the present invention is the CD30 receptor.

As used herein, the term "effector cell" refers to an immune cell which is involved in the effector phase of an immune response, as opposed to the cognitive and activation phases of an immune response. Exemplary immune cells include a cell of a myeloid or lymphoid origin, e.g., lymphocytes (e.g., B cells and T cells including cytolytic T cells (CTLs)), killer cells, natural killer cells, macrophages, monocytes, eosinophils, neutrophils, polymorphonuclear cells, granulocytes, mast cells, and basophils. Some effector cells express specific Fc receptors and carry out specific immune functions. In preferred embodiments, an effector cell is capable of inducing antibody-dependent cell-mediated cytotoxicity (ADCC), e.g., a neutrophil capable of inducing ADCC. For example, monocytes and macrophages, which express FcR are involved in specific killing of target cells and presenting antigens to other components of the immune system, or binding to cells that
present antigens. In other embodiments, an effector cell can phagocytose a target antigen or target cell. The expression of a particular FcR on an effector cell can be regulated by humoral factors such as cytokines. For example, expression of FcαRI has been found to be up-regulated by G-CSF or GM-CSF. This enhanced expression increases the effector function of FcαRI-bearing cells against targets. An effector cell can phagocytose or lyse a target antigen or a target cell.

"Target cell" refers to any cell or pathogen whose elimination would be beneficial in a subject (e.g., a human or animal) and that can be targeted by a composition (e.g., antibody) of the invention. For example, the target cell can be a cell expressing or overexpressing CD30.

The term "antibody-dependent cellular cytotoxicity" or "ADCC" refers to a cell-mediated cytotoxic reaction in which a CD30+ target cell with bound anti-CD30 antibody is recognized by an effector cell bearing Fc receptors and is subsequently lysed without requiring the involvement of complement.

As used herein, the term "enhances ADCC" (e.g., referring to cells) is intended to include any measurable increase in cell lysis when contacted with an anti-CD30 antibody lacking fucosyl and xylosyl residues as compared to the cell killing of the same cell in contact with a fucosylated and xylosylated anti-CD30 antibody in the presence of effector cells (for example, at a ratio of target cells:effector cells of 1:50), e.g., an increase in cell lysis by at least about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 150%, 200%, 250%, 300%, or 325%.

The term "antibody" as referred to herein includes whole antibodies and any antigen binding fragment (i.e., "antigen-binding portion") or single chains thereof. An "antibody" refers to a glycoprotein comprising at least two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds, or an antigen binding portion thereof. Each heavy chain is comprised of a heavy chain variable region (abbreviated herein as V\textsubscript{H}) and a heavy chain constant region. The heavy chain constant region is comprised of three domains, C\textsubscript{H1}, C\textsubscript{H2} and C\textsubscript{H3}. Each light chain is comprised of a light chain variable region (abbreviated herein as V\textsubscript{L}) and a light chain constant region. The light chain constant region is comprised of one domain, C\textsubscript{L}. The V\textsubscript{H} and V\textsubscript{L} regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDR), interspersed with regions that are more conserved, termed framework regions (FR). Each V\textsubscript{H} and V\textsubscript{L} is composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus.
in the following order: FRI, CDR1, FR2, CDR2, FR3, CDR3, FR4. The variable regions of
the heavy and light chains contain a binding domain that interacts with an antigen. The
constant regions of the antibodies may mediate the binding of the immunoglobulin to host
tissues or factors, including various cells of the immune system (e.g., effector cells) and the
first component (Clq) of the classical complement system.

The term "antigen-binding portion" of an antibody (or simply "antibody portion"), as
used herein, refers to one or more fragments of an antibody that retain the ability to
specifically bind to an antigen (e.g., CD30). It has been shown that the antigen-binding
function of an antibody can be performed by fragments of a full-length antibody. Examples
of binding fragments encompassed within the term "antigen-binding portion" of an antibody
include (i) a Fab fragment, a monovalent fragment consisting of the V_L, V_H, C_L and C_H I
domains; (ii) a F(ab')_2 fragment, a bivalent fragment comprising two Fab fragments linked by
da disulfide bridge at the hinge region; (iii) a Fab' fragment, which is essentially an Fab with
part of the hinge region (see, FUNDAMENTAL IMMUNOLOGY (Paul ed., 3.sup.rd ed.
1993); (iv) a Fd fragment consisting of the V_H and C_H I domains; (v) a Fv fragment
consisting of the V_L and V_H domains of a single arm of an antibody, (vi) a dAb fragment
(Ward et al., (1989) Nature 341:544-546), which consists of a V_H domain; (vii) an isolated
complementarity determining region (CDR); and (viii) a nanobody, a heavy chain variable
region containing a single variable domain and two constant domains. Furthermore, although
the two domains of the Fv fragment, V_L and V_H, are coded for by separate genes, they can be
joined, using recombinant methods, by a synthetic linker that enables them to be made as a
single protein chain in which the V_L and V_H regions pair to form monovalent molecules
(known as single chain Fv (scFv); see e.g., Bird et al. (1988) Science 242:423-426; and
are also intended to be encompassed within the term "antigen-binding portion" of an
antibody. These antibody fragments are obtained using conventional techniques known to
those with skill in the art, and the fragments are screened for utility in the same manner as are
intact antibodies.

The term "recombinant human antibody", as used herein, includes all human
antibodies that are prepared, expressed, created or isolated by recombinant means, such as (a)
antibodies isolated from an animal (e.g., a mouse) that is transgenic or transchromosomal for
human immunoglobulin genes or a hybridoma prepared therefrom (described further below),
(b) antibodies isolated from a host cell transformed to express the human antibody, e.g., from
a transfectoma, (c) antibodies isolated from a recombinant, combinatorial human antibody
library, and (d) antibodies prepared, expressed, created or isolated by any other means that involve splicing of human immunoglobulin gene sequences to other DNA sequences. Such recombinant human antibodies have variable regions in which the framework and CDR regions are derived from human germline immunoglobulin sequences. In certain embodiments, however, such recombinant human antibodies can be subjected to in vitro mutagenesis (or, when an animal transgenic for human Ig sequences is used, in vivo somatic mutagenesis) and thus the amino acid sequences of the \( V_H \) and \( V_L \) regions of the recombinant antibodies are sequences that, while derived from and related to human germline \( V_H \) and \( V_L \) sequences, may not naturally exist within the human antibody germline repertoire in vivo.

The terms "monoclonal antibody" or "monoclonal antibody composition" as used herein refer to a preparation of antibody molecules of single molecular composition. A monoclonal antibody composition displays a single binding specificity and affinity for a particular epitope.

The phrases "an antibody recognizing an antigen" and "an antibody specific for an antigen" are used interchangeably herein with the term "an antibody which binds specifically to an antigen."

The term "human antibody derivatives" refers to any modified form of the human antibody, e.g., a conjugate of the antibody and another agent or antibody.

The term "human antibody", as used herein, is intended to refer to antibodies having variable regions in which both the framework and CDR regions are derived from human germline immunoglobulin sequences. Furthermore, if the antibody contains a constant region, the constant region also is derived from human germline immunoglobulin sequences. The human antibodies of the invention may include amino acid residues not encoded by human germline immunoglobulin sequences (e.g., mutations introduced by random or site-specific mutagenesis in vitro or by somatic mutation in vivo).

The term "human monoclonal antibody" refers to antibodies displaying a single binding specificity which have variable regions in which both the framework and CDR regions are derived from human germline immunoglobulin sequences. In one embodiment, the human monoclonal antibodies are produced by a hybridoma which includes a B cell obtained from a transgenic nonhuman animal, e.g., a transgenic mouse, having a genome comprising a human heavy chain transgene and a light chain transgene fused to an immortalized cell. The term "human monoclonal antibody", as used herein, also includes all human antibodies that are prepared, expressed, created or isolated by recombinant means, such as (a) antibodies isolated from an animal (e.g., a mouse) that is transgenic or
transchromosomal for human immunoglobulin genes or a hybridoma prepared therefrom (described further below), (b) antibodies isolated from a host cell transformed to express the human antibody, e.g., from a transfectoma, (c) antibodies isolated from a recombinant, combinatorial human antibody library, and (d) antibodies prepared, expressed, created or isolated by any other means that involve splicing of human immunoglobulin gene sequences to other DNA sequences. Such recombinant human antibodies have variable regions in which the framework and CDR regions are derived from human germline immunoglobulin sequences. In certain embodiments, however, such recombinant human antibodies can be subjected to in vitro mutagenesis (or, when an animal transgenic for human Ig sequences is used, in vivo somatic mutagenesis) and thus the amino acid sequences of the $V_H$ and $V_L$ regions of the recombinant antibodies are sequences that, while derived from and related to human germline $V_H$ and $V_L$ sequences, may not naturally exist within the human antibody germline repertoire in vivo.

An "isolated antibody," as used herein, is intended to refer to an antibody which is substantially free of other antibodies having different antigenic specificities (e.g., an isolated antibody that specifically binds to CD30 is substantially free of antibodies that specifically bind antigens other than CD30). An isolated antibody that specifically binds to an epitope, isoform or variant of human CD30 may, however, have cross-reactivity to other related antigens, e.g., from other species (e.g., CD30 species homologs). Moreover, an isolated antibody may be substantially free of other cellular material and/or chemicals. In one embodiment of the invention, a combination of "isolated" monoclonal antibodies having different specificities are combined in a well defined composition.

The term "humanized antibody" is intended to refer to antibodies in which CDR sequences derived from the germline of another mammalian species, such as a mouse, have been grafted onto human framework sequences. Additional framework region modifications may be made within the human framework sequences.

The term "chimeric antibody" is intended to refer to antibodies in which the variable region sequences are derived from one species and the constant region sequences are derived from another species, such as an antibody in which the variable region sequences are derived from a mouse antibody and the constant region sequences are derived from a human antibody.

As used herein, an antibody that "specifically binds to human CD30" is intended to refer to an antibody that binds to human CD30 with a $K_D$ of $1 \times 10^7$ M or less, more
preferably 5 x 10⁻⁸ M or less, more preferably 3 x 10⁻⁸ M or less, more preferably 1 x 10⁻⁸ M or less, even more preferably 5 x 10⁻⁹ M or less.

The term "does not substantially bind" to a protein or cells, as used herein, means does not bind or does not bind with a high affinity to the protein or cells, i.e. binds to the protein or cells with a K_D of 1 x 10⁻⁶ M or more, more preferably 1 x 10⁻⁵ M or more, more preferably 1 x 10⁻⁴ M or more, more preferably 1 x 10⁻³ M or more, even more preferably 1 x 10⁻² M or more.

The term "K_{ass}" or "K_a" as used herein, is intended to refer to the association rate of a particular antibody-antigen interaction, whereas the term "K_{dis}" or "K_{diss}" as used herein, is intended to refer to the dissociation rate of a particular antibody-antigen interaction. The term "K_D," as used herein, is intended to refer to the dissociation constant, which is obtained from the ratio of Kato K_a (i.e., K_a/K_{dis}) and is expressed as a molar concentration (M). K_D values for antibodies can be determined using methods well established in the art. A preferred method for determining the K_D of an antibody is by using surface plasmon resonance, preferably using a biosensor system such as a Biacore® system.

As used herein, the term "high affinity" for an IgG antibody refers to an antibody having a K_D of 1 x 10⁻⁷ M or less, more preferably 5 x 10⁻⁸ M or less, even more preferably 1x10⁻⁸ M or less, even more preferably 5 x 10⁻⁹ M or less and even more preferably 1 x 10⁻⁹ M or less for a target antigen. However, "high affinity" binding can vary for other antibody isotypes. For example, "high affinity" binding for an IgM isotype refers to an antibody having a K_D of 10⁻⁸ M or less, more preferably 10⁻⁷ M or less, even more preferably 10⁻⁸ M or less.

The term "epitope" means a protein determinant capable of specific binding to, or specific binding by, an antibody. Epitopes usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and usually have specific three dimensional structural characteristics, as well as specific charge characteristics. Conformational and nonconformational epitopes are distinguished in that the binding to the former but not the latter is lost in the presence of denaturing solvents.

As used herein, "specific binding" refers to antibody binding to a predetermined antigen. Typically, the antibody binds with a dissociation constant (K_D) of 10⁻⁷ M or less, and binds to the predetermined antigen with a K_D that is at least two-fold less than its K_D for binding to a non-specific antigen (e.g., BSA, casein) other than the predetermined antigen or a closely-related antigen. The phrases "an antibody recognizing an antigen" and "an antibody
specific for an antigen" are used interchangeably herein with the term "an antibody which binds specifically to an antigen".

As used herein, "isotype" refers to the antibody class (e.g., IgM or IgGl) that is encoded by heavy chain constant region genes.

The term "vector," as used herein, is intended to refer to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid", which refers to a circular double-stranded DNA loop into which additional DNA segments may be ligated. Another type of vector is a viral vector, wherein additional DNA segments may be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (e.g., bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (e.g., non-episomal mammalian vectors) can be integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "recombinant expression vectors" (or simply, "expression vectors"). In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. In the present specification, "plasmid" and "vector" may be used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors, such as viral vectors (e.g., replication defective retroviruses, adenoviruses and adeno-associated viruses), which serve equivalent functions.

The term "recombinant host cell" (or simply "host cell"), as used herein, is intended to refer to a cell into which a recombinant expression vector has been introduced. It should be understood that such terms are intended to refer not only to the particular subject cell but to the progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term "host cell" as used herein. Recombinant host cells include, for example, Lemna cells, CHO cells, transfectomas, and lymphocytic cells.

As used herein, the term "subject" includes any human or nonhuman animal. The term "nonhuman animal" includes all vertebrates, e.g., mammals and non-mammals, such as nonhuman primates, sheep, dogs, cats, horses, cows, chickens, amphibians, reptiles, etc.

The terms "transgenic, nonhuman animal" refers to a nonhuman animal having a genome comprising one or more human heavy and/or light chain transgenes or
txanschromosomes (either integrated or non-integrated into the animal's natural genomic DNA) and which is capable of expressing fully human antibodies. For example, a transgenic mouse can have a human light chain transgene and either a human heavy chain transgene or human heavy chain transchromosome, such that the mouse produces human anti-CD30 antibodies when immunized with CD30 antigen and/or cells expressing CD30. The human heavy chain transgene can be integrated into the chromosomal DNA of the mouse, as is the case for transgenic, e.g., HuMAb mice, or the human heavy chain transgene can be maintained extrachromosomally, as is the case for transchromosomal (e.g., KM) mice as described in WO 02/43478. Such transgenic and transchromosomal mice are capable of producing multiple isotypes of human monoclonal antibodies to CD30 (e.g., IgG, IgA and/or IgE) by undergoing V-D-J recombination and isotype switching.

Various aspects of the invention are described in further detail in the following subsections.

**Human Monoclonal Anti-CD30 Antibodies**

Preferred antibodies of the invention include human anti-CD30 monoclonal antibodies. Examples of human anti-CD30 monoclonal antibodies include the 5F11, 17Gl, and 2H9 antibodies, isolated and structurally characterized as described in PCT Publication WO 03/059282, U.S. Pat. Publ. No. 2004/0006215 and Lahive and Cockfield LLP attorney docket No. MXI-333-1, the contents of which are hereby incorporated by reference in their entirety. The \( V_H \) amino acid sequences of 5F11, 17Gl, and 2H9 are shown in SEQ ID NOs: 1, 2, and 3, respectively. The \( V_L \) amino acid sequences of 5F11, 17Gl, and 2H9 are shown in SEQ ID NOs: 4, 5, and 6, respectively.

Given that each of these antibodies can bind to CD30, the \( V_H \) and \( V_L \) sequences can be "mixed and matched" to create other anti-CD30 binding molecules of the invention. CD30 binding of such "mixed and matched" antibodies can be tested using the binding assays well known in the art, such as FACS analysis and ELISA assays. Preferably, when \( V_H \) and \( V_L \) chains are mixed and matched, a \( V_H \) sequence from a particular \( V_H/V_L \) pairing is replaced with a structurally similar \( V_H \) sequence. Likewise, preferably a \( V_L \) sequence from a particular \( V_H/V_L \) pairing is replaced with a structurally similar \( V_L \) sequence. For example, the \( V_H \) sequences of 5F11 and 2H9 are particularly amenable for mixing and matching, since these antibodies use \( V_H \) sequences derived from the same germline sequence (\( V_H \) 4-34) and thus they exhibit structural similarity.

In particular embodiments, the invention provides a defucosylated and dexylosylated monoclonal antibody, or antigen binding portion thereof, comprising:
(a) a heavy chain variable region comprising an amino acid sequence selected from
the group consisting of SEQ ID NOs: 1, 2, and 3; and
(b) a light chain variable region comprising an amino acid sequence selected from the
group consisting of SEQ ID NO: 4, 5, and 6;

wherein the antibody specifically binds human CD30.

Preferred heavy and light chain combinations include:
(a) a heavy chain variable region comprising the amino acid sequence of
SEQ ID NO: 1; and (b) a light chain variable region comprising the amino acid sequence of
SEQ ID NO: 4; or

(a) a heavy chain variable region comprising the amino acid sequence of
SEQ ID NO: 2; and (b) a light chain variable region comprising the amino acid sequence of
SEQ ID NO: 5; or

(a) a heavy chain variable region comprising the amino acid sequence of
SEQ ID NO: 3; and (b) a light chain variable region comprising the amino acid sequence of
SEQ ID NO: 6.

In another aspect, the invention provides defucosylated and dxylosylated antibodies
that comprise the heavy chain and light chain CDR1s, CDR2s and CDR3s of 5F11, 17Gl,
and 2H9, or combinations thereof. The amino acid sequences of the V<sub>H</sub> CDR1s
of 5F11, 17Gl, and 2H9 are shown in SEQ ID NOs: 7, 8, and 9, respectively. The amino acid
sequences of the V<sub>H</sub> CDR2s of 5F11, 17Gl, and 2H9 are shown in SEQ ID NOs: 10, 11, and
12, respectively. The amino acid sequences of the V<sub>H</sub> CDR3s of 5F11, 17Gl, and 2H9 are
shown in SEQ ID NOs: 13, 14, and 15, respectively. The amino acid sequences of the V<sub>k</sub>
CDR1s of 5F11, 17Gl, and 2H9 are shown in SEQ ID NOs: 16, 17, and 18, respectively. The
amino acid sequences of the V<sub>k</sub> CDR2s of 5F11, 17Gl, and 2H9 are shown in SEQ ID
NOs: 19, 20, and 21, respectively. The amino acid sequences of the V<sub>k</sub> CDR3s of 5F11, 17Gl,
and 2H9 are shown in SEQ ID NOs: 22, 23, and 24, respectively. The CDR regions
are delineated using the Kabat system (Kabat, E. A., et al. (1991) Sequences of Proteins of
Publication No. 91-3242).

Given that each of these antibodies can bind to CD30 and that antigen-binding
specificity is provided primarily by the CDR1, 2 and 3 regions, the V<sub>H</sub> CDR1, 2 and 3
sequences and V<sub>k</sub> CDR1, 2 and 3 sequences can be "mixed and matched" (i.e., CDRs from
different antibodies can be mixed and match, although each antibody must contain a V<sub>H</sub>
CDR1, 2 and 3 and a V<sub>k</sub> CDR1, 2 and 3) to create other anti-CD30 binding molecules of the
invention. CD30 binding of such "mixed and matched" antibodies can be tested using binding assays known in the art, for example, FACS analysis and ELISA assays. Preferably, when V\textsubscript{H} CDR sequences are mixed and matched, the CDR1, CDR2 and/or CDR3 sequence from a particular V\textsubscript{H} sequence is replaced with a structurally similar CDR sequence(s).

Likewise, when V\textsubscript{L} CDR sequences are mixed and matched, the CDR1, CDR2 and/or CDR3 sequence from a particular V\textsubscript{L} sequence preferably is replaced with a structurally similar CDR sequence(s). It will be readily apparent to the ordinarily skilled artisan that novel V\textsubscript{H} and V\textsubscript{L} sequences can be created by substituting one or more V\textsubscript{H} and/or V\textsubscript{L} CDR region sequences with structurally similar sequences from the CDR sequences disclosed herein for monoclonal antibodies antibodies 5Fl1, 17Gl, and 2H9.

Accordingly, in another preferred embodiment, the invention provides a defucosylated and defucosylated monoclonal antibody, or antigen binding portion thereof comprising:

(a) a heavy chain variable region CDR1 comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 7, 8, and 9;

(b) a heavy chain variable region CDR2 comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 10, 11, and 12;

(c) a heavy chain variable region CDR3 comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 13, 14, and 15;

(d) a light chain variable region CDR1 comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 16, 17, and 18;

(e) a light chain variable region CDR2 comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 19, 20, and 21; and

(f) a light chain variable region CDR3 comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 22, 23, and 24;

wherein the antibody specifically binds CD30.

In a preferred embodiment, the antibody comprises:

(a) a heavy chain variable region CDR1 comprising SEQ ID NO: 7;

(b) a heavy chain variable region CDR2 comprising SEQ ID NO: 10;

(c) a heavy chain variable region CDR3 comprising SEQ ID NO: 13;

(d) a light chain variable region CDR1 comprising SEQ ID NO: 16;

(e) a light chain variable region CDR2 comprising SEQ ID NO: 19; and

(f) a light chain variable region CDR3 comprising SEQ ID NO: 22.

In another preferred embodiment, the antibody comprises:

(a) a heavy chain variable region CDR1 comprising SEQ ID NO: 8;
(b) a heavy chain variable region CDR2 comprising SEQ ID NO: 11;
(c) a heavy chain variable region CDR3 comprising SEQ ID NO: 14;
(d) a light chain variable region CDR1 comprising SEQ ID NO: 17;
(e) a light chain variable region CDR2 comprising SEQ ID NO: 20; and
(f) a light chain variable region CDR3 comprising SEQ ID NO: 24.

In another preferred embodiment, the antibody comprises:
(a) a heavy chain variable region CDR1 comprising SEQ ID NO: 9;
(b) a heavy chain variable region CDR2 comprising SEQ ID NO: 12;
(c) a heavy chain variable region CDR3 comprising SEQ ID NO: 15;
(d) a light chain variable region CDR1 comprising SEQ ID NO: 18;
(e) a light chain variable region CDR2 comprising SEQ ID NO: 21; and
(f) a light chain variable region CDR3 comprising SEQ ID NO: 24.

It is well known in the art that the CDR3 domain, independently from the CDR1 and/or CDR2 domain(s), alone can determine the binding specificity of an antibody for a cognate antigen and that multiple antibodies can predictably be generated having the same binding specificity based on a common CDR3 sequence. See, for example, Klimka et al, British J. of Cancer 83(2):252-260 (2000) (describing the production of a humanized anti-CD30 antibody using only the heavy chain variable domain CDR3 of murine anti-CD30 antibody Ki-4); Beiboer et al, J. Mol Biol. 296:833-849 (2000) (describing recombinant epithelial glycoprotein-2 (EGP-2) antibodies using only the heavy chain CDR3 sequence of the parental murine MOC-31 anti-EGP-2 antibody); Rader et al., Proc. Natl. Acad. Sd. U.S.A. 95_:8910-8915 (1998) (describing a panel of humanized anti-integrin αβ3 antibodies using a heavy and light chain variable CDR3 domain of a murine anti-integrin αβ3 antibody LM609 wherein each member antibody comprises a distinct sequence outside the CDR3 domain and capable of binding the same epitope as the parent muring antibody with affinities as high or higher than the parent murine antibody); Barbas et al., J. Am. Chem. Soc. 11^:2161-2162 (1994) (disclosing that the CDR3 domain provides the most significant contribution to antigen binding); Barbas et al, Proc. Natl. Acad. ScL U.S.A. 92:2529-2533 (1995) (describing the grafting of heavy chain CDR3 sequences of three Fabs (SI-I, SI-40, and SI-32) against human placental DNA onto the heavy chain of an anti-tetanus toxoid Fab thereby replacing the existing heavy chain CDR3 and demonstrating that the CDR3 domain alone conferred binding specificity); and Ditzel et al, J. Immunol 157:739-749 (1996) (describing grafting studies wherein transfer of only the heavy chain CDR3 of a parent polyspecific Fab LNA3 to a heavy chain of a monospecific IgG tetanus toxoid-binding Fab
p313 antibody was sufficient to retain binding specificity of the parent Fab). Each of these
references is hereby incorporated by reference in its entirety.

Accordingly, the present invention provides monoclonal antibodies comprising one
or more heavy and/or light chain CDR3 domains from an antibody derived from a human or
non-human animal, wherein the monoclonal antibody is capable of specifically binding to
CD30. Within certain aspects, the present invention provides monoclonal antibodies
comprising one or more heavy and/or light chain CDR3 domain from a non-human antibody,
such as a mouse or rat antibody, wherein the monoclonal antibody is capable of specifically
binding to CD30. Within some embodiments, such inventive antibodies comprising one or
more heavy and/or light chain CDR3 domain from a non-human antibody (a) are capable of
competing for binding with; (b) retain the functional characteristics; (c) bind to the same
epitope; and/or (d) have a similar binding affinity as the corresponding parental non-human
antibody.

Within other aspects, the present invention provides monoclonal antibodies
comprising one or more heavy and/or light chain CDR3 domain from a human antibody,
such as, for example, a human antibody obtained from a non-human animal, wherein the
human antibody is capable of specifically binding to CD30. Within other aspects, the present
invention provides monoclonal antibodies comprising one or more heavy and/or light chain
CDR3 domain from a first human antibody, such as, for example; a human antibody obtained
from a non-human animal, wherein the first human antibody is capable of specifically
binding to CD30 and wherein the CDR3 domain from the first human antibody replaces a
CDR3 domain in a human antibody that is lacking binding specificity for CD30 to generate a
second human antibody that is capable of specifically binding to CD30. Within some
embodiments, such inventive antibodies comprising one or more heavy and/or light chain
CDR3 domain from the first human antibody (a) are capable of competing for binding with;
(b) retain the functional characteristics; (c) bind to the same epitope; and/or (d) have a similar
binding affinity as the corresponding parental first human antibody. In preferred
embodiments, the first human antibody is 5Fl 1, 17Gl or 2H9.

Anti-CD30 Antibodies Lacking Fucosyl and Xylosyl Residues and Having Enhanced ADCC
Activity

The present invention also relates to a defucosylated and dexylosylated anti-CD30
antibody with enhanced antibody directed cellular cytotoxicity (ADCC) against cells
expressing CD30 as compared to the fucosylated and xylosylated form of the antibody. In a
preferred embodiment, a defucosylated and dexylosylated antibody of the invention induces
ADCC of L1236 cells *in vitro* wherein the fucosylated and xylosylated form of the antibody does not induce ADCC, under conditions of an antibody concentration of 0.1 µg/ml and a target cell to effector cell ratio of 1:50. In another preferred embodiment, a defucosylated and dexylosylated antibody of the invention enhances ADCC of L540, L428 and Karpas cells *in vitro* compared to the fucosylated and xylosylated form of the antibody, under conditions of an antibody concentration of 0.1 µg/ml and a target cell to effector cell ratio of 1:50.

The increased ADCC activity of a defucosylated and dexylosylated antibody of the invention can be quantitated, for example, as an increase in percent cell lysis, as compared to the fucosylated and xylosylated form of the antibody, when ADCC activity is measured under the same conditions for the two forms (*e.g.*, same antibody concentrations and same target to effector cell ratios). Preferably, a defucosylated and dexylosylated anti-CD30 antibody of the invention increases the percent lysis of CD30+ cells as compared to the fucosylated form of the antibody at least 1.25 fold (i.e., the ratio of the % lysis of the defucosylated and dexylosylated form to the fucosylated and xylosylated form is at least 1.25), more preferably at least 2 fold, even more preferably at least 2.5 fold and even more preferably at least 3 fold. In various embodiments, the defucosylated and dexylosylated form of the antibody increases percent lysis of CD30+ cells as compared to the fucosylated form of the antibody from 1.25 to 3.25 fold, preferably 1.5 to 3.25 fold, even more preferably 1.61 to 3.25 fold, even more preferably 2.15 to 3.25 fold, and even more preferably 2.63 to 3.25 fold, preferably under conditions where the antibody is at a concentration of 25 µg/ml and the target to effector cell ratio is 1:50.

Additionally or alternatively, the increased ADCC activity of a defucosylated and dexylosylated antibody of the invention can be quantitated, for example, as an increased potency as measured by a decrease in the EC50 value for the defucosylated and dexylosylated form, as compared to the fucosylated and xylosylated form. This can be quantitated by the ratio of the EC50 for the fucosylated and xylosylated form to the defucosylated and dexylosylated form. Preferably, the EC50 ratio of the fucosylated and xylosylated form to the defucosylated and dexylosylated form for ADCC of CD30+ cells is at least 3 (*i.e.*, the EC50 of the defucosylated and dexylosylated form is 3-fold lower than the EC50 of the fucosylated and xylosylated form), more preferably, at least 4, even more preferably at least 5, at least 7, at least 10, at least 15 or at least 20. In various embodiments, the EC50 ratio of the fucosylated and xylosylated form to the defucosylated and dexylosylated form for ADCC of CD30+ cells is from 2 to 27.1, more preferably from 4 to 27.1, even more preferably from
4.7 to 27.1, even more preferably from 7.8 to 27.1, and even more preferably from 11.1 to 27.1. Preferably, the EC50 values are determined in ADCC assays that use a target to effector cell ratio of 1:50 and antibody concentrations from 0.0001 µg/ml to 10 µg/ml or higher.

Examples of CD30+ cell lines that can be used in the ADCC assays of the invention and against which a defucosylated and dexylosylated antibody of the invention exhibits enhanced ADCC activity, as compared to the fucosylated and xylosylated form of the antibody, include L540 cells (human Hodgkin's lymphoma; DSMZ Deposit No. ACC 72), L428 cells (human Hodgkin's lymphoma; DSMZ Deposit No. ACC 197), L1236 cells (human Hodgkin's lymphoma; DSMZ Deposit No. ACC 530), and Karpas cells (human T cell lymphoma; DSMZ Deposit No. ACC 31). The enhanced ADCC effect by defucosylated and dexylosylated anti-CD30 antibodies may result in ADCC activity on CD30+ cells at antibody concentrations where ADCC would not be observed with the fucosylated and xylosylated form of the antibody. For example, in an in vitro ADCC assay with a target-effector cell ratio of 1:50, ADCC due to a defucosylated and dexylosylated anti-CD30 antibody is observed with the CD30+ cell line L1236 at concentrations as low as 0.005 µg/ml, whereas no ADCC activity is detected with the fucosylated and xylosylated anti-CD30 antibody at concentrations as high as 0.1 µg/ml.

Defucosylation and Dexylosylation of Anti-CD30 Antibodies

Anti-CD30 antibodies (e.g., murine, chimeric, humanized and human antibodies) are known in the art, and may be used in the present invention. In one embodiment, the anti-CD30 antibody of the present invention is modified such that the antibody is lacking in fucosyl residues. An antibody can be made that is lacking in fucosyl residues by one of a variety of methods. For example, the antibody can be expressed, using recombinant DNA technology, in a cell with an altered glycosylation mechanism such that addition of fucosyl residues to carbohydrate chains is inhibited. Additionally or alternatively, an antibody can be defucosylated through chemical removal of the fucosyl residue.

In one embodiment, the antibody is expressed in a cell that is lacking in a fucosyltransferase enzyme such that the cell line produces proteins lacking fucosyl in their carbohydrates. For example, the cell lines Ms704, Ms705, and Ms709 lack the fucosyltransferase gene, FUT8 (alpha (1,6) fucosyltransferase), such that antibodies expressed in the Ms704, Ms705, and Ms709 cell lines lack fucosyl on their carbohydrates. The Ms704, Ms705, and Ms709 FUTS−/− cell lines were created by the targeted disruption of
the FUT8 gene in CHO/DG44 cells using two replacement vectors (see U.S. Patent Publication No. 200401 10704 by Yamane et al. and Yamane-Ohnuki et al. (2004) Biotechnol Bioeng 87:614-22). As another example, EP 1,176,195 by Hanai et al. describes a cell line with a functionally disrupted FUT8 gene, which encodes a fucosyl transferase, such that antibodies expressed in such a cell line exhibit hypofucosylation by reducing or eliminating the alpha 1,6 bond-related enzyme. Hanai et al also describe cell lines which naturally have a low enzyme activity for adding fucosyl to the N-acetylglucosamine that binds to the Fc region of the antibody or does not have the enzyme activity, for example the rat myeloma cell line YB2/0 (ATCC CRL 1662). PCT Publication WO 03/035835 by Presta describes a variant CHO cell line, Led 3 cells, with reduced ability to attach fucosyl to Asn(297)-linked carbohydrates, also resulting in hypofucosylation of antibodies expressed in that host cell (see also Shields, RX. et al. (2002) J. Biol. Chem. 277:26733-26740). PCT Publication WO 99/54342 by Umana et al. describes cell lines engineered to express glycoprotein-modifying glycosyl transferases (e.g., beta(1,4)-N-acetylglucosaminyltransferase III (GnTHI)) such that antibodies expressed in the engineered cell lines exhibit increased bisecting GlcNac structures which results in increased ADCC activity of the antibodies (see also Umana et al. (1999) Nat. Biotech. 17:176-180).

In another embodiment, an anti-CD30 antibody is expressed and the fucosyl residue(s) is cleaved using a fucosidase enzyme. For example, the fucosidase alpha-L-fucosidase removes fucosyl residues from antibodies (Tarentino, A.L. et al. (1975) Biochem. 14:5516-23).

Additionally, in other embodiments, other forms of glycosylation of an antibody are also modified. For example, an aglycosylated antibody can be made (i.e., the antibody lacks glycosylation). Such carbohydrate modifications can be accomplished by, for example, altering one or more sites of glycosylation within the antibody sequence. For example, one or more amino acid substitutions can be made that result in elimination of one or more variable region framework glycosylation sites to thereby eliminate glycosylation at that site. Such aglycosylation may increase the affinity of the antibody for antigen. Such an approach is described in further detail in U.S. Patent Nos. 5,714,350 and 6,350,861 by Co et al.

In another embodiment, the anti-CD30 antibody of the present invention is modified such that the antibody is lacking in fucosyl and xylosyl residues. An antibody can be made that is lacking in fucosyl and xylosyl residues by one of a variety of methods. For example, the antibody can be expressed, using recombinant DNA technology, in a plant cell with an altered glycosylation mechanism such that addition of fucosyl and xylosyl residues to
carbohydrate chains is inhibited. Additionally or alternatively, an antibody can be defucosylated and dxylosylated through chemical removal of the fucosyl and xylosyl residue.

A number of plant species have been targeted for use in "molecular farming" of mammalian proteins of pharmaceutical interest. Higher plants, particularly higher plants that serve as expression systems for recombinant proteins for pharmaceutical use, that have been stably transformed to produce glycoproteins with an altered 7V-glycosylation pattern using the methods described herein may be genetically modified to produce any recombinant protein of interest. Thus, in one aspect, the invention provides methods for producing monoclonal antibodies in higher plants, wherein the monoclonal antibodies have an iV-glycosylation pattern that reflects a reduction in the amount of β\(1\),2-linked xylosyl residues and α\(l\),3-linked fucosyl residues within the N-linked glycans, and compositions comprising recombinant monoclonal antibodies produced using plant hosts genetically modified in the manner set forth herein. Methods for production of antibodies in a plant system are disclosed in _________[Alston & Bird LLP attorney docket No.: 040989/322372] and _________[Alston & Bird LLP attorney docket No.: 040989/322364], filed on even date herewith, both of which are expressly incorporated herein by reference. In some embodiments, the plant host of interest is a member of the duckweed family. In some embodiments, the plant serving as the host for recombinant production of the monoclonal antibody is a member of the Lemnaceae family, for example, a Lemma plant. Lemma is a small, aquatic, higher plant that has been developed for the production of recombinant therapeutic proteins. The Lemma Expression System (LEX System\textsuperscript{SM}) enables rapid, clonal expansion of transgenic plants, secretion of transgenic proteins, high protein yields, full containment, and has the additional advantage of low operating and capital costs. Numerous proteins including mAbs have been successfully produced in Lemma with expression levels routinely in the range of 6-8% of the total soluble protein (TSP). These expression levels, in combination with Lemma's high protein content and fast growth rate (36 hr doubling time), enable production of > 1g of mAb per kg biomass in a robust and well-controlled format.

Plants produce glycoproteins with complex iV-glycans having a core bearing two N-acetylglicosamine (GlcNAc) residues that is similar to that observed in mammals. However, in plant glycoproteins this core is substituted by a β\(1\),2-linked xylosyl residue (core xylosyl), which residue does not occur in humans, Lewis\textsuperscript{a} epitopes, and an α\(l\),3-linked fucosyl (core α\(l\),3-fucosyl) instead of an α\(l\),6-linked core fucosyl as in mammals (see, for example, Lerouge et al (1998) Plant Mol. Biol. 38:3 1-48 for a review). Both the α\(l\),3-fucosyl and β\(1\),2-xylosyl residues reportedly are, at least partly, responsible for the immunogenicity of plant glycoproteins in mammals (see, for example, Ree et al. (2000) J. Biol. Chem.
15:11451-11458; Bardor et al. (2003) Glycobiol. 13:427-434; Garcia-Casado et al. (1996) Glycobiol. 6:471-477). Therefore removal of these potentially allergenic sugar residues from mammalian glycoproteins recombinantly produced in plants would overcome concerns about the use of these proteins as pharmaceuticals for treatment of humans.

In addition, plants do not naturally contain a β1,4-galactosyltransferase (GaIT) enzyme, which is responsible for transfer of Gal from UDP-Gal to GlcNAc residues in N-linked glycans. Mammalian GaIT cDNA has been successfully expressed in plant cells, resulting in partially galactosylated N-glycans similar to antibodies produced by hybridoma cells (Bakker et al. (2001) Proc Nat Acad Sd, USA 98:2899-2904). Thus, in one embodiment of the invention, antibodies may be produced which lack Gal in the N-glycan structure. In another embodiment, antibodies may be produced in plant cells which contain Gal in the N-glycan structure.

Accordingly, the present invention provides methods for producing a recombinant monoclonal antibody, including a monoclonal antibody having improved effector function, where the antibody is recombinantly produced within a plant having an altered N-glycosylation pattern of endogenous and heterologous glycoproteins produced therein such that these glycoproteins exhibit a reduction in the amount of the plant-specific β1,2-linked xylosyl residues and/or α1,3-linked fucosyl residues attached to the IV-glycans thereof. Where the antibodies have reduced amounts α1,3-fucosyl residues attached to the N-glycans thereof, the antibodies may have increased ADCC activity relative to antibodies produced in a control plant that has not been genetically modified to inhibit expression or function of FucT.

The transgenic higher plants of the invention are capable of producing a glycoprotein product that has a substantially homogenous glycosylation profile for the CA glycan species, and which is characterized by its substantial homogeneity for the CA glycoform. The CA glycan species is also referred to as the "GnGn" or GlcNAc2Man3GlcNAc2 IV-glycan species. This advantageously results in plant host expression systems that have increased production consistency, as well as reduced chemical, manufacturing, and control (CMC) risk associated with the production of these glycoprotein compositions. In a preferred embodiment, the produced glycoprotein product contains at least 70% having a homogeneous glycoform. In another preferred embodiment, the produced glycoprotein product contains at least 80% having a homogeneous glycoform. In yet another preferred embodiment, the produced glycoprotein product contains at least 90%, 95%, 96%, 97%, 98%, 99% or 100% having a
homogeneous glycoform. In one embodiment, the glycoprotein compositions of
the invention comprise IV-linked glycans that are predominately of the GO glycan structure. The
GO glycoform of the antibody compositions of the present invention advantageously provides
an antibody composition that has increased ADCC activity in association with the absence of
fucosyl residues. Furthermore, the GO glycoform lacks the terminal Gal residues present in
antibodies having the G2 glycoform. As such, these substantially homogeneous antibody
compositions of the invention having predominately the GO glycoform have increased
ADCC/CDC ratios.

Methods for altering the 6f-glycosylation pattern of proteins in higher plants include
stably transforming the plant with at least one recombinant nucleotide construct that provides
for the inhibition of expression of 61,3-fucosyltransferase (FucT) and 61,2-xylosyltransferase
(XyIT) in a plant. Methods for production of antibodies in a plant system are disclosed in
herewith, both of which are expressly incorporated herein by reference. Inhibition of the
expression of FucT or XyIT, or both, may be obtained by a number of methods well known
in the art, including, but not limited to, antisense suppression, double-stranded RNA
(dsRNA) interference, hairpin RNA (hpRNA) interference or intron-containing hairpin RNA
(ihpRNA) interference. For dsRNA interference, a sense RNA molecule like that described
above for cosuppression and an antisense RNA molecule that is fully or partially
complementary to the sense RNA molecule are expressed in the same cell, resulting in
inhibition of the expression of the corresponding endogenous messenger RNA. For hpRNA
interference, the expression cassette is designed to express an RNA molecule that hybridizes
with itself to form a hairpin structure that comprises a single-stranded loop region and a base-
paired stem. The base-paired stem region comprises a sense sequence corresponding to all or
part of the endogenous messenger RNA encoding the gene whose expression is to be
inhibited, and an antisense sequence that is fully or partially complementary to the sense
sequence. Thus, the base-paired stem region of the molecule generally determines the
specificity of the RNA interference. hpRNA molecules are highly efficient at inhibiting the
expression of endogenous genes, and the RNA interference they induce is inherited by
subsequent generations of plants. See, for example, Chuang and Meyerowitz (2000) Proc.
plants of the invention having FucT and XyIT expression silenced in the manner set forth herein can be further modified in their glycosylation machinery such that they express a galactosyltransferase and efficiently attach the terminal galactose residue to the JV-glycans of endogenous and heterologous glycoproteins produced therein.

Transformation protocols as well as protocols for introducing nucleotide sequences into plants may vary depending on the type of plant or plant cell or nodule, that is, monocot or dicot, targeted for transformation. Suitable methods of introducing nucleotide sequences into plants or plant cells or nodules include microinjection (Crossway et al. (1986) Biotechniques 4:320-334), electroporation (Riggs et al. (1986) Proc. Natl. Acad. Set. USA 83:5602-5606), Agrobacterium-mediated transformation (U.S. Patent Nos. 5,563,055 and 5,981,840, both of which are herein incorporated by reference), direct gene transfer (Paszkowski et al. (1984) EMBOJ. 3:2717-2722), ballistic particle acceleration (see, e.g., U.S. Patent Nos. 4,945,050; 5,879,918; 5,886,244; and 5,932,782 (each of which is herein incorporated by reference); and Tomes et al. (1995) "Direct DNA Transfer into Intact Plant Cells via Microprojectile Bombardment," in Plant Cell, Tissue, and Organ Culture: Fundamental Methods, ed. Gamborg and Phillips (Springer-Verlag, Berlin); McCabe et al. (1988) Biotechnology 6:923-926). The cells that have been transformed may be grown into plants in accordance with conventional ways. See, for example, McCormick et al. (1986) Plant Cell Reports 5:81-84.

Characterization of Absence of Fucosyl or Xylosyl Residues on Anti-CD30 Antibodies

Antibodies of the invention lack fucosyl and xylosyl residues, for example in the Fc portion carbohydrate chain. Antibodies can be tested for the absence of fucosyl or xylosyl residues using standard techniques known in the art, such as APTS capillary electrophoresis laser induced fluorescence. Briefly, the N-linked oligosaccharides of the purified anti-CD30 antibody can be released by adding the peptide N-glycanase (Prozyme) and incubating overnight. The carbohydrates are resuspended and derivatized with 8-aminopyrene-1,3,6-trisulfonate (APTS) under mild reductive animation conditions in which desialylation and loss of fucosyl or xylosyl residues is minimized. The reaction adducts are analyzed by capillary electrophoresis with a laser-induced fluorescence detector (Beckman Coulter). An absence of fucosyl or xylosyl can be observed by a shift in the electrophoresis compared to the same antibody containing fucosyl or xylosyl. Another technique for testing the absence of fucosyl or xylosyl on anti-CD30 antibodies is a monosaccharide analysis using HPLC. Suitable assays to determine CD30 binding are further described in the Examples.

Characterization of Antibody Dependent Cell Killing of CD30+ Cells
Anti-CD30 antibodies can be tested for their ability to mediate phagocytosis and killing of cells expressing CD30. In one embodiment, a defucosylated and dexylosylated anti-CD30 antibody enhances killing of cells expressing CD30 in comparison to the same antibody containing fucosyl and xylosyl when compared at the same concentration. In another embodiment, a defucosylated and dexylosylated anti-CD30 antibody induces killing of cells expressing CD30 where the same antibody containing fucosyl and xylosyl does not induce cell killing at the same concentration.

The ADCC activity of a monoclonal antibody can be tested in established in vitro assays. As an example, a chromium release ADCC assay may be used. Briefly, peripheral blood mononuclear cells (PBMCs), or other effector cells, from healthy donors can be purified by Ficoll Hypaque density centrifugation, followed by lysis of contaminating erythrocytes. Washed PBMCs can be suspended in RPMI supplemented with 10% heat-inactivated fetal calf serum and mixed with $^{51}$Cr labeled cells expressing CD30, at various ratios of effector cells to tumor cells (effector cells:tumor cells). Anti-CD30 antibody can then be added at various concentrations. An isotype matched antibody can be used as a negative control. Assays can be carried out for 4-18 hours at 37° C. Samples can be assayed for cytolysis by measuring $^{51}$Cr release into the culture supernatant. Anti-CD30 monoclonal can also be tested in combinations with each other to determine whether cytolysis is enhanced with multiple monoclonal antibodies.

Fc-receptor (FcR) mediated effector cell function has been shown to be important for the in vivo activity of many therapeutic mAbs. The FcR expressed on NK cells and macrophages responsible for ADCC activity is FcγRIIIa. Macrophage mediated phagocytosis has been shown to be an important mechanism in the depletion of B cells in vivo (Tedder et al. (2006) Springer Semin Immunol 28.:351-64). The removal of fucose residues from various mAbs produced in other expression systems has been shown previously to increase FcR binding and enhance ADCC function. Thus, in one embodiment, the antibodies of the present invention have an increased binding affinity for FcγRIIIa.

An alternative assay that can be used to test for anti-CD30 antibody ability to mediate phagocytosis and killing of cells expressing CD30 is a time resolved fluorometry assay. Briefly, CD30 expressing cells are loaded with an acetoxymethyl ester of fluorescence enhancing ligand (BATDA), which penetrates cell membranes. Inside the cell, the ester bonds are hydrolyzed and the compound can no longer pass the cell membrane. Anti-CD30 antibody can then be added at various concentrations. Following cytolysis, an europium
solution (Perkin Elmer) is added and any free ligand binds the europium to form a highly fluorescent and stable chelate (EuTDA) that can be read on a microplate reader (Perkin Elmer). The measured signal correlates with the amount of lysed cells.

Anti-CD30 antibodies also can be tested in an in vivo model (e.g., in mice) to determine their efficacy in mediating phagocytosis and killing of cells expressing CD30, e.g., tumor cells. These antibodies can be selected, for example, based on the following criteria, which are not intended to be exclusive:

1) binding to live cells expressing CD30;
2) high affinity of binding to CD30;
3) binding to a unique epitope on CD30 (to eliminate the possibility that monoclonal antibodies with complimentary activities when used in combination would compete for binding to the same epitope);
4) opsonization of cells expressing CD30;
5) mediation in vitro of growth inhibition, phagocytosis and/or killing of cells expressing CD30 in the presence of human effector cells.

Preferred monoclonal antibodies of the invention meet one or more of these criteria. In a particular embodiment, the monoclonal antibodies are used in combination, e.g., as a pharmaceutical composition comprising two or more anti-CD30 monoclonal antibodies or fragments thereof. For example, anti-CD30 monoclonal antibodies having different, but complementary activities can be combined in a single therapy to achieve a desired therapeutic or diagnostic effect. An illustration of this would be a composition containing an anti-CD30 monoclonal antibody that mediates highly effective killing of target cells in the presence of effector cells, combined with another anti-CD30 monoclonal antibody that inhibits the growth of cells expressing CD30.

Characterization of Binding to CD30

Antibodies of the invention can be tested for binding to CD30 by, for example, standard assays known in the art, such as ELISA, FACS analysis and/or Biacore analysis. In a typical ELISA assay, briefly, microtiter plates are coated with purified CD30 at 0.25 µg/ml in PBS, and then blocked with 5% bovine serum albumin in PBS. Dilutions of antibody are added to each well and incubated for 1-2 hours at 37°C. The plates are washed with PBS/Tween and then incubated with secondary reagent (e.g., for human antibodies or a goat-anti-human IgG Fc-specific polyclonal reagent) conjugated to alkaline phosphatase for 1
hour at 37°C. After washing, the plates are developed with pNPP substrate (1 mg/ml), and analyzed at OD of 405-650.

In order to demonstrate binding of monoclonal antibodies to live cells expressing the CD30, flow cytometry can be used. In a typical (but non-limiting) example of a flow cytometry protocol, cell lines expressing CD30 (grown under standard growth conditions) are mixed with various concentrations of monoclonal antibodies in PBS containing 0.1% BSA and 20% mouse serum, and incubated at 37°C for 1 hour. After washing, the cells are reacted with Fluorescein-labeled secondary antibody (e.g., anti-human IgG antibody) under the same conditions as the primary antibody staining. The samples can be analyzed by a FACScan instrument using light and side scatter properties to gate on single cells. An alternative assay using fluorescence microscopy may be used (in addition to or instead of) the flow cytometry assay. Cells can be stained exactly as described above and examined by fluorescence microscopy. This method allows visualization of individual cells, but may have diminished sensitivity depending on the density of the antigen.

Anti-CD30 antibodies can be further tested for reactivity with CD30 antigen by Western blotting. For example, cell extracts from cells expressing CD30 can be prepared and subjected to sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. After electrophoresis, the separated antigens are transferred to nitrocellulose membranes, blocked with 20% mouse serum, and probed with the monoclonal antibodies to be tested. Antibody binding can be detected using anti-species specific secondary antibody linked to alkaline phosphatase and developed with BCIP/NBT substrate tablets (Sigma Chem. Co., St. Louis, MO). Other techniques for evaluating the binding ability of antibodies towards CD30 are known in the art, including RIAs and Biacore analysis. Suitable assays to determine CD30 binding are described in detail in the Examples.

Antibody Physical Properties

The antibodies of the present invention may be further characterized by the various physical properties of the anti-CD30 antibodies. Various assays maybe used to detect and/or differentiate different classes of antibodies based on these physical properties.

In some embodiments, antibodies of the present invention may contain one or more glycosylation sites in either the light or heavy chain variable region. The presence of one or more glycosylation sites in the variable region may result in increased immunogenicity of the antibody or an alteration of the pK of the antibody due to altered antigen binding (Marshall et al (1972) Annu Rev Biochem 41:673-702; Gala FA and Morrison SL (2004) J Immunol
Glycosylation has been known to occur at motifs containing an N-X-S/T sequence. Variable region glycosylation may be tested using a Glycoblot assay, which cleaves the antibody to produce a Fab, and then tests for glycosylation using an assay that measures periodate oxidation and Schiff base formation. Alternatively, variable region glycosylation may be tested using Dionex light chromatography (Dionex-LC), which cleaves saccharides from a Fab into monosaccharides and analyzes the individual saccharide content. In some instances, it is preferred to have an anti-CD30 antibody that does not contain variable region glycosylation. This can be achieved either by selecting antibodies that do not contain the glycosylation motif in the variable region or by mutating residues within the glycosylation motif using standard techniques well known in the art.

In a preferred embodiment, the antibodies of the present invention do not contain asparagine isomerism sites. A deamidation or isoaspartic acid effect may occur on N-G or D-G sequences, respectively. The deamidation or isoaspartic acid effect results in the creation of isoaspartic acid which decreases the stability of an antibody by creating a kinked structure off a side chain carboxy terminus rather than the main chain. The creation of isoaspartic acid can be measured using an iso-quant assay, which uses a reverse-phase HPLC to test for isoaspartic acid.

Each antibody will have a unique isoelectric point (pI), but generally antibodies will fall in the pH range of between 6 and 9.5. The pI for an IgGl antibody typically falls within the pH range of 7-9.5 and the pI for an IgG4 antibody typically falls within the pH range of 6-8. Antibodies may have a pI that is outside this range. Although the effects are generally unknown, there is speculation that antibodies with a pI outside the normal range may have some unfolding and instability under in vivo conditions. The isoelectric point may be tested using a capillary isoelectric focusing assay, which creates a pH gradient and may utilize laser focusing for increased accuracy (Janini et al (2002) Electrophoresis 23_:1605-11; Ma et al. (2001) Chromatographia 53:S75-89; Hunt et al (1998) J ChromatogrA 800:355-67). In some instances, it is preferred to have an anti-CD30 antibody that contains a pI value that falls in the normal range. This can be achieved either by selecting antibodies with a pI in the normal range, or by mutating charged surface residues using standard techniques well known in the art.

Each antibody will have a melting temperature that is indicative of thermal stability (Krishnamurthy R and Manning MC (2002) Curr Pharm Biotechnol 3:361-7)l. A higher
thermal stability indicates greater overall antibody stability in vivo. The melting point of an antibody may be measured using techniques such as differential scanning calorimetry (Chen et al. (2003) Pharm Res 20:1952-60; Ghirlando et al. (1999) Immunol Lett 68:47-52). $T_{M1}$ indicates the temperature of the initial unfolding of the antibody. $T_{M2}$ indicates the temperature of complete unfolding of the antibody. Generally, it is preferred that the $T_{M1}$ of an antibody of the present invention is greater than 60°C, preferably greater than 65°C, even more preferably greater than 70°C. Alternatively, the thermal stability of an antibody may be measured using circular dichroism (Murray et al. (2002) J. Chromatogr A 1002:343-9).

In a preferred embodiment, antibodies are selected that do not rapidly degrade. Fragmentation of an anti-CD30 antibody may be measured using capillary electrophoresis (CE) and MALDI-MS, as is well understood in the art (Alexander AJ and Hughes DE (1995) Anal Chem 67:3626-32).

In another preferred embodiment, antibodies are selected that have minimal aggregation effects. Aggregation may lead to triggering of an unwanted immune response and/or altered or unfavorable pharmacokinetic properties. Generally, antibodies are acceptable with aggregation of 25% or less, preferably 20% or less, even more preferably 15% or less, even more preferably 10% or less and even more preferably 5% or less. Aggregation may be measured by several techniques well known in the art, including size-exclusion column (SEC) high performance liquid chromatography (HPLC), and light scattering to identify monomers, dimers, trimers or multimers.

Chimeric or Humanized Anti-CD30 Antibodies

In certain embodiments, an anti-CD30 antibody of the invention is a chimeric or humanized antibody. Such antibodies can be prepared using mouse anti-CD30 antibodies that are available in the art and established procedures for converting a mouse antibody to a chimeric or humanized antibody. Non-limiting examples of such mouse anti-CD30 antibodies include the AC10, HeFi-I, Ber-H2, Ki-I, Ki-4, HRS-3, Irac, HRS-4, M44, M67 and Ber-H8 monoclonal antibodies. Moreover, humanized anti-CD30 antibodies are described in PCT Publication WO 02/4661.

Antibodies Having Particular Germline Sequences
In certain embodiments, an antibody of the invention comprises a heavy chain variable region from a particular germline heavy chain immunoglobulin gene and/or a light chain variable region from a particular germline light chain immunoglobulin gene.

For example, in a preferred embodiment, the invention provides a defucosylated monoclonal antibody, or an antigen-binding portion thereof, comprising a heavy chain variable region that is the product of or derived from a human $V_H$ 4-34 gene, wherein the antibody specifically binds to human CD30. In another preferred embodiment, the invention provides a defucosylated monoclonal antibody, or an antigen-binding portion thereof, comprising a heavy chain variable region that is the product of or derived from a human $V_H$ 3-07 gene, wherein the antibody specifically binds CD30. In another preferred embodiment, the invention provides a defucosylated monoclonal antibody, or an antigen-binding portion thereof, comprising a light chain variable region that is the product of or derived from a human $V_K$ L15 gene, wherein the antibody specifically binds to human CD30. In another preferred embodiment, the invention provides a defucosylated monoclonal antibody, or an antigen-binding portion thereof, comprising a light chain variable region that is the product of or derived from a human $V_K$ L6 gene, wherein the antibody specifically binds to human CD30.

In yet another preferred embodiment, the invention provides a defucosylated monoclonal antibody, or an antigen-binding portion thereof, wherein the antibody:

(a) comprises a heavy chain variable region that is the product of or derived from a human $V_H$ 4-34 or 3-07 gene (which encodes the amino acid sequence set forth in SEQ ID NOs: 25 and 26, respectively);

(b) comprises a light chain variable region that is the product of or derived from a human $V_K$ L15, A27, or L6 gene (which encode the amino acid sequences set forth in SEQ ID NOs: 27, 28, and 29, respectively); and

(c) specifically binds to human CD30.

A preferred $V_H$ and $V_K$ germline combination is $V_H$ 4-34 and $V_K$ L15. An example of an antibody having $V_H$ and $V_K$ of $V_H$ 4-34 and Vk L15, respectively, is the 5Fl 1 antibody. Another preferred $V_H$ and $V_K$ germline combination is $V_H$ 3-07 and $V_K$ A27. An example of an antibody having $V_H$ and $V_K$ of $V_H$ 3-07 and Vk A27, respectively, is the 17Gl antibody.
Another preferred V<sub>H</sub> and V<sub>K</sub> germline combination is V<sub>H</sub> 4-34 and V<sub>K</sub> L6. An example of an antibody having V<sub>H</sub> and V<sub>K</sub> of V<sub>H</sub> 4-34 and V<sub>K</sub> L6, respectively, is the 2H9 antibody.

As used herein, a human antibody comprises heavy or light chain variable regions that is "the product of" or "derived from" a particular germline sequence if the variable regions of the antibody are obtained from a system that uses human germline immunoglobulin genes. Such systems include immunizing a transgenic mouse carrying human immunoglobulin genes with the antigen of interest or screening a human immunoglobulin gene library displayed on phage with the antigen of interest. A human antibody that is "the product of" or "derived from" a human germline immunoglobulin sequence can be identified as such by comparing the amino acid sequence of the human antibody to the amino acid sequences of human germline immunoglobulins (e.g., using the Vbase database) and selecting the human germline immunoglobulin sequence that is closest in sequence (i.e., greatest % identity) to the sequence of the human antibody. A human antibody that is "the product of" or "derived from" a particular human germline immunoglobulin sequence may contain amino acid differences as compared to the germline sequence, due to, for example, naturally-occurring somatic mutations or intentional introduction of site-directed mutation. However, a selected human antibody typically is at least 90% identical in amino acids sequence to an amino acid sequence encoded by a human germline immunoglobulin gene and contains amino acid residues that identify the human antibody as being human when compared to the germline immunoglobulin amino acid sequences of other species (e.g., murine germline sequences). In certain cases, a human antibody may be at least 95%, or even at least 96%, 97%, 98%, or 99% identical in amino acid sequence to the amino acid sequence encoded by the germline immunoglobulin gene.

Typically, a human antibody derived from a particular human germline sequence will display no more than 10 amino acid differences from the amino acid sequence encoded by the human germline immunoglobulin gene. In certain cases, the human antibody may display no more than 5, or even no more than 4, 3, 2, or 1 amino acid difference from the amino acid sequence encoded by the germline immunoglobulin gene.

**Homologous Antibodies**

In yet another embodiment, a defucosylated antibody of the invention comprises heavy and light chain variable regions comprising amino acid sequences that are homologous to the amino acid sequences of the preferred antibodies described herein, and wherein the antibodies retain the desired functional properties of the anti-CD30 antibodies of the invention.
For example, the invention provides a defucosylated monoclonal antibody, or antigen binding portion thereof, comprising a heavy chain variable region and a light chain variable region, wherein:

(a) the heavy chain variable region comprises an amino acid sequence that is at least 80% homologous to an amino acid sequence selected from the group consisting of SEQ ID NOs: 1, 2, and 3;

(b) the light chain variable region comprises an amino acid sequence that is at least 80% homologous to an amino acid sequence selected from the group consisting of SEQ ID NOs: 4, 5, and 6; and

(c) the antibody specifically binds to human CD30.

In other embodiments, the V_H and/or V_L amino acid sequences may be 85%, 90%, 95%, 96%, 97%, 98% or 99% homologous to the sequences set forth above. An antibody having V_H and V_L regions having high (i.e., 80% or greater) homology to the V_H and V_L regions of the sequences set forth above, can be obtained by mutagenesis (e.g., site-directed or PCR-mediated mutagenesis) of one or more nucleic acid molecules encoding SEQ ID NOs: 1-6, followed by testing of the encoded altered antibody for retained function (i.e., binding to CD30) using the binding assays described herein. Nucleic acid molecules encoding SEQ ID NOs: 1-6 are shown in SEQ ID NOs: 30-35.

As used herein, the percent homology between two amino acid sequences is equivalent to the percent identity between the two sequences. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences (i.e., % homology = # of identical positions/total # of positions x 100), taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences. The comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm, as described in the non-limiting examples below.

The percent identity between two nucleotide sequences can be determined using the GAP program in the GCG software package (available at www.gcg.com), using a NWSgapdna.CMP matrix and a gap weight of 40, 50, 60, 70, or 80 and a length weight of 1, 2, 3, 4, 5, or 6. The percent identity between two nucleotide or amino acid sequences can also determined using the algorithm of E. Meyers and W. Miller (Comput. Appl. Biosci., 4:1 1-17 (1988)) which has been incorporated into the ALIGN program (version 2.0), using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4. In addition, the percent identity between two amino acid sequences can be determined using
the Needleman and Wunsch (J. Mol. Biol 48:444-453 (1970)) algorithm which has been incorporated into the GAP program in the GCG software package (available at www.gcg.com), using either a Blossum 62 matrix or a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6.

The percent identity between two amino acid sequences can be determined using the algorithm of E. Meyers and W. Miller (Comput. Appl. Biosci., 4:11-17 (1988)) which has been incorporated into the ALIGN program (version 2.0), using a PAM1 20 weight residue table, a gap length penalty of 12 and a gap penalty of 4. In addition, the percent identity between two amino acid sequences can be determined using the Needleman and Wunsch (J. Mol. Biol. 48:444-453 (1970)) algorithm which has been incorporated into the GAP program in the GCG software package (available at www.gcg.com), using either a Blossum 62 matrix or a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6.

Additionally or alternatively, the protein sequences of the present invention can further be used as a "query sequence" to perform a search against public databases to, for example, identify related sequences. Such searches can be performed using the XBLAST program (version 2.0) of Altschul, et al. (1990) J. Mol. Biol. 215.:403-10. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to the antibody molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul et al., (1997) Nucleic Acids Res. 25(17):3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) can be used. See www.ncbi.nkn.nih.gov.

Antibodies with Conservative Modifications

In certain embodiments, a defucosylated antibody of the invention comprises a heavy chain variable region comprising CDR1, CDR2 and CDR3 sequences and a light chain variable region comprising CDR1, CDR2 and CDR3 sequences, wherein one or more of these CDR sequences comprise specified amino acid sequences based on the preferred antibodies described herein (e.g., 5Fl 1, 17Gl, and 2H9), or conservative modifications thereof, and wherein the antibodies retain the desired functional properties of the anti-CD30 antibodies of the invention. Accordingly, the invention provides a defucosylated monoclonal antibody, or antigen binding portion thereof, comprising a heavy chain variable region comprising CDR1, CDR2, and CDR3 sequences and a light chain variable region comprising CDR1, CDR2, and CDR3 sequences, wherein:
(a) the heavy chain variable region CDR3 sequence comprises the amino acid sequence of SEQ ID NO: 9, 12, or 15, and conservative modifications thereof;

(b) the light chain variable region CDR3 sequence comprises the amino acid sequence of SEQ ID NO: 18, 21, or 24, and conservative modifications thereof; and

(c) the antibody specifically binds to human CD30.

In a preferred embodiment, the heavy chain variable region CDR2 sequence comprises the amino acid sequence of SEQ ID NO: 8, 11, or 14, and conservative modifications thereof; and the light chain variable region CDR2 sequence comprises the amino acid sequence of SEQ ID NO: 17, 20, or 23, and conservative modifications thereof.

In another preferred embodiment, the heavy chain variable region CDR1 sequence comprises the amino acid sequence of SEQ ID NO: 7, 10, or 13, and conservative modifications thereof; and the light chain variable region CDR1 sequence comprises the amino acid sequence of SEQ ID NO: 16, 19, or 22, and conservative modifications thereof.

As used herein, the term "conservative sequence modifications" is intended to refer to amino acid modifications that do not significantly affect or alter the binding characteristics of the antibody containing the amino acid sequence. Such conservative modifications include amino acid substitutions, additions and deletions. Modifications can be introduced into an antibody of the invention by standard techniques known in the art, such as site-directed mutagenesis and PCR-mediated mutagenesis. Conservative amino acid substitutions are ones in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine, tryptophan), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine). Thus, one or more amino acid residues within the CDR regions of an antibody of the invention can be replaced with other amino acid residues from the same side chain family and the altered antibody can be tested for retained function (i.e., the functions set forth in (c) above) using the functional assays described herein.

Alternatively, in another embodiment, mutations can be introduced randomly along all or part of an anti-CD30 antibody coding sequence, such as by saturation mutagenesis, and the resulting modified anti-CD30 antibodies can be screened for binding activity.
Antibodies that Bind to the Same Epitope as Anti-CD30 Antibodies of the Invention

In another embodiment, the invention provides defucosylated antibodies that bind to the same epitope as do the various anti-CD30 antibodies of the invention provided herein, such as other human antibodies that bind to the same epitope as the 5Fl 1, 17Gl or 2H9 antibodies described herein. Such additional antibodies can be identified based on their ability to cross-compete (e.g., to competitively inhibit the binding of, in a statistically significant manner) with other antibodies of the invention, such as 5Fl 1, 17Gl or 2H9, in standard CD30 binding assays. The ability of a test antibody to inhibit the binding of, e.g., 5Fl 1, 17Gl or 2H9 to human CD30 demonstrates that the test antibody can compete with that antibody, for binding to human CD30; such an antibody may, according to non-limiting theory, bind to the same or a related (e.g., a structurally similar or spatially proximal) epitope on human CD30 as the antibody with which it competes. In a preferred embodiment, the defucosylated antibody that binds to the same epitope on human CD30 as 5Fl 1, 17Gl or 2H9 is a human monoclonal antibody. Such human monoclonal antibodies can be prepared and isolated as described in PCT Publication WO 03/059282.

Engineered and Modified Antibodies

A defucosylated antibody of the invention further can be prepared using an antibody having one or more of the V_H and/or V_L sequences disclosed herein as starting material to engineer a modified antibody, which modified antibody may have altered properties from the starting antibody. An antibody can be engineered by modifying one or more amino acid residues within one or both variable regions (i.e., V_H and/or V_L), for example within one or more CDR regions and/or within one or more framework regions. Additionally or alternatively, an antibody can be engineered by modifying residues within the constant region(s), for example to alter the effector function(s) of the antibody.

One type of variable region engineering that can be performed is CDR grafting. Antibodies interact with target antigens predominantly through amino acid residues that are located in the six heavy and light chain complementarity determining regions (CDRs). For this reason, the amino acid sequences within CDRs are more diverse between individual antibodies than sequences outside of CDRs. Because CDR sequences are responsible for most antibody-antigen interactions, it is possible to express recombinant antibodies that mimic the properties of specific naturally occurring antibodies by constructing expression vectors that include CDR sequences from the specific naturally occurring antibody grafted onto framework sequences from a different antibody with different properties (see, e.g., Riechmann, L. et al. (1998) Nature 332:323-327; Jones, P. et al. (1986) Nature 321:522-525;

Accordingly, another embodiment of the invention pertains to a defucosylated monoclonal antibody, or antigen binding portion thereof, comprising a heavy chain variable region comprising CDR1, CDR2, and CDR3 sequences comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 7, 8, and 9, SEQ ID NOs: 10, 11, and 12, and SEQ ID NOs: 13, 14, and 15, respectively, and a light chain variable region comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 16, 17, and 18, SEQ ID NOs: 19, 20, and 21 and SEQ ID NOs: 22, 23, and 24, respectively. Thus, such antibodies contain the $V_H$ and $V_L$ CDR sequences of monoclonal antibodies 5Fl1, 17Gl, or 2H9 yet may contain different framework sequences from these antibodies.

Such framework sequences can be obtained from public DNA databases or published references that include germline antibody gene sequences. For example, germline DNA sequences for human heavy and light chain variable region genes can be found in the "VBase" human germline sequence database (available on the Internet at www.mrc-cpe.cam.ac.uk/vbase), as well as in Kabat, E. A., et al. (1991) Sequences of Proteins of Immunological Interest, Fifth Edition, U.S. Department of Health and Human Services, NIH Publication No. 91-3242; Tomlinson, I. M., et al. (1992) "The Repertoire of Human Germline $V_H$ Sequences Reveals about Fifty Groups of $V_H$ Segments with Different Hypervariable Loops" J. Mol. Biol. 24:827-836; the contents of each of which are expressly incorporated herein by reference. As another example, the germline DNA sequences for human heavy and light chain variable region genes can be found in the Genbank database. For example, the following heavy chain germline sequences found in the HCo7 HuMAb mouse are available in the accompanying Genbank accession numbers: 1-69 (NGJ0109 0109, NT_024637 and BC070333), 3-33 (NG_0010109 and NT_024637) and 3-7 (NGJ0109 0109 and NT_024637). As another example, the following heavy chain germline sequences found in the HCo 12 HuMAb mouse are available in the accompanying Genbank accession numbers: 1-69 (NGJ0109, NT_024637 and BC070333), 5-51 (NG_0010109 and NT_024637), 4-34 (NG_0010109 and NT_024637), 3-30.3 (CAJ556644) and 3-23 (AJ406678).

Antibody protein sequences are compared against a compiled protein sequence database using one of the sequence similarity searching methods called the Gapped BLAST.
(Altschul et al. (1997) Nucleic Acids Research 25:3389-3402), which is well known to those skilled in the art. BLAST is a heuristic algorithm in that a statistically significant alignment between the antibody sequence and the database sequence is likely to contain high-scoring segment pairs (HSP) of aligned words. Segment pairs whose scores cannot be improved by extension or trimming is called a hit. Briefly, the nucleotide sequences of VBASE origin (ybase.mrc-cpe.cam.ac.uk/vbasel/list2.php) are translated and the region between and including FRI through FR3 framework region is retained. The database sequences have an average length of 98 residues. Duplicate sequences which are exact matches over the entire length of the protein are removed. A BLAST search for proteins using the program blastp with default, standard parameters except the low complexity filter, which is turned off, and the substitution matrix of BLOSUM62, filters for top 5 hits yielding sequence matches. The nucleotide sequences are translated in all six frames and the frame with no stop codons in the matching segment of the database sequence is considered the potential hit. This is in turn confirmed using the BLAST program tblastx, which translates the antibody sequence in all six frames and compares those translations to the VBASE nucleotide sequences dynamically translated in all six frames.

The identities are exact amino acid matches between the antibody sequence and the protein database over the entire length of the sequence. The positives (identities + substitution match) are not identical but amino acid substitutions guided by the BLOSUM62 substitution matrix. If the antibody sequence matches two of the database sequences with same identity, the hit with most positives would be decided to be the matching sequence hit.

Preferred framework sequences for use in the antibodies of the invention are those that are structurally similar to the framework sequences used by selected antibodies of the invention, e.g., similar to the V_H 4-34 or 3-07 sequences (SEQ ID NO: 25 or 26) and/or the V_k L15, A27 or L6 framework sequence (SEQ ID NO: 27, 28, or 29) used by preferred monoclonal antibodies of the invention. The V_H CDRI, 2 and 3 sequences, and the V_k CDRI, 2 and 3 sequences, can be grafted onto framework regions that have the identical sequence as that found in the germline immunoglobulin gene from which the framework sequence derive, or the CDR sequences can be grafted onto framework regions that contain one or more mutations as compared to the germline sequences. For example, it has been found that in certain instances it is beneficial to mutate residues within the framework regions to maintain or enhance the antigen binding ability of the antibody (see e.g., U.S. Patent Nos. 5,530,101; 5,585,089; 5,693,762 and 6,180,370 to Queen et al).
Another type of variable region modification is to mutate amino acid residues within the $V_h$ and/or $V_k$ CDR1, CDR2 and/or CDR3 regions to thereby improve one or more binding properties (e.g., affinity) of the antibody of interest. Site-directed mutagenesis or PCR-mediated mutagenesis can be performed to introduce the mutation(s) and the effect on antibody binding, or other functional property of interest, can be evaluated in in vitro or in vivo assays as described herein and provided in the Examples. Preferably conservative modifications (as discussed above) are introduced. The mutations may be amino acid substitutions, additions or deletions, but are preferably substitutions. Moreover, typically no more than one, two, three, four or five residues within a CDR region are altered.

Accordingly, in another embodiment, the invention provides defucosylated anti-CD30 monoclonal antibodies, or antigen binding portions thereof, comprising a heavy chain variable region comprising: (a) a $V_h$ CDR1 region comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 7, 8, and 9, or an amino acid sequence having one, two, three, four or five amino acid substitutions, deletions or additions as compared to an amino acid sequence selected from the group consisting of SEQ ID NO: 7, 8, and 9; (b) a $V_h$ CDR2 region comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 10, 11, and 12, or an amino acid sequence having one, two, three, four or five amino acid substitutions, deletions or additions as compared to an amino acid sequence selected from the group consisting of SEQ ID NO: 10, 11, and 12; (c) a $V_h$ CDR3 region comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 13, 14, and 15, or an amino acid sequence having one, two, three, four or five amino acid substitutions, deletions or additions as compared to an amino acid sequence selected from the group consisting of SEQ ID NO: 13, 14, and 15; (d) a $V_k$ CDR1 region comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 16, 17 and 18, or an amino acid sequence having one, two, three, four or five amino acid substitutions, deletions or additions as compared to an amino acid sequence selected from the group consisting of SEQ ID NO: 16, 17, and 18; (e) a $V_k$ CDR2 region comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 19, 20, and 21, or an amino acid sequence having one, two, three, four or five amino acid substitutions, deletions or additions as compared to an amino acid sequence selected from the group consisting of SEQ ID NO: 19, 20, and 21; and (f) a $V_k$ CDR3 region comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 22, 23, and 24, or an amino acid sequence having one, two, three, four or five amino acid substitutions, deletions or additions as compared to an amino acid sequence selected from the group consisting of SEQ ID NO: 22, 23, and 24.
Engineered antibodies of the invention include those in which modifications have been made to framework residues within $V_H$ and/or $V_K$, e.g. to improve the properties of the antibody. Typically such framework modifications are made to decrease the immunogenicity of the antibody. For example, one approach is to "backmutate" one or more framework residues to the corresponding germline sequence. More specifically, an antibody that has undergone somatic mutation may contain framework residues that differ from the germline sequence from which the antibody is derived. Such residues can be identified by comparing the antibody framework sequences to the germline sequences from which the antibody is derived. For example, for 5Fl1, amino acid residue #83 (within FR3) of $V_{His}$ is an asparagine whereas this residue in the corresponding $V_H$ 4-34 germline sequence is a serine. To return the framework region sequences to their germline configuration, the somatic mutations can be "backmutated" to the germline sequence by, for example, site-directed mutagenesis or PCR-mediated mutagenesis (e.g., residue 83 of FR3 of the $V_H$ of 5Fl1 can be "backmutated" from asparagine to serine. Such "backmutated" antibodies are also intended to be encompassed by the invention.

Another type of framework modification involves mutating one or more residues within the framework region, or even within one or more CDR regions, to remove T cell epitopes to thereby reduce the potential immunogenicity of the antibody. This approach is also referred to as "deimmunization" and is described in further detail in U.S. Patent Publication No. 20030153043 by Carr et al.

In addition or alternative to modifications made within the framework or CDR regions, antibodies of the invention may be engineered to include modifications within the Fc region, typically to alter one or more functional properties of the antibody, such as serum half-life, complement fixation, Fc receptor binding, and/or antigen-dependent cellular cytotoxicity. Furthermore, an antibody of the invention may be chemically modified (e.g., one or more chemical moieties can be attached to the antibody) or be modified to alter its glycosylation, again to alter one or more functional properties of the antibody. Each of these embodiments is described in further detail below. The numbering of residues in the Fc region is that of the EU index of Kabat.

In one embodiment, the hinge region of $CH\_1$ is modified such that the number of cysteine residues in the hinge region is altered, e.g., increased or decreased. This approach is described further in U.S. Patent No. 5,677,425 by Bodmer et al. The number of cysteine residues in the hinge region of $CH\_1$ is altered to, for example, facilitate assembly of the light and heavy chains or to increase or decrease the stability of the antibody.
In another embodiment, the Fc hinge region of an antibody is mutated to decrease the biological half life of the antibody. More specifically, one or more amino acid mutations are introduced into the CH2-CH3 domain interface region of the Fc-hinge fragment such that the antibody has impaired Staphylococcal protein A (SpA) binding relative to native Fc-hinge domain SpA binding. This approach is described in further detail in U.S. Patent 6,165,745 by Ward et al.

In another embodiment, the antibody is modified to increase its biological half life. Various approaches are possible. For example, one or more of the following mutations can be introduced: T252L, T254S, T256F, as described in U.S. Patent No. 6,277,375 to Ward. Alternatively, to increase the biological half life, the antibody can be altered within the CH1 or CL region to contain a salvage receptor binding epitope taken from two loops of a CH2 domain of an Fc region of an IgG, as described in U.S. Patent Nos. 5,869,046 and 6,121,022 by Presta et al.

In yet other embodiments, the Fc region is altered by replacing at least one amino acid residue with a different amino acid residue to alter the effector function(s) of the antibody. For example, one or more amino acids selected from amino acid residues 234, 235, 236, 237, 297, 318, 320 and 322 can be replaced with a different amino acid residue such that the antibody has an altered affinity for an effector ligand but retains the antigen-binding ability of the parent antibody. The effector ligand to which affinity is altered can be, for example, an Fc receptor or the C1 component of complement. This approach is described in further detail in U.S. Patent Nos. 5,624,821 and 5,648,260, both by Winter et al.

In another example, one or more amino acids selected from amino acid residues 239, 311 and 322 can be replaced with a different amino acid residue such that the antibody has altered C1q binding and/or reduced or abolished complement dependent cytotoxicity (CDC). This approach is described in further detail in U.S. Patent Nos. 6,194,551 by Idusogie et al.

In another example, one or more amino acid residues within amino acid positions 231 and 239 are altered to thereby alter the ability of the antibody to fix complement. This approach is described further in PCT Publication WO 94/29351 by Bodmer et al.

In yet another example, the Fc region is modified to increase the ability of the antibody to mediate antibody dependent cellular cytotoxicity (ADCC) and/or to increase the affinity of the antibody for an Fcγ receptor by modifying one or more amino acids at the following positions: 238, 239, 248, 249, 252, 254, 255, 256, 258, 265, 267, 268, 269, 270, 272, 276, 278, 280, 283, 285, 286, 289, 290, 292, 293, 294, 295, 296, 298, 301, 303, 305,
307, 309, 312, 315, 320, 322, 324, 326, 327, 329, 330, 331, 333, 334, 335, 337, 338, 340, 360, 373, 376, 378, 382, 388, 389, 398, 414, 416, 419, 430, 434, 435, 437, 438 or 439. This approach is described further in PCT Publication WO 00/42072 by Presta. Moreover, the binding sites on human IgG1 for FcγRI, FcγRII, FcγRIII and FcRn have been mapped and variants with improved binding have been described (see Shields, R.L. et al. (2001) J. Biol. Chem. 276:6591-6604). Specific mutations at positions 256, 290, 298, 333, 334 and 339 were shown to improve binding to FcγRIII. Additionally, the following combination mutants were shown to improve FcγRIII binding: T256A/S298A, S298A/E333A, S298A/K224A and S298A/E333A/K334A.

Another modification of the antibodies herein that is contemplated by the invention is pegylation. An antibody can be pegylated to, for example, increase the biological (e.g., serum) half life of the antibody. To pegylate an antibody, the antibody, or fragment thereof, typically is reacted with polyethylene glycol (PEG), such as a reactive ester or aldehyde derivative of PEG, under conditions in which one or more PEG groups become attached to the antibody or antibody fragment. Preferably, the pegylation is carried out via an acylation reaction or an alkylation reaction with a reactive PEG molecule (or an analogous reactive water-soluble polymer). As used herein, the term "polyethylene glycol" is intended to encompass any of the forms of PEG that have been used to derivatize other proteins, such as mono (Cl-ClO) alkoxy- or aryloxy-polyethylene glycol or polyethylene glycol-maleimide.

In certain embodiments, the antibody to be pegylated is an aglycosylated antibody. Methods for pegylating proteins are known in the art and can be applied to the antibodies of the invention. See for example, EP 0 154 316 by Nishimura et al. and EP 0 401 384 by Ishikawa et al.

Methods of Engineering Antibodies

As discussed above, the defucosylated anti-CD30 antibodies having V_H and V_K sequences disclosed herein can be used to create new anti-CD30 antibodies by modifying the VH and/or V_K sequences, or the constant region(s) attached thereto. Thus, in another aspect of the invention, the structural features of an anti-CD30 antibody of the invention, e.g. 5Fl1, 17Gl, or 2H9, are used to create structurally related defucosylated anti-CD30 antibodies that retain at least one functional property of the antibodies of the invention, such as binding to human CD30. For example, one or more CDR regions of 5Fl1, 17Gl, or 2H9, or mutations thereof, can be combined recombinantly with known framework regions and/or other CDRs
to create additional, recombinantly-engineered, anti-CD30 antibodies of the invention, as discussed above. Other types of modifications include those described in the previous section. The starting material for the engineering method is one or more of the $V_H$ and/or $V_K$ sequences provided herein, or one or more CDR regions thereof. To create the engineered antibody, it is not necessary to actually prepare (i.e., express as a protein) an antibody having one or more of the $V_H$ and/or $V_K$ sequences provided herein, or one or more CDR regions thereof. Rather, the information contained in the sequence(s) is used as the starting material to create a "second generation" sequence(s) derived from the original sequence(s) and then the "second generation" sequence(s) is prepared and expressed as a protein.

Accordingly, in another embodiment, the invention provides a method for preparing an anti-CD30 antibody comprising:

(a) providing: (i) a heavy chain variable region antibody sequence comprising a CDR1 sequence selected from the group consisting of SEQ ID NOs: 7, 8, and 9, a CDR2 sequence selected from the group consisting of SEQ ID NOs: 10, 11, and 12 and/or a CDR3 sequence selected from the group consisting of SEQ ID NOs: 13, 14, and 15; and/or (ii) a light chain variable region antibody sequence comprising a CDR1 sequence selected from the group consisting of SEQ ID NOs: 16, 17, and 18, a CDR2 sequence selected from the group consisting of SEQ ID NOs: 19, 20, and 21 and/or a CDR3 sequence selected from the group consisting of SEQ ID NOs: 22, 23, and 24;

(b) altering at least one amino acid residue within the heavy chain variable region antibody sequence and/or the light chain variable region antibody sequence to create at least one altered antibody sequence; and

(c) expressing the altered antibody sequence as a protein.

Standard molecular biology techniques can be used to prepare and express the altered antibody sequence. The altered antibody sequence so prepared can then be made in defucosylated form using the methods disclosed herein to obtain a defucosylated altered anti-CD30 antibody.

The functional properties of the altered antibodies can be assessed using standard assays available in the art and/or described herein, such as those set forth in the Examples (e.g., flow cytometry, binding assays, ADCC assays).

In certain embodiments of the methods of engineering antibodies of the invention, mutations can be introduced randomly or selectively along all or part of an anti-CD30 antibody coding sequence and the resulting modified anti-CD30 antibodies can be screened for binding activity and/or other functional properties as described herein. Mutational
methods have been described in the art. For example, PCT Publication WO 02/092780 by Short describes methods for creating and screening antibody mutations using saturation mutagenesis, synthetic ligation assembly, or a combination thereof. Alternatively, PCT Publication WO 03/074679 by Lazar et al. describes methods of using computational screening methods to optimize physiochemical properties of antibodies.

**Nucleic Acid Molecules Encoding Antibodies of the Invention**

Another aspect of the invention pertains to nucleic acid molecules that encode the antibodies of the invention. The term "nucleic acid molecule" as used herein, is intended to include DNA molecules and RNA molecules. A nucleic acid molecule may be single-stranded or double-stranded, but preferably is double-stranded DNA. The nucleic acids may be present in whole cells, in a cell lysate, or in a partially purified or substantially pure form. A nucleic acid is "isolated" or "rendered substantially pure" when purified away from other cellular components or other contaminants, e.g., other cellular nucleic acids or proteins, by standard techniques, including alkaline/SDS treatment, CsCl banding, column chromatography, agarose gel electrophoresis and others well known in the art. See, F. Ausubel, *et al.*, ed. (1987) *Current Protocols in Molecular Biology*, Greene Publishing and Wiley Interscience, New York. A nucleic acid of the invention can be, for example, DNA or RNA and may or may not contain intronic sequences. In a preferred embodiment, the nucleic acid is a cDNA molecule.

Nucleic acids of the invention can be obtained using standard molecular biology techniques. For antibodies expressed by hybridomas (e.g., hybridomas prepared from transgenic mice carrying human immunoglobulin genes as described further below), cDNAs encoding the light and heavy chains of the antibody made by the hybridoma can be obtained by standard PCR amplification or cDNA cloning techniques. For antibodies obtained from an immunoglobulin gene library (e.g., using phage display techniques), nucleic acid encoding the antibody can be recovered from the library.

Preferred nucleic acids molecules of the invention are those encoding the VH and VL sequences of the 5Fl 1, 17Gl, and 2H9 monoclonal antibodies. The DNA sequence encoding the VH sequence of 5Fl 1 is shown in SEQ ID NO: 30. The DNA sequence encoding the VL sequence of 5Fl 1 is shown in SEQ ID NO: 31. The DNA sequence encoding the VH sequence of 17Gl is shown in SEQ ID NO: 33. The DNA sequence encoding the VL sequence of 17Gl is shown in SEQ ID NO: 34. The DNA sequence encoding the VH
sequence of 2H9 is shown in SEQ ID NO: 32. The DNA sequence encoding the VL sequence of 2H9 is shown in SEQ ID NO: 35.

Once DNA fragments encoding VH and VL segments are obtained, these DNA fragments can be further manipulated by standard recombinant DNA techniques, for example to convert the variable region genes to full-length antibody chain genes, to Fab fragment genes or to a scFv gene. In these manipulations, a VL- or VH-encoding DNA fragment is operatively linked to another DNA fragment encoding another protein, such as an antibody constant region or a flexible linker. The term "operatively linked", as used in this context, is intended to mean that the two DNA fragments are joined such that the amino acid sequences encoded by the two DNA fragments remain in-frame.

The isolated DNA encoding the VH region can be converted to a full-length heavy chain gene by operatively linking the VH-encoding DNA to another DNA molecule encoding heavy chain constant regions (CH1, CH2 and CH3). The sequences of human heavy chain constant region genes are known in the art (see e.g., Kabat, E. A., el al. (1991) Sequences of Proteins of Immunological Interest, Fifth Edition, U.S. Department of Health and Human Services, NTH Publication No. 91-3242) and DNA fragments encompassing these regions can be obtained by standard PCR amplification. The heavy chain constant region can be an IgG1, IgG2, IgG3, IgG4, IgA, IgE, IgM or IgD constant region, but most preferably is an IgG1 or IgG4 constant region. For a Fab fragment heavy chain gene, the VH-encoding DNA can be operatively linked to another DNA molecule encoding only the heavy chain CH1 constant region.

The isolated DNA encoding the VL region can be converted to a full-length light chain gene (as well as a Fab light chain gene) by operatively linking the VL-encoding DNA to another DNA molecule encoding the light chain constant region, CL. The sequences of human light chain constant region genes are known in the art (see e.g., Kabat, E. A., et al. (1991) Sequences of Proteins of Immunological Interest, Fifth Edition, U.S. Department of Health and Human Services, NIH Publication No. 91-3242) and DNA fragments encompassing these regions can be obtained by standard PCR amplification. The light chain constant region can be a kappa or lambda constant region, but most preferably is a kappa constant region.

To create a scFv gene, the VH- and VL-encoding DNA fragments are operatively linked to another fragment encoding a flexible linker, e.g., encoding the amino acid sequence (Gly4 -Ser)₃, such that the VH and VL sequences can be expressed as a contiguous single-chain protein, with the VL and VH regions joined by the flexible linker.

The nucleic acid compositions of the present invention, while often in a native sequence (except for modified restriction sites and the like), from either cDNA, genomic or mixtures may be mutated, thereof in accordance with standard techniques to provide gene sequences. For coding sequences, these mutations, may affect amino acid sequence as desired. In particular, DNA sequences substantially homologous to or derived from native V, D, J, constant, switches and other such sequences described herein are contemplated (where "derived" indicates that a sequence is identical or modified from another sequence).

Production of Monoclonal Antibodies of the Invention

Monoclonal antibodies (mAbs) of the present invention can be produced by a variety of techniques, including conventional monoclonal antibody methodology e.g., the standard somatic cell hybridization technique of Kohler and Milstein (1975) Nature 256: 495. Although somatic cell hybridization procedures are preferred, in principle, other techniques for producing monoclonal antibody can be employed e.g., viral or oncogenic transformation of B lymphocytes.

The preferred animal system for preparing hybridomas is the murine system. Hybridoma production in the mouse is a very well-established procedure. Immunization protocols and techniques for isolation of immunized splenocytes for fusion are known in the art. Fusion partners (e.g., murine myeloma cells) and fusion procedures are also known.

In various embodiments, the antibody can be, for example, human antibodies, humanized antibodies or chimeric antibodies.

Chimeric or humanized antibodies of the present invention can be prepared based on the sequence of a murine monoclonal antibody prepared as described above. DNA encoding the heavy and light chain immunoglobulins can be obtained from the murine hybridoma of interest and engineered to contain non-murine (e.g., human) immunoglobulin sequences using standard molecular biology techniques. For example, to create a chimeric antibody, the murine variable regions can be linked to human constant regions using methods known in the art (see e.g., U.S. Patent No. 4,816,567 to Cabilly et al.). To create a humanized antibody, the murine CDR regions can be inserted into a human framework using methods known in the art (see e.g., U.S. Patent No. 5,225,539 to Winter, and U.S. Patent Nos. 5,530,101; 5,585,089; 5,693,762 and 6,180,370 to Queen et al.). A variety of mouse anti-CD30 antibodies are known in the art that can be used to create chimeric or humanized anti-
CD30 antibodies, for example, AClO, HeFi-I, Ber-H2, Ki-I, HRS-3, Irac, HRS-4, M44, M67, and Ber-H8.

In a preferred embodiment, the antibodies of the invention are human monoclonal antibodies. Such human monoclonal antibodies directed against CD30 can be generated using transgenic or transchromosomal mice carrying parts of the human immune system rather than the mouse system. These transgenic and transchromosomal mice include mice referred to herein as HuMab mice and KM mice, respectively, and are collectively referred to herein as "human Ig mice."


In another embodiment, human antibodies of the invention can be raised using a mouse that carries human immunoglobulin sequences on transgenes and transchromosomes, such as a mouse that carries a human heavy chain transgene and a human light chain
transchromosome. Such mice, referred to herein as "KM mice", are described in detail in PCT Publication WO 02/43478 to Ishida et al.

Still further, alternative transgenic animal systems expressing human immunoglobulin genes are available in the art and can be used to raise anti-CD30 antibodies of the invention. For example, an alternative transgenic system referred to as the Xenotnouse (Abgenix, Inc.) can be used; such mice are described in, for example, U.S. Patent Nos. 5,939,598; 6,075,181; 6,114,598; 6, 150,584 and 6,162,963 to Kucherlapati et al.

Moreover, alternative transchromosomic animal systems expressing human immunoglobulin genes are available in the art and can be used to raise anti-CD30 antibodies of the invention. For example, mice carrying both a human heavy chain transchromosome and a human light chain tranchromosome, referred to as "TC mice" can be used; such mice are described in Tomizuka et al. (2000) Proc. Natl. Acad. SciL USA 97:722-727.

Furthermore, cows carrying human heavy and light chain transchromosomes have been described in the art (Kuroiwa et al. (2002) Nature Biotechnology 20:889-894) and can be used to raise anti-CD30 antibodies of the invention.

Human monoclonal antibodies of the invention can also be prepared using phage display methods for screening libraries of human immunoglobulin genes. Such phage display methods for isolating human antibodies are established in the art. See for example: U.S. Patent Nos. 5,223,409; 5,403,484; and 5,571,698 to Ladner et al.; U.S. Patent Nos. 5,427,908 and 5,580,717 to Dower et al.; U.S. Patent Nos. 5,969,108 and 6,172,197 to McCafferty et al.; and U.S. Patent Nos. 5,885,793; 6,521,404; 6,544,731; 6,555,313; 6,582,915 and 6,593,081 to Griffiths et al.

Human monoclonal antibodies of the invention can also be prepared using SCID mice into which human immune cells have been reconstituted such that a human antibody response can be generated upon immunization. Such mice are described in, for example, U.S. Patent Nos. 5,476,996 and 5,698,767 to Wilson et al. Immunization of Human Ig Mice

When human Ig mice are used to raise human antibodies of the invention, such mice can be immunized with a purified or enriched preparation of CD30 antigen and/or recombinant CD30, or an CD30 fusion protein, as described by Lonberg, N. et al. (1994) Nature 368(6474): 856-859; Fishwild, D. et al. (1996) Nature Biotechnology 14: 845-851; and PCT Publication WO 98/24884 and WO 01/14424. Preferably, the mice will be 6-16 weeks of age upon the first infusion. For example, a purified or recombinant preparation (5-50 µg) of CD30 antigen can be used to immunize the human Ig mice intraperitoneally.
Detailed procedures to generate fully human monoclonal antibodies to CD30 are described in PCT Publication WO 03/059282. Cumulative experience with various antigens has shown that the transgenic mice respond when initially immunized intraperitoneally (IP) with antigen in complete Freund's adjuvant, followed by every other week IP immunizations (up to a total of 6) with antigen in incomplete Freund's adjuvant. However, adjuvants other than Freund's are also found to be effective. In addition, whole cells in the absence of adjuvant are found to be highly immunogenic. The immune response can be monitored over the course of the immunization protocol with plasma samples being obtained by retroorbital bleeds. The plasma can be screened by ELISA (as described below), and mice with sufficient titers of anti-CD30 human immunoglobulin can be used for fusions. Mice can be boosted intravenously with antigen 3 days before sacrifice and removal of the spleen. It is expected that 2-3 fusions for each immunization may need to be performed. Between 6 and 24 mice are typically immunized for each antigen. Usually both HCo7 and HCo12 strains are used. In addition, both HCo7 and HCo12 transgene can be bred together into a single mouse having two different human heavy chain transgenes (HCo7/HCo12).

**Generation of Hybridomas Producing Human Monoclonal Antibodies of the Invention**

To generate hybridomas producing human monoclonal antibodies of the invention, splenocytes and/or lymph node cells from immunized mice can be isolated and fused to an appropriate immortalized cell line, such as a mouse myeloma cell line. The resulting hybridomas can be screened for the production of antigen-specific antibodies. For example, single cell suspensions of splenic lymphocytes from immunized mice can be fused to one-sixth the number of P3X63-Ag8.653 nonsecreting mouse myeloma cells (ATCC, CRL 1580) with 50% PEG. Alternatively, the single cell suspension of splenic lymphocytes from immunized mice can be fused using an electric field based electrofusion method, using a CytoPulse large chamber cell fusion electroporator (CytoPulse Sciences, Inc., Glen Burnie, Maryland). Cells are plated at approximately 2 x 10^5 in flat bottom microtiter plate, followed by a two week incubation in selective medium containing 20% fetal Clone Serum, 18% "653" conditioned media, 5% origen (IGEN), 4 mM L-glutamine, 1 mM sodium pyruvate, 5mM HEPES, 0.055 mM 2-mercaptoethanol, 50 units/ml penicillin, 50 mg/ml streptomycin, 50 mg/ml gentamycin and 1X HAT (Sigma; the HAT is added 24 hours after the fusion).

After approximately two weeks, cells can be cultured in medium in which the HAT is replaced with HT. Individual wells can then be screened by ELISA for human monoclonal IgM and IgG antibodies. Once extensive hybridoma growth occurs, medium can be observed usually after 10-14 days. The antibody secreting hybridomas can be replated, screened again,
and if still positive for human IgG, the monoclonal antibodies can be subcloned at least twice by limiting dilution. The stable subclones can then be cultured in vitro to generate small amounts of antibody in tissue culture medium for characterization.

To purify human monoclonal antibodies, selected hybridomas can be grown in two-liter spinner-flasks for monoclonal antibody purification. Supernatants can be filtered and concentrated before affinity chromatography with protein A-sepharose (Pharmacia, Piscataway, N.J.). Eluted IgG can be checked by gel electrophoresis and high performance liquid chromatography to ensure purity. The buffer solution can be exchanged into PBS, and the concentration can be determined by OD280 using 1.43 extinction coefficient. The monoclonal antibodies can be aliquoted and stored at -80°C.

Generation of Transfectomas Producing Monoclonal Antibodies of the Invention

Antibodies of the invention also can be produced in a host cell transfectoma using, for example, a combination of well known recombinant DNA techniques and gene transfection methods (e.g., Morrison, S. (1985) Science 229:1202-).

For example, to express the antibodies, or antibody fragments thereof, DNAs encoding partial or full-length light and heavy chains, can be obtained by standard molecular biology techniques (e.g., PCR amplification or cDNA cloning using a hybridoma that expresses the antibody of interest) and the DNAs can be inserted into expression vectors such that the genes are operatively linked to transcriptional and translational control sequences. In this context, the term "operatively linked" is intended to mean that an antibody gene is ligated into a vector such that transcriptional and translational control sequences within the vector serve their intended function of regulating the transcription and translation of the antibody gene. The expression vector and expression control sequences are chosen to be compatible with the expression host cell used. The antibody light chain gene and the antibody heavy chain gene can be inserted into separate vector or, more typically, both genes are inserted into the same expression vector. The antibody genes are inserted into the expression vector by standard methods (e.g., ligation of complementary restriction sites on the antibody gene fragment and vector, or blunt end ligation if no restriction sites are present). The light and heavy chain variable regions of the antibodies described herein can be used to create full-length antibody genes of any antibody isotype by inserting them into expression vectors already encoding heavy chain constant and light chain constant regions of the desired isotype such that the \(V_H\) segment is operatively linked to the \(C_H\) segment(s) within the vector and the \(V_K\) segment is operatively linked to the \(C_L\) segment within the vector. Additionally or alternatively, the recombinant expression vector can encode a signal
peptide that facilitates secretion of the antibody chain from a host cell. The antibody chain
gene can be cloned into the vector such that the signal peptide is linked in-frame to the amino
terminus of the antibody chain gene. The signal peptide can be an immunoglobulin signal
peptide or a heterologous signal peptide (i.e., a signal peptide from a non-immunoglobulin
protein).

In addition to the antibody chain genes, the recombinant expression vectors of the
invention carry regulatory sequences that control the expression of the antibody chain genes
in a host cell. The term "regulatory sequence" is intended to include promoters, enhancers
and other expression control elements (e.g., polyadenylation signals) that control the
transcription or translation of the antibody chain genes. Such regulatory sequences are
described, for example, in Goeddel (Gene Expression Technology. Methods in Enzymology
185, Academic Press, San Diego, CA (1990)). It will be appreciated by those skilled in the
art that the design of the expression vector, including the selection of regulatory sequences,
may depend on such factors as the choice of the host cell to be transformed, the level of
expression of protein desired, etc. Preferred regulatory sequences for mammalian host cell
expression include viral elements that direct high levels of protein expression in mammalian
cells, such as promoters and/or enhancers derived from cytomegalovirus (CMV), Simian
Virus 40 (SV40), adenovirus, (e.g., the adenovirus major late promoter (AdMLP) and
polyoma. Alternatively, nonviral regulatory sequences may be used, such as the ubiquitin
promoter or β-globin promoter. Still further, regulatory elements composed of sequences
from different sources, such as the SRa promoter system, which contains sequences from the
SV40 early promoter and the long terminal repeat of human T cell leukemia virus type 1

In addition to the antibody chain genes and regulatory sequences, the recombinant
expression vectors of the invention may carry additional sequences, such as sequences that
regulate replication of the vector in host cells (e.g., origins of replication) and selectable
marker genes. The selectable marker gene facilitates selection of host cells into which the
vector has been introduced (see, e.g., U.S. Pat. Nos. 4,399,216, 4,634,665 and 5,179,017, all
by Axel et al.). For example, typically the selectable marker gene confers resistance to
drugs, such as G418, hygromycin or methotrexate, on a host cell into which the vector has
been introduced. Preferred selectable marker genes include the dihydrofolate reductase
(DHFR) gene (for use in dhfr- host cells with methotrexate selection/amplification) and the
neo gene (for G418 selection).
For expression of the light and heavy chains, the expression vector(s) encoding the heavy and light chains is transfected into a host cell by standard techniques. The various forms of the term "transfection" are intended to encompass a wide variety of techniques commonly used for the introduction of exogenous DNA into a prokaryotic or eukaryotic host cell, e.g., electroporation, calcium-phosphate precipitation, DEAE-dextran transfection and the like. Although it is theoretically possible to express the antibodies of the invention in either prokaryotic or eukaryotic host cells, expression of antibodies in eukaryotic cells, and most preferably mammalian host cells, is the most preferred because such eukaryotic cells, and in particular mammalian cells, are more likely than prokaryotic cells to assemble and secrete a properly folded and immunologically active antibody. Prokaryotic expression of antibody genes has been reported to be ineffective for production of high yields of active antibody (Boss, M. A. and Wood, C. R. (1985) Immunology Today 6:12-13).

Preferred host cells for expressing the recombinant antibodies of the invention include cells which modify the fucosylation of an expressed antibody. For example, the host cell may be a cell that is lacking in a fucosyltransferase enzyme such that the host cell produces proteins lacking fucosyl in their carbohydrates, or a host cell that expresses glycoprotein-modifying glycosyl transferases such that expressed antibodies in the host cell have increased bisecting GlcNac structures that prevents fucosylation. Other mammalian host cells for expressing the recombinant antibodies include Chinese Hamster Ovary (CHO cells) (including dhrr- CHO cells, described in Urlaub and Chasin, (1980) Proc. Natl. Acad. Sd. USA 77:4216-4220, used with a DHFR selectable marker, e.g., as described in R. J. Kaufman and P. A. Sharp (1982) Mol Biol. 159:601-621), NSO myeloma cells, COS cells and SP2 cells. In particular, for use with NSO myeloma cells, another preferred expression system is the GS gene expression system disclosed in WO 87/04462, WO 89/01036 and EP 338,841. When recombinant expression vectors encoding antibody genes are introduced into mammalian host cells, the antibodies are produced by culturing the host cells for a period of time sufficient to allow for expression of the antibody in the host cells or, more preferably, secretion of the antibody into the culture medium in which the host cells are grown.

Antibodies can be recovered from the culture medium using standard protein purification methods.

Other preferred hosts for expressing the recombinant antibodies of the invention include cells which modify the fucosylation and xylosylation of an expressed antibody. For example, the host cell may be a plant cell that is lacking in a fucosyltransferase and xylosyltransferase enzyme such that the host cell produces proteins lacking fucosyl and...
xylosyl in their carbohydrates. Methods for altering the α-L-glycosylation pattern of proteins in higher plants include stably transforming the plant with at least one recombinant nucleotide construct that provides for the inhibition of expression of αL.3-fucosyltransferase (FucT) and β1,2-xylosyltransferase (XyIT) in a plant. Methods for production of antibodies in a plant system are disclosed in [Alston & Bird LLP attorney docket No.: 040989/322372] and [Alston & Bird LLP attorney docket No.: 040989/322364], filed on even date herewith, both of which are expressly incorporated herein by reference.

Immunocojugates

In another aspect, the present invention features a defucosylated and dexylosylated anti-CD30 antibody, or a fragment thereof, conjugated to a therapeutic moiety, such as a cytotoxin, a drug (e.g., an immunosuppressant) or a radiotoxin. Such conjugates are referred to herein as "immunoconjugates". Immunoconjugates that include one or more cytotoxins are referred to as "immunotoxins." A cytotoxin or cytotoxic agent includes any agent that is detrimental to (e.g., kills) cells. Examples include taxol, cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, tenoposide, vincristine, vinblastine, colchicine, doxorubicin, daunorubicin, dihydroxy anthracin dione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, and puromycin and analogs or homologs thereof. Therapeutic agents also include, for example, antimitabolites (e.g., methotrexate, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-fluorouracil decarbazine), alkylating agents (e.g., mechlorethamine, thioepa chlorambucil, melphalan, carmustine (BSNU) and lomustine (CCNU), cyclophosphamide, busulfan, dibromomannitol, streptozotocin, mitomycin C, and cis-dichlorodiamine platinum (II) (DDP) cisplatin), anthracyclines (e.g., daunorubicin (formerly daunomycin) and doxorubicin), antibiotics (e.g., dactinomycin (formerly actinomycin), bleomycin, mithramycin, and anthramycin (AMC)), and anti-mitotic agents (e.g., vincristine and vinblastine).

Other preferred examples of therapeutic cytotoxins that can be conjugated to an antibody of the invention include duocarmycins, calicheamicins, maytansines and auristatins, and derivatives thereof. An example of a calicheamicin antibody conjugate is commercially available (Mylotarg®; American Home Products).

Cytoxins can be conjugated to antibodies of the invention using linker technology available in the art. Examples of linker types that have been used to conjugate a cytotoxin to
an antibody include, but are not limited to, hydrazones, thioethers, esters, disulfides and peptide-containing linkers. A linker can be chosen that is, for example, susceptible to cleavage by low pH within the lysosomal compartment or susceptible to cleavage by proteases, such as proteases preferentially expressed in tumor tissue such as cathepsins (e.g., cathepsins B, C, D).


Antibodies of the present invention also can be conjugated to a radioactive isotope to generate cytotoxic radiopharmaceuticals, also referred to as radioimmunoconjugates. Examples of radioactive isotopes that can be conjugated to antibodies for use diagnostically or therapeutically include, but are not limited to, iodine$^{131}$, indium$^{111}$, yttrium$^{90}$ and lutetium$^{177}$. Method for preparing radioimmunoconjugates are established in the art. Examples of radioimmunoconjugates are commercially available, including Zevalin™ (IDEC Pharmaceuticals) and Bexxar™ (Corixa Pharmaceuticals), and similar methods can be used to prepare radioimmunoconjugates using the antibodies of the invention.

The antibody conjugates of the invention can be used to modify a given biological response, and the drug moiety is not to be construed as limited to classical chemical therapeutic agents. For example, the drug moiety may be a protein or polypeptide possessing a desired biological activity. Such proteins may include, for example, an enzymatically active toxin, or active fragment thereof, such as abrin, ricin A, pseudomonas exotoxin, or diphtheria toxin; a protein such as tumor necrosis factor or interferon-γ; or, biological response modifiers such as, for example, lymphokines, interleukin-1 ("IL-1"), interleukin-2 ("IL-2"), interleukin-6 ("IL-6"), granulocyte macrophage colony stimulating factor ("GM-CSF"), granulocyte colony stimulating factor ("G-CSF"), or other growth factors.

Techniques for conjugating such therapeutic moiety to antibodies are well known, see, e.g., Arnon et al., "Monoclonal Antibodies For Immunotargeting Of Drugs In Cancer Therapy", in Monoclonal Antibodies And Cancer Therapy, Reisfeld et al. (eds.), pp. 243-56 (Alan R. Liss, Inc. 1985); Hellstrom et al., "Antibodies For Drug Delivery", in Controlled Drug Delivery (2nd Ed.). Robinson et al. (eds.), pp. 623-53 (Marcel Dekker, Inc. 1987);

Bispecific Molecules

In another aspect, the present invention features bispecific molecules comprising an anti-CD30 antibody, or a fragment thereof, of the invention. An antibody of the invention, or antigen-binding portions thereof, can be derivatized or linked to another functional molecule, e.g., another peptide or protein (e.g., another antibody or ligand for a receptor) to generate a bispecific molecule that binds to at least two different binding sites or target molecules. The antibody of the invention may in fact be derivatized or linked to more than one other functional molecule to generate multispecific molecules that bind to more than two different binding sites and/or target molecules; such multispecific molecules are also intended to be encompassed by the term "bispecific molecule" as used herein. To create a bispecific molecule of the invention, an antibody of the invention can be functionally linked (e.g., by chemical coupling, genetic fusion, noncovalent association or otherwise) to one or more other binding molecules, such as another antibody, antibody fragment, peptide or binding mimic, such that a bispecific molecule results.

Accordingly, the present invention includes bispecific molecules comprising at least one first binding specificity for CD30 and a second binding specificity for a second target epitope. In a particular embodiment of the invention, the second target epitope is an Fc receptor, e.g., human FcγRI (CD64) or a human Fcα receptor (CD89). Therefore, the invention includes bispecific molecules capable of binding both to FcγR or FcαR expressing effector cells (e.g., monocytes, macrophages or polymorphonuclear cells (PMNs)), and to target cells expressing CD30. These bispecific molecules target CD30 expressing cells to effector cell and trigger Fc receptor-mediated effector cell activities, such as phagocytosis of an CD30 expressing cells, antibody dependent cell-mediated cytotoxicity (ADCC), cytokine release, or generation of superoxide anion.

In an embodiment of the invention in which the bispecific molecule is multispecific, the molecule can further include a third binding specificity, in addition to an anti-Fc binding
specificity and an anti-CD30 binding specificity. In one embodiment, the third binding specificity is an anti-enhancement factor (EF) portion, e.g., a molecule which binds to a surface protein involved in cytotoxic activity and thereby increases the immune response against the target cell. The "anti-enhancement factor portion" can be an antibody, functional antibody fragment or a ligand that binds to a given molecule, e.g., an antigen or a receptor, and thereby results in an enhancement of the effect of the binding determinants for the Fc receptor or target cell antigen. The "anti-enhancement factor portion" can bind an Fc receptor or a target cell antigen. Alternatively, the anti-enhancement factor portion can bind to an entity that is different from the entity to which the first and second binding specificities bind. For example, the anti-enhancement factor portion can bind a cytotoxic T-cell (e.g. via CD2, CD3, CD8, CD28, CD4, CD40, ICAM-I or other immune cell that results in an increased immune response against the target cell).

In one embodiment, the bispecific molecules of the invention comprise as a binding specificity at least one antibody, or an antibody fragment thereof, including, e.g., an Fab, Fab', F(ab')₂, Fv, Fd, dAb or a single chain Fv. The antibody may also be a light chain or heavy chain dimer, or any minimal fragment thereof such as a Fv or a single chain construct as described in U.S. Patent No. 4,946,778 to Ladner et al., the contents of which is expressly incorporated by reference.

In one embodiment, the binding specificity for an Fcγ receptor is provided by a monoclonal antibody, the binding of which is not blocked by human immunoglobulin G (IgG). As used herein, the term "IgG receptor" refers to any of the eight γ-chain genes located on chromosome 1. These genes encode a total of twelve transmembrane or soluble receptor isoforms which are grouped into three Fcγ receptor classes: FcγRI (CD64), Fcγ RH(CD32), and FcγRIII (CD 16). In one preferred embodiment, the Fcγ receptor a human high affinity FcγRI. The human FcγRI is a 72 kDa molecule, which shows high affinity for monomeric IgG (10⁶ - 10⁹ M⁻¹).

The production and characterization of certain preferred anti-Fcγ monoclonal antibodies are described in PCT Publication WO 88/00052 and in U.S. Patent No. 4,954,617 to Fanger et al., the teachings of which are fully incorporated by reference herein. These antibodies bind to an epitope of FcγRI, FcγRH or FcγRIII at a site which is distinct from the Fcγ binding site of the receptor and, thus, their binding is not blocked substantially by physiological levels of IgG. Specific anti-FcγRI antibodies useful in this invention are mAb 22, mAb 32, mAb 44, mAb 62 and mAb 197. The hybridoma producing mAb 32 is available
from the American Type Culture Collection, ATCC Accession No. HB9469. In other 
embodiments, the anti-Fcγ receptor antibody is a humanized form of monoclonal antibody 22 
(H22). The production and characterization of the H22 antibody is described in Graziano, 
Tempest et al. The H22 antibody producing cell line was deposited at the American Type 
Culture Collection under the designation HA022CL1 and has the accession no. CRL 11177.

In still other preferred embodiments, the binding specificity for an Fc receptor is provided by an antibody that binds to a human IgA receptor, e.g., an Fc-alpha receptor (Fcα 
RI (CD89)), the binding of which is preferably not blocked by human immunoglobulin A 
(IgA). The term "IgA receptor" is intended to include the gene product of one α-gene (Fcα 
RT) located on chromosome 19. This gene is known to encode several alternatively spliced transmembrane isoforms of 55 to 110 kDa. FcγRI (CD89) is constitutively expressed on monocytes/macrophages, eosinophilic and neutrophilic granulocytes, but not on non-effector cell populations. FcαRI has medium affinity (≈ 5 x 10^7 M^-1) for both IgA1 and IgA2, which is increased upon exposure to cytokines such as G-CSF or GM-CSF (Morton, H.C. et al. 

FcαRI and FcγRI are preferred trigger receptors for use in the bispecific molecules of the invention because they are (1) expressed primarily on immune effector cells, e.g., monocytes, PMNs, macrophages and dendritic cells; (2) expressed at high levels (e.g., 5,000-100,000 per cell); (3) mediators of cytotoxic activities (e.g., ADCC, phagocytosis); and (4) mediate enhanced antigen presentation of antigens, including self-antigens, targeted to them.

While human monoclonal antibodies are preferred, other antibodies which can be 
employed in the bispecific molecules of the invention are murine, chimeric and humanized monoclonal antibodies.

The bispecific molecules of the present invention can be prepared by conjugating the constituent binding specificities, e.g., the anti-FcR and anti-CD30 binding specificities, using methods known in the art. For example, each binding specificity of the bispecific molecule can be generated separately and then conjugated to one another. When the binding specificities are proteins or peptides, a variety of coupling or cross-linking agents can be used for covalent conjugation. Examples of cross-linking agents include protein A, carbodiimide, N-succinimidyl-S-acetyl-thioacetate (SATA), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), o-

When the binding specificities are antibodies, they can be conjugated via sulphydryl bonding of the C-terminus hinge regions of the two heavy chains. In a particularly preferred embodiment, the hinge region is modified to contain an odd number of sulphydryl residues, preferably one, prior to conjugation.

Alternatively, both binding specificities can be encoded in the same vector and expressed and assembled in the same host cell. This method is particularly useful where the bispecific molecule is a mAb x mAb, mAb x Fab, Fab x F(ab')2 x ligand x Fab fusion protein. A bispecific molecule of the invention can be a single chain molecule comprising one single chain antibody and a binding determinant, or a single chain bispecific molecule comprising two binding determinants. Bispecific molecules may comprise at least two single chain molecules. Methods for preparing bispecific molecules are described for example in U.S. Patent Numbers 5,260,203; 5,455,030; 4,881,175; 5,132,405; 5,091,513; 5,476,786; 5,013,653; 5,258,498; and 5,482,858, all of which are expressly incorporated herein by reference.

Binding of the bispecific molecules to their specific targets can be confirmed by, for example, enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), FACS analysis, bioassay (e.g., growth inhibition), or Western Blot assay. Each of these assays generally detects the presence of protein-antibody complexes of particular interest by employing a labeled reagent (e.g., an antibody) specific for the complex of interest. For example, the FcR-antibody complexes can be detected using e.g., an enzyme-linked antibody or antibody fragment which recognizes and specifically binds to the antibody-FcR complexes. Alternatively, the complexes can be detected using any of a variety of other immunoassays. For example, the antibody can be radioactively labeled and used in a radioimmunoassay (RIA) (see, for example, Weintraub, B., Principles of Radioimmunoassays, Seventh Training Course on Radioligand Assay Techniques, The Endocrine Society, March, 1986, which is incorporated by reference herein). The radioactive
isotope can be detected by such means as the use of a y counter or a scintillation counter or by autoradiography.

**Pharmaceutical Compositions**

In another aspect, the present invention provides a composition, *e.g.*, a pharmaceutical composition, containing one or a combination of monoclonal antibodies, or antigen-binding portion(s) thereof, of the present invention, formulated together with a pharmaceutically acceptable carrier. Such compositions may include one or a combination of (*e.g.*, two or more different) antibodies, or immunoconjugates of the invention. For example, a pharmaceutical composition of the invention can comprise a combination of antibodies (or immunoconjugates) that bind to different epitopes on the target antigen or that have complementary activities.

Pharmaceutical compositions of the invention also can be administered in combination therapy, *i.e.*, combined with other agents. For example, the combination therapy can include a defucosylated anti-CD30 antibody of the present invention combined with at least one other anti-neoplastic, anti-inflammatory or immunosuppressive agent. Such therapeutic agents include, among others, steroidal and nonsteroidal anti-inflammatory drugs (NSAIDS), *e.g.*, aspirin and other salicylates, such as ibuprofen (Motrin, Advil), naproxen (Naprosyn), sulindac (Clinoril), diclofenac (Voltaren), piroxicam (Feldene), ketoprofen (Orudis), diflunisal (Dolobid), nabumetone (Relafen), etodolac (Lodine), oxaprozin (Daypro), indomethacin (Indocin), and aspirin in high doses. Other examples of therapeutic agents that can be used in combination therapy are described in greater detail below in the section on uses of the antibodies of the invention.

As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like that are physiologically compatible. Preferably, the carrier is suitable for intravenous, intramuscular, subcutaneous, parenteral, spinal or epidermal administration (*e.g.*, by injection or infusion). Depending on the route of administration, the active compound, *i.e.*, antibody or immunoconjugate, may be coated in a material to protect the compound from the action of acids and other natural conditions that may inactivate the compound.

The pharmaceutical compounds of the invention may include one or more pharmaceutically acceptable salts. A "pharmaceutically acceptable salt" refers to a salt that retains the desired biological activity of the parent compound and does not impart any undesired toxicological effects (*see e.g.*, Berge, S.M., *et al.* (1977) *J. Pharm. Sd.* 66:1-19).
Examples of such salts include acid addition salts and base addition salts. Acid addition salts
include those derived from nontoxic inorganic acids, such as hydrochloric, nitric, phosphoric,
sulfuric, hydrobromic, hydroiodic, phosphorous and the like, as well as from nontoxic
organic acids such as aliphatic mono- and dicarboxylic acids, phenyl-substituted alkanoic
acids, hydroxy alkanoic acids, aromatic acids, aliphatic and aromatic sulfonic acids and the
like. Base addition salts include those derived from alkaline earth metals, such as sodium,
potassium, magnesium, calcium and the like, as well as from nontoxic organic amines, such
as N,N'-dibenzylethylenediamine, N-methylglucamine, chloroprocaine, choline,
diethanolamine, ethylenediamine, procaine and the like.

A pharmaceutical composition of the invention also may include a pharmaceutically
acceptable anti-oxidant. Examples of pharmaceutically acceptable antioxidants include: (1)
water soluble antioxidants, such as ascorbic acid, cysteine hydrochloride, sodium bisulfate,
sodium metabisulfite, sodium sulfite and the like; (2) oil-soluble antioxidants, such as
ascorbil palmitate, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT),
lecithin, propyl gallate, alpha-tocopherol, and the like; and (3) metal chelating agents, such as
citric acid, ethylenediamine tetraacetic acid (EDTA), sorbitol, tartaric acid, phosphoric acid,
and the like.

Examples of suitable aqueous and nonaqueous carriers that may be employed in the
pharmaceutical compositions of the invention include water, ethanol, polyols (such as
glycerol, propylene glycol, polyethylene glycol, and the like), and suitable mixtures thereof,
vegetable oils, such as olive oil, and injectable organic esters, such as ethyl oleate. Proper
fluidity can be maintained, for example, by the use of coating materials, such as lecithin, by
the maintenance of the required particle size in the case of dispersions, and by the use of
surfactants.

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

Pharmaceutically acceptable carriers include sterile aqueous solutions or dispersions
and sterile powders for the extemporaneous preparation of sterile injectable solutions or
dispersion. The use of such media and agents for pharmaceutically active substances is
known in the art. Except insofar as any conventional media or agent is incompatible with the
active compound, use thereof in the pharmaceutical compositions of the invention is
contemplated. Supplementary active compounds can also be incorporated into the
compositions.

Therapeutic compositions typically must be sterile and stable under the conditions of
manufacture and storage. The composition can be formulated as a solution, microemulsion,
emulsion, liposome, or other ordered structure suitable to high drug concentration. The carrier can be a
solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, 
glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable
mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating
such as lecithin, by the maintenance of the required particle size in the case of dispersion and
by the use of surfactants. In many cases, it will be preferable to include isotonic agents, for
example, sugars, polyalcohols such as mannitol, sorbitol, or sodium chloride in the
composition. Prolonged absorption of the injectable compositions can be brought about by
including in the composition an agent that delays absorption, for example, monostearate salts
and gelatin.

Sterile injectable solutions can be prepared by incorporating the active compound in
the required amount in an appropriate solvent with one or a combination of ingredients
enumerated above, as required, followed by sterilization microfiltration. Generally,
dispersions are prepared by incorporating the active compound into a sterile vehicle that
contains a basic dispersion medium and the required other ingredients from those enumerated
above. In the case of sterile powders for the preparation of sterile injectable solutions, the
preferred methods of preparation are vacuum drying and freeze-drying (lyophilization) that
yield a powder of the active ingredient plus any additional desired ingredient from a
previously sterile-filtered solution thereof.

The amount of active ingredient which can be combined with a carrier material to
produce a single dosage form will vary depending upon the subject being treated, and the
particular mode of administration. The amount of active ingredient which can be combined
with a carrier material to produce a single dosage form will generally be that amount of the
composition which produces a therapeutic effect. Generally, out of one hundred per cent,
this amount will range from about 0.01 per cent to about ninety-nine percent of active
ingredient, preferably from about 0.1 per cent to about 70 per cent, most preferably from
about 1 per cent to about 30 per cent of active ingredient in combination with a pharmaceutically acceptable carrier.

Dosage regimens are adjusted to provide the optimum desired response (e.g., a therapeutic response). For example, a single bolus may be administered, several divided doses may be administered over time or the dose may be proportionally reduced or increased as indicated by the exigencies of the therapeutic situation. It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subjects to be treated; each unit contains a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active compound for the treatment of sensitivity in individuals.

For administration of the antibody, the dosage ranges from about 0.0001 to 100 mg/kg, and more usually 0.01 to 5 mg/kg, of the host body weight. For example dosages can be 0.3 mg/kg body weight, 1 mg/kg body weight, 3 mg/kg body weight, 5 mg/kg body weight or 10 mg/kg body weight or within the range of 1-10 mg/kg. An exemplary treatment regime entails administration once per week, once every two weeks, once every three weeks, once every four weeks, once a month, once every 3 months or once every three to 6 months. Preferred dosage regimens for a defucosylated anti-CD30 antibody of the invention include 1 mg/kg body weight or 3 mg/kg body weight via intravenous administration, with the antibody being given using one of the following dosing schedules: (i) every four weeks for six dosages, then every three months; (ii) every three weeks; (iii) 3 mg/kg body weight once followed by 1 mg/kg body weight every three weeks.

In some methods, two or more monoclonal antibodies with different binding specificities are administered simultaneously, in which case the dosage of each antibody administered falls within the ranges indicated. Antibody is usually administered on multiple occasions. Intervals between single dosages can be, for example, weekly, monthly, every three months or yearly. Intervals can also be irregular as indicated by measuring blood levels of antibody to the target antigen in the patient. In some methods, dosage is adjusted to
achieve a plasma antibody concentration of about 1-1000 µg/ml and in some methods about 25-300 µg /ml.

Alternatively, antibody can be administered as a sustained release formulation, in which case less frequent administration is required. Dosage and frequency vary depending on the half-life of the antibody in the patient. In general, human antibodies show the longest half life, followed by humanized antibodies, chimeric antibodies, and nonhuman antibodies. The dosage and frequency of administration can vary depending on whether the treatment is prophylactic or therapeutic. In prophylactic applications, a relatively low dosage is administered at relatively infrequent intervals over a long period of time. Some patients continue to receive treatment for the rest of their lives. In therapeutic applications, a relatively high dosage at relatively short intervals is sometimes required until progression of the disease is reduced or terminated, and preferably until the patient shows partial or complete amelioration of symptoms of disease. Thereafter, the patient can be administered a prophylactic regime.

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

A "therapeutically effective dosage" of an anti-CD30 antibody of the invention preferably results in a decrease in severity of disease symptoms, an increase in frequency and duration of disease symptom-free periods, or a prevention of impairment or disability due to the disease affliction. For example, for the treatment of cancerous tumors, a "therapeutically effective dosage" preferably inhibits cell growth or tumor growth by at least about 20%, more preferably by at least about 40%, even more preferably by at least about 60%, and still more preferably by at least about 80% relative to untreated subjects. The ability of a compound to inhibit tumor growth can be evaluated in an animal model system predictive of
efficacy in human tumors. Alternatively, this property of a composition can be evaluated by examining the ability of the compound to inhibit, such inhibition in vitro by assays known to the skilled practitioner. A therapeutically effective amount of a therapeutic compound can decrease tumor size, or otherwise ameliorate symptoms in a subject. One of ordinary skill in the art would be able to determine such amounts based on such factors as the subject's size, the severity of the subject's symptoms, and the particular composition or route of administration selected.

A composition of the present invention can be administered via one or more routes of administration using one or more of a variety of methods known in the art. As will be appreciated by the skilled artisan, the route and/or mode of administration will vary depending upon the desired results. Preferred routes of administration for antibodies of the invention include intravenous, intramuscular, intradermal, intraperitoneal, subcutaneous, spinal or other parenteral routes of administration, for example by injection or infusion. The phrase "parenteral administration" as used herein means modes of administration other than enteral and topical administration, usually by injection, and includes, without limitation, intravenous, intramuscular, intraarterial, intrathecal, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticular, subcapsular, subarachnoid, intraspinal, epidural and intrasternal injection and infusion. Alternatively, a defucosylated antibody of the invention can be administered via a non-parenteral route, such as a topical, epidermal or mucosal route of administration, for example, intranasally, orally, vaginally, rectally, sublingually or topically.

The active compounds can be prepared with carriers that will protect the compound against rapid release, such as a controlled release formulation, including implants, transdermal patches, and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Many methods for the preparation of such formulations are patented or generally known to those skilled in the art. See, e.g., Sustained and Controlled Release Drug Delivery Systems, J.R. Robinson, ed., Marcel Dekker, Inc., New York, 1978.

Therapeutic compositions can be administered with medical devices known in the art. For example, in a preferred embodiment, a therapeutic composition of the invention can be administered with a needleless hypodermic injection device, such as the devices disclosed in U.S. Patent Nos. 5,399,163; 5,383,851; 5,312,335; 5,064,413; 4,941,880; 4,790,824; or 4,596,556. Examples of well-known implants and modules useful in the present invention
include: U.S. Patent No. 4,487,603, which discloses an implantable micro-infusion pump for dispensing medication at a controlled rate; U.S. Patent No. 4,486,194, which discloses a therapeutic device for administering medicants through the skin; U.S. Patent No. 4,447,233, which discloses a medication infusion pump for delivering medication at a precise infusion rate; U.S. Patent No. 4,447,224, which discloses a variable flow implantable infusion apparatus for continuous drug delivery; U.S. Patent No. 4,439,196, which discloses an osmotic drug delivery system having multi-chamber compartments; and U.S. Patent No. 4,475,196, which discloses an osmotic drug delivery system. These patents are incorporated herein by reference. Many other such implants, delivery systems, and modules are known to those skilled in the art.

In certain embodiments, the defucosylated antibodies of the invention can be formulated to ensure proper distribution in vivo. For example, the blood-brain barrier (BBB) excludes many highly hydrophilic compounds. To ensure that the therapeutic compounds of the invention cross the BBB (if desired), they can be formulated, for example, in liposomes. For methods of manufacturing liposomes, see, e.g., U.S. Patents 4,522,811: 5,374,548; and 5,399,331. The liposomes may comprise one or more moieties which are selectively transported into specific cells or organs, thus enhance targeted drug delivery (see, e.g., V.V. Ranade (1989) J. Clin. Pharmacol. 29:685). Exemplary targeting moieties include folate or biotin (see, e.g., U.S. Patent 5,416,016 to Low et al.); manniosides (Umezawa et al., (1988) Biochem. Biophys. Res. Commun. 153:1038); antibodies (P.G. Bloeman et al. (1995) FEBS Lett. 357:140; M. Owais et al. (1995) Antimicrob. Agents Chemother. 39:180); surfactant protein A receptor (Briscoe et al. (1995) Am. J. Physiol. 1233:134); pl20 (Schreier et al. (1994) J. Biol. Chem. 269:9090); see also K. Keinanen; MX. Laukkanen (1994) FEBS Lett. 346:123; JJ. Killion; I.J. Fidler (1994; Immunomethods ±213.

Uses and Methods of the Invention

The defucosylated antibodies, antibody compositions and methods of the present invention have numerous in vitro and in vivo diagnostic and therapeutic utilities involving the diagnosis and treatment of disorders involving CD30 expression. For example, these molecules can be administered to cells in culture, e.g. in vitro or ex vivo, or to human subjects, e.g., in vivo, to treat, prevent and to diagnose a variety of disorders. As used herein, the term "subject" is intended to include human and non-human animals. Non-human animals includes all vertebrates, e.g., mammals and non-mammals, such as non-human primates, sheep, dogs, cats, cows, horses, pigs, chickens, avians, amphibians, and reptiles. Preferred subjects include human patients having disorders characterized by CD30
expression. When antibodies to CD30 are administered together with another agent, the two can be administered in either order or simultaneously.

Other antibodies can be used in combination with anti-CD30 antibodies of the present invention to produce an additive or synergistic effect. These include molecules on the surface of dendritic cells which activate DC function and antigen presentation. Anti-CD40 antibodies are able to substitute effectively for T cell helper activity (Ridge, J. et al. (1998) Nature 393: 474-478) and can be used in conjunction with CD30 antibodies. Activating antibodies to T cell costimulatory molecules such as CTLA-4 (e.g., US Patent No. 5,811,097), OX-40 (Weinberg, A. et al. (2000) Immunol Lett 76: 2160-2169), 4-1BB (Melero, I. et al. (1997) Nature Medicine 3: 682-685 (1997), and ICOS (Hutloff, A. et al (1999) Nature 397: 262-266) may also provide for increased levels of T cell activation.

Suitable routes of administering the antibody compositions (e.g., antibody or immunoconjugate) of the invention in vivo and in vitro are well known in the art and can be selected by those of ordinary skill. For example, the antibody compositions can be administered by injection (e.g., intravenous or subcutaneous). Suitable dosages of the molecules used will depend on the age and weight of the subject and the concentration and/or formulation of the antibody composition.

In one embodiment, the antibodies of the invention can be initially tested for binding activity associated with therapeutic or diagnostic use in vitro. For example, compositions of the invention can be tested using ELISA and flow cytometric assays. Moreover, the activity of these molecules in triggering at least one effector-mediated effector cell activity, including inhibiting the growth of and/or killing of cells expressing CD30 can be assayed. Protocols for assaying for effector cell-mediated ADCC are described in the Examples below.

A. Detection Methods

In one embodiment, the antibodies of the invention can be used to detect levels of CD30, or levels of cells which contain CD30 on their membrane surface, which levels can then be linked to certain disease symptoms.

In a particular embodiment, the invention provides methods for detecting the presence of CD30 antigen in a sample, or measuring the amount of CD30 antigen, comprising contacting the sample, and a control sample, with a defucosylated antibody, or an antigen binding portion thereof, which specifically binds to CD30, under conditions that allow for formation of a complex between the antibody or portion thereof and CD30. The formation of a complex is then detected, wherein a difference complex formation between the sample compared to the control sample is indicative the presence of CD30 antigen in the sample.
For example, standard detection methods, well-known in the art, such as ELISA and flow cytometric assays, can be performed using the compositions of the invention.

Accordingly, in one aspect, the invention further provides methods for detecting the presence of CD30 (e.g., human CD30 antigen) in a sample, or measuring the amount of CD30, comprising contacting the sample, and a control sample, with an antibody of the invention, or an antigen binding portion thereof, which specifically binds to CD30, under conditions that allow for formation of a complex between the antibody or portion thereof and CD30. The formation of a complex is then detected, wherein a difference in complex formation between the sample compared to the control sample is indicative of the presence of CD30 in the sample.

The compositions of the invention can also be used to target cells expressing CD30, for example for labeling such cells. For such use, the binding agent can be linked to a molecule that can be detected. Thus, the invention provides methods for localizing ex vivo or in vitro cells expressing CD30. The detectable label can be, e.g., a radioisotope, a fluorescent compound, an enzyme, or an enzyme co-factor.

**B. Inhibition of Growth of CD30+ Cells**

The antibodies can be used to inhibit or block CD30 function which, in turn, can be linked to the prevention or amelioration of certain disease symptoms, thereby implicating CD30 as being involved in the disease. Differences in CD30 expression during a disease state as compared to a non-disease state can be determined by contacting a test sample from a subject suffering from the disease and a control sample with the anti-CD30 antibody under conditions that allow for the formation of a complex between the antibody and CD30. Any complexes formed between the antibody and CD30 are detected and compared in the sample and the control.

For example, the antibodies can be used to elicit in vivo or in vitro one or more of the following biological activities: to inhibit the growth of and/or kill a cell expressing CD30; to mediate phagocytosis or ADCC of a cell expressing CD30 in the presence of human effector cells; to inhibit shedding of soluble CD30, to block CD30 ligand binding to CD30, to inhibit IL-4 expression or to mediate expression of the Th2 phenotype, e.g., at low dosages. As discussed herein, the defucosylated antibodies of the invention exhibit enhanced ADCC activity as compared to the fucosylated form of the antibody.

Accordingly, in another aspect, the invention provides a method of inhibiting growth of CD30+ cells comprising contacting said cells with a defucosylated anti-CD30 antibody under conditions sufficient to induce antibody-dependent cellular cytotoxicity (ADCC) of said
cells. The cells can be, for example, tumor cells. In a preferred embodiment, the anti-CD30 antibody is a human antibody.

In one embodiment, the antibodies, or binding portions thereof, of the present invention can be used to modulate CD30 levels on target cells, such as by capping and eliminating receptors on the cell surface. Mixtures of anti-Fc receptor antibodies can also be used for this purpose.

Target-specific effector cells, e.g., effector cells linked to compositions of the invention can also be used as therapeutic agents. Effector cells for targeting can be human leukocytes such as macrophages, neutrophils or monocytes. Other cells include eosinophils, natural killer cells and other IgG- or IgA-receptor bearing cells. If desired, effector cells can be obtained from the subject to be treated. The target-specific effector cells, can be administered as a suspension of cells in a physiologically acceptable solution. The number of cells administered can be in the order of $10^8 - 10^9$ but will vary depending on the therapeutic purpose. In general, the amount will be sufficient to obtain localization at the target cell, e.g., a tumor cell expressing CD30, and to effect cell killing by, e.g., phagocytosis. Routes of administration can also vary.

Therapy with target-specific effector cells can be performed in conjunction with other techniques for removal of targeted cells. For example, anti-tumor therapy using the compositions of the invention and/or effector cells armed with these compositions can be used in conjunction with chemotherapy. Additionally, combination immunotherapy may be used to direct two distinct cytotoxic effector populations toward tumor cell rejection.

C. Use of Immunoconjugates and Combination Therapy

In one embodiment, immunoconjugates of the invention can be used to target compounds (e.g., therapeutic agents, labels, cytotoxins, radiotoxins immunosuppressants, etc.) to cells which have CD30 cell surface receptors by linking such compounds to the antibody. For example, an anti-CD30 antibody can be conjugated to any of the toxin compounds described in US Patent Nos. 6, 281, 354 and 6,548,530, US patent publication Nos. 20030050331, 20030064984, 20030073852, and 20040087497, published in WO 03/022806, WO05/112919 or disclosed in [the U.S. Patent application corresponding to Darby & Darby LLP attorney docket No. 0203496-WOO, filed on April 7, 2006], which are hereby incorporated by reference in their entirety. Thus, the invention also provides methods for localizing ex vivo or in vitro cells expressing CD30 (e.g., with a detectable label, such as a radioisotope, a fluorescent compound, an enzyme, or an enzyme co-factor). Alternatively,
the immunoconjugates can be used to kill cells which have CD30 cell surface receptors by targeting cytotoxins or radiotoxins to CD30, such as to CD30-expressing tumor cells to thereby eliminate the tumor cell, or to CD30-expressing antigen-presenting cells to thereby eliminate the APCs as a means to inhibit immune responses (e.g., in autoimmune disorders).

In other embodiments, the subject can be additionally treated with an agent that modulates, e.g., enhances or inhibits, the expression or activity of Fcγ or Fcγ receptors by, for example, treating the subject with a cytokine. Preferred cytokines for administration during treatment include of granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon-γ (IFN-γ), and tumor necrosis factor (TNF).

In another embodiment, the subject can be additionally treated with a lymphokine preparation. Cancer cells which do not highly express CD30 can be induced to do so using lymphokine preparations. Lymphokine preparations can cause a more homogeneous expression of CD30 among cells of a tumor which can lead to a more effective therapy.

Lymphokine preparations suitable for administration include interferon-gamma, tumor necrosis factor, and combinations thereof. These can be administered intravenously. Suitable dosages of lymphokine are 10,000 to 1,000,000 units/patient.

In another embodiment, patients treated with antibody compositions of the invention can be additionally administered (prior to, simultaneously with, or following administration of an antibody of the invention) with another therapeutic agent, such as a cytotoxic or radiotoxic agent, which enhances or augments the therapeutic effect of the human antibodies, or another antibody. The antibody can be linked to the agent (as an immunocomplex) or can be administered separate from the agent. In the latter case (separate administration), the antibody can be administered before, after or concurrently with the agent or can be co-administered with other known therapies, e.g., an anti-cancer therapy, e.g., radiation. Such therapeutic agents include, among others, anti-neoplastic agents such as doxorubicin (adriamycin), cisplatin bleomycin sulfate, carmustine, chlorambucil, and cyclophosphamide hydroxyurea which, by themselves, are only effective at levels which are toxic or subtoxic to a patient. Cisplatin is intravenously administered as a 100 mg/ml dose once every four weeks and adriamycin is intravenously administered as a 60-75 mg/ml dose once every 21 days. Co-administration of the anti-CD30 antibodies, or antigen binding fragments thereof, of the present invention with chemotherapeutic agents provides two anti-cancer agents which operate via different mechanisms which yield a cytotoxic effect to human tumor cells. Such
co-administration can solve problems due to development of resistance to drugs or a change in the antigenicity of the tumor cells which would render them unreactive with the antibody.

D. Treatment of Autoimmune Diseases

The compositions can be used in vitro or in vivo to treat diseases mediated by or involving CD30, for example, diseases characterized by expression, typically overexpression, of CD30 such as autoimmune diseases mediated by macrophages, activated neutrophils, dendritic cells or NK cells, such as thyroid autoimmune diseases, such as Graves' Disease and Hashimoto's thyroiditis, autoimmune diabetes and multiple sclerosis (Ruggeri et al. (2006) Histol Histopathol. 21:249-56; Chiarle et al. (2003) Pathologica 95:229-30; Chakrabarty et al. (2003) CUn Exp Immunol.133:318-225; Watanabe et al. (2003) Thyroid 11:259-63; Pellegrini et al. (2005) Neuroimmunomodulation 12:220-34). Soluble CD30 is regularly shed from the surface of cells expressing CD30 and elevated sCD30 levels have been reported in the serum of patients with a variety of tumorigenie and autoimmune disorders. Accordingly, yet another use for the antibodies of the invention includes the prevention or treatment of diseases involving blocking or inhibiting of shedding of sCD30.

By contacting the antibody with CD30 (e.g., by administering the antibody to a subject), the ability of CD30 to induce such activities is inhibited and, thus, the associated disorder is treated. The antibody composition can be administered alone or along with another therapeutic agent, such as an immunosuppressant which acts in conjunction with or synergistically with the antibody composition to treat or prevent the CD30 mediated disease. Preferred antibodies bind to epitopes which are specific to CD30 and, thus, advantageously inhibit CD30 induced activities, but do not interfere with the activity of structurally related surface antigens. The compositions can be used to treat any diseases mediated by CD30 expressing cells, including, but not limited to, autoimmune hemolytic anemia (AIHA), rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), Systemic Sclerosis, Atopic Dermatitis, Graves' disease, Hashimoto's thyroiditis, Wegner's granulomatosis, Omen's syndrome, chronic renal failure, idiopathic thrombocytopenic purpura (ITP), inflammatory bowel disease (IBD; including Crohn's Disease, Ulcerative Colitis and Celiac's Disease), insulin dependent diabetes mellitus (IDDM), acute infectious mononucleosis, HIV, herpes virus associated diseases, multiple sclerosis (MS), transplantation rejection, allergy or Graft versus Host Disease (GVHD), hemolytic anemia, thyroiditis, stiff man syndrome, pemphigus vulgaris and myasthenia gravis (MG).

E. Treatment of Cancer
In another embodiment, the present invention provides a method of inhibiting the growth of CD30+ tumor cells (Le., tumor cells expressing CD30) in a subject, in which a defiicosylated anti-CD30 antibody of the invention is administered to the subject such that growth of the CD30+ tumor cells is inhibited. For human subjects, the antibody preferably is a humanized or human antibody. In a preferred embodiment, the tumor cells are Hodgkin's Disease tumor cells. In another preferred embodiment, the tumor cells are anaplastic large-cell lymphomas (ALCL) tumor cells. In other embodiments, the tumor cells may be from a disease selected from the group consisting of non-Hodgkin's lymphoma, Burkitt's lymphoma, cutaneous T-cell lymphomas, nodular small cleaved-cell lymphomas, lymphocytic lymphomas, peripheral T-cell lymphomas, Lennert's lymphomas, immunoblastic lymphomas, T-cell leukemia/lymphomas (ATLL), adult T-cell leukemia (T-ALL), enteroblastic/centrocytic (cb/cc) follicular lymphomas cancers, diffuse large cell lymphomas of B lineage, angioimmunoblastic lymphadenopathy (AILD)-like T cell lymphoma, adult T-cell lymphoma (ATL), HIV associated body cavity based lymphomas, Embryonal Carcinomas, undifferentiated carcinomas of the rhino-pharynx (e.g., Schmincke's tumor), Castleman's disease, Kaposi's Sarcoma and other CD30+ T-cell lymphomas and CD30+ B-cell lymphomas.

The method involves administering to a subject an antibody composition of the present invention in an amount effective to treat or prevent the disorder. The antibody composition can be administered alone or along with another therapeutic agent, such as a cytotoxic or a radiotoxic agent which acts in conjunction with or synergistically with the antibody composition to treat or prevent the disease associated with CD30 expression.

Kits

Also within the scope of the invention are kits comprising an antibody of the invention and instructions for use. The kit can further contain one or more additional reagents, such as an immunostimulatory reagent, a cytotoxic agent or a radiotoxic agent, or one or more additional antibodies of the invention (e.g., an antibody having a complementary activity which binds to an epitope in the CD30 antigen distinct from the first antibody). Kits typically include a label indicating the intended use of the contents of the kit. The term label includes any writing, or recorded material supplied on or with the kit, or which otherwise accompanies the kit.

The present invention is further illustrated by the following examples which should not be construed as further limiting. The contents of all figures and all references, patents
and published patent applications cited throughout this application are expressly incorporated herein by reference.

EXAMPLES

5 Example 1: Preparation and Characterization of Defucpsylated Anti-CD30 Monoclonal Antibody

In this example, a fully human anti-CD30 monoclonal antibody was expressed in a cell line lacking a fucosyl transferase enzyme such that the cell line produces proteins lacking fucosyl in their carbohydrates. The defucosylated antibody was tested against a fucosylated anti-CD30 antibody (expressed in a different cell line that contains the fucosyl transferase enzyme) to determine structural and characteristic differences between the antibodies, using a variety of chemical analysis techniques, including capillary electrophoresis, comparison of amino acid sequence, mass differences by mass spectroscopy and charge variation by capillary isoelectric focusing.

The anti-CD30 fully human monoclonal antibody 5Fl 1 was originally described in PCT Publication WO 03/059282. The amino acid and nucleotide sequences of the 5Fl 1 heavy chain is shown in Figure IA and the amino acid and nucleotide sequences of the 5Fl 1 light chain are shown in Figure IB. The 5Fl 1 heavy and light chain variable sequences were subcloned into an expression vector. The 5Fl 1 kappa variable region cDNA, including its signal sequence and an optimal Kozak sequence, was subcloned in frame with the human kappa constant region. The 5Fl 1 heavy chain variable region cDNA, including its signal sequence and an optimal Kozak sequence, was subcloned in frame with the human γ1 heavy constant region. Both light and heavy chain expression were driven by human ubiquitin C promoters (Nenoi, M. et al. Gene 175:179, 1996). This expression vector is described in further detail in U.S. Patent Application Serial No. 60/500,803, the contents of which are expressly incorporated herein by reference.

The expression vector was transfected into the FUT8<sup>+</sup> host cell line Ms704 by DNA electroporation. The Ms704 FUT8<sup>+</sup> cell line was created by the targeted disruption of the FUT8 gene in CHO/DG44 cells using two replacement vectors, and is more fully described in U.S. Patent Publication 20040110704 by Yamane et al. and Yarnane-Ohnuki et al. (2004) Biotechnol Bioeng 87:614-22. The Ms704 cells were adapted to growth in suspension culture in growth medium, EX-CELL™ 325 PF CHO Medium (JRH #14335) supplemented
with 100 µM hypoxanthine with 16 µM thymidine (Invitrogen #11067-030) and 6 mM L-glutamine (Invitrogen #25030-081).

The vector DNA to be used for electroporation was ethanol precipitated and resuspended in 10 mM Tris 7.6, 1 mM EDTA. 1, 5, 10, 15 or 20 µg DNA was utilized for twenty electroporations, four electroporations per DNA concentration. The Ms704 cells were prepared for transfection by washing the cells in a sucrose-buffered solution (SBS) and resuspending the cells at 1 X 10^7 cells/ml SBS solution. 400 µl cells were mixed with construct DNA and electroporated utilizing settings at 230 volts, 400 microfaradays capacitance and 13 ohms resistance (BTX Molecular Delivery Systems #600 electro cell manipulator). The cells were removed from the electroporation cuvettes and 20 ml growth medium was added. The cells were plated into a 96 well dish using 200 µl cells per well, approximately 4 X 10^4 cells/well. 2 days after the electroporation, 150 µl of medium was removed from each well and replaced with 150 µl selection medium, growth medium with 400 µg/ml G418 (Invitrogen #10131-035). Every three to seven days, 150 µl of selection medium per well was replaced with fresh selection medium. CHO DG44 host cells (FUT 8 +/-) were electroporated with the identical 5Fl 1 construct using a similar procedure and CHO DG44 transfectants expressing recombinant 5Fl 1 antibody containing fucosylated carbohydrates were established.

The highest producing Ms704 and CHO DG44 clones were expanded and recombinant 5Fl 1 antibody was purified from cell culture supernatants by Protein A affinity chromatography.

Comparative analysis of N-linked oligosaccharides derived from the Ms704 and the CHO DG44 derived anti-CD30 monoclonal antibody samples was done by capillary electrophoresis laser induced fluorescence (cLIF) (Chen and Evangelista (1998) Electrophoresis 15:1892). The N-linked oligosaccharides of the purified antibody were released by adding the peptide N-glycanase (Prozyme) and incubating overnight. The protein was ethanol precipitated, and the carbohydrate containing supernatant was transferred to a new tube and dried using a Speedvac. The carbohydrates were resuspended and derivatized with 8-aminopyrene-l,3,6-trisulfonate (APTS) under mild reductive animation conditions in which desialylation and loss of fucosyl residues was minimized. The reaction adducts were analyzed by capillary electrophoresis with a laser-induced fluorescence detector (Beckman Coulter) (Ma and Nashabeh (1999) Anal. Chem. 21:5185). Differences in the oligosaccharide profile were observed between the antibody obtained from the Ms704 cell
line as compared to the CHO DG44 cell line, consistent with an absence of fucosyl residues in the Ms704 derived anti-CD30 antibodies.

To confirm the absence of fucosyl residues on the antibody expressed in Ms704 cells, monosaccharide composition analysis was performed. The results are shown below in Table 1:

**Table 1: Monosaccharide Analysis**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Protein Amount (µg)</th>
<th>Monosaccharide</th>
<th>Amount Found (pmol)</th>
<th>mol Sugar/mol Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-CD30 + fucosyl</td>
<td>29 µg</td>
<td>Fucosyl</td>
<td>206.0</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Galactosamine</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Glucosamine</td>
<td>847.6</td>
<td>4.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Galactose</td>
<td>85.8</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mannose</td>
<td>547.0</td>
<td>2.9</td>
</tr>
<tr>
<td>Anti-CD30 - fucosyl</td>
<td>23 µg</td>
<td>Fucosyl</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Galactosamine</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Glucosamine</td>
<td>655.2</td>
<td>4.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Galactose</td>
<td>89.7</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mannose</td>
<td>488.8</td>
<td>3.2</td>
</tr>
</tbody>
</table>

The results of the monosaccharide analysis confirm that the antibody expressed in Ms704 cells lacks fucosyl residues.

Aside from the difference in oligosaccharides shown by capillary electrophoresis and monosaccharide analysis, the Ms704 and CHO DG44 derived anti-CD30 antibody protein samples were essentially identical. Analysis of N-terminal protein sequence revealed an identical N-terminal amino acid sequence. Mass spectroscopy of the light chain of the Ms704 and CHO DG44 derived anti-CD30 antibodies yielded masses of 23,740 and 23,742, respectively, which were within the error of the instrument. The two antibodies were also tested using a standard capillary isoelectric focusing kit assay (Beckman Coulter) and showed that the two antibody samples had an identical isoelectric point at 8.6. These studies indicate that the protein component of the antibody samples derived from the Ms704 and the CHO DG44 cells are essentially identical with the exception of the defucosylation of the carbohydrate component of the Ms704 derived antibodies.
Example 2: Assessment of ADCC Activity of Defucosylated Anti-CD30 Antibody

The anti-CD30 monoclonal antibody 5Fl 1 is capable of killing CD30+ cells through the recruitment of an effector cell population via antibody dependent cellular cytotoxicity (ADCC). In this example, defucosylated 5FH (defuc-5Fl) monoclonal antibodies were tested for the ability to kill CD30+ cell lines in the presence of effector cells in a cytotoxicity chromium release assay.

Human effector cells were prepared from whole blood as follows. Human peripheral blood mononuclear cells were purified from heparinized whole blood by standard Ficoll-paque separation. The cells were resuspended in EPMII 640 media containing 10% FBS and 200 U/ml of human IL-2 and incubated overnight at 37°C. The following day, the cells were collected and washed once in culture media and resuspended at 1 x 10^7 cells/ml. Two million target CD30+ cells were incubated with 200 µCi ^51^Cr in 1 ml total volume for 1 hour at 37°C. The target cells were washed once, resuspended in 1 ml of media, and incubated at 37°C for an additional 30 minutes. After the final incubation, the target cells were washed once and brought to a final volume of 1x10^5 cells/ml.

The CD30+ cell lines L540 (human Hodgkin's lymphoma; DSMZ Deposit No. ACC 72), L428 (human Hodgkin's lymphoma; DSMZ Deposit No. ACC 197), L1236 (human Hodgkin's lymphoma; DSMZ Deposit No. ACC 530) and Karpas (human T cell lymphoma; DSMZ Deposit No. ACC 31) cell lines were initially tested for binding to both the fucosylated 5FI 1 (fuc-5Fl 1) and defuc-5Fl 1 using a standard FACS analysis. Each target cell displayed similar binding profiles through a range of antibody concentrations for both fuc-5Fl 1 and defuc-5Fl 1. The level of CD30 expression, as determined by mean fluorescence intensity, was highest in L540, followed by Karpas, L428, and the lowest CD30 expression was on L1236 cells.

The L540, L428, L1236 and Karpas cells were tested in a modified ^51^Cr antibody dependent cellular cytotoxicity (ADCC) assay as follows. Each target cell line (100 µl of labeled CD30+ cells) was incubated with 50 µl of effector cells and 50 µl of antibody. A target to effector ratio of 1:50 was used throughout the experiments. In all studies, the following negative controls were also run: a) target and effector cells without antibody, b) target cells without effector cells, c) wells containing target and effector cells in the presence of 1% Triton X-100, and d) human IgGl isotype control. Following a 4 hour incubation at
37° C, the supernatants were collected and counted on a gamma Counter (Cobra II auto-
gamma from Packard Instruments) with a reading window of 240-400 keV. The counts per
minute were plotted as a function of antibody concentration and the data was analyzed by
non-linear regression, sigmoidal dose response (variable slope) using Prism software (San
Diego, CA). Cell cytotoxicity curves for the L540, L428, L1236 and Karpas cell lines using
varying concentrations of fuc-5Fl 1 and defuc-5Fl 1 are shown in Figures 4-7, respectively.

The percent lysis was determined by the following equation:

\[
\text{% Lysis} = \frac{(\text{Sample CPM} - \text{no antibody CPM})/\text{TritonX CPM-No antibody CPM}) \times 100}
\]

The % Lysis was tested at an antibody concentration of 25 µg/ml and a target to effector cell
ratio of 1:50. EC50 values also were calculated for each target cell. The results are
summarized in Table 2 below.

<table>
<thead>
<tr>
<th>Target cell</th>
<th>% Lysis</th>
<th>% Lysis</th>
<th>% Lysis ratio</th>
<th>EC50 (µg/ml)</th>
<th>EC50 (µg/ml)</th>
<th>EC50 ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fucosyl+</td>
<td>Fucosyl-</td>
<td>Fucosyl+ : fucosyl-</td>
<td>Fucosyl+</td>
<td>Fucosyl-</td>
<td></td>
</tr>
<tr>
<td>L540</td>
<td>42</td>
<td>68</td>
<td>1.61</td>
<td>0.042</td>
<td>0.009</td>
<td>4.7</td>
</tr>
<tr>
<td>Karpas</td>
<td>19</td>
<td>50</td>
<td>2.63</td>
<td>0.250</td>
<td>0.032</td>
<td>7.8</td>
</tr>
<tr>
<td>L428</td>
<td>20</td>
<td>43</td>
<td>2.15</td>
<td>0.100</td>
<td>0.009</td>
<td>11.1</td>
</tr>
<tr>
<td>L1236</td>
<td>4</td>
<td>13</td>
<td>3.25</td>
<td>1.218</td>
<td>0.045</td>
<td>27.1</td>
</tr>
</tbody>
</table>

Defuc-5F11 showed from 1.61 times (for L540 cells) to 3.25 times (for L1236 cells) greater
percent cell lysis as compared to the fuc-5Fl 1 antibody. This increased potency of the defuc-
5Fl 1 results in measurable cell lysis at antibody concentrations where the fuc-5Fl 1 has no
measurable effect. For example, on L1236 cells, which have a low level of expression of
CD30, defuc-5Fl 1 at 0.1 µg/ml results in a 10% specific lysis, whereas fuc-5Fl 1 at the same
concentration has no measurable effect (see Figure 6). Defuc-5F1 1 was 4.7 times (for L540
cells) to 27.1 times (for L1236 cells) more potent in ADCC activity than the fuc-5Fl 1
antibody, as measured by ratio of EC50 values.
Example 3: Assessment of ADCC Activity of Anti-CD30 Antibody

In this example, anti-CD30 monoclonal antibodies were tested for the ability to kill CD30+ cell lines in the presence of effector cells via antibody dependent cellular cytotoxicity (ADCC) in a fluorescence cytotoxicity assay. Human effector cells were prepared as described above and the ADCC assay performed as indicated above. As can be seen in Figure 9, when using the defucosylated anti-CD30 antibody there was increased ADCC activity as compared with parental anti-CD30 antibody. In addition, the defucosylated anti-CD30 antibody was more potent than the parental antibody as evidenced by the reduced EC50 as compared to the parental anti-CD30 antibody. The antibody was also more efficacious as evidenced by the fact that the maximum percent lysis was higher for the defucosylated anti-CD30 antibody. With either antibody, the anti-CD16 (3G8) antibody effectively inhibited the ADCC suggesting that this lysis was mediated by CD16.

Example 4: Increased ADCC With Human Effector Cells

ADCC assays were performed as described above. In this experiment, however, mouse effector cells were compared with human effector cells. As can be seen in Figure 10, while there was no increased ADCC comparing parental anti-CD30 antibody with defucosylated antibody when mouse effector cells were used, when human effector cells were examined, there was a notable increase in ADCC with the defucosylated antibody as compared to the parental anti-CD30 antibody.

Example 5: ADCC assay comparing parental and defucosylated antibody using effector cells from cynomolgus monkeys

Whole blood was obtained from cynomolgus monkeys. Red blood cell lysed cynomolgus peripheral blood cells were stimulated with 50 U/ml rIL-2 and cultured in RPMII 640 media containing 10% FBS overnight at 37°C. On the day of the study, cynomolgus cells were resuspended in assay buffer (RPMII 640, 10% FBS, 2.5 mM probenecid) at 1x10^7 cells/mL. CD30 positive target cells, Karpas 299, were labeled, washed three times with wash buffer (PBS, 2.5mM probenecid, 20mM HEPES), and adjusted to 1x10^5 cells/mL for 1:50 target to effector cell ratio. The ADCC assay was performed as described above. We compared the activity of parental anti-CD30 antibody to defucosylated antibody using effector cells purified from cynomolgus blood. Modest ADCC activity was seen with the parental antibody (from around 7-10% cell lysis at 10 µg/mL). In contrast, the
defucosylated antibody induced significantly higher percent lysis (from around 10-30% cell lysis at 10 µg/mL) and a reduced EC50 (see Figure 11).

Example 6: Scatchard analysis of binding affinity of anti-CD30 monoclonal antibodies to L540 cells, activated human and cynomolgus peripheral blood cells

The binding affinity of the parental and defucosylated anti-CD30 antibodies was determined. We compared the binding affinity of the two antibodies to CD30 positive L540 cells as well as PHA/Con A-activated human or cynomolgus peripheral blood mononuclear cells.

Human or cynomolgus peripheral blood cells were stimulated with 2µg/ml PHA, 10 µg/ml Con A, and 50 U/ml rIL-2 and cultured in RPMI 640 media containing 10% fetal bovine serum (FBS) at 1x10^6 cells/ml density for 3 days. On the day of the study, the cells were washed and adjusted to 2x10^7 cells/ml in binding buffer (RPMI1640 +10% FBS). As a control, CD30 positive L540 cells (adjusted to 4-8x10^6 cells/ml) were used in these studies since they express high levels of the antigen. The cells were placed on ice until the initiation of the experiment. Millipore glass fiber filter plates (MAFBN0B50) were coated with 1% nonfat dry milk in water and stored a 4°C overnight. The plates were washed three times with 0.2 ml of binding buffer. Fifty microliters of buffer alone was added to the maximum binding wells (total binding). Twenty-five microliters of buffer alone was added to the control wells. Varying concentration of 125I-anti-CD30 antibody was added to all wells in a volume of 25 µl. Varying concentrations of unlabeled antibody at 300-400 fold excess were added in a volume of 25 µl to control wells (non-specific binding) and 25 µl of CD30 positive L540 cells or stimulated human or cynomolgus peripheral blood cells in binding buffer were added to all wells. The plates were incubated for 2 hours at 200 RPM on a shaker at 4°C. At the completion of the incubation the Millipore plates were washed twice with 0.2 ml of cold wash buffer (RPMI 640, 10% FBS, 500 mM NaCl). The filters were removed and counted in a gamma counter. Evaluation of equilibrium binding was performed using single site binding parameters with the Prism software (San Diego, CA).

Using the above Scatchard binding assay, the K_D of the parental CD30 antibody for L540 cells was approximately 1.4 nM while the defucosylated antibody had a K_D of 1.9 nM (Table 3). This indicates that there was little change in affinity with removal of fucosyl. These studies were repeated using primary cells rather than a cell line. In addition, the affinity on cells which express significantly fewer receptors per cell was tested. Activated human peripheral blood cells were
prepared as indicated above and the $K_D$ was found to be 1.1 and 2.7 nM for parental and defucosylated anti-CD30 antibody, respectively.

Finally, the binding affinity of the parental and defucosylated antibody for PHA, Con A, and rIL-2 activated cynomolgus peripheral blood mononuclear cells was compared. The $K_D$ was found to be approximately 0.47 nM and 0.83 nM for parental and defucosylated antibody, respectively.

### Table 3 Scatchard Analysis

<table>
<thead>
<tr>
<th>Sample</th>
<th>L540</th>
<th>Human</th>
<th>Cynomolgus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parental CD30</td>
<td>$K_D$ (nM ave)</td>
<td>1.37</td>
<td>1.08</td>
</tr>
<tr>
<td></td>
<td>Receptors Per Cell (ave)</td>
<td>2496082</td>
<td>45654</td>
</tr>
<tr>
<td>Defucosylated CD30</td>
<td>$K_D$ (nM ave)</td>
<td>1.93</td>
<td>2.66</td>
</tr>
<tr>
<td></td>
<td>Receptors Per Cell (ave)</td>
<td>3024600</td>
<td>74258</td>
</tr>
</tbody>
</table>

### Example 7: Production of Anti-CD30 Monoclonal Antibody Having Improved Receptor Binding and Increased ADCC Activity

This example outlines the expression of human anti-CD30 mAbs in *Lemna*. Anti-CD30 fully human monoclonal antibodies were originally described in PCT Publication WO 03/059282, which is hereby incorporated by reference. Optimization of anti-CD30 mAb glycosylation was accomplished by co-expression with an RNAi construct targeting the endogenous expression of $\alpha$-1,3-fucosyltransferase (FucT) and $\beta$-1,2-xylosyltransferase (XyIT) genes in a manner similar to that noted in the examples above for mAbl. The resultant anti-CD30 mAb produced in *Lemna* having its native glycosylation machinery engineered to suppress FucT and XyIT expression contained a single major $N$-glycan species without any trace of plant-specific $\alpha$T-glycans. In addition to the $N$-glycan homogeneity, glyco-optimized anti-CD30 mAbs were also shown to have enhanced antibody-dependent cell-mediated cytotoxicity (ADCC) and effector cell receptor binding activity when compared to CHO-expressed anti-CD30 mAbs.
METHODS

Strains and Reagents.

Novablue competent Escherichia coli cells were used for all recombinant DNA work (EMD Biosciences, San Diego, CA). Restriction endonucleases and DNA modification enzymes were obtained from New England Biolabs (Ipswich, MA). Oligonucleotides were obtained from Integrated DNA technologies (Coralville, IA). Waters Oasis HLB and MCX columns (1 cc), 2,5-dihydroxybenzoic acid (DHB), and α-cyano-4-hydroxycinnamic acid (CHCA) were from Waters Corporation (Milford, MA). Purified dabsylated, tetrapeptide, GnGn iV-glycan acceptors (GnGn-dabsyl-peptide) and iV-glycosidase A were from EMD Biosciences. Carbograph SPE columns (4 cc) were from Grace Davidson Discovery Sciences (Deerfield, IL). Uridine-5'-diphospho-D-xylosyl (UDP-Xyl) was purchased from Carbosource Services (Athens, GA). Acetonitrile (Optima grade) was from Fisher Scientific (Summerville, NY). Ammonium acetate was from MP Biochemicals (Irvine, CA). Maltooligosaccharides (MD6-1) were from V-Labs Inc. (Covington, CA). Monosaccharide standards were from Dionex (Sunnyvale, CA). BATDA (bis(acetoxymethyl)2,2′:6′,2″-terpyridine-6,6″-dicarboxylate) and Europium solution were from Perkin-Elmer (Wellesley, MA). Guanosine-5′-diphospho-L-fucosyl (GDP-Fuc), iV-acetylglucosamine (GlcNAc), 2-aminobenzoic acid (2-AA) and all other materials were from Sigma (St. Louis, MO).

Construction of mAb and RNAi expression vectors.

The heavy (H) and light (L) chain variable region cDNA sequences of fully human mAb kappa antibody 5FI 1 derived from a transgenic Medarex HuMAb-Mouse® were determined and the full length 5FI 1 human mAb antibody was produced recombinantly by a Chinese hamster ovary cell line, CHO DG44, using standard techniques. Optimized genes for H and L chains were designed to have Lenvα-preferred codon usage (63%-67% GC content) and contain the rice α-amylase signal sequence (GenBank M24286) fused to the 5′ end of their coding sequences. Restriction endonuclease sites were added for cloning into Agrobacterium binary vectors (EcoRI (5′) / Sad (3′), H-chain) and (Sail (5′) / HindIII (3′), L-chain). Synthetic genes were constructed and provided by Picoscript (Houston, TX).

A chimeric hairpin RNA was designed to target silencing of endogenous Lemma genes encoding α 1,3-fucosyltransferase (based on the coding sequence for L. minor FucT isoform #1, see GenBank DQ789145) and β-1,2-xylodultransferase (based on the coding sequence for L. minor XyIT isoform #2, see GenBank DQ789146). The chimeric FucT + XyIT hairpin RNA was designed to have 602 bp of double stranded FucT sequence, 626 bp
of double stranded XyIT sequence, and 500 bp of spacer sequence. The sense strand portion 
of the hairpin RNA cassette encompasses the FucT forward fragment and XyIT forward 
fragment, a spacer sequence. The antisense strand portion of the hairpin RNA encompasses 
the XyIT reverse fragment and FucT reverse fragment. The chimeric hairpin RNA was 
constructed by PCR amplifying FucT and XyIT forward and reverse gene fragments from 
*Lemna minor* cDNA and sequentially cloning them into pT7blue (EMD Biosciences) 
creating plasmid XF02 in T7-4. The FucT forward gene fragment was amplified with DNA 
primers BLX 686 (5'-ATGGTGAATCTCAAGA TCAAC-3') (SEQ ID NO:36) and 
BLX690 (5' ATGTCTAGAATG CAGCAGCAAGTGCACC-3') (SEQ ID NO:37) 
producing a 620 bp product with terminal Sail (5') and Xbal (3') cloning sites. The XyIT 
forward gene fragment was amplified with DNA primers BLX 700 (5'-ATGACTAGTTGC 
GAAGCTACTTCCGCAACAGCS') (SEQ ID NO:38) and BLX694 (5' 
ATGGGATCCGAATCTCAAGA TCAAC-3') (SEQ ID NO:39) producing a 1144 
bp product with terminal Spel (5') and BamHI (3') cloning sites. The XyIT reverse gene 
fragment was amplified with DNA primers BLX 695 (5' 
ATGGGATCCGAATCTCAAGA TCAAC-3') (SEQ ID NO:40) and BLX696 
(5' ATGGGA ATGGGTACCATGCAGCAGCAAGTGCACC-S') (SEQ ID NO:41) producing a 644 
bp product with terminal BamHI (5') and Kpn1 (3') cloning sites. The FucT reverse gene 
fragment was amplified with DNA primers BLX 691 (5' 
ATGGGATCCGAATCTCAAGA TCAAC-3') (SEQ ID NO:42) and BLX692 (5' 
ATGGGATCCGAATCTCAAGA TCAAC-3') (SEQ ID NO:43) producing a 620 bp 
product with terminal Kpn1 (5') and Sac (3') cloning sites.

Independent expression cassettes containing promoter, gene of interest, and Nos 
terminator were created for the optimized 5Fl 1 H and L chains and the chimeric RNAi.

Expression cassettes were cloned into a modification of the *Agrobacteriwn* binary vector 
PBMSP3 (obtained from Dr. Stan Gelvin, Purdue University) with the appropriate restriction 
sites. The H chain was fused to the modified chimeric octopine and mannopine synthase 
promoter with *Lemna gibba* 5'RbcS leader. The L-chain was fused to the high expression, 
constitutive *Lemna minor* polyubiquitin promoter (LmUbq). The chimeric RNAi cassette, 
taken from plasmid XF02 in T7-4, was fused to the high expression, constitutive *Spirode1a 
polyrhiza* polyubiquitin promoter (SpUbq). The three expression cassettes were cloned into 
the modified pBMSP3 binary vector in tandem orientation creating plasmid MDXA04. 
*Transformation and plant line screening.*
Using *Agrobacterium tumefaciens* C58Z707, transgenic plants representing individual clonal lines were generated from rapidly growing *Lemma minor* nodules according to the procedure of Yamamoto *et al.* For transgenic screening, individual clonal lines were preconditioned for 1 week at 150 to 200 µmol m⁻² s⁻¹ in vented plant growth vessels containing SH media without sucrose. Fifteen to twenty preconditioned fronds were then placed into vented containers containing fresh SH media, and allowed to grow for two weeks. Tissue and media samples from each line were frozen and stored at −70°C.

**ELISA analysis of mAb produced in *Lemma***

*Lemma* tissue (100 mg) was homogenized using a FastPrep FP 120 bead mill (Thermo Electron Corporation). Supernatants were diluted to 1 µg/mL and assayed using the IgG Quantitation ELISA kit (Bethyl Laboratories). For the assay, microliter plates were coated with goat anti-human IgG at a concentration of 10 µg/mL, and mAb was detected by horseradish peroxidase (HRP)-conjugated goat anti-human IgG diluted 1:100,000. Standard curves were created with Human Reference IgG supplied with the ELISA kit. The sensitivity of the ELISA was 7.8 ng/mL. All samples were analyzed in duplicate.

**Preparation of *Lemma* microsomal membranes and assaying for core β-1,2-xylosyltransferase and α-1,3-fucosyltransferase activities.**

*Lemma* tissue (100 mg) from each line was homogenized in 1 mL of cold homogenization buffer (50 mM 4-[2-hydroxyethyl]-l-piperazineethanesulfonic acid [HEPES], pH 7.5, 0.25 M sucrose, 2 mM ethylenediaminetetraacetic acid [EDTA], 1 mM 1,4-dithiothreitol [DTT]) for 40 s in a FastPrep FP 120 bead mill (Thermo Electron Corporation, Waltham, MA). The homogenate was centrifuged at 1,000 g for 5 min at 4°C. The supernatant was removed and centrifuged at 18,000×g for 90 min at 4°C. The resulting pellet was resuspended in 20 µL of cold reaction buffer (0.1 M 2-[4-morpholino]ethanesulfonic acid [Mes], pH 7.0, 0.1% [v/v] Triton X-100, 10 mM MnCl₂) and kept on ice or stored at −80°C until use.

Core β-1,2-xylosyltransferase and α-1,3-fucosyltransferase activities were measured simultaneously in 4 µL of microsomal membranes prepared from each RNAi line by incubating with 125 mM GlcNAc, 6.25 mM UDP-Xyl, 6.25 mM GDP-Fuc, 12.5 mM MnCl₂, and 1.5 nmol of GnGn-dabsyl-peptide acceptor for 2 h at 37°C as described previously. The reaction was terminated by a brief centrifugation and incubation at 4°C and the products were analyzed by positive reflectron mode MALDI-TOF MS.

**Purification of 5FJL LEX and LEX<sup>op</sup> mAbs.**
Plant tissue was homogenized with 50 mM sodium phosphate, 0.3 M sodium chloride, and 10 mM EDTA, pH 7.2 using a Silverson high shear mixer. The homogenate was acidified to pH 4.5 with 1 M citric acid, and centrifuged at 7,500g for 30 min at 4°C. The pH of the supernatant was adjusted to pH 7.2 with 2 M Tris, prior to filtration using 0.22 µm filters. The material was loaded directly on mAbSelect SuRe protein A resin (GE Healthcare) equilibrated with a solution containing 50 mM sodium phosphate, 0.3 M sodium chloride, and 10 mM EDTA, pH 7.2. After loading, the column was washed to baseline with the equilibration buffer followed by an intermediate wash with 5 column volumes of 0.1 M sodium acetate, pH 5.0. Bound antibody was eluted with 10 column volumes of 0.1 M sodium acetate, pH 3.0. The protein A eluate was immediately neutralized with 2 M 2-amino-2-[hydroxymethyl]-1,3-propanediol (Tris). For aggregate removal, the protein A eluate was adjusted to pH 5.5 and applied to a ceramic hydroxyapatite type I (Bio-Rad) column equilibrated with 25 mM sodium phosphate, pH 5.5. After washing the column with 5 column volumes of equilibration buffer, the antibody was eluted in a single step-elution using 0.25 M sodium phosphate, pH 5.5. Fractions containing antibody by A280 were pooled and stored at -80°C.

Tissue extract and protein A flow through samples were prepared for SDS-PAGE under reducing and non-reducing conditions by addition of 2x SDS sample buffer ± 5% [v/v] 2-mercaptoethanol. Protein A eluate and hydroxyapatite eluate samples were diluted to a protein concentration of 0.5mg/mL followed by addition of 2x SDS sample buffer ± 5% [v/v] 2-mercaptoethanol. Samples were incubated at 95°C for 2 minutes prior to electrophoresis using 4-20% Tris-Glycine gradient gels (Invitrogen, Carlsbad, CA). Markl2 Molecular weight markers (Invitrogen) and a 5Fl 1 reference standard were included on the gels. Gels were stained with Colloidal Blue stain.

Purification of N-linked glycans.

Protein A purified monoclonal antibodies (1 mg) from wild-type and RNAi *Lemna* plant lines were dialyzed extensively against water and lyophilized to dryness. Samples were resuspended in 100 µL of 5% (v/v) formic acid, brought to 0.05 mg/ml pepsin, and incubated at 37°C overnight. The samples were heat inactivated at 95°C for 15 min and dried. Pepsin digests were resuspended in 100 µL of 100 mM sodium acetate, pH 5.0 and incubated with 1 mU of JV-glycosidase A at 37°C overnight. The released iV-glycans were isolated using 4 cc Carbograph SPE columns and dried.
Dried iV-glycans were further purified using 1 cc Waters Oasis MCX cartridges. Columns were prepared by washing with 3 column volumes of methanol followed by 3 column volumes of 5% (v/v) formic acid. iV-glycans, resuspended in 1 mL of 5% (v/v) formic acid, were loaded onto the prepared columns. The unbound fraction as well as 2 additional column volume washes of 5% (v/v) formic acid were collected, pooled, and dried.

Derivatization of oligosaccharides with 2-aminobenzoic acid (2-AA).

Purified AT-glycans or maltooligosaccharides were labeled with 2-AA and purified using 1 cc Waters Oasis HLB cartridges according to Anumula and Dhume, 1998\textsuperscript{50}. Labeled iV-glycans and maltooligosaccharides were resuspended in 50 µL of water and analyzed by negative mode MALDI-TOF MS and NP-HPLC-QTOF MS.

MALDI-TOF Mass Spectrometry.

MALDI-TOF MS was conducted using a Waters MALDI Micro MX (Millford, MA). Analysis of β-1,2-xylosyltransferase/ α-1,3-fucosyltransferase reaction products was conducted by mixing 0.5 µL of each reaction supernatant with 0.5 µL of 10 mg/mL CHCA in 0.05% (v/v) TFA, 50% (v/v) acetonitrile on a target plate. Xylosylated ([M+H]\(^+\) = 2192.85 Da) or fucosylated ([M+H]\(^+\) = 2206.87 Da) GnGn-dabsyl-peptide products were detected in positive reflectron mode. Ion counts of 200 combined spectra from each sample were normalized against that of β-1,4-galactosylated, GnGn-dabsyl-peptide ([M+H]\(^+\) = 2222.87 Da) present as a contaminant (<5%) in the original GnGn-dabsyl-peptide mixture from EMD Biosciences.

2-AA labeled iV-glycans or maltooligosaccharides (0.5 µL) were diluted with water, mixed with 0.5 µL of 10 mg/ml DHB matrix in 70% (v/v) acetonitrile, spotted onto a target plate and analyzed in negative reflectron mode.

NP-HPLC-Q-TOF MS analysis of 2-AA labeled N-glycans.

2-AA labeled iV-glycans or maltooligosaccharides were brought to 80% (v/v) acetonitrile and separated on a Waters 2695 HPLC system fitted with a TSK-Gel Amide-80 (2 mm x 25 cm, 5 µm) column (Tosoh Biosciences, Montgomeryville, PA). 2-AA labeled carbohydrates were detected and analyzed using a Waters 2475 fluorescence detector (230 nm excitation, 425 nm emission) and a Waters Q-TOF API US quadropole-time of flight (QTOF) mass spectrometer fitted on-line with the HPLC system.

Separations were conducted at 0.2 mL/min, 40°C, using 10 mM ammonium acetate, pH 7.3 (solvent A) and 10 mM ammonium acetate, pH 7.3, 80% (v/v) acetonitrile (solvent B). Sample elution was carried out at 0% A isocratic for 5 min, followed by a linear increase
to 10% A at 8 min, and a linear increase to 30% A at 48 min. The column was washed with 100% A for 15 min and equilibrated at 0% A for 15 min prior to the next injection.

QTOF analysis was conducted in negative ion mode with source and desolvation temperatures of 100°C and 300°C, respectively, and capillary and cone voltages of 2,100 and 30 V, respectively. Mass spectra shown are the result of combining ≥40 individual scans per labeled JV-glycan.

Monosaccharide analysis by HPAEC-PAD.

mAb samples (200 μg) were subjected to acid hydrolysis using 2 N TFA at 100°C for 3 h. Samples were dried by vacuum centrifugation at ambient temperature and reconstituted in 150 μL water prior to analysis by HPAE-PAD (Dionex). An aliquot (25 μL) of the reconstituted sample was applied to a CarboPac PA10 column (4 x 250 mm) with a pre-column Amino Trap (Dionex). Separation of monosaccharides was accomplished with a mobile phase of 3.5 mM KOH, using an EG40 eluent generator. Monosaccharide peak identity and relative abundance were determined using monosaccharide standards.

Thermal stability of mAb.

A MicroCal (Northampton, MA) VP-Capillary differential scanning calorimetry (DSC) instrument was used to determine thermal stability of glycol-optimized and wild-type mAbs. Purified mAb samples were dialyzed in 20 mM NaH₂PO₄, pH 7.4, 150 mM NaCl (PBS) overnight. Thermal denaturation data was collected by heating the samples at a concentration of 300 μg/mL from 35 to 95°C at a scan rate of 1°C/min using PBS as the reference buffer. The feedback and gain were set to low. The baseline-corrected and normalized data was fit to a non-2-state model using Origin v7.0 software.

FcR binding activity of mAb.

The experiment was conducted using a BIACORE (Biacore AB, Uppsala, Sweden) instrument using surface plasmon resonance technology. mAbs, 2 μg/mL, were captured on the antigen coated surface (recombinant human CD30). Several concentrations of both the Val¹⁵⁸ and Phe¹⁵⁸ allotypes of FcγIIIa, starting from 6 μM, were flowed over the captured antibodies for 3 min. The binding signal as a function of FcγIIIa was fit to a one-site binding model using GraphPad Prism (v4.01) software to obtain the Kᵦ values. HBS-EP buffer (10 mM HEPES, 0.15 M NaCl, 3 mM EDTA and 0.005% (v/v) P20 at pH 7.4) was used throughout the experiment. Binding of the mAbs to buffer or FcγIIIa to blank surfaces were used as negative controls.
Assay for antigen binding affinity.

CD30-expressing L540 cells (DSMZ Cell Culture Collection # ACC 72) were used as antigen positive cells to assay for binding. Aliquots of 2 x 10^5 cells/well were incubated for 30 min at 4°C with 100 µL of primary antibody at the indicated concentrations. Cells were washed twice in PBS with 2% (v/v) fetal bovine serum (FBS) before addition of goat anti-human mAb, FITC-labeled secondary antibody (Jackson ImmunoResearch, West Grove, PA) at 1:500 dilution in 100 µL/well for 30 min at 4°C. Cells were washed twice in PBS with 2% (v/v) FBS and assayed by flow cytometry using a FACS Calibur instrument (Becton Dickinson, Franklin Lakes, NJ). EC_{50} values of 5Fl 1 CHO, LEX and LEX^0_br mAb binding to CD30 on L540 cells were determined from binding curves utilizing GraphPad Prism 3.0 software.

ADCC assay.

Human peripheral-blood mononuclear effector cells were purified from heparinized whole blood by standard Ficoll-Paque separation. Cells (2 x 10^6) were washed in PBS and sent for genotyping. The remaining effector cells were then resuspended at 1 x 10^6 cells/mL in RPMI 1640 medium containing 10% (v/v) FBS and 50 U/mL of human IL-2 (Research Diagnostics, Concord, MA) and incubated overnight at 37°C. The effector cells were washed once in culture medium and resuspended at 1 x 10^7 cells/mL prior to use. L540 target cells at 1 x 10^6 cells/mL in RPMI 1640 medium containing 10% (v/v) FBS and 5 mM probenecid were labeled with 20 µM BATDA (bis(acetoxymethyl)2,2',6',2''-terpyridine-6,6' ' -dicarboxylate) for 20 min at 37°C. Target cells were washed three times in PBS supplemented with 20 mM HEPES and 5 mM probenecid, resuspended at 1 x 10^5 cells/mL and added to effector cells in 96-well plates (1 x 10^4 target cells and 5 x 10^8 effector cells/well) at a final target to effector ratio of 1:50. Maximal release was obtained by incubation of target cells in 3% (v/v) Lysol and spontaneous release obtained by incubation in cell culture medium alone. After 1 h incubation at 37°C, 20 µL of supernatant was harvested from each well and added to wells containing 180 µL of Europium solution. The reaction was read with a Perkin Elmer Fusion Alpha TRF reader using a 400 µsec delay and 330/80, 620/10 excitation and emission filters respectively. The counts per second were plotted as a function of antibody concentration and the data was analyzed by non-linear regression, sigmoidal dose response (variable slope) using GraphPad Prism 3.0 software. The percent specific lysis was calculated as: (experimental release - spontaneous release)/(maximal release - spontaneous release) x 100. In all studies, human nxAbl isotype
control was included and compared to 5Fl 1 CHO, LEX, and LEX\textsuperscript{Opt} mAbs. Other controls included target and effector cells with no mAb, target cells with no effector cells and target and effector cells in the presence of 3% (v/v) Lysol.

**RESULTS**

*Expression of SFll mAb in the LEX system.*

5Fl 1 (also known as MDX-060) is an anti-CD30 antibody being developed for the treatment of Hodgkins lymphoma and anaplastic large cell lymphoma. Two binary vectors were constructed for the expression of 5Fl 1 in the LEX system. Expression vector MDXAO\textsubscript{1} contained codon optimized genes encoding heavy (H) and light (L) chains of SFll while vector MDXA0\textsubscript{4} contained genes encoding H and L chains in addition to a chimeric FucT/XylT RNAi gene. Independent transgenic lines were generated for both the MDXAO\textsubscript{1} (165 lines) and MDXA0\textsubscript{4} (195 lines) expression vectors. For simplicity, MDXAO\textsubscript{1} derived mAbs (wild-type $\alpha$-glycosylation), and MDXA0\textsubscript{4} derived mAbs (containing the FucT/XylT RNAi construct) will be referred to as 5Fl 1 LEX and 5Fl 1 LEX\textsuperscript{Opt}, respectively, in the discussions below.

Transgenic plant lines were first screened for mAb expression with an IgG ELISA. LEX\textsuperscript{Opt} lines with high levels of mAb expression were assayed further for FucT and XylT activity. Transferase activities in the majority of the high expressing 5Fl 1 LEX\textsuperscript{Opt} lines were reduced to levels of the negative control indicating effective silencing in the majority of the assayed lines (Figure 12). 5Fl 1 LEX\textsuperscript{Opt} lines did not exhibit any morphological or growth differences compared to wild-type *Lemma* plants (data not shown). These results suggest that RNAi silencing of the FucT/XylT genes has no effect on plant viability.

Thermal stabilities of the 5Fl 1 CHO, LEX, and LEX\textsuperscript{Opt} mAbs were determined using differential scanning calorimetry (DSC). All three mAbs exhibited similar melting curve kinetics (data not shown) and melting transition point temperatures (Table 4 below), further demonstrating the structural integrity of the Ze/wm\textsubscript{w} produced 5Fl 1 LEX and LEX\textsuperscript{Opt} mAbs compared to the 5Fl 1 CHO mAb. SDS-PAGE analysis under non-reducing (Figure 13A) and reducing conditions (Figure 13B) showed that mAbs from the 5Fl 1 LEX\textsuperscript{Opt} and 5Fl 1 CHO lines had similar protein profiles.
Table 4. Comparison of the thermal stabilities of 5Fl 1 CHO, 5Fl 1 LEX, and glyco-optimized 5Fl 1 LEX\textsuperscript{opt} mAbs by differential scanning calorimetry (DSC).

<table>
<thead>
<tr>
<th>Antibody</th>
<th>T\textsubscript{m1} (°C)</th>
<th>T\textsubscript{m2} (°C)</th>
<th>T\textsubscript{m3} (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5Fl1 CHO</td>
<td>72</td>
<td>75</td>
<td>84</td>
</tr>
<tr>
<td>5Fl1 LEX</td>
<td>71</td>
<td>75</td>
<td>84</td>
</tr>
<tr>
<td>5Fl1 LEX\textsuperscript{opt}</td>
<td>72</td>
<td>76</td>
<td>84</td>
</tr>
</tbody>
</table>

N-glycan structures of SFll CHO, LEX, and LEX\textsuperscript{opt} mAbs.

N-glycan oligosaccharides were released from 5Fl 1 CHO, 5Fl 1 LEX, and 5Fl 1 LEX\textsuperscript{opt} derived mAbs and analyzed by MALDI-TOF and normal phase (NP) HPLC-QTOF MS. Negative reflectron mode MALDI-TOF MS analysis of 2-AA derivatized N-glycans from 5Fl1 CHO lines indicated the presence of four major N-glycans with m/z values corresponding to 2-AA labeled GnGnF\textsuperscript{6} (nomenclature derived from www.proglycan.com), Man5, Gna\textsubscript{iso}F\textsuperscript{6}, and AAF\textsuperscript{6} (Figure 14). NP-HPLC separated the Gna\textsubscript{iso}F\textsuperscript{6} N-glycan into its two isoforms (Gal attached to the \(\alpha\)-1,6-Man or \(\alpha\)-1,3-Man arm) bringing the total number of major N-glycans found on 5Fl 1 CHO to five (Figure 15). MS/MS fragmentation of the peaks was not conducted to confirm the identity of each isoform; however, the higher abundance of the earlier peak suggested that Gal was attached to the \(\alpha\)-1,6-Man arm of this N-glycan\textsuperscript{38}. On-line negative mode QTOF MS analysis gave m/z values corresponding to doubly charged GnGnF\textsuperscript{6}, Man5, Gna\textsubscript{iso}F\textsuperscript{6} (both isoforms), and AAF\textsuperscript{6}, confirming the MALDI-TOF MS results (Table 5 below). Peak integration of the fluorescent trace revealed that GnGnF\textsuperscript{6}, Man5, A GnF\textsuperscript{6}, GnAF\textsuperscript{6}, and AAF\textsuperscript{6} constituted 50.8, 2.5, 26.1, 10.7 and 6.8\%, respectively, of the total N-glycan structures from 5Fl 1 CHO. The remaining 3.1\% of N-glycans were found to be a mixture of GnGn, GmM\textsubscript{iso}F\textsuperscript{6}, GmM\textsubscript{iso}F\textsuperscript{30}, and MM with no structure higher than 1.2\% of the total (data not shown).
Table 5. Summary of observed MALDI-TOF and QTOF MS masses of the major 2-AA labeled JV-glycans from MDXA-060 mAbs produced by CHO cells (CHO), wild-type *Lemma* (LEX) or glyco-optimized *Lemma* lines expressing the RNAi construct (LEX\textsuperscript{Opt}).

<table>
<thead>
<tr>
<th>N-glycan name\textsuperscript{a}</th>
<th>Proposed Structure</th>
<th>Theoretical m/z</th>
<th>Observed MALDI-TOF\textsuperscript{c}</th>
<th>Observed Q-TOF\textsuperscript{c}</th>
<th>% Peak Area\textsuperscript{c}</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CHO</strong></td>
<td></td>
<td></td>
<td>[M-H]\textsuperscript{+}/[M-2H]\textsuperscript{2+}</td>
<td>[M-H]\textsuperscript{+}</td>
<td>[M-2H]\textsuperscript{2+}</td>
</tr>
<tr>
<td>GnGnF\textsuperscript{6}-2AA</td>
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<td>1582.590</td>
<td>1582.455</td>
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<td>Man5-2AA</td>
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<td>1354.479</td>
<td>1354.392</td>
<td>676.7343</td>
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<tr>
<td>GnA\textsubscript{iso}F\textsuperscript{6}-2AA</td>
<td>or</td>
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<td>1744.492</td>
<td>871.7970</td>
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<td>AAF\textsuperscript{6}-2AA</td>
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<td>1906.695</td>
<td>1906.567</td>
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<td><strong>LEX</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
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<td></td>
<td>1436.532</td>
<td>1436.549</td>
<td>717.7894</td>
<td>8.40</td>
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<td>1568.581</td>
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<td>1436.532</td>
<td>1436.523</td>
<td>717.7993</td>
<td>95.8</td>
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</table>

\textsuperscript{a}IV-glycan names are based on Proglycan (www.proglycan.com) nomenclature. 2AA, 2-aminobenzoic acid.

\textsuperscript{b}The symbols of the proposed N-glycan structures are as follows: ■, \(N\)-acetylgalactosamine; ○, mannose; ◆, galactose; ▲, xylosyl; ▼, α-1,3-fucosyl; ▽, α-1,6-fucosyl; p, 2-aminobenzoic acid.

MALDI-TOF MS analysis of wild-type 5Fl 1 LEX mAb revealed the presence of three major species with m/z values corresponding to GnGnXF\textsuperscript{3}, GnGnX and GnGn (Figure 14). NP-HPLC followed by on-line QTOF MS analysis showed three major fluorescent peaks with m/z values corresponding to doubly charged GnGnXF\textsuperscript{3}, GnGnX and GnGn, again confirming the MALDI-TOF MS results (Figure 15; Table 5). Integration of the fluorescent
peaks indicated that GnGnXF\textsuperscript{3}, GnGnX and GnGn constituted 67.4, 17.2 and 8.4\%, respectively, of the total JV-glycans derived from 5Fl 1 LEX mAb's. The remaining 7\% of N-glycans were determined to be a mixture of MM, GnMi\textsubscript{is}, MMXF\textsuperscript{3}, GnGnF\textsuperscript{3}, GnMj\textsubscript{is}XF\textsuperscript{3}, Man6, Man7, Gn(FA)\textsubscript{iso}XF\textsuperscript{3}, Man8 and Man9 with no N-glycan greater than 2\% of the total (data not shown). Similar results were seen with mAbs isolated from two independently transformed 5Fl 1 LEX lines (data not shown).

In contrast to the 5Fl 1 LEX mAb, iV-glycans from the 5Fl 1 LEX\textsuperscript{0 \#} mAb possessed GnGn as the major AT-glycan species by both MALD-TOF and NP-HPLC-QTOF MS analysis (Figures 14 and 15; Table 5). GnGn comprised 95.8\% of the total iV-glycans with the remaining 4.2\% of N-glycans determined to be MM, GnMi\textsubscript{is}, GnAi\textsubscript{is}, Man6, Man7 and Man8 with no one structure greater than 1.2\% of the total iV-glycans. None of the LEX\textsuperscript{0 \#} N-glycans contained fucosyl (Fuc) or xylosyl (Xyl). These results demonstrated that co-expression of an RNAi construct targeting \textit{Lemma} FucT and XyIT resulted in the complete elimination of Fuc and Xyl-containing iV-glycans from 5Fl 1 LEX\textsuperscript{0 \#} mAbs and produced highly homogeneous mAb glycoforms. Similar results were obtained with two independent 5Fl 1 LEX\textsuperscript{0 \#} mAbs (data not shown).

The absence of Fuc or Xyl on 5Fl 1 LEX\textsuperscript{0 \#} mAb AT-glycans was further confirmed by monosaccharide analysis (Table 6 below). Monosaccharides were released from 5Fl 1 CHO, LEX and LEX\textsuperscript{0 \#} mAbs by acid hydrolysis and analyzed by high performance anion exchange chromatography (HPAEC) coupled to pulsed amperometric detection (PAD). The monosaccharide ratios for Man and GlcNAc residues were similar for CHO and wild-type LEX mAbs and correlated well with expected values. LEX mAbs were significantly decreased in Gal and Fuc content and had a significant increase in Xyl when compared to CHO-derived mAbs. Monosaccharide analysis of \textit{Lemma} derived mAbs revealed that while Fuc and Xyl were present on wild-type LEX TV-glycans, they were not detected on LEX\textsuperscript{0 \#} mAbs. These results confirmed the oligosaccharide profiling data and further suggested that RNAi silencing of \textit{Lemma} XyIT and FucT activity changed the \textit{Lemma} iV-glycosylation pathway to produce mAbs devoid of plant-specific iV-glycans.
Table 6. Monosaccharides released from 5Fl1 CHO, LEX, and LEX<sup>0,pt</sup> mAbs by acid hydrolysis and analyzed by HPAEC-PAD. The monosaccharide content from each mAb was determined by normalizing against carbohydrate controls.

<table>
<thead>
<tr>
<th>Monosaccharide</th>
<th>5F11 CHO pmol (% total)</th>
<th>5F11 LEX pmol (% total)</th>
<th>5F11 LEX&lt;sup&gt;0,pt&lt;/sup&gt; pmol (% total)</th>
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</thead>
<tbody>
<tr>
<td>Fuc</td>
<td>254 (20)</td>
<td>232 (13)</td>
<td>0</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>605 (47)</td>
<td>773 (45)</td>
<td>1,003 (67)</td>
</tr>
<tr>
<td>Gal</td>
<td>75 (6)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Man</td>
<td>355 (27)</td>
<td>491 (29)</td>
<td>501 (33)</td>
</tr>
<tr>
<td>Xyl</td>
<td>0</td>
<td>226 (13)</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>1,289 (100)</td>
<td>1,722 (100)</td>
<td>1,504 (100)</td>
</tr>
</tbody>
</table>

Functional activity of 5Fl1 CHO, LEX, and LEX<sup>0,pt</sup> mAbs.

Antigen binding properties of the 5Fl1 CHO, 5Fl1 LEX, and 5Fl1 LEX<sup>0,pt</sup> mAbs were determined using CD30 expressing L540 cells. All three mAbs had nearly identical binding curves (Figure 15). EC50 concentrations were determined to be 0.180 μg/mL, 0.227 μg/mL, and 0.196 μg/mL for 5Fl1 CHO, LEX, and LEX<sup>0,pt</sup>, respectively (Figure 16), indicating that antigen binding for all three mAbs were similar.

FcR binding of CHO, LEX and LEX<sup>0,pt</sup> mAbs was determined by incubating mAbs with effector cells expressing two different human FcRγllla allotypes (Phe<sup>158</sup> or Val<sup>158</sup>).

5Fl1 LEX had a 1.7-fold increase in FcRγlllaPhe<sup>158</sup> and a 0.4-fold decrease in FcRγlllaVal<sup>158</sup> binding compared to the CHO-derived mAb, demonstrating that receptor binding for CHO and LEX mAbs were similar. In contrast, LEX<sup>0,pt</sup> mAbs had a 27 and 15-fold higher affinity for FcRγlllaPhe<sup>158</sup> and FcRγlllaVal<sup>158</sup>, respectively, than CHO mAbs (Figure 17). These results suggested that RNAi silencing of the *Lemma* FucT and XyIT activities in LEX<sup>0,pt</sup> lines produced mAbs with enhanced FcR binding.

ADCC activities of the CHO, LEX and LEX<sup>0,pt</sup> mAbs were determined by incubating mAbs with either homozygous (FcRγlllaPhe<sup>158</sup>) or heterozygous (FcRγlllaPhe/Val<sup>158</sup>)
human effector cells and BATDA (bis(acetoxymethyl)2,2',6,2"-te \( \phi \) yridine-6,6"-dicarboxylate) labeled L540 target cells (Figure 17). 5Fl 1 LEX mAbs (31%) had the same maximal percent cell lysis as CHO mAbs (31%) using heterozygous Fc\( \gamma \)llaPhe/Val 158 human effector cells (Figure 18) with similar EC_{50} values (0.04210 and 0.05887, respectively). Maximal percent cell lysis for LEX mAbs (27%) was slightly increased compared to CHO mAbs (15%) using homozygous Fc\( \gamma \)RIlla Phe/Phe 158 effector cells, with EC_{50} values of 0.05759 and 0.03368, respectively. Importantly, LEX\(^{0.91} \) mAbs had significantly increased ADCC activity compared to 5Fl 1 CHO and LEX mAbs, irrespective of the donor genotype. This was assessed by both an increase in potency and efficacy.

Maximal percent lysis for 5Fl 1 Lex\(^{0.91} \) was 45% for both experiments. The EC_{50} value of 0.01306 was 3 to 5 times lower than 5Fl 1 LEX and 5Fl 1 CHO mAbs, respectively, for Fc\( \gamma \)Rma Val/Phe 158 effector cells. The EC_{50} value of 0.01990 was 2 to 3 times lower for the Fc\( \gamma \)RIlla Phe/Phe 158 effector cells. These results demonstrate that removal of Fuc and Xyl-containing \( \mathcal{N} \)-glycans from 5Fl 1 LEX\(^{0.91} \) mAbs caused an enhancement in ADCC activity and hence can improve their therapeutic potential.

**RP-HPLC-Q-TOFMS analysis of intact IgG for 5Fl 1 LEX and 5Fl 1 LEX\(^{0.91} \).**

Protein A purified IgG's (50 \( \mu \)g) were desalted using the Waters 2695 HPLC system fitted with a Poros Rl-10 column (2 mm x 30 mm; Applied Biosystems). IgG's were detected and analyzed using a Waters 2487 dual wavelength UV detector (280 nm) and the Waters Q-TOF API US. Separations were conducted at 0.15 mL/min, 60°C, using 0.05% (v/v) trifluoroacetic acid (TFA; solvent A) and 0.05% (v/v) TFA, 80% (v/v) acetonitrile (solvent B). Sample elution was carried out using a linear increase from 30 to 50% B for 5 min, an increase to 80% B for 5 min. The solvent ratio remained at 80% B for an additional 4 min, followed by a wash with 100% B for 1 min and equilibration of the column with 30% B for 15 min prior to the next run.

Q-TOF analysis was conducted in positive ion mode with source and desolvation temperatures of 100°C and 300°C, respectively, and capillary and cone voltages of 3.0 and 60 V, respectively. Data are the result of combining >100 individual scans and deconvolution to the parent mass spectrum using MaxEnt 1.


Figure 19 shows intact mass analysis of the 5Fl 1 LEX mAb compositions produced in wild-type L. minor comprising the MDXAOI construct. When XyIT and FucT expression
are not suppressed in \textit{L. minor}, the recombinantly produced 5Fl 1 LEX mAb composition comprises at least 7 different glycoforms, with the GOXF\(^3\) glycoform being the predominate species present. Note the absence of a peak representing the GO glycoform.

Figure 20 shows glycan mass analysis of the heavy chain of the 5Fl 1 LEX mAb produced in wild-type \textit{L. minor} comprising the MDXAO1 construct. When XyIT and FucT expression are not suppressed in \textit{L. minor}, the predominate iV-glycan species present is GOXF\(^3\), with additional major peaks reflecting the GOX species. Note the minor presence of the GO glycan species.

Figure 21 shows intact mass analysis of the 5Fl 1 LEX\(^0\) mAb compositions produced in transgenic \textit{L. minor} comprising the MDXA04 construct. When XyIT and FucT expression are suppressed in \textit{L. minor}, the intact mAb composition contains only GON-glycans. In addition, the composition is substantially homogeneous for the GO glycoform (peak 2), wherein both glycosylation sites are occupied by the GOiV-glycan species, with two minor peaks reflecting trace amounts of precursor glycoforms (peak 1, showing mAb having an Fc region wherein the C\(_{H2}\) domain of one heavy chain has a GO glycan species attached to Asn 297, and the C\(_{H2}\) domain of the other heavy chain is unglycosylated; and peak 3, showing mAb having an Fc region wherein the Asn 297 glycosylation site on each of the C\(_{H2}\) domains has a GO glycan species attached, with a third GO glycan species attached to an additional glycosylation site within the mAb structure).

Figure 22 shows glycan mass analysis of the heavy chain of the 5Fl 1 LEX\(^0\) mAb produced in transgenic \textit{L. minor} comprising the MDXAO4 construct. When XyIT and FucT expression are suppressed in \textit{L. minor}, the only readily detectable \(\alpha\)-glycan species attached to the Asn 297 glycosylation sites of the C\(_{H2}\) domains of the heavy chains is GO.

**DISCUSSION**

Glyco-optimization of 5Fl 1 was accomplished by co-expression with an RNAi cassette aimed at silencing the endogenous \textit{Lemma} FucT and XyIT genes. This simultaneous silencing of both FucT and XyIT genes was achieved using a single RNAi transcript. The absence of Fuc and XyI on the LEX\(^0\) mAb was confirmed by MALDI-TOF, NP-HPLC-QTOF MS, and monosaccharide analysis of \(\alpha\)-glycans purified from the 5Fl 1 LEX\(^0\) mAb.

These analyses corroborate the lack of transferase activity observed in microsomal membranes. Importantly, \(>95\%\) of the \(\alpha\)-glycans released from LEX\(^0\) mAbs were of a single structure, GnGn, indicating that this strategy had the added benefit of producing mAbs with a homogeneous JV-glycan profile. 5Fl 1 LEX and LEX\(^0\) mAbs were found to be indistinguishable with regard to thermal stability and antigen binding compared to 5Fl 1
CHO. Electrophoretic analysis was also found to be similar for all three mAbs. In fact, the only structural differences detected were in the mAb N-glycan profiles.

Without being bound by theory, the ability of the 5Fl 1 LEX\(^0\) lines to produce mAbs with a single major JV-glycan species may be based on the more uniform mAb glycoform distribution found in wild-type \textit{Lemma}. AT-glycans released from mAbs purified from wild-type tobacco, alfalfa, and moss show that mAb glycoform heterogeneity in plants with wild-type JV-glycosylation can range from five (alfalfa) to eight (tobacco) different major structures. 5Fl 1 LEX possesses only three major JV-glycan structures (GnGn, GnGnX and GnGnXF). This simple array of AT-glycans on mAbs produced by wild-type \textit{Lemma} may provide a more amenable starting point for glyco-optimization leading to greater homogeneity than that observed in other systems.

Fc-receptor mediated effector cell function has been shown to be important for the \textit{in vivo} activity of many therapeutic mAbs. In this study, the ADCC activity of 5Fl 1 CHO, 5Fl 1 LEX, and 5Fl 1 LEX\(^0\) mAbs was compared. Since the FcR expressed on NK cells and macrophages responsible for ADCC activity is FcγRII\(\alpha\), the binding of the various mAbs to this receptor was also compared. The results discussed above show that 5Fl 1 LEX\(^0\) mAb has an increased binding affinity (15-25 fold) and maximal binding (4-5 fold) to FcγRIIIa as well as enhanced ADCC activity compared to 5Fl 1 CHO and 5Fl 1 LEX mAbs. The removal of α-1,6-linked Fuc from various mAbs produced in other expression systems has been shown previously to increase FcR binding and enhance ADCC function. The results presented herein suggest that removal of the α-1,3-linked Fuc from the 5Fl 1 LEX\(^0\) mAbs has the same effect on mAb function as the removal of α-1,6-linked Fuc.

In this study, two naturally occurring polymorphic isoforms of FcγRIIIa at residue 158\(^{41}\), Val\(^{158}\) and Phe\(^{158}\), were evaluated. 5Fl11 LEX\(^{0,pt}\) shows a higher binding affinity to FcγRII\(\gamma\)a-Val\(^{158}\) compared to FcγRIIIa-Phe\(^{158}\) as has been observed with other IgGl mAbs. The fact that an increase in binding with 5Fl 1 LEX\(^0\) was observed with both isoforms is important since differential binding to Val\(^{158}\) over Phe\(^{158}\) was found to be predictive of the clinical and immunological responsiveness of certain patient groups receiving anti-CD20 treatment. This increase in binding could lead to more positive clinical outcomes over a broad population base.

A similar increase in ADCC activity was also observed. In this study, the 5Fl 1 LEX\(^{opt}\) mAb showed an increase in cell lysis and a decrease in the EC\(50\) value, resulting in an increase in efficacy and potency when compared to 5Fl 1 CHO. This corresponds to a 20-
fold increase in activity, determined by taking the maximum percent lysis of 5Fl 1 CHO and calculating the concentration of 5Fl 1 LEX0 mAb giving rise to the same percent cell lysis. As with the FcγRIIIa binding study, the increase in ADCC activity was observed with both a homozygous FcγRIHaPhe/Phe158 and a heterozygous FcγRIIIa Phe/Val158 effector cell donor.

The robustness of this glyco-optimization strategy has been demonstrated with multiple independent Lemna plant lines expressing the 5Fl 1 LEX0 mAb as well as with other mAbs expressed in the Lemna expression system. Furthermore, there is no apparent difference in plant phenotype or growth rate compared with wild-type Lemna plants. Unlike mammalian cell culture systems where JV-glycan heterogeneity can change with culture conditions, growth scale and growth period, the glycan uniformity observed with LEX015 mAbs has been shown to be consistent under a variety of growth conditions and scales (data not shown).

In conclusion, an RNAi strategy was used to produce a glyco-optimized anti-CD30 antibody in the Lemna expression system. The resulting mAb consists of a single, major iV-glycan structure; without any evidence of Fuc and XyI. In addition, the resulting optimized mAb has increased ADCC activity and FcγRIIIa binding activity compared to a CHO-derived mAb. The homogeneous glycosylation profile obtained on mAbs produced in a Lemna expression system having this FucT+XylT gene knockout strategy makes it possible to express mAbs with increased production consistency.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents of the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.
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<th>SEQUENCE</th>
<th>SEQ ID NO:</th>
<th>SEQUENCE</th>
</tr>
</thead>
<tbody>
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<td>VK CDR2 a.a. 5F11</td>
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<td>VK CDR1 a.a. 2H9</td>
<td></td>
<td>VK n.t. 2H9</td>
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</table>
We claim:

1. An isolated glycosylated anti-CD30 antibody, wherein the glycosylation comprises substantially a single glycoform and which lacks fucosyl and xylosyl residues.

2. The antibody of claim 1, wherein the antibody does not contain galactosyl residues.

3. A glycoprotein composition comprising an anti-CD30 antibody composition comprising a substantially homogeneous iV-glycosylation profile, wherein at least 90% of the iV-glycans species present in said profile are GlcNAc2Man3GlcNAc2 (GO), said profile comprising a trace amount of precursor A1-glycan species, wherein said precursor iV-glycan species is selected from the group consisting of Man3GlcNAc2, GlcNaclMan3GlcNAc2 wherein GlcNacl is attached to the 1,3 mannose arm (MGn), GlcNaclMan3GlcNAc2 wherein GlcNacl is attached to the 1,6 mannose arm (GnM), and any combination thereof.

4. The glycoprotein composition of claim 3, wherein said antibody composition comprises an Fc region selected from the group consisting of an IgGl, IgG2, IgG3, and IgG4 region.

5. The antibody composition of claim 3, which is a human antibody.

6. The antibody composition of claim 3, which is a humanized or chimeric antibody.

7. The antibody composition of claim 6, wherein the humanized or chimeric antibody is prepared from a mouse anti-CD30 antibody selected from the group consisting of: ACIO, HeFi-I, Ber-H2, Ki-I, Ki-4, HRS-3, Irac, HRS-4, M44, M67 and Ber-H8.

8. The glycoprotein composition of claim 3, wherein said antibody composition is a monoclonal antibody.

9. The glycoprotein composition of claim 3, wherein said antibody composition exhibits increased binding affinity for an FcγRIII.

10. The glycoprotein composition of claim 3, wherein the antibody composition enhances antibody dependent cellular cytotoxicity of cells expressing cell surface CD30.
11. The glycoprotein composition of claim 3, wherein said antibody composition exhibits increased macrophage mediated phagocytosis.

12. A pharmaceutical composition comprising the glycoprotein composition of any one of claims 3 through 11.

13. A host cell comprising the glycoprotein composition of claim 3.

14. The host cell of claim 13, wherein said host cell is a plant host cell.

15. The host cell of claim 14, wherein said plant host cell is a duckweed cell.

16. The glycoprotein composition of claim 3, wherein the antibody composition comprises a human heavy chain variable region and a human light chain variable region, wherein:
(a) the human heavy chain variable region comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 1, 2 and 3; and
(b) the human light chain variable region comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 4, 5 and 6.

17. The glycoprotein composition of claim 16, wherein the antibody composition heavy chain variable region comprises the amino acid sequence of SEQ ID NO: 1 and the light chain variable region comprises the amino acid sequence of SEQ ID NO: 4.

18. The glycoprotein composition of claim 16, wherein the antibody composition heavy chain variable region comprises the amino acid sequence of SEQ ID NO: 2 and the light chain variable region comprises the amino acid sequence of SEQ ID NO: 5.

19. The glycoprotein composition of claim 16, wherein the antibody composition heavy chain variable region comprises the amino acid sequence of SEQ ID NO: 3 and the light chain variable region comprises the amino acid sequence of SEQ ID NO: 6.

20. The glycoprotein composition of claim 3, wherein the antibody composition comprises:
(a) a human heavy chain variable region CDR1 comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 7, 8, and 9;
(b) a human heavy chain variable region CDR2 comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 10, 11, and 12;
(c) a human heavy chain variable region CDR3 comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 13, 14, and 15;
(d) a human light chain variable region CDR1 comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 16, 17, and 18;
(e) a human light chain variable region CDR2 comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 19, 20, and 21; and
(f) a human light chain variable region CDR3 comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 22, 23, and 24.

21. The glycoprotein composition of claim 20, wherein the antibody composition comprises:
(a) a human heavy chain variable region CDR1 comprising SEQ ID NO: 7;
(b) a human heavy chain variable region CDR2 comprising SEQ ID NO: 10;
(c) a human heavy chain variable region CDR3 comprising SEQ ID NO: 13;
(d) a human light chain variable region CDR1 comprising SEQ ID NO: 16;
(e) a human light chain variable region CDR2 comprising SEQ ID NO: 19; and
(f) a human light chain variable region CDR3 comprising SEQ ID NO: 22.

22. The glycoprotein composition of claim 20, wherein the antibody composition comprises:
(a) a human heavy chain variable region CDR1 comprising SEQ ID NO: 8;
(b) a human heavy chain variable region CDR2 comprising SEQ ID NO: 11;
(c) a human heavy chain variable region CDR3 comprising SEQ ID NO: 14;
(d) a human light chain variable region CDR1 comprising SEQ ID NO: 17;
(e) a human light chain variable region CDR2 comprising SEQ ID NO: 20; and
(f) a human light chain variable region CDR3 comprising SEQ ID NO: 23.

23. The glycoprotein composition of claim 20, wherein the antibody composition comprises:
(a) a human heavy chain variable region CDR1 comprising SEQ ID NO: 9;
(b) a human heavy chain variable region CDR2 comprising SEQ ID NO: 12;
(c) a human heavy chain variable region CDR3 comprising SEQ ID NO: 15;
(d) a human light chain variable region CDR1 comprising SEQ ID NO: 18;
(e) a human light chain variable region CDR2 comprising SEQ ID NO:21; and
(f) a human light chain variable region CDR3 comprising SEQ ID NO:24.

24. The glycoprotein composition of claim 3, wherein the antibody composition comprises a
   heavy chain variable region that is a product of or derived from a human V_H 4-34 or V_H 3-07
gene.

25. The glycoprotein composition of claim 3, wherein the antibody composition comprises a
   light chain variable region that is a product of or derived from a human Vk L15, A27 or L6
gene.

26. The glycoprotein composition of claim 25, wherein the antibody composition comprises a
   heavy chain variable region that is a product of or derived from a human V_H 4-34 or V_H 3-07
gene and a light chain variable region that is a product of or derived from a human V_k L15,
   A27 or L6 gene.

27. A host cell comprising immunoglobulin heavy and light chain genes encoding an anti-
   CD30 antibody, wherein said host cell lacks a fucosyltransferase and a xylosyltransferase
   such that the anti-CD30 antibody expressed by said host cell lacks fucosyl and xylosyl
   residues.

28. The host cell of claim 27, wherein the host cell is a plant cell.

29. The host cell of claim 28, wherein the plant cell is a member of the Lemnaceae family.

30. The host cell of claim 28, wherein the plant cell is Lemna minor.

31. The host cell of claim 27, wherein the immunoglobulin heavy and light chain genes are
   human immunoglobulin heavy and light chain genes.

32. The host cell of claim 27, wherein said fucosyltransferase is FucT and said
   xylosyltransferase is XyIT.
33. A method of inhibiting growth of CD30+ cells comprising contacting said cells with an anti-CD30 antibody comprising substantially a single glycoform and which lacks fucosyl and xylosyl residues under conditions sufficient to induce antibody-dependent cellular cytotoxicity (ADCC) of said cells.

34. The method of claim 33, wherein said cells are tumor cells.

35. The method of claim 33, wherein said anti-CD30 antibody is a human antibody.

36. A method of inhibiting growth of tumor cells expressing CD30 in a subject, comprising administering to the subject an anti-CD30 antibody comprising substantially a single glycoform and which lacks fucosyl and xylosyl residues in an amount effective to inhibit growth of tumor cells expressing CD30 in the subject.

37. The method of claim 36, wherein said anti-CD30 antibody is a human antibody.

38. The method of claim 36, wherein said tumor cells are Hodgkin's Disease (HD) tumor cells.

39. The method of claim 36, wherein said tumor cells are anaplastic large-cell lymphoma (ALCL) tumor cells.

40. The method of claim 36, wherein the tumor cells are of from a disease selected from the group consisting of non-Hodgkin's lymphoma, Burkitt's lymphoma, cutaneous T-cell lymphomas, nodular small cleaved-cell lymphomas, lymphocytic lymphomas, peripheral T-cell lymphomas, Lennert's lymphomas, immunoblastic lymphomas, T-cell leukemia/lymphomas (ATLL), adult T-cell leukemia (T-ALL), entroblastic/centrocytic (cb/cc) follicular lymphomas cancers, diffuse large cell lymphomas of B lineage, angioimmunoblastic lymphadenopathy (AILD)-like T cell lymphoma, adult T-cell lymphoma (ATL), HIV associated body cavity based lymphomas, Embryonal Carcinomas, undifferentiated carcinomas of the rhino-pharynx (e.g., Schmincke's tumor), Castleman's disease, Kaposi's Sarcoma, CD30+ T-cell lymphomas and CD30+ B-cell lymphomas.
41. A method of treating an autoimmune disorder in a subject, comprising administering to the subject an anti-CD30 antibody comprising substantially a single glycoform and which lacks fucosyl and xylosyl residues in an amount effective to treat an autoimmune disorder in the subject.
Anti-CD30 5F11 VH

V-segment: Locus: 4-34
D segment: Locus: 7-27
J segment: JH4b

Q V Q L Q Q W G A G L L K P S E T L
1 CAG GTG CAG CTA CAG CAG TGG GCC GCA GGA CTG TTG AAG CCT TCG GAG ACC CTG

CDR1

S L T C A V Y G G S F S A Y Y W S W
55 TCC CTC ACC TGC GCT GTC TAT GGT GGG TCC TTC AGT GCT TAC TAC TGG AGC TGG

CDR2

I R Q P P G K G L E W I G D I N H G
109 ATC CGC CAG CCC CCA GGG AAG GGG CTG GAG TGG ATT GGG GAC ATC AAT CAT GGT

~~~~~~~~~~~~~~~~~~~~~~~~~~~~

G G T N Y N P S L K S R V T I S V D
163 GGA GGC ACC AAC TAC AAC CCG TCC CTC AAG AGT CGA GTC ACC ATA TCA GTA GAC

~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

T S K N Q F S L K L N S V T A A D T
217 ACG TCC AAC AAG AAC CAG TCC CTG AAG CTG AAC TCT GTA ACC GCC GCG GAC ACG

CDR3

A V Y Y C A S L T A Y W G Q G S L V
271 GCT GTG TAT TAC TGT GCG AGC CTA ACT GCC TAC TGG GGC CAG GGA AGC CTG GTC

[Diagram]

D7-27/DHQ52

JH4b

T V S S
325 ACC GTC TCC TCA

Fig. 1A
Anti-CD30 5F11 VL

V-segment: Locus: L15
J segment: JK5

1 GAC ATC CAG ATG ACC CAG TCT CCA ACC TCA CTG TCT GCA TCT GTA GGA GAC AGA

CDR1

V T I T C R A S Q G I S S W L T W Y

55 GTC ACC ATC ACT TGT CGG GCG AGT CAG GGT ATT AGC AGC TGG TTA ACC TGG TAT

CDR2

Q Q K P E K A P K S L I Y A A S S L

109 CAG CAG AAA CCA GAG AAA GCC CCT AAG TCC CTG ATC TAT CCT GCT GCA TCC AGT TTG

CDR3

Q S G V P S R F S G S G S G T D F T

163 CAA AGT GGG GTC CCA TCA AGG TTC AGC GGC AGT GGA TCT GGG ACA GAT TTC ACT

CDR3

L T I S S L Q P E D F A T Y Y C Q Q

217 CTC ACC ATC AGC AGC CTG CAG CCT GAA GAT TTT GCA ACT TAT TAC TGC CAA CAG

CDR3

Y D S Y P I T F G Q G T R L E I K

271 TAT GAT AGT TAC CCT ATC ACC TTC GGC CAA GGG ACA CGA CTG GAG ATT AAA

Fig. 1B
Anti-CD30 17G1 VH

V-segment:  Locus: 3-07
D segment:  Not Found
J segment:  JH2

E V Q L V E S G G G L V Q P G G S L
1 GAG GTG CAG TTG GTG GAG TCT GGG GGA GGC TTG GTC CAG CCT GGG GGG TCC CTG

CDR1

R L S C V A S G F T F S N S W M S W
55 AGA CTC TCC TGT GTA GCC TCT GGA TTC AGT AAC TCT TGG ATG AGC TGG

CDR2

V R Q A P G K G L E W V A N I N E D
109 GTC CGC CAG GCT CCA GGG AAA GGG CTG GAG TGG GTG GCC AAC ATA AAC GAA GAT

CDR3

G S E K F Y V D S V K G R F T F S R
163 GGA AGT GAG AAA TTC TAT GTG GAC TCT GTG AAG GGC CGA TTC ACC TTC TCC AGA

D N A E N S L Y L Q M N S L R A E D
217 GAC AAC GCC GAG AAC TCA CTG TAT CTG CAA ATG AAC AGC CTG AGA GCC GAG GAC

CDR3

T A V Y Y C A R V H W Y F H L W G R
271 ACG GCT GTG TAT TAC TGT GGC AGG GTT CAT TGG TAC TTC CAT CTC TGG GCC CGT

G T L V T V S S
325 GCC ACC CTG GTC ACT GTC TCC TCA

Fig. 2A
Anti-CD30 17G1 VL
V-segment: Locus: A27
J segment: JK1

E I V L T Q S P G T L S L S P G E R
1 GAA ATT GTG TTG ACG CAG TCT CCA GCC ACC CTG TCT TTG TCT CCA GGG GAA AGA

CDR1

A T L S C R A S Q S V S S S Y L A W
55 GCC ACC CTC TCC TGC AGG GCC AGT CAG AGT GTT AGC AGC AGC TAC TTA GCC TGG

CDR2

Y Q Q K P G Q A P R L L I Y G A S S
109 TAC CAG CAG AAA CCT GCC CAG GCT CCC AGG CTC CTC ATC TAT GGT GCA TCC AGC

CDR2

R A T G I P D R F S G S G S G T D F
163 AGG GCC ACT GGC ATC CCA GAC AGG TTC AGT GCC AGT GGG TCT GGG ACA GAC TTC

CDR3

T L T I S S L E P E D F A V Y Y C Q
217 ACT CTC ACC ATC AGC AGC CTG GAG CCT GAA GAT TTT GCA GTG TAT TAC TGT CAG

CDR3

Q Y G S S P W T F G Q G T K V E I K
271 CAG TAT GGT AGC TCA CCG TGG AGC TTC GCC CAA GGG ACC AAG GTG GAA ATC AAA

Fig. 2B
Anti-2H9 CD30 VH

V-segment: Locus: 4–34
D segment: Locus: 5–12
J segment: JH2

Q V Q L Q Q W G A G L L K P S E T L
1 CAG GTG CAG CTA CAG CAG TGG GCC GCA GGA CTG TGG AAG CCT TCG GAG ACC CTG

CDR1

S L T C A V Y G G S F S G Y Y W S W
55 TCC CTC ACC TGC GCT GTC TAT GGT GGG TCC TTC AGT GGT TAC TAC TGG AGC TGG

CDR2

I R Q P P G K G L E W I G E I N H S
109 ATC GCC CAG CCC CCA GGG AAG GGG CTG GAG TGG ATT GGG GAA ATC AAT CAT AGT

CDR3

G S T K Y T P S L K S R V T I S V D
163 GGA AGC ACC AAG TAC ACC CCG TCC CTC AAG AGC CGA GTC ACC ATA TCA GTA GAC

TS K H Q F S L K L S S V T A A D T
217 ACG TCC AAG CAC CAA TTC TCC CTG AAG CTG AGC TCT GTG ACC GCC GCG GAC ACG

CDR3

A V Y Y C A R E T V Y Y F D L W G R
271 GCT GTG TAT TAC TGT GCG AGA GAG ACT GTC TAC TAC TTC GAT CTC TGG GCC CGT

Fig. 3A
Anti-2H9 CD30 VL
V-segment: L6
J segment: JK1

1 GAA ATT GTG TTG ACA CAG TCT CCA GCC ACC CTG TCT TTG TCT CCA GGG GAA AGA

CDR1

ATL S C R A S Q S V S S N L A W Y

55 GCC ACC CTC TCC TGC AGG GCC AGT CAG AGT GTA AGC AGC AAC TTA GCC TGG TAC

CDR2

QQKPGQAPRLLILYDASNR

109 CRA CAG AAA CCT GGC CAG GCT CCC AGG CTC CTC ATC TAT GAT GCA TCC AAC AGG

CDR2

ATGIILLSGSGSGFTDFT

163 GCC ACT GGC ATC CCA GCC AGG CTC AGT GGC AGT GGG TCT GGG ACA GAC TTC ACT

CDR3

LTISSLEPWFAVYYCQQ

217 CTC ACC ATC AGC AGC CTA GAG CCT GAA GAT TTT GCA GTT TAT TAC TGT CAA CAG

CDR3

RSNWPFWTFTGQGTTKVEI

271 CGT AGC AAC TGG CGG TGG AGC TTC GCC CAA GGG ACC AAG GTG GAA ATC AAA

→ JK1

Fig. 3B
ADCC: Cr
L540 w/ CD30

Fig. 4
ADCC: Cr-51
L428 w/ αCD30

- CD30
- CD30 defucose
- 1D4 (IgG1)

Fig. 5
ADCC: Cr-51
L1236 w/ αCD30

- CD30
- CD30 defucosyl
- 1D4 (IgG1)

Fig. 6
ADCC: Cr
Karpas w/ CD30

Fig. 7
Heavy Chain Germline Sequences

4-34 Germline: QVQLQQWGA GSLLPSETLSLTCAYGGSFSGYYSW

            CDR1

            CDR2

WIRQPPGKGLEWIGEINHSGSTNYNPSLKSRTVIS

            CDR3

VDTSKNSFSLKLSVTAADTAVYYCAR

            CDR1

3-07 Germline: EQLVESGGGLVQPGGSLRLSCAASGFTFSSYWS

            CDR2

VRQPAGKGLEWVKANIKQDGSEKYYVDSVKGRFTISR

            CDR3

DNAKNSLYLQMSLRAEDTAVYYCAR

Fig. 8A
Light Chain Germline Sequences

L6 germline: EIVLTQSPATLSLSSPGERATLSRCRASQSVSSYLA

WYQQKPGQAPRLIIYDAASNRRATGIPARFSGSGS

TDFTLTISSLPEPEDFAVYYCQQRSNW

A27 germline: EIVLTQSPGTLSSLSPGERATLS RASQSVSSSYLA WYQQKPGQAPRLIIY

GASSRAT

GIPDRFSGSGSTDFTLTISLPEPEDFAVYYCQQYGSS

L15 germline: DIQMTQPSSLSASVGDRTITC RASQGISSWLA WYQQKPEAKSPLY

AASSLQS

GYPFSRSFRSGSTDFTLTISSLQPEDFATYYCQQYNSY

Fig. 8B
Comparison of ADCC assay with mouse and human effector cells

Mouse effector cells
1001-BP 081704

Human effector cells
1001-BP 081704

Fig. 10
ADCC assay using cynomolgus blood

Experiment #1
ADCC CD30 with Karpas 299 and Cyno Cells
1:50 T/E Ratio
50U/ml IL2 Overnight Treatment

Fig. 11
MDXA04 transgenic lines

Fig. 12
Fig. 15

19/26

MDX-060
CHO

MDX-060
LEX

MDX-060
LEX Opt

Fig. 15
Fig. 16
**Fig. 17**

### Table: Binding Constants (nM)

<table>
<thead>
<tr>
<th></th>
<th>MDX-060 CHO</th>
<th>MDX-060 LEX</th>
<th>MDX-060 LEX&lt;sup&gt;Opt&lt;/sup&gt;</th>
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<tr>
<td>Human FcR</td>
<td>177</td>
<td>427</td>
<td>12</td>
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<tr>
<td>CD16-Val</td>
<td>1091</td>
<td>641</td>
<td>41</td>
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<tr>
<td>CD16-Phe</td>
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</tr>
</tbody>
</table>
Fig. 18
MDXA01 Intact Mass Analysis

Fig. 19
Heavy chain analysis of MDXA01

$H_c^-$
m/z = 49550.7

$H_c^-$
m/z = 49682.7

$H_c$
m/z = 48252.2

$H_c^-$
m/z = 50430.7

Fig. 20
MDXA04 Intact Mass Analysis

062010_MDXA04_2 273 (10.151) M1 [E=126389, k=10] (G6, 0.750, 2585.4000, 0.10, L33, R33); Sm (SG, 2x4.00); Cm (246.479)

1: TOF MS ES+

G0

G0X

G0XF³

mass

Fig. 21
Heavy chain analysis of MDXA04

$m/z = 49553.4$

$m/z = 48252.9$

Fig. 22