



(22) Date de dépôt/Filing Date: 2004/05/07
(41) Mise à la disp. pub./Open to Public Insp.: 2005/01/06
(45) Date de délivrance/Issue Date: 2018/07/31
(62) Demande originale/Original Application: 2 525 251
(30) Priorité/Priority: 2003/05/09 (US60/469,451)

(51) Cl.Int./Int.Cl. *C07K 16/28* (2006.01),
A61K 39/395 (2006.01), *A61P 35/00* (2006.01),
A61P 37/06 (2006.01), *C07K 16/46* (2006.01),
C12N 15/13 (2006.01)

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(54) Titre : ANTICORPS SPECIFIQUES DE CD20 ET LEURS METHODES D'UTILISATION
(54) Title: CD20-SPECIFIC ANTIBODIES AND METHODS EMPLOYING SAME

(57) **Abrégé/Abstract:**

The present invention provides monoclonal antibodies and antigen-binding fragments thereof that specifically bind to CD20, as well as pharmaceutical compositions comprising the same The invention further provides methods of using the monoclonal antibodies, antigen-binding fragments, and pharmaceutical compositions, for example, in methods of depleting B cells or in treating B cell disorders Also provided are cells, nucleic acids and methods for producing the monoclonal antibodies

ABSTRACT

The present invention provides monoclonal antibodies and antigen-binding fragments thereof that specifically bind to CD20, as well as pharmaceutical compositions comprising the same. The invention further provides methods of using the monoclonal antibodies, antigen-binding fragments, and pharmaceutical compositions, for example, in methods of depleting B cells or in treating B cell disorders. Also provided are cells, nucleic acids and methods for producing the monoclonal antibodies.

CD20-SPECIFIC ANTIBODIES AND METHODS EMPLOYING SAME

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FIELD OF THE INVENTION

The present invention relates to monoclonal antibodies directed to CD20 and methods of making and using the same.

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BACKGROUND OF THE INVENTION

B lymphocytes are the origin of humoral immunity, represent a substantial portion of hematopoietic malignancies, and contribute to autoimmunity.

Consequently, cell surface molecules expressed by B cells and their malignant counterparts are important targets for immunotherapy. CD20, a B cell-specific member of the MS4A gene family, is expressed on the surface of immature and mature B cells and their malignant counterparts (Tedder and Engel (1994) *Immunol. Today* 15:450-454).

A limited analysis of CD20 transcripts in mouse cell lines and tissues suggests that mouse CD20 is also B cell-specific (Tedder, et al. (1988) *J. Immunol.* 141:4388). Both human and mouse CD20 cDNAs encode a membrane-embedded protein with hydrophobic regions of sufficient length to pass through the membrane four times (Tedder, et al. (1988) *J. Immunol.* 141:4388; Tedder, et al. (1988) *Proc. Natl. Acad. Sci. USA.* 85:208; Einfeld, et al. (1988) *EMBO J.* 7:711; Stamenkovic and Seed (1988) *J. Exp. Med.* 167:1975). Mouse and human CD20 are well conserved (73%) in amino acid sequence, particularly the transmembrane and long amino- and carboxyl-

terminal cytoplasmic domains (Tedder, et al. (1988) *J. Immunol.* 141:4388). The cytoplasmic domains are serine- and threonine-rich with multiple consensus sequences for phosphorylation. Human CD20 is not glycosylated, but three isoforms (33-, 35- and 37,000 M_r) result from the differential
5 phosphorylation of a single protein on different serine and threonine residues (Tedder, et al. (1988) *Molec. Immunol.* 25:1321; Tedder and Schlossman (1988) *J. Biol. Chem.* 263:10009; Valentine, et al. (1987) *Proc. Natl. Acad. Sci. U.S.A.* 84:8085).

CD20 plays a role in the regulation of human B cell activation,
10 proliferation and Ca²⁺ transport (Tedder, et al. (1985) *J. Immunol.* 135:973; Bubien, et al. (1993) *J. Cell Biol.* 121:1121). Antibody ligation of CD20 can generate transmembrane signals that result in enhanced CD20 phosphorylation (Tedder and Schlossman (1988) *J. Biol. Chem.* 263:10009), induction of *c-myc* and *B-myb* oncogene expression (Smeland, et al. (1985)
15 *Proc. Natl. Acad. Sci. U.S.A.* 82:6255; Golay, et al. (1992) *J. Immunol.* 149:300), induced serine/threonine and tyrosine phosphorylation of cellular proteins (Deans, et al. (1993) *J. Immunol.* 151:4494), increased CD18, CD58 and MHC class II molecule expression (White, et al. (1991) *J. Immunol.* 146:846; Clark and Shu (1987) *J. Immunol.* 138:720), and protein tyrosine
20 kinase activation that induces B cell adhesion (Kansas and Tedder (1991) *J. Immunol.* 147:4094). CD20 ligation promotes transmembrane Ca²⁺ transport (Bubien, et al. (1993) *J. Cell Biol.* 121:1121), but does not usually lead to increased intracellular calcium ([Ca²⁺]_i)³ levels (Bubien, et al. (1993) *J. Cell Biol.* 121:1121; Tedder, et al. (1986) *Eur. J. Immunol.* 16:881; Golay, et al.
25 (1985) *J. Immunol.* 135:3795), except after extensive crosslinking (Deans, et al. (1993) *J. Immunol.* 151:4494). Antibody binding to CD20 inhibits B cell progression from the G₁ phase into the S/G₂+M stages of cell cycle following mitogen stimulation, and inhibits mitogen-induced B cell differentiation and antibody secretion (Tedder, et al. (1985) *J. Immunol.* 135:973; Tedder, et al.
30 (1986) *Eur. J. Immunol.* 16; Golay, et al. (1985) *J. Immunol.* 135:3795; Golay and Crawford (1987) *Immunology* 62:279). Extensive CD20 cross-linking can also influence apoptosis (Holder, et al. (1995) *Eur. J. Immunol.* 25:3160; Shan, et al. (1998) *Blood* 91:1644). These divergent observations may be

explained in part by the finding that CD20 is a component of an oligomeric complex that forms a membrane transporter or Ca^{2+} ion channel that is activated during cell cycle progression (Bubien, et al. (1993) *J. Cell Biol.* 121:1121; Kanzaki, et al. (1995) *J. Biol. Chem.* 270:13099; Kanzaki, et al. (1997) *J. Biol. Chem.* 272:14733; Kanzaki, et al. (1997) *J. Biol. Chem.* 272:4964). Despite this, B cell development and function in a line of CD20-deficient (CD20^{-/-}) mice is reported to be normal (O'Keefe, et al. (1998) *Immunogenetics* 48:125).

The majority of human B cell-lineage malignancies express CD20 (Anderson, et al. (1984) *Blood* 63:1424). Chimeric or radiolabeled monoclonal antibody-based therapies directed against CD20 have been used for non-Hodgkin's lymphoma (Press, et al. (2001) *Hematology*:221-240; Kaminski, et al. (1993) *N. Engl. J. Med.* 329:459-465; Weiner (1999) *Semin. Oncol.* 26:43-51; Onrust, et al. (1999) *Drugs* 58:79-88; McLaughlin, et al. (1998) *Oncology* 12:1763-1769). Clinical studies indicate that anti-CD20 monoclonal antibody therapy also ameliorates the manifestations of rheumatoid arthritis, idiopathic thrombocytopenic purpura and hemolytic anemia, as well as other immune-mediated diseases (Silverman and Weisman (2002) *Arthritis Rheum.* 48:1484-1492; Edwards and Cambridge (2001) *Rheumatology* 40:1-7).

Competing hypotheses are employed to explain the therapeutic efficacy of anti-CD20 monoclonal antibodies *in vivo*. In one model, CD20 serves as a membrane-embedded target for monoclonal antibody-mediated depletion of B cells through activation of the innate immune system or the initiation of effector mechanisms (Reff, et al. (1994) *Blood* 83:435-445; Maloney, et al. (1997) *Blood* 90:2188-2195; Maloney, et al. (1997) *J. Clin. Oncol.* 15:3266-3274).

Rituximab, a chimeric human IgG1 anti-human CD20 monoclonal antibody is highly effective in inducing classical pathway complement (C) activation and C-dependent cytotoxicity of freshly isolated lymphoma cells and B cell lines (Reff, et al. (1994) *Blood* 83:435-445; Golay, et al. (2001) *Blood* 98:3383-3389; Cragg, et al. (2003) *Blood* 101:1045-1052; Di Gaetano, et al. (2003) *J. Immunol.* 171:1581-1587; Bellosillo, et al. (2001) *Blood* 98:2771-2777). Rituximab also activates C *in vivo* in both patients (van der Kolk, et al.

(2001) *Br. J. Hematol.* 115:807-811) and primates (Kennedy, et al. (2003) *Blood* 101:1071-1079). Furthermore, tumor cell expression of C regulatory proteins, including CD59, is associated with resistance to anti-CD20 therapy (Golay, et al. (2001) *Blood* 98:3383-3389; Treon, et al. (2001) *J. Immunotherapy* 24:263-271). Although many consider C-dependent cytotoxicity to be the major pathway used by Rituximab antibody to deplete human lymphoma cells *in vitro* and *in vivo* (Golay, et al. (2001) *Blood* 98:3383-3389; Cragg, et al. (2003) *Blood* 101:1045-1052; Di Gaetano, et al. (2003) *J. Immunol.* 171:1581-1587; Golay, et al. (2000) *Blood* 95:3900-3908; Di Gaetano, et al. (2001) *Br. J. Hematol.* 114:800-809; Weiner (2003) *Blood* 101:788), others have found that the susceptibility of tumor cells to C-mediated lysis and expression of C inhibitors CD46, CD55, and CD59 on tumor cells does not predict the outcome of Rituximab therapy (Weng and Levy (2001) *Blood* 98:1352-1357). Other antibody-dependent effects also appear important since a chimeric anti-CD20 monoclonal antibody of an isotype different than that used clinically does not deplete normal B cells in non-human primates (Anderson, et al. (1997) *Biochem. Soc. Transac.* 25:705-708) and the anti-tumor effect of anti-CD20 monoclonal antibody depends in part on immune activation through Fc receptors (FcγR) for IgG (Clynes, et al. (2000) *Nature Med.* 6:443-446). Alternatively, anti-CD20 monoclonal antibody treatment alters transmembrane Ca²⁺ transport and B cell function, which disrupts progression through cell cycle (Tedder and Engel (1994) *Immunol. Today* 15:450-454) and can induce B cell apoptosis (Shan, et al. (1998) *Blood* 91:1644-1652; Demidem, et al. (1997) *Cancer Biother. Radiopharm.* 12:177-186).

It is difficult to differentiate between these hypotheses *in vivo* due to the complexities of carrying out mechanistic studies in humans undergoing immunotherapy (Edwards and Cambridge (2001) *Rheumatology* 40:1-7). Moreover, human studies primarily focus on changes in blood, which contains <2% of the B cells outside of the bone marrow. Thus, it is difficult to accurately ascertain the effects of anti-CD20 therapies on the majority of B cells, which are found in peripheral lymphoid tissues.

Needed in the art are improved reagents and methods for altering B cell function, in particular in B cell disorders such as B cell malignancies and

autoimmune diseases. Also needed are new anti-CD20 monoclonal antibodies with different immunoreactive characteristics than conventional monoclonal antibodies directed against CD20.

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SUMMARY OF THE INVENTION

The present invention is based, in part on the production and identification of novel monoclonal antibodies that react with CD20 having desirable characteristics.

Accordingly, in one embodiment, the invention provides a monoclonal antibody (mAb) or antigen-binding fragment thereof that specifically binds to CD20 wherein the density of binding of the mAb or antigen-binding fragment to B cells is at least two-fold higher than the density of binding to one or more conventional mAbs, such as mAb 1F5, to B cells and/or their malignant counterparts.

As another aspect, the invention provides a mAb or antigen-binding fragment thereof that specifically binds to CD20, wherein binding of the mAb or antigen-binding fragment to CD20 on B cells (and/or their malignant counterparts) results in upregulation of binding sites for the mAb or antigen-binding fragment on the B cells.

As a further aspect, the invention provides a mAb or antigen-binding fragment thereof that specifically binds to CD20, wherein the mAb or antigen-binding fragment binds to the same antigenic determinant as a mAb selected from the group consisting of HB20-3, HB20-4, HB20-25 and MB20-11. In particular embodiments, the mAb is selected from the group consisting of HB20-25 and MB20-11. In other embodiments, the antigen-binding fragment is selected from the group consisting of an antigen-binding fragment of HB20-25 and MB20-11.

In another aspect, the invention provides a mAb or antigen-binding fragment thereof that specifically binds to CD20, wherein the mAb or antigen-binding fragment comprises a heavy chain CDR3 region from a mAb selected from the group consisting of HB20-3, HB20-4, HB20-25 and MB20-11 or a heavy chain CDR3 region having at least 80% amino acid sequence similarity to the heavy chain CDR3 region of HB20-3, HB20-4, HB20-25 or MB20-11.

In still other embodiments, the invention provides a mAb or antigen-binding fragment thereof that specifically binds to CD20, wherein the mAb or antigen-binding fragment comprises a light chain CDR3 region from a mAb selected from the group consisting of HB20-3, HB20-4 and HB20-25 or a light chain CDR3 region having at least 80% amino acid sequence similarity to the light chain CDR3 region of HB20-3, HB20-4 or HB20-25.

In further embodiments, the invention provides a mAb or antigen-binding fragment thereof that specifically binds to CD20, wherein the mAb or antigen-binding fragment comprises a CDR3 region from a mAb selected from the group consisting of HB20-3, HB20-4 and HB20-25 or a CDR3 region having at least 80% amino acid sequence similarity to the CDR3 region of HB20-3, HB20-4 or HB20-25.

In still further embodiments, the invention provides a mAb or antigen-binding fragment thereof that specifically binds to CD20, wherein the mAb or antigen-binding fragment comprises CDR1, CDR2 and CDR3 regions from a mAb selected from the group consisting of HB20-3, HB20-4 and HB20-25 or CDR1, CDR2 and CDR3 regions having at least 80% amino acid sequence similarity to the CDR1, CDR2 and CDR3 regions, respectively, of HB20-3, HB20-4 or HB20-25.

As yet another aspect, the invention provides a mAb or antigen-binding fragment thereof that specifically binds to CD20, wherein the mAb or antigen-binding fragment is selected from the group consisting of:

(a) a mAb or antigen-binding fragment comprising a heavy chain comprising the heavy chain variable region of SEQ ID NO:3 (HB20-3), SEQ ID NO:5 (HB20-4), SEQ ID NO:9 (HB20-25) or SEQ ID NO:21 (HB20-11) or a heavy chain variable region that has at least 80% amino acid sequence similarity with the amino acid sequence of SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:9 or SEQ ID NO:21;

(b) a mAb or antigen-binding fragment comprising a light chain comprising a light chain variable region of SEQ ID NO:31 (HB20-3), SEQ ID NO:33 (HB20-4), or SEQ ID NO:37 (HB20-25) or a light chain variable region that has at least 80% amino acid sequence similarity with the amino acid sequence of SEQ ID NO:31, SEQ ID NO:33, or SEQ ID NO:37; and

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(c) a mAb or antigen-binding fragment comprising a heavy chain and a light chain according to (a) and (b).

As yet another aspect, the invention provides a mAb or antigen-binding fragment thereof that specifically binds to CD20, wherein the mAb or antigen-binding fragment selected from the group consisting of:

(a) a mAb or antigen-binding fragment comprising a heavy chain comprising the heavy chain variable region of SEQ ID NO:3 (HB20-3) or a heavy chain variable region that has at least 80% amino acid sequence similarity with the amino acid sequence of SEQ ID NO:3 and a light chain comprising the light chain variable region of SEQ ID NO:31 (HB20-3) or a light chain variable region that has at least 80% amino acid sequence similarity with the amino acid sequence of SEQ ID NO:31;

(b) a mAb or antigen-binding fragment comprising a heavy chain comprising the heavy chain variable region of SEQ ID NO:5 (HB20-4) or a heavy chain variable region that has at least 80% amino acid sequence similarity with the amino acid sequence of SEQ ID NO:5 and a light chain comprising the light chain variable region of SEQ ID NO:33 (HB20-4) or a light chain variable region that has at least 80% amino acid sequence similarity with the amino acid sequence of SEQ ID NO:33; and

(c) a mAb or antigen-binding fragment comprising a heavy chain comprising the heavy chain variable region of SEQ ID NO:9 (HB20-25) or a heavy chain variable region that has at least 80% amino acid sequence similarity with the amino acid sequence of SEQ ID NO:9 and a light chain comprising the light chain variable region of SEQ ID NO:37 (HB20-25) or a light chain variable region that has at least 80% amino acid sequence similarity with the amino acid sequence of SEQ ID NO:37.

In other particular embodiments, the invention provides a mAb or antigen-binding fragment thereof that specifically binds to CD20, wherein the mAb or antigen-binding fragment comprises the heavy chain variable region from a mAb selected from the group consisting of HB20-3 (SEQ ID NO:3), HB20-4 (SEQ ID NO:5), HB20-25 (SEQ ID NO:9) and MB20-11 (SEQ ID NO:21).

In yet further embodiments, the invention provides a mAb or antigen-binding fragment thereof that specifically binds to CD20, wherein the mAb or

antigen-binding fragment comprises the light chain variable region from a mAb selected from the group consisting of HB20-3 (SEQ ID NO:31), HB20-4 (SEQ ID NO:33) and HB20-25 (SEQ ID NO:37).

As a further aspect, the invention provides a mAb or antigen-binding
5 fragment thereof that specifically binds to mouse CD20.

Also provided are pharmaceutical compositions comprising the mAbs and antigen-binding fragments of the invention.

As one embodiment, the invention provides a pharmaceutical composition comprising a mAb or antigen-binding fragment thereof which
10 specifically binds to the same antigenic determinant as a mAb selected from the group consisting of HB20-1, HB20-3, HB20-4 and HB20-25.

The invention also provides cell lines for producing the mAbs and antigen-binding fragments of the invention.

As a further aspect, the invention provides a method of depleting B
15 cells in a mammalian subject, comprising administering a mAb or antigen-binding fragment or a pharmaceutical composition of the invention to the mammalian subject in an amount effective to deplete B cells and/or their malignant counterparts.

As yet a further aspect, the invention provides a method of treating a B
20 cell disorder, comprising administering to a mammalian subject having a B cell disorder a treatment-effective amount of a mAb or antigen-binding fragment thereof that specifically binds to CD20, wherein the mAb or antigen-binding fragment has a treatment-effective dosage range of 125 mg/m² or less that results in at least a 75% depletion in circulating B cells and/or their
25 malignant counterparts.

As still a further aspect, the invention provides a method of treating a B cell disorder, comprising administering to a mammalian subject having a B cell disorder a treatment-effective amount of a mAb or antigen-binding fragment or a pharmaceutical composition of the invention.

30 In particular embodiments of the foregoing methods, the B cell disorder is a B cell malignancy or an autoimmune disease.

As a further aspect, the invention provides a method of treating a B cell disorder, comprising administering to a mammalian subject having a B cell disorder a treatment-effective amount of: (i) a mAb or antigen-binding

fragment or a pharmaceutical composition of the invention, and (ii) a compound that enhances monocyte or macrophage function.

As another aspect, the invention provides a method of producing a mAb that specifically binds to CD20, comprising: (a) immunizing a CD20^{-/-} mammal with CD20 or an antigenically effective fragment thereof under conditions sufficient to elicit an antibody response; (b) harvesting antibody producing cells from the mammal; (c) fusing the antibody producing cells with immortalized cells in culture to form monoclonal antibody-producing hybridoma cells; (d) culturing the hybridoma cells under conditions sufficient for production of monoclonal antibodies; and (e) recovering from the culture monoclonal antibodies that specifically bind to CD20.

As still another aspect, the invention provides a method of producing a mAb that specifically binds to CD20, comprising: (a) immunizing a CD20^{-/-} mammal with CD20 or an antigenically effective fragment thereof under conditions sufficient to elicit an antibody response; (b) harvesting a cell that produces an antibody that specifically binds to CD20 from the mammal; (c) isolating an immunoglobulin coding gene from the antibody-producing cell; (d) introducing the immunoglobulin coding gene into a cell to produce a transformed cell; (e) culturing the transformed cell under conditions sufficient for transcription and translation of the immunoglobulin gene and production of a monoclonal antibody; and (e) recovering from the culture monoclonal antibodies that specifically bind to CD20.

The invention further provides for the use of a nucleic acid, vector, mAb or antigen-binding fragment or pharmaceutical composition of the invention for use in depleting B cells (and/or their malignant counterparts) and/or for the treatment of a B cell disorder.

As other aspects, the invention further provides isolated nucleic acids encoding the heavy and light chains of the mAbs and antigen-binding fragments of the invention. Further provided are vectors and cells comprising the isolated nucleic acids.

These and other aspects of the invention are set forth in more detail in the description of the invention below.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-1M illustrate targeted disruption of the *Cd20* gene. **Figure 1A**, Genomic clones encoding the 3' end of the *Cd20* gene. **Figure 1B**, Intron-exon organization of wild-type *Cd20* containing exons 5-8 (filled squares). Exon numbers are based on human *CD20* structure (Tedder et al., (1989) *J. Immunol.* 142:2560-2568). **Figure 1C**, Targeting vector structure. **Figure 1D**, Structure of the *Cd20* allele after homologous recombination, with the *EcoRV* restriction site in exon 6 deleted. **Figure 1E**, Southern blot analysis of genomic DNA from two wild-type and four *CD20*^{-/-} littermates digested with *EcoRV*, transferred to nitrocellulose and hybridized with the 5' DNA probe indicated in **Figure 1D**. **Figure 1F**, PCR amplification of genomic DNA from wild-type and *CD20*^{-/-} littermates using primers that bind in exons 6 (5' of *EcoRV* site) and 7. G3PDH amplification is shown as a positive control. **Figure 1G**, PCR amplification of cDNA generated from splenic RNA of wild-type and *CD20*^{-/-} littermates. Each reaction mixture contained a sense primer that hybridized with sequences encoded by exon 3 and two antisense primers that hybridized with exon 6 or *Neo*^r gene promoter sequences. DNA amplified with exon 3 and 6 primers was 445 bp long, while exon 3 and *Neo* primers amplified a 749 bp fragment. **Figure 1H**, Reactivity of the MB20-13 monoclonal antibody with *CD20* cDNA-transfected (thick line) or untransfected (dashed line) 300.19 cells or CHO cells. The thin lines represent *CD20* cDNA-transfected cells stained with secondary antibody alone or an isotype-control monoclonal antibody. Immunofluorescence staining was visualized by flow cytometry analysis. **Figure 1I**, Immunofluorescence staining of splenocytes from *CD20*^{-/-} or wild-type littermates with MB20-7 (visualized using a phycoerythrin-conjugated, anti-mouse IgG2b antibody) and anti-CD19 (FITC-conjugated) monoclonal antibodies with flow cytometry analysis. Quadrants delineated by squares indicate negative and positive populations of cells as determined using unreactive monoclonal antibody controls. **Figure 1H** and **Figure 1I** results are representative of those obtained with twelve anti-mouse *CD20* monoclonal antibodies. **Figure 1J**, B lymphocyte distribution in *CD20*^{-/-} and wild-type littermates. The gated cell populations correspond to the cells

described in **Table 4** and represent results obtained using groups of 10 littermates. **Figure 1K**, Mitogen responses of CD20^{-/-} B cells. Purified spleen B cells (2×10^5 /well) from CD20^{-/-} and wild-type littermates were cultured with anti-IgM F(ab')₂ antibody fragments, anti-IgM antibody plus IL-4, or LPS.

5 Values are means ($\pm$ SEM) from triplicate cultures and represent results obtained in four independent experiments. **Figure 1L**, Mean ($\pm$ SEM) serum Ig levels for 6 CD20^{-/-} (filled histograms) and wild-type (open histograms) littermates as measured by isotype-specific ELISA. **Figure 1M**, T cell-dependent humoral immune responses. Two CD20^{-/-} (filled circles, solid
10 lines) and wild-type (open squares, dashed lines) mice were immunized with DNP-KLH on days 0 and 21, with serum collected at the times indicated. Serum anti-DNP antibodies were determined by isotype-specific ELISA. Mean CD20^{-/-} (solid line) and wild-type (dashed line) antibody levels are shown.

15 **Figures 2A-2I** show CD20 expression during B cell development. **Figure 2A**, Immunofluorescence staining of mouse lymphoblastoid cell lines using the MB20-7 (thick line) or isotype-control (dashed line) monoclonal antibodies. Single-cell suspensions of lymphocytes isolated from bone marrow (**Figure 2B**), blood (**Figure 2C**), peripheral lymph nodes (PLN; **Figure**
20 **2D**), spleens (**Figure 2E**) and peritoneal cavities (**Figure 2F**) of wild-type C57BL/6 mice were examined by two-color immunofluorescence staining with flow cytometry analysis. **Figure 2G**, CD20 expression by bone marrow B cell subpopulations assessed by four-color flow cytometry analysis. Pro-B cells were identified as CD43⁺ B220^{lo} cells with the forward- and side-scatter
25 properties of lymphocytes. Pre-B cells were IgM⁻ CD43⁻ B220^{lo} cells. Immature and mature CD43⁻ B cells were divided into three factions (I, II and III) based on relative IgM and B220 densities. Background fluorescence staining was assessed using isotype-matched control monoclonal antibodies as negative controls (dotted lines). **Figure 2H**, CD20 expression by T1, T2 or
30 mature (M) spleen B cells as defined by relative HSA and CD21 expression densities. **Figure 2I**, CD20 expression by T1, T2, marginal zone (MZ) and mature (M) spleen B cells defined by CD23 expression, and relative IgM and

CD21 densities. All results are representative of those obtained with ≥ 3 two-month old wild-type mice.

Figures 3A-3C show the biochemical characterization of mouse CD20 and CD20^{-/-} B cells. **Figure 3A**, CD20 immunoprecipitated (arrows) from surface-biotinylated Raji (human) and A20 (mouse) B cell lines using the HB20-8 (PB4; human CD20) and MB20-1 (mouse CD20) monoclonal antibodies, respectively. Immunoprecipitations with isotype-matched control monoclonal antibodies (C antibody) are shown. The dashed vertical line in the reduced gel panel indicates that the results came from separate gels run in parallel. **Figure 3B**, Western blot analysis of CD20 expression. Lysates of Raji (1×10^6 cells/lane), A20 and the 300.19 B cell lines or purified mouse splenic B cells (5×10^6 cells/lane) were boiled under reducing conditions, separated by SDS-PAGE and transferred to nitrocellulose before probing with the MB20-1 monoclonal antibody. **Figure 3C**, CD20 phosphorylation in primary B cells and B cell lines incubated with and without PMA. A20 cells (2×10^7), LPS-activated mouse splenic B cells (2×10^7) and Raji cells (1×10^7) cultured in phosphate-free media were incubated in media containing $^{32}\text{PO}_4$ for 90 minutes. Half of each culture was incubated with PMA (200 ng/mL) for 30 minutes before detergent lysis of the cells.

Figures 4A-4D shows altered Ca^{2+} responses in CD20^{-/-} B cells. Ca^{2+} responses induced by IgM (**Figure 4A**), CD19 ligation (**Figure 4B**), or thapsigargin (**Figure 4C**) in indo-1-loaded B cells from CD20^{-/-} and wild-type littermates. At 1 minute (arrow), optimal concentrations of goat anti-IgM F(ab')_2 antibody fragments, anti-CD19 monoclonal antibody or thapsigargin were added, with or without EGTA present. Increased ratios of indo-1 fluorescence indicate increased $[\text{Ca}^{2+}]_i$. Results are representative of those from at least six experiments. **Figure 4D**, CD19 expression by splenocytes from CD20^{-/-} (thin line) and wild-type (thick line) littermates was assessed by immunofluorescence staining using phycoerythrin-conjugated anti-CD19 monoclonal antibody with flow cytometry analysis. The dashed line represents staining of wild-type splenocytes with a control monoclonal antibody.

Figures 5A-5B shows protein tyrosine phosphorylation in purified splenic B cells of CD20^{-/-} and wild-type littermates. **Figure 5A**, B cells (2 x 10⁷/sample) were incubated with F(ab')₂ anti-IgM antibody fragments for the times shown and detergent lysed. Proteins were resolved by SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-phosphotyrosine (anti-pTyr) antibody. The blot was stripped and reprobed with anti-SHP-1 antibody as a control for equivalent protein loading. **Figure 5B**, Tyrosine phosphorylation of signaling molecules by CD20^{-/-} B cells. Purified splenic B cells from wild-type and CD20^{-/-} littermates were stimulated with F(ab')₂ anti-mouse IgM antibody (40 µg/mL) for the indicated times. Detergent lysates of cells were utilized for Western blot analysis with anti-phosphotyrosine antibodies to assess protein phosphorylation. The blots were subsequently stripped and reprobed with anti-ERK2 antibody to confirm equivalent protein loading between samples. The migration of molecular weight markers (kDa) is shown for each panel. All results represent those obtained in at least three separate experiments.

Figures 6A-6C shows reactivity of anti-CD20 monoclonal antibodies with mouse spleen B cells. **Figure 6A**, Fluorescence intensity of CD19⁺ cells stained with representative anti-CD20 (solid lines) or isotype-matched control (dashed line) monoclonal antibodies (10 µg/mL). **Figure 6B**, Mean fluorescence intensity (MFI) of anti-CD20 monoclonal antibody staining over a range of monoclonal antibody concentrations. Arrows indicate mean intensities of monoclonal antibody staining when used at 0.5 µg/mL. **Figure 6C**, Fluorescence intensity of CD19⁺ cells stained with anti-CD20 (solid lines) or isotype-matched control (dashed line) monoclonal antibodies (0.5 µg/mL). In all cases, monoclonal antibody staining was visualized using PE-conjugated isotype-specific secondary antibodies with flow cytometry analysis. Results represent those obtained in ≥3 experiments.

Figures 7A-7D show B cell depletion *in vivo*. **Figure 7A**, Representative B cell depletion from blood (day 2) and spleen (day 7)

following MB20-11 or isotype-matched control monoclonal antibody treatment of wild-type or CD20^{-/-} mice as determined by immunofluorescent staining with flow cytometry analysis. Numbers indicate the percentage of gated B220⁺ B cells. **Figure 7B**, Total numbers ($\pm$ SEM) of blood (day 2 or 7, per mL) and spleen (day 7) B cells following treatment of ≥ 2 wild-type littermates with MB20 or isotype-control monoclonal antibodies. Significant differences between mean results for MB20 or isotype-control monoclonal antibody treated mice are indicated; * $p < 0.05$, ** $p < 0.01$. **Figure 7C**, Blood and spleen B cell numbers ($\pm$ SEM) in wild-type littermates 7 days after treatment with MB20-11 monoclonal antibody at different doses (≥ 2 mice per data point). Significant differences between untreated (0) and monoclonal antibody-treated mice are indicated; ** $p < 0.01$. **Figure 7D**, Blood and spleen B cell numbers ($\pm$ SEM) in wild-type mice after MB20-11 (closed circles) or isotype-control (open circles) monoclonal antibody treatment on day 0 (≥ 5 mice per group). The value shown after time 0 represents data obtained at 1 hour.

Figures 8A-8E show B cell depletion is Fc γ R-dependent. **Figure 8A**, Blood B cell depletion after MB20-11 (closed circles) or isotype-control (open circles) monoclonal antibody treatment of FcR γ ^{-/-}, Fc γ RI^{-/-}, Fc γ RII^{-/-} and Fc γ RIII^{-/-} mice on day 0. Values indicate mean circulating B cell numbers ($\pm$ SEM, per mL) before (time 0) and 1 hour or 2, 4 or 7 days after monoclonal antibody treatment (≥ 5 mice per time point). **Figure 8B**, Representative spleen B cell depletion 7 days following monoclonal antibody treatment. Numbers indicate the percentage of B220⁺ lymphocytes within the indicated gates. **Figure 8C**, Mean spleen B cell numbers ($\pm$ SEM) 7 days after MB20-11 (closed bars) or isotype-control (open bars) monoclonal antibody treatment (≥ 5 mice per group). Numbers indicate the mean relative percentage of B220⁺ lymphocytes in anti-CD20 monoclonal antibody treated mice compared with control monoclonal antibody treated littermates. **Figure 8D**, B cell depletion after MB20-1 (closed circles) or isotype-control (open circles) monoclonal antibody treatment of FcR γ ^{-/-} littermates on day 0 compared with MB20-1 (closed squares) or isotype-control (open squares) monoclonal antibody treatment of wild-type littermates on day 0. Representative spleen B cell depletion 7 days after MB20-1 or control monoclonal antibody treatment of

- FcR $\gamma^{-/-}$ littermates. Numbers indicate the percentage of B220⁺ lymphocytes. Bar graphs represent mean spleen B cell numbers ($\pm$ SEM) 7 days after MB20-1 or isotype-control monoclonal antibody treatment of FcR $\gamma^{-/-}$ (filled bars) or wild-type (open bars) mice (≥ 5 mice per group). **Figure 8E**, Blood and spleen (day 7) B cell depletion after MB20-18 (closed circles) or isotype-control (open circles) monoclonal antibody treatment of FcR $\gamma^{-/-}$ littermates on day 0 compared with MB20-18 (closed squares) or isotype-control (open squares) monoclonal antibody treatment of wild-type littermates on day 0. Histograms represent mean spleen B cell numbers ($\pm$ SEM) 7 days after MB20-18 or isotype-control monoclonal antibody treatment of FcR $\gamma^{-/-}$ (filled bars) or wild-type (open bars) mice (≥ 5 mice per group). **Figure 8A-E**, Significant differences between mean results for MB20 or isotype-control monoclonal antibody treated mice are indicated; * $p < 0.05$, ** $p < 0.01$.
- Figures 9A-9D** show that B cell depletion *in vivo* is C-independent. **Figure 9A**, *In vitro* C-dependent cytotoxicity of MB20 monoclonal antibodies for spleen B cells. Values represent the mean ($\pm$ SEM) percentage of B220⁺ cells that were propidium iodide positive (PI⁺) in ≥ 3 experiments. **Figure 9B**, B cell depletion after MB20-11 (closed circles) or isotype-control (open circles) monoclonal antibody treatment of C3 $^{-/-}$, C4 $^{-/-}$ or C1q $^{-/-}$ mice on day 0. Blood values indicate mean circulating B cell numbers ($\pm$ SEM, per mL) before (time 0) and 1 hour or 2, 4 or 7 days after monoclonal antibody treatment (≥ 5 mice per time point). Representative spleen B cell frequencies and mean B cell numbers ($\pm$ SEM) 7 days after MB20-11 (closed bars) and isotype-control (open bars) monoclonal antibody treatment (≥ 5 mice per group). **Figure 9C-D**, Blood and spleen B cell depletion after MB20-1 or MB20-18 (closed circles) or isotype-control (open circles) monoclonal antibody treatment of C3 $^{-/-}$ mice on day 0 compared with MB20-1 or MB20-18 (closed squares) or isotype-control (open squares) monoclonal antibody treatment of wild-type mice on day 0. Representative spleen B cell depletion 7 days following MB20-1 or control monoclonal antibody treatment of C3 $^{-/-}$ littermates. Numbers indicate the percentage of B220⁺ lymphocytes within the indicated gates. Bar graphs represent mean spleen B cell numbers ($\pm$ SEM) 7 days after MB20-1 or isotype-control monoclonal antibody treatment of C3 $^{-/-}$ (filled bars) or wild-type

(open bars) mice (≥ 5 mice per group). **Figure 9A-D**, Significant differences between mean results for MB20 or isotype-control monoclonal antibody treated mice are indicated; * $p < 0.05$, ** $p < 0.01$.

5 **Figures 10A-10B** show that monocytes mediate B cell depletion. Wild-type mice were treated with clodronate (CLOD) as shown (arrows) to deplete macrophages, while other mice had genetic deficiencies in leukocyte subpopulations. **Figure 10A**, Blood B cell depletion after MB20-11 (closed circles) or isotype-control (open circles) monoclonal antibody treatment on
10 day 0. For clodronate-treated mice, blood B cell numbers were determined 1 hour and 2, 4 and 7 days following monoclonal antibody treatment, with the vertical dashed line indicating time 0 monoclonal antibody treatment. For CSF1^{op} mice, circulating B cell numbers were not quantified 1 hour following monoclonal antibody treatment because of the small size of these mice and
15 the risk for mortality. B cell numbers at 1 hour time points are shown for the other mouse genotypes. **Figure 10B**, Representative flow cytometry analysis and mean spleen B cell numbers ($\pm$ SEM) 7 days after MB20-11 (closed bars) or isotype-control (open bars) monoclonal antibody treatment (≥ 5 mice per group). Significant differences between mean results from isotype-control or
20 MB20 monoclonal antibody treated cells are indicated; * $p < 0.05$, ** $p < 0.01$.

Figures 11A and 11B show the amino acid sequence alignment of known mouse anti-human CD20 monoclonal antibodies (**Table 1**). **Figure 11A**, Heavy chain amino acid numbering and designation of the origins of the
25 coding sequence for each monoclonal antibody V, D and J region is according to the conventional methods (Kabat, et al. (1991) Sequences of Proteins of Immunological Interest. U. S. Government Printing Office, Bethesda, MD) where amino acid positions 1-94 and complementarity-determining regions CDR1 and 2 are encoded by a V_H gene. A dash indicates a gap inserted in
30 the sequence to maximize alignment of similar amino acid sequences. Gaps in the sequences were introduced between V_H, D and J segments for clarity. **Figure 11B**, Light chain V_k amino acid sequence analysis of anti-CD20 monoclonal antibodies. Amino acid numbering and designation of the origins of the coding sequence for each monoclonal antibody is according to the

conventional methods (Kabat, et al. (1991) Sequences of Proteins of Immunological Interest. U. S. Government Printing Office, Bethesda, MD). The amino acid following the predicted signal sequence cleavage site is numbered 1. A dash indicates a gap inserted in the sequence to maximize
5 alignment of similar amino acid sequences. Gaps in the sequences were introduced between Vk and J segment sequences for clarity.

Figure 12 depicts UPGMA (unweighted pair group method using arithmetic averages) trees of deduced monoclonal antibody heavy and light
10 chain sequences for known mouse anti-human CD20 monoclonal antibodies shown in **Figure 11**. For comparative purposes, three mouse anti-human CD20 monoclonal antibodies, HB20-03, -04 and -25 monoclonal antibodies are shown. Relative horizontal tree branch length is a measure of sequence relatedness. For example, the heavy and light chains of the known mouse
15 anti-human CD20 monoclonal antibodies are more similar to each other than they are to the sequences of the HB20-03, -04 and -25 monoclonal antibodies, which are most similar to each other. In the third panel, the heavy and light chain sequences were joined to form a contiguous H+L chain sequence prior to sequence analysis. This analysis shows that the
20 combination of heavy and light chains are not related between the known anti-CD20 monoclonal antibodies and the HB20-3, -4 and -25 monoclonal antibodies. The UPGMA tree was generated using Geneworks version 2.0 (IntelliGenetics, Inc., Mountain View, CA).

Figure 13 shows amino acid sequence comparisons of deduced monoclonal antibody heavy chain V(D)J sequences for known mouse anti-human CD20 monoclonal antibodies shown in **Figure 11** and the HB20 and MB20 series of monoclonal antibodies reactive with human and mouse CD20
25 (**Table 1**). Data are shown as an UPGMA tree of deduced monoclonal antibody heavy chain sequences. Relative horizontal tree branch length is a measure of sequence relatedness. Heavy chains were grouped (A-G) based on sequence similarities as indicated on the right.
30

Figures 14A-14N show the nucleotide and predicted amino acid sequences for heavy chain V_H -D- J_H junctional sequences of the HB20 and MB20 series of monoclonal antibodies reactive with human and mouse CD20 (**Table 1**). **Figure 14A**, amino acid (SEQ ID NO:1) and nucleotide (SEQ ID NO:2) sequences for HB20-01, HB20-02 and HB20-06; **Figure 14B**, amino acid (SEQ ID NO:3) and nucleotide (SEQ ID NO:4) sequences for HB20-03; **Figure 14C**, amino acid (SEQ ID NO:5) and nucleotide (SEQ ID NO:6) sequences for HB20-04; **Figure 14D**, amino acid (SEQ ID NO:7) and nucleotide (SEQ ID NO:8) sequences for HB20-05; **Figure 14E**, amino acid (SEQ ID NO:9) and nucleotide (SEQ ID NO:10) sequences for HB20-25; **Figure 14F**, amino acid (SEQ ID NO:11) and nucleotide (SEQ ID NO:12) sequences for MB20-01 and MB20-13; **Figure 14G**, amino acid (SEQ ID NO:13) and nucleotide (SEQ ID NO:14) sequences for MB20-02; **Figure 14H**, amino acid (SEQ ID NO:15) and nucleotide (SEQ ID NO:16) sequences for MB20-07; **Figure 14I**, amino acid (SEQ ID NO:17) and nucleotide (SEQ ID NO:18) sequences for MB20-08; **Figure 14J**, amino acid (SEQ ID NO:19) and nucleotide (SEQ ID NO:20) sequences for MB20-10; **Figure 14K**, amino acid (SEQ ID NO:21) and nucleotide (SEQ ID NO:22) sequences for MB20-11; **Figure 14L**, amino acid (SEQ ID NO:23) and nucleotide (SEQ ID NO:24) sequences for MB20-14; **Figure 14M**, amino acid (SEQ ID NO:25) and nucleotide (SEQ ID NO:26) sequences for MB20-16; and **Figure 14N**, amino acid (SEQ ID NO:27) and nucleotide (SEQ ID NO:28) sequences for MB20-18. Sequences that overlap with the 5' PCR primers are indicated by double underlining and may vary from the actual DNA sequence since redundant primers were used to amplify each sequence (**Table 1**). Approximate junctional borders between V, D and J sequences are designated in the sequences by vertical bars (|). Deduced sequences homologous to known D region DNA sequences are single underlined. Lower case nucleotides indicate either nucleotide additions at junctional borders or potential sites for somatic hypermutation.

Figure 15 shows the amino acid sequence alignment for heavy chain V_H -D- J_H junctional sequences of known mouse anti-human CD20 monoclonal

antibodies and the HB20 and MB20 series of monoclonal antibodies reactive with human and mouse CD20. Each monoclonal antibody is grouped relative to its homology with other monoclonal antibody sequences. The relative rank order of sequences shown was based on relatedness to the 2B8 (Rituximab) monoclonal antibody sequence. Heavy chain amino acid numbering and designation of the origins of the coding sequence for each monoclonal antibody V, D and J region is according to the conventional methods (Kabat, et al. (1991) Sequences of Proteins of Immunological Interest. U. S. Government Printing Office, Bethesda, MD) where amino acid positions 1-94 and CDR1 and 2 are encoded by a V_H gene. A dot indicates identity between each monoclonal antibody and the consensus amino acid sequence for all monoclonal antibodies. A dash indicates a gap inserted in the sequence to maximize alignment of similar amino acid sequences. Gaps in the sequences were introduced between V_H and D segments for clarity. CDR regions are boxed for clarity.

Figure 16 shows amino acid sequence comparisons of deduced monoclonal antibody light chain VJ sequences for known mouse anti-human CD20 monoclonal antibodies shown in **Figure 11** and the HB20 and MB20 series of monoclonal antibodies reactive with human and mouse CD20. Data are shown as an UPGMA tree of deduced monoclonal antibody light chain V and J sequences. Relative horizontal tree branch length is a measure of sequence relatedness. Light chains were grouped (A-G) based on sequence similarities as indicated on the right.

25

Figures 17A-17N show nucleotide and predicted amino acid sequences for light chain V-J sequences of the HB20 and MB20 series of monoclonal antibodies reactive with human and mouse CD20 (**Table 1**). Sequences that overlap with the 5' PCR primers are indicated by double underlining and may vary from the actual DNA sequence since redundant primers were used to amplify each sequence (**Table 1**). **Figure 17A**, amino acid (SEQ ID NO:29) and nucleotide (SEQ ID NO:30) sequences for HB20-01, HB20-02 and HB20-06; **Figure 17B**, amino acid (SEQ ID NO:31) and nucleotide (SEQ ID NO:32) sequences for HB20-03; **Figure 17C**, amino acid

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(SEQ ID NO:33) and nucleotide (SEQ ID NO:34) sequences for HB20-04; **Figure 17D**, amino acid (SEQ ID NO:35) and nucleotide (SEQ ID NO:36) sequences for HB20-05; and **Figure 17E**, amino acid (SEQ ID NO:37) and nucleotide (SEQ ID NO:38) sequences for HB20-25; **Figure 17F**, amino acid
 5 (SEQ ID NO:39) and nucleotide (SEQ ID NO:40) sequences for MB20-01; **Figure 17G**, amino acid (SEQ ID NO:41) and nucleotide (SEQ ID NO:42) sequences for MB20-02; **Figure 17H**, amino acid (SEQ ID NO:43) and nucleotide (SEQ ID NO:44) sequences for MB20-03; **Figure 17I**, amino acid (SEQ ID NO:45) and nucleotide (SEQ ID NO:46) sequences for MB20-07;
 10 **Figure 17J**, amino acid (SEQ ID NO:47) and nucleotide (SEQ ID NO:48) sequences for MB20-08; **Figure 17K**, amino acid (SEQ ID NO:49) and nucleotide (SEQ ID NO:50) sequences for MB20-10; **Figure 17L**, amino acid (SEQ ID NO:51) and nucleotide (SEQ ID NO:52) sequences for MB20-13; **Figure 17M**, amino acid (SEQ ID NO:53) and nucleotide (SEQ ID NO:54)
 15 sequences for MB20-14; and **Figure 17N**, amino acid (SEQ ID NO:55) and nucleotide (SEQ ID NO:56) sequences for MB20-18. Lower case nucleotides indicate either nucleotide additions at junctional borders or potential sites for somatic hypermutation. "N" indicates where a nucleotide in the sequence was ambiguous and the corresponding amino acid was therefore unknown.

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Figure 18 shows an amino acid sequence alignment for light chain VJ sequences of known mouse anti-human CD20 monoclonal antibodies and the HB20 and MB20 (**Table 1**) series of monoclonal antibodies reactive with human and mouse CD20. Each monoclonal antibody is grouped relative to its
 25 homology with other monoclonal antibody sequences. The relative rank order of sequences shown was based on relatedness to the consensus light chain sequence for all anti-CD20 monoclonal antibodies. Light chain amino acid numbering and designation of the origins of the coding sequence for each monoclonal antibody V and J region is according to the conventional methods
 30 (Kabat, et al. (1991) Sequences of Proteins of Immunological Interest. U. S. Government Printing Office, Bethesda, MD). A dot indicates identity between each monoclonal antibody and the consensus amino acid sequence for all monoclonal antibodies. A dash indicates a gap inserted in the sequence to maximize alignment of similar amino acid sequences. Gaps in the sequences

were introduced between V and J segments for clarity. CDR regions are boxed for clarity.

Figure 19 depicts UPGMA analysis of deduced monoclonal antibody heavy and light chain sequences for known mouse anti-human CD20 monoclonal antibodies and the HB20 and MB20 series of monoclonal antibodies reactive with human and mouse CD20. The heavy V(D)J and light (VJ) chain sequences were joined to form a contiguous H+L chain sequence prior to sequence analysis. Heavy and light chain pairs were grouped based on sequence similarities (**Figure 13** and **Figure 16**) between heavy and light chains as indicated on the right.

Figures 20A-B show that the density of anti-CD20 monoclonal antibody binding to the cell surface of B cells regulates the effectiveness of anti-CD20 monoclonal antibody-induced B cell depletion. B cell depletion in heterozygous CD20^{+/-} mice that express 50% of the normal density of cell surface CD20 was examined in comparison with wild-type littermates. Both sets of littermates were treated i.v. with either 10 or 250 µg of MB20-11 monoclonal antibody (filled bars) or isotype-matched control (open bars) monoclonal antibody (n≥3 mice/group) with blood (per mL) (**Figure 20A**) and spleen (total) (**Figure 20B**) B220⁺ B cell numbers quantified on day 7 by flow cytometry. Values represent mean (±SEM) B cell numbers with the percentage of B cells remaining in MB20-11 monoclonal antibody-treated mice relative to control monoclonal antibody-treated littermates shown. Significant differences between mean results for each group of mice are indicated; *p<0.05, **p<0.01. The MB20-11 monoclonal antibody effectively cleared circulating and spleen B cells in CD20^{+/-} and wild-type littermates when used at 250 µg. However, when the MB20-11 monoclonal antibody was used at 10 µg, only a fraction of the B cells were depleted in CD20^{+/-} mice, while the vast majority of B cells were depleted in wild-type littermates.

Figures 21A-21B show that binding of the MB20-11 monoclonal antibody to CD20 increases cell surface CD20 density, **Figure 21A**). Increased MB20-11 monoclonal antibody binding revealed by indirect

immunofluorescence staining of purified mouse spleen B cells that were incubated with either an isotype control (C) or MB20-11 monoclonal antibody (10 µg/mL) for the indicated times before staining with fluorochrome-conjugated goat anti-mouse IgG2a secondary antibody, with subsequent flow
5 cytometry analysis. For the 0 time point, the cells were incubated on ice with monoclonal antibody for 30 minutes before washing and staining with the secondary antibody. **Figure 21B**, Representative time course for MB20-11 monoclonal antibody-binding to cell surface CD20 in comparison with the MB20-18 monoclonal antibody. Each value represents the mean fluorescence
10 channel number for fluorescence staining of purified spleen B cells as described in **Figure 21A**. These results are representative of those obtained in ≥3 independent experiments.

Figures 22A-B show that HB20-3, -4, and -25 monoclonal antibodies
15 bind to cell surface CD20 at a higher density than known anti-CD20 monoclonal antibodies. Reactivity of human blood lymphocytes (**Figure 22A**) and the Raji B lymphoblastoid cell line (**Figure 22B**) with 1F5, HB20-3 and B1 anti-CD20 monoclonal antibodies (solid lines) or secondary antibody alone (dashed line) is shown. The anti-CD20 monoclonal antibodies were used at
20 concentrations that were predetermined to be saturating and to give optimal staining: 1F5 as ascites fluid diluted 1:200; HB20-3 as tissue culture supernatant fluid from the HB20-3 hybridoma; or B1 monoclonal antibody at either 10 µg/mL of purified monoclonal antibody or as tissue culture supernatant fluid. In all cases, monoclonal antibody staining was visualized
25 using PE-conjugated isotype-specific secondary antibodies with flow cytometry analysis. Results represent those obtained in ≥3 experiments.

Figures 23A-23B shows that i.v. (**Figure 23A**) or subcutaneous (s.c.; **Figure 23B**) and administration of the MB20-11 monoclonal antibody
30 effectively depletes circulating and tissue B cells *in vivo*. Wild-type mice were treated either s.c. or i.v. with the MB20-11 monoclonal antibody at the indicated doses. Values represent mean (±SEM) blood (per mL) or spleen (total) B220⁺ B cell numbers on day 7 (n≥2) as assessed by flow cytometry.

Significant differences between mean results for each group of mice are indicated; * $p < 0.05$, ** $p < 0.01$.

DETAILED DESCRIPTION OF THE INVENTION

5 The present invention is based in part on the production of a panel of monoclonal antibodies (mAbs) that specifically bind to human CD20 that have distinct binding properties and other characteristics as compared with conventional anti-CD20 mAbs (e.g., 1F5 or 2B8). In particular, the mAbs and antigen-binding fragments of the invention can be distinguished from
10 conventional anti-CD20 antibodies on a molecular level, for example, by the nucleotide and amino acid sequence of the light and/or heavy chain variable regions or particular segments of the variable regions such as the complementarity determining regions ("CDRs").

 In particular embodiments, the mAbs and antigen-binding fragments of
15 the invention can bind to B cells at a higher density than conventional mAbs, which property is advantageous for methods of depleting B cells, for therapeutic or diagnostic methods, or for use as a laboratory reagent (for example, to identify B cells or to purify B cells).

 The invention also provides mAbs and antigen binding fragments
20 thereof that specifically bind to mouse CD20.

 Further provided are anti-CD20 mAbs that are generated from antibody producing cells (e.g., B cells) isolated from a CD20^{-/-} mammal (e.g., a mouse).

 The present invention will now be described with reference to the accompanying drawings, in which preferred embodiments of the invention are
25 shown. This invention can be embodied in different forms and should not be construed as limited to the embodiments set forth herein. Rather, these embodiments are provided so that this disclosure will be thorough and complete, and will fully convey the scope of the invention to those skilled in the art. For example, features illustrated with respect to one embodiment can
30 be incorporated into other embodiments, and features illustrated with respect to a particular embodiment can be deleted from that embodiment. In addition, numerous variations and additions to the embodiments suggested herein will

be apparent to those skilled in the art in light of the instant disclosure, which do not depart from the instant invention.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. The terminology used in the description of the invention herein is for the purpose of describing particular embodiments only and is not intended to be limiting of the invention.

As used in the description of the invention and the appended claims, the singular forms "a," "an" and "the" are intended to include the plural forms as well, unless the context clearly indicates otherwise.

Except as otherwise indicated, standard methods can be used for the production of antibodies or antigen-binding fragments thereof, manipulation of nucleic acid sequences, production of transformed cells, and the like according to the present invention. Such techniques are known to those skilled in the art. See, e.g., SAMBROOK *et al.*, MOLECULAR CLONING: A LABORATORY MANUAL 2nd Ed. (Cold Spring Harbor, NY, 1989); F. M. AUSUBEL *et al.* CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (Green Publishing Associates, Inc. and John Wiley & Sons, Inc., New York).

Anti-CD20 mAbs, Antigen-Binding Fragments and Cell Lines.

As one aspect, the invention provides mAbs and antigen-binding fragments thereof that specifically bind to CD20. As used herein the terms "mAb that specifically binds to CD20" and "anti-CD20 mAb" and similar language are interchangeable. In particular embodiments, the mAb or antigen-binding fragment specifically binds to human CD20 and/or mouse CD20. The mAb or antigen-binding fragment can bind to any region of the CD20 protein, but in representative embodiments, binds to an extracellular region of CD20.

The term "antibody" or "antibody molecule" in the various grammatical forms as used herein refers to an immunoglobulin molecule (including IgG, IgE, IgA, IgM, IgD) and/or immunologically active portions of immunoglobulin molecules, *i.e.*, molecules that contain an antibody combining site or paratope

WO 2005/000901

PCT/US2004/014326

and can bind antigen. An "antibody combining site" or "antigen binding site" is that structural portion of an antibody molecule comprised of heavy and light chain variable and hypervariable (CDR) regions that specifically binds antigen. As is known in the art, particular properties of antibodies relate to immunoglobulin isotype. In representative embodiments, the antibody or antigen-binding fragment is an IgG2a, an IgG1 or an IgG2b isotype molecule. The antibody or fragment can further be from any species of origin including avian (e.g., chicken, turkey, duck, geese, quail, etc.) and mammalian (e.g., human, non-human primate, mouse, rat, rabbit, cattle, goat, sheep, horse, pig, dog, cat, etc.) species.

The term "monoclonal antibody" or "mAb" as used herein refers to an antibody obtained from a population of substantially homogenous antibodies, i.e., the individual antibodies comprising the population are identical except for the possibility of naturally occurring mutations that may be present in minor amounts. mAbs are highly specific and are directed against a single antigenic determinant (i.e., epitope) on the antigen. This characteristic contrasts with polyclonal antibody preparations, which typically include antibodies directed against different antigenic determinants.

The terms "antibody" and "mAb" are used here in the broadest sense and specifically covers multispecific antibodies (e.g., bi-specific antibodies), naked antibodies, antibody conjugates, and antibody fragments as long as they exhibit the desired biological activity. Further, the terms "antibody" and "mAb" encompass intact (i.e., complete) immunoglobulin molecules or an antigen-binding fragment of an antibody that contains the paratope, including Fab, Fab', F(ab')₂ and Fv fragments, diabodies, linear antibodies, single-chain antibody molecules, and multispecific antibodies formed from antibody fragments.

Single-chain Fv or "sFv" antibody fragments comprise the antibody heavy and light chain variable domains, where these domains are present in a single polypeptide chain. Generally, the Fv polypeptide further comprises a polypeptide linker between the heavy chain variable and light chain variable regions, which enables the sFv to form the desired structure for antigen binding. For a review of sFv, see Pluckthun, *The Pharmacology of Monoclonal Antibodies*, vol. 133, Rosenberg and Moore eds. Springer-Verlag,

New York, pp. 269-315 (1994). The production of single chain antibodies has been described in the art, see e.g., U.S. Pat. No. 5,260, 203. In one exemplary method of producing a single chain antibody, combinatorial immunoglobulin phagemid libraries are prepared from RNA isolated from the spleen of an immunized animal, and phagemids expressing appropriate antibodies are selected by panning on endothelial tissue. The advantages of this approach over conventional hybridoma techniques are that approximately 10^4 times as many antibodies can be produced and screened in a single round, and that new specificities are generated by H and L chain combination in a single chain, which further increases the chance of finding appropriate antibodies.

The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy chain variable domain connected to a light chain variable domain in the same polypeptide chain. By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are known in the art, see e.g., EP 404,097; WO 93/11161; and Hollinger et al., *Proc. Natl. Acad. Sci* 90:6444-6448 (1993).

The expression "linear antibodies" as used herein refers to antibodies comprising a pair of tandem Fd segments ($V_H-C_H1-V_H-C_H1$) that form a pair of antigen binding sites. Linear antibodies can be bispecific or monospecific and are described in more detail in Zapata et al., *Protein Eng.* 8:1057-1062 (1995).

Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, e.g., Morimoto et al., *J. Biochem. Biophys. Methods* 24:107-117 (1992) and Brennan et al., *Science* 229:81 (1985)). However, these fragments can now be produced directly by recombinant nucleic acid technology in transformed host cells. For example, Fab'-SH fragments can be directly recovered from *E. coli* and chemically coupled to form $F(ab')_2$ fragments (Carter et al., *Bio/Technology* 10:163-167 (1992)). Alternatively, the $F(ab')_2$ is formed using the leucine zipper GCN4 to promote assembly of the $F(ab')_2$ molecule. According to another approach, Fv, Fab or

F(ab')₂ fragments can be isolated directly from recombinant host cell culture. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner.

Exemplary mAbs of the present invention include HB20-1, HB20-2,
5 HB20-3, HB20-4, HB20-5, HB20-6, HB20-25, MB20-1, MB20-2, MB20-3, MB20-6, MB20-7, MB20-8, MB20-10, MB20-11, MB20-13, MB20-14, MB20-16 and MB20-18 as disclosed herein. HB20-1, HB20-2, HB20-3, HB20-4, HB20-5 and HB20-6 have previously been designated as HB13a, HB13b, HB13c, HB13d, HB13e and HB13f, respectively.

10 The invention further encompasses functional equivalents of the mAbs and antigen-binding fragments specifically disclosed herein that have substantially similar nucleic acid and/or amino acid sequences of the heavy chain, light chain, heavy chain variable region, light chain variable region and/or CDR1, CDR2 and/or CDR3 regions (as described in more detail below) as
15 compared with the corresponding chain or region of an antibody specifically described herein and specifically bind to CD20, and optionally exhibit one or more of the other functional properties of the antibodies and antibody fragments specifically described herein (e.g., density of binding, efficacy of B cell depletion). In one illustrative embodiment, the mAbs and antigen-binding
20 fragments of the invention bind to the same antigenic determinant (*i.e.*, epitope) as the mAbs and antigen-binding fragments specifically described herein.

It is routine for those skilled in the art to determine, without undue experimentation, whether an antibody has the specificity of a mAb disclosed herein by epitope mapping. For example, the nucleic acid and/or amino acid
25 sequence can be determined of one or more of the heavy and/or light chain CDR region(s) or the heavy and/or light chain variable region(s) of the antibodies in question. Antibody molecules having identical, or functionally equivalent, amino acid residue sequences in these regions have the same or similar binding specificity. Methods of assessing and comparing the similarity
30 of the variable and CDR regions to determine functional equivalency are known to those skilled in the art.

Another method of determining whether a monoclonal antibody has the same specificity as an antibody described herein is by comparison of the antibody paratope three-dimensional structures as predicted by computer

modeling based on amino acid sequence. An epitope-antibody paratope interaction typically involves four forces: van der Waal's forces (dipole-dipole interactions), hydrogen bonds, hydrophobic interactions, and ionic (coulombic) bonding. Non-covalent binding stabilizes the antibody-antigen complex and holds it together. The interaction is determined by the 3D structure of both molecules. Therefore, a prediction of the 3D structure of the antibody paratope, epitope, and/or epitope-antibody paratope complex permits immunospecificity comparison to other antibodies.

Alternatively, or in addition, epitope mapping can be performed by using a technique based on fragmentation of the antigen to which the antibody binds, either randomly or by specific genetic construction, and determining the reactivity of the fragments obtained with the antibody. Fragmentation can also be performed on the nucleic acid level, for example by PCR technique, followed by transcription and translation into protein *in vitro* in the presence of radioactive amino acids. For further details see, for example, Harlow and Lane, Using Antibodies, a Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1999, pp. 390-392.

According to a further method of epitope mapping, a set of overlapping peptides is synthesized, each corresponding to a small linear segment of the protein antigen, and arrayed on a solid phase. The panel of peptides is then probed with the test antibody, and bound antibody is detected using an enzyme-labeled secondary antibody. (Harlow and Lane, *supra*, pp. 393-396.)

An additional method well known in the art for epitope mapping is antibody selection from a random synthetic or a phage display peptide library. For example, phage display libraries can be constructed by cloning complex mixtures of peptide-encoding oligonucleotides into the amino terminus of the minor coat protein gene of the f1-type ssDNA phage. Such phage display libraries are commercially available, for example, from New England Biolabs. The libraries can be amplified as stocks, and then an aliquot sufficient to represent multiple copies of each independent clone is mixed with the antibody of interest. Antibody-bound phage are collected by a procedure called "biopanning," and unbound phage are removed. The bound phage are eluted and used to infect bacteria, and the selected stock is amplified.

Individual plaques of the final selected stock are grown and checked for specific antibody reactivity, e.g. by ELISA, and the DNA around the insert site is sequenced. Analysis of the sequence encoding the peptide to which the antibody binds defines the specificity of the antibody. For further details see, 5 e.g., Smith and Scott, *Methods Enzymol.* 217:228-257 (1993), and Harlow and Lane, *supra*, pp. 397-398.

Another, albeit less reliable, way to determine if a mAb has the same specificity as a mAb described herein is by ascertaining whether the former prevents the latter from binding to the target molecule (e.g., CD20). If the 10 mAb being tested competes with a mAb as described herein, as shown by a decrease in binding by the mAb in standard competition assays for binding to the target molecule, then it is possible that the two mAbs bind to the same, or a closely related, epitope. However, this is not a definitive test. The actual epitopes to which the tested mAb and the mAbs disclosed herein bind may 15 still be different, even though the tested antibody is capable of decreasing binding to the target molecule by an antibody disclosed herein. For example, binding by the test mAb to its antigenic determinant can mask the antigenic determinant of a mAb antibody described herein and prevent its binding simply due to the physical bulk of the test mAb, rather than by binding the 20 same epitope. Therefore, more precise procedures (e.g., amino acid sequencing of the variable region and 3D modeling) are often employed in conjunction with competition methods to confirm specificity.

Still another way to determine whether a mAb might have the specificity of a mAb described herein is to pre-incubate a mAb disclosed 25 herein with the target molecule (e.g., CD20), and then add the mAb being tested to determine if the mAb being tested is inhibited in its ability to bind the target. If the mAb being tested is inhibited then it is possible that it has the same, or functionally equivalent, epitope specificity as the mAb disclosed herein. However, this procedure is subject to the same limitations as the 30 competition studies discussed above, and as such, is not necessarily determinative of identical specificity.

In particular embodiments of the invention, the mAb or antigen-binding fragment thereof specifically binds to CD20, wherein the density of binding of the mAb or antigen-binding fragment to CD20 and/or B cells is at least about 30%,

40%, 50%, 60%, 75%, 85%, two-fold, three-fold, four-fold or even five-fold or greater than binding of conventional anti-CD20 mAbs (e.g., 2H7, B9E9, 1H4, 2B8, 1F5 and/or Leu-16 antibodies) to CD20 and/or B cells. In addition, the mAbs of the invention can be more therapeutically effecting in depleting malignant B cells that express CD20 at lower densities. These conventional antibodies are available to those skilled in the art (see, e.g., Shan, et al. (1999) *J. Immunol.* 162:6589-6595; Schultz, et al. (2000) *Cancer Res.* 60:6663-6669; and Haisma, et al. (1998) *Blood* 92:184-190; Stashenko, et al. (1980) *J. Immunol.* 125:1678). While not wishing to be limited by any particular theory of the invention, the density of antibody binding can be attributable to the accessibility or availability of the epitopes bound by the antibody. Thus, according to this embodiment, it appears that the antibodies and antigen-binding fragments of the invention are directed against epitopes that have increased accessibility on the cell surface as compared with one or more of the conventional antibodies described above. The antibodies and antigen-binding fragments according to this embodiment of the invention are advantageous for therapeutic applications because they can induce B cell depletion at lower dosages than conventional antibodies. Those skilled in the art will appreciate that degree of enhancement in the density of binding as compared with conventional antibodies can vary according to the target, e.g., the cell line used. In one illustrative embodiment, the mAb or antigen-binding fragment upregulates the binding sites (i.e., accessibility of the epitope) on B cells, which results in a higher density of binding of the mAb or antigen-binding fragment to the B cells and/or their malignant counterparts.

Methods of determining the density of antibody binding to cells are known to those skilled in the art (see, e.g., Sato et al., *J. Immunology* 165:6635-6643 (2000); which discloses a method of assessing cell surface density of CD19). Other standard methods include Scatchard analysis. For example, the antibody or fragment can be isolated, radiolabeled, and the specific activity of the radiolabeled antibody determined. The antibody is then contacted with a target cell expressing CD20. The radioactivity associated with the cell can be measured and, based on the specific activity, the amount of antibody or antibody fragment bound to the cell determined.

Alternatively, fluorescence activated cell sorting (FACS) analysis can be employed. Generally, the antibody or antibody fragment is bound to a target cell expressing CD20. A second reagent that binds to the antibody is then added, for example, a flouochrome labeled anti-immunoglobulin antibody.

- 5 Flouochrome staining can then be measured and used to determine the density of antibody or antibody fragment binding to the cell.

As another suitable method, the antibody or antibody fragment can be directly labeled with a detectable label, such as a fluorophore, and bound to a target cell. The ratio of label to protein is determined and compared with
10 standard beads with known amounts of label bound thereto. Comparison of the amount of label bound to the cell with the known standards can be used to calculate the amount of antibody bound to the cell.

In other embodiments of the invention, the functionally equivalent antibody or fragment has the same or a similar efficacy for depleting B cells and/or treating a B cell disorder as an antibody or fragment described herein.
15 This aspect of the invention is described in more detail below. To illustrate, in representative embodiments, a functionally equivalent antibody or fragment achieves at least about a 25%, 35%, 50%, 75%, 85%, 90%, 95% or 98% or more depletion in circulating and/or tissue B cells for at least about 5, 7, 14, 21,
20 30, 45, 60, 120 or 180 days or longer at a dosage of about 125 mg/m², 75 mg/m², 37.5 mg/m², 10 mg/m², 3.75 mg/m², 1 mg/m², 0.75 mg/m², 0.375 mg/m², 0.1 mg/m², 0.05 mg/m², 0.001 mg/m², 0.0005 mg/m² or less. Other particular dosages, degree of depletion, and depletion times are described in more detail below.

25 In representative embodiments of the invention, a mAb or antigen-binding fragment of the invention comprises a heavy chain or light chain of a mAb as described herein. In other exemplary embodiments, the mAb or antigen-binding fragment of the invention comprises a heavy chain variable region and/or a light chain variable region from a mAb as described herein. In still other
30 embodiments, the mAb or antigen-binding fragment comprises a heavy chain V and/or D and/or J region and/or a light chain V and/or J region from a mAb disclosed herein. In still other representative embodiments, the mAb or antigen-binding fragment comprises a heavy chain CDR1 and/or CDR2 and/or CDR3 region and/or a light chain CDR1 and/or CDR2 and/or CDR3 region of a mAb

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described herein. According to this embodiment, the mAb or antigen-binding fragment can comprise the CDR1, CDR2 and CDR3 regions (heavy and light chain) from a mAb as described herein.

In particular embodiments, the anti-human CD20 antibodies or antigen binding fragments thereof specifically bind to CD20 and have a heavy chain CDR3 region comprising the amino acid sequence of FYXYXXX¹YGAX²XXY, wherein X can be any amino acid, and wherein X¹ can be any amino acid and is preferably a Y or an S, and wherein X² can be any amino acid and is preferably an M or an L and wherein F is a Phenylalanine, Y is a Tyrosine, G is a Glycine, A is an Alanine, M is a Methionine, L is a Leucine and S is a Serine. The CDRs are defined as shown in Figures 15 and 18.

In certain embodiments, the anti-human CD20 antibodies or antigen binding fragments thereof further comprise a heavy chain CDR1 region comprising the amino acid sequence NXXXX wherein X can be any amino acid and N is Asparagine.

In another embodiment, the anti-human anti-CD20 antibodies or antigen binding fragments thereof further comprise a light chain CDR3 region comprising the amino sequence of XHFWXX³XWX, wherein X can be any amino acid sequence, H is a Histidine, F is a Phenylalanine, W is a Tryptophan and X³ can be any amino acid and is preferably a T or an I, wherein T is Threonine and I is Isoleucine.

Further, the mAbs and antigen-binding fragments of the invention encompass those that have substantial sequence similarity, for example, at least about 70%, 75%, 80%, 85%, 90%, 95%, 97% or more amino acid sequence similarity with the amino acid sequences specified above (*e.g.*, the heavy or light chain, heavy and/or light chain variable region, V, D, and/or J regions or CDR(s)). Alternatively, the nucleic acids encoding these regions have at least about 70%, 75%, 80%, 85%, 90%, 95%, 97% or more nucleotide sequence similarity with the nucleotide sequences of the corresponding regions of the antibodies described herein.

Those skilled in the art will appreciate that certain modifications can be made to the amino acid and nucleic acid sequences disclosed herein within the scope of the invention. For example, the sequences can be modified as a result of cloning or amplification procedures or other laboratory manipulations of the

nucleic acid or protein molecules, to provide an enhanced affinity and/or density of binding to CD20 and/or B cells, and/or to enhance interactions with Fc receptors.

In particular embodiments, the mAb or antigen-binding fragment (a) comprises a heavy chain comprising the heavy chain variable region of a mAb specifically disclosed herein or a heavy chain variable region that has substantial amino acid sequence similarity (as described above) with the amino acid sequence of a heavy chain variable region of a mAb specifically disclosed herein; (b) comprises a light chain comprising the light chain variable region of a mAb disclosed herein or a light chain variable region that has substantial amino acid sequence similarity with the amino acid sequence of light chain variable region of a mAb specifically disclosed herein; or (c) a mAb or antigen-binding fragment comprising a heavy chain and a light chain according to (a) and (b) above. In particular embodiments, a mAb or antigen-binding fragment of the invention comprises both a heavy chain comprising the heavy chain variable region of a mAb specifically disclosed herein or a heavy chain variable region that has substantial amino acid sequence similarity therewith and, further, a light chain comprising a light chain variable region from the same mAb disclosed herein or a light chain variable region that has substantial amino acid sequence similarity therewith.

As is known in the art, a number of different programs can be used to identify whether a nucleic acid or polypeptide has sequence identity or similarity to a known sequence. Sequence identity and/or similarity can be determined using standard techniques known in the art, including, but not limited to, the local sequence identity algorithm of Smith & Waterman, *Adv. Appl. Math.* **2**, 482 (1981), by the sequence identity alignment algorithm of Needleman & Wunsch, *J. Mol. Biol.* **48**, 443 (1970), by the search for similarity method of Pearson & Lipman, *Proc. Natl. Acad. Sci. USA* **85**, 2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Drive, Madison, WI), the Best Fit sequence program described by Devereux *et al.*, *Nucl. Acid Res.* **12**, 387-395 (1984), preferably using the default settings, or by inspection.

An example of a useful algorithm is PILEUP. PILEUP creates a multiple sequence alignment from a group of related sequences using progressive, pairwise alignments. It can also plot a tree showing the clustering relationships used to create the alignment. PILEUP uses a
5 simplification of the progressive alignment method of Feng & Doolittle, *J. Mol. Evol.* **35**, 351-360 (1987); the method is similar to that described by Higgins & Sharp, *CABIOS* **5**, 151-153 (1989).

Another example of a useful algorithm is the BLAST algorithm, described in Altschul et al., *J. Mol. Biol.* **215**, 403-410, (1990) and Karlin et al.,
10 *Proc. Natl. Acad. Sci. USA* **90**, 5873-5787 (1993). A particularly useful BLAST program is the WU-BLAST-2 program, which was obtained from Altschul et al., *Methods in Enzymology*, **266**, 460-480 (1996). WU-BLAST-2 uses several search parameters, which are preferably set to the default values. The parameters are dynamic values and are established by the
15 program itself depending upon the composition of the particular sequence and composition of the particular database against which the sequence of interest is being searched; however, the values can be adjusted to increase sensitivity.

An additional useful algorithm is gapped BLAST as reported by
20 Altschul et al., (1997) *Nucleic Acids Res.* **25**, 3389-3402.

A percentage amino acid sequence identity value can be determined by the number of matching identical residues divided by the total number of residues of the "longer" sequence in the aligned region. The "longer" sequence is the one having the most actual residues in the aligned region
25 (gaps introduced by WU-Blast-2 to maximize the alignment score are ignored).

The alignment can include the introduction of gaps in the sequences to be aligned. In addition, for sequences which contain either more or fewer amino acids than the polypeptides specifically disclosed herein, it is
30 understood that in one embodiment, the percentage of sequence identity will be determined based on the number of identical amino acids in relation to the total number of amino acids. Thus, for example, sequence identity of sequences shorter than a sequence specifically disclosed herein, will be determined using the number of amino acids in the shorter sequence, in one

embodiment. In percent identity calculations relative weight is not assigned to various manifestations of sequence variation, such as, insertions, deletions, substitutions, *etc.*

In one embodiment, only identities are scored positively (+1) and all forms of sequence variation including gaps are assigned a value of "0", which obviates the need for a weighted scale or parameters as described below for sequence similarity calculations. Percent sequence identity can be calculated, for example, by dividing the number of matching identical residues by the total number of residues of the "shorter" sequence in the aligned region and multiplying by 100. The "longer" sequence is the one having the most actual residues in the aligned region.

In other embodiments, a mAb, antigen-binding fragment, or specified region thereof having "substantial sequence similarity" to a mAb or corresponding antigen-binding fragment or specified region specifically described herein is encoded by a nucleic acid that hybridizes to the corresponding segment of the nucleic acids specifically disclosed herein under standard conditions as known by those skilled in the art and encode a functionally equivalent mAb or antigen-binding fragment as defined herein.

To illustrate, hybridization of such nucleic acid sequences can be carried out under conditions of reduced stringency, medium stringency or even stringent conditions (*e.g.*, conditions represented by a wash stringency of 35-40% Formamide with 5x Denhardt's solution, 0.5% SDS and 1x SSPE at 37°C; conditions represented by a wash stringency of 40-45% Formamide with 5x Denhardt's solution, 0.5% SDS, and 1x SSPE at 42°C; and/or conditions represented by a wash stringency of 50% Formamide with 5x Denhardt's solution, 0.5% SDS and 1x SSPE at 42°C, respectively) to the sequences specifically disclosed herein. See, *e.g.*, Sambrook et al., *Molecular Cloning, A Laboratory Manual* (2d Ed. 1989) (Cold Spring Harbor Laboratory).

It will be appreciated by those skilled in the art that there can be variability in the nucleic acids that encode the mAbs and antigen-binding fragments of the present invention due to the degeneracy of the genetic code. The degeneracy of the genetic code, which allows different nucleic acid sequences to code for the same polypeptide, is well known in the art.

Further variation in the nucleic acid sequence can be introduced by the presence (or absence) of non-translated sequences, such as intronic sequences and 5' and 3' untranslated sequences.

Now that the inventors have produced and characterized a panel of anti-
 5 CD20 mAbs having desirable characteristics, it would be routine for those skilled in the art to produce similar or improved antibodies and fragments. For example, the sequences of the heavy and/or light chain variable regions (or portions thereof, such as one or more of the CDRs) can be used as a starting point for the identification of other antibodies with desired properties. As one approach, a
 10 phage library can be generated that comprises variants of the sequences disclosed herein. The phage library can be selected on the basis of any desirable characteristic, e.g., CD20 reactivity, density of binding, efficacy of B cell depletion, efficacy of treating a B cell disorder, and the like.

Furthermore, to modify the amino acid and nucleic acid sequences of the
 15 mAbs and antigen-binding fragments thereof specifically disclosed herein, amino acid substitutions can be based on any characteristic known in the art, including the relative similarity or differences of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. In particular embodiments, conservative substitutions are made in the amino acid
 20 sequence. As used herein, a "conservative amino acid substitution" is a substitution whose probability of occurring in nature is greater than about ten times the probability of that substitution occurring by chance (e.g., as defined by the computational methods described by Dayhoff et al., *Atlas of Protein Sequence and Structure*, 1971, pages 95-96 and Figures 9-10).

25 In making amino acid substitutions, the hydropathic index of amino acids can be considered. The importance of the hydropathic amino acid index in conferring interactive biologic function on a protein is generally understood in the art (see, Kyte and Doolittle, (1982) *J. Mol. Biol.* **157**:105). It is accepted that the relative hydropathic character of the amino acid contributes to the secondary
 30 structure of the resultant protein, which in turn defines the interaction of the protein with other molecules, for example, enzymes, substrates, receptors, DNA, antibodies, antigens, and the like.

Each amino acid has been assigned a hydropathic index on the basis of its hydrophobicity and charge characteristics (Kyte and Doolittle, *Id.*), and these are: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine
 5 (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

It is also understood in the art that the substitution of amino acids can be made on the basis of hydrophilicity. U.S. Patent No. 4,554,101 states that the
 10 greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with a biological property of the protein.

As detailed in U.S. Patent No. 4,554,101, the following hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (± 3.0);
 15 aspartate ($+3.0 \pm 1$); glutamate ($+3.0 \pm 1$); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (-0.4); proline (-0.5 ± 1); alanine (-0.5); histidine (-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); tryptophan (-3.4).

In other embodiments, functionally equivalent mAbs and antigen binding
 20 fragments of the invention encompass those comprising one or more of the specified regions above (*e.g.*, heavy or light chains, heavy and/or light chain variable regions or portions thereof) from the mAbs or antigen-binding fragments disclosed herein having no more than 14, 12, 10, 8, 6, 5, 4, 3, 2 or 1 amino acid substitutions, deletions and/or insertions. In particular embodiments, a mAb or
 25 antigen-binding fragment of the invention comprises a CDR1, CDR2 and/or CDR3 region, wherein each CDR region comprises no more than 5, 4, 3, 2 or 1 amino acid substitutions, deletions and/or insertions. In an exemplary embodiment, the CDR1, CDR2 and/or CDR3 region each comprises no more than 5, 4, 3, 2 or 1 conservative amino acid substitutions.

30 The antibodies or fragments can additionally have more than one antigen specificity, *e.g.*, can be a bispecific antibody. The bispecific antibody can, for example, additionally bind to another CD20 epitope. In addition, the bispecific antibody can have binding specificity for other antigens, such as, CD19, CD22, CD52, CD3, CD28, or HLA-DR10 (Lym-1); or for Fc receptors,

e.g. CD16, CD64 and CD89; T cell receptors (e.g., the zeta chain of the T cell receptor complex) or for other cell surface molecules such as receptors such as cytokine, hormone or growth factor receptors.

The antibodies and fragments thereof can further be a "chimeric" antibody. Chimeric antibodies and antigen-binding fragments comprise portions from two or more different species (e.g., mouse and human). Chimeric antibodies can be produced with mouse variable regions of desired specificity spliced into human constant domain gene segments (see, e.g., U.S. Patent No. 4,816,567). In this manner, non-human (e.g., mouse) antibodies can be modified to make them more suitable for human clinical application.

The mAbs of the invention can further be "humanized" or "CDR grafted" forms of non-human (e.g., mouse) mAbs, which can offer advantages as therapeutic agents for humans over murine mAbs, particularly because they are not cleared from the circulation in humans as rapidly as mouse antibodies, and do not generally provoke an adverse immune reaction when administered to human subjects. Generally, a humanized antibody has one or more amino acid residues introduced into it from a non-human source. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Methods of preparing humanized antibodies are generally well known in the art, and can readily be applied to the mAbs disclosed herein. For example, humanization can be essentially performed following the method of Winter and co-workers (Jones et al., *Nature*, 321:522-525 (1986); Riechmann et al., *Nature*, 332:323-327 (1988); Verhoeyen et al., *Science*, 239:1534-1536 (1988)), by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. In particular embodiments, humanized forms of non-human (e.g., mouse) antibodies are human antibodies (recipient antibody) in which hypervariable (CDR) region residues of the recipient antibody are replaced by hypervariable region residues from a non-human species (donor antibody) such as a mouse, rat, rabbit, or nonhuman primate having the desired specificity, affinity, and binding capacity. In some instances, framework region residues of the human immunoglobulin are also replaced by corresponding non-human residues (so called "back mutations"). The

positions for such backmutations can be determined by sequence and structural analysis, or by analysis of the variable regions' three-dimensional structure using a computer model. In addition, phage display libraries can be used to vary amino acids at chosen positions within the antibody sequence.

5 The properties of a humanized antibody are also affected by the choice of the human framework. Furthermore, humanized and chimerized antibodies can be modified to comprise residues that are not found in the recipient antibody or in the donor antibody, in order to further improve antibody properties, such as affinity. In general, the humanized antibody will comprise all or

10 substantially all of at least one, two or even all three CDR domains that correspond to the CDR domains of a non-human immunoglobulin and all or substantially all of the framework region residues are those of a human immunoglobulin sequence. The humanized antibody optionally will also comprise at least a portion of an immunoglobulin constant region (Fc),

15 typically that of a human immunoglobulin. For further details, see Jones et al., *Nature* 321:522-525 (1986); and Reichmann et al., *Nature* 332:323-329 (1988). Thus, provided in one embodiment of the invention is a mAb that is humanized by grafting to introduce components of human immunoglobulins without substantially interfering with the ability of the antibody to bind antigen

20 (*i.e.*, CD20).

The mAbs or antigen-binding fragments of the invention can be naked antibodies or antigen-binding fragments that are not conjugated to other agents, for example, therapeutic agent. Alternatively, the mAb or antigen-binding fragment can be conjugated to a therapeutic agent (*i.e.*, to form an

25 immunoconjugate) such as a cytotoxic agent, a small molecule compound, a hormone, growth factor, cytokine, enzyme, RNase, ribozyme or a nucleic acid molecule including coding sequences, antisense RNA and RNAi.

Illustrative cytotoxic agents include but are not limited to protein toxins such as ricin, diphtheria toxin, *Staphylococcal* enterotoxin, *Pseudomonas*

30 exotoxin, abrin or other ribosomal inactivating proteins. These proteins can be linked to the antibody or antibody fragment either chemically using a chemical cross-linking agent or by recombinant nucleic acid technology by constructing a fusion protein that encodes all or part of the protein toxin. Other illustrative cytotoxic agents include high-energy radioisotopes such as

⁹⁰Y, ¹³¹I or ¹¹¹In. Further cytotoxic agents include cytotoxic and cystostatic drugs such as methotrexate, chlorambucil, adriamycin, daunorubicin and vincristine.

Alternatively, the mAb or antigen-binding fragment can be detectably labeled. Exemplary detectable labels include radiolabels, heavy metals, chromophores, flourophores and enzymes where the end-product of the enzymatic reaction is detectable. Detectably labeled antibodies and antigen-binding fragments can be used, for example, in diagnostic and laboratory methods.

The anti-CD20 mAbs can be made by any standard method known in the art, such as, for example, by the hybridoma method (Koehler and Milstein, *Nature* 256:495-497 (1975); and Goding, *Monoclonal Antibodies: Principles and Practice*, pp. 59-103, (Academic Press, 1986)), or by recombinant techniques, disclosed, for example, in U.S. Patent No. 4,816,567 and by Wood *et al.*, *Nature* 314:446-9 (1985).

Monoclonal antibodies are typically produced by clones of a single cell, such as hybridoma cells, that produce a homogenous population of antibody molecules that have the same antibody combining site. The hybridoma cell is formed by fusing an antibody-producing cell and a myeloma or other self-perpetuating cell line. The preparation of such antibodies was first described by Kohler and Milstein, *Nature* 256:495-497 (1975). Additional methods are described by Zola, *Monoclonal Antibodies: a Manual of Techniques*, CRC Press, Inc. (1987). The hybridoma supernates so prepared can be screened for the presence of antibody molecules that bind to CD20 and/or have other desirable characteristics as described herein.

Generally to produce a hybridoma that produces an anti-CD20 mAb, a myeloma or other self-perpetuating cell line is fused with lymphocytes obtained from the spleen, lymph nodes or other antibody producing cells, of a mammal hyperimmunized against CD20 (see, e.g., Kearney *et al.*, *J. Immunol.*, 123:1548-50 (1979)).

In one embodiment, the myeloma cell line used to prepare a hybridoma is from the same species as the lymphocytes. A suitable mouse myeloma for use in the present invention is the NS-1 myeloma cell lines available from the

American Type Culture Collection, Manassas, Virginia, United States of America.

Splenocytes are typically fused with myeloma cells using polyethylene glycol (PEG) 1500. Fused hybrids are selected by their sensitivity to HAT.

- 5 Hybridomas producing a disclosed monoclonal antibody are identified using the enzyme linked immunosorbent assay (ELISA) and fluorescence-activated cell sorting (FACS) described herein.

The antibody producing cells can be obtained from an inbred mouse strain, such as the C57BL/6 strain. In other embodiments, the antibody
10 producing cell is from a CD20^{-/-} mammal, for example a CD20^{-/-} mouse. In one representative embodiment, the CD20^{-/-} mouse is originally derived from strain 129 mice. The CD20^{-/-} mammal can be produced using techniques known to one of skill in the art and as described herein, see, e.g., Hogan et al. (1986) *Manipulating the Mouse Embryo: A Laboratory Manual*, Cold Spring
15 Harbor Laboratory Press, Cold Spring Harbor, N.Y.

The anti-CD20 mAbs of the present invention may be fully human. Methods of preparing fully human antibodies are known in the art and include the use of transgenic animals and phage display techniques.

It is now also possible to produce transgenic animals (e.g., mice) that
20 are capable, upon immunization, of producing a repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of
25 the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, e.g. Jakobovits et al., *Proc. Natl. Acad. Sci. USA* 90, 2551-255 (1993); Jakobovits et al., *Nature* 362, 255-258 (1993).

Mendez et al. (*Nature Genetics* 15: 146-156 (1997)) have further
30 improved the technology and have generated a line of transgenic mice designated as "Xenomouse II" that, when challenged with an antigen, generates high affinity fully human antibodies. This was achieved by germ-line integration of megabase human heavy chain and light chain loci into mice with deletions in the endogenous J_H segment as described above. The

Xenomouse II harbors 1,020 kb of human heavy chain locus containing approximately 66 V_H genes, complete D_H and J_H regions and three different constant regions (μ , δ and χ), and also harbors 800 kb of human κ locus containing 32 V _{κ} genes, J _{κ} segments and C _{κ} genes. The antibodies
5 produced in these mice closely resemble those seen in humans in all respects, including gene rearrangement, assembly, and repertoire. The human antibodies are preferentially expressed over endogenous antibodies due to deletions in the endogenous J_H segment that prevents gene rearrangement in the murine locus.

10 Other methods of producing a mAb are also known. See, for example, the method of isolating mAbs from an immunological repertoire as described by Sastry, et al., *Proc Natl Acad Sci USA* 86:5728-5732 (1989); and Huse et al., *Science* 246:1275-1281 (1989).

Alternatively, phage display technology (McCafferty *et al.*, *Nature* 348,
15 552-553 (1990)) can be used to produce human antibodies and antibody fragments *in vitro*, from immunoglobulin variable (V) domain gene repertoires from unimmunized donors. According to this technique, antibody V domain genes are cloned in-frame into either a major or minor coat protein gene of a filamentous bacteriophage, such as M13 or fd, and displayed as functional
20 antibody fragments on the surface of the phage particle. Because the filamentous particle contains a single-stranded DNA copy of the phage genome, selections based on the functional properties of the antibody also result in selection of the gene encoding the antibody exhibiting those properties. Thus, the phage mimics some of the properties of the B-cell.
25 Phage display can be performed in a variety of formats; see, *e.g.*, Johnson, Kevin S. and Chiswell, David J., *Current Opinion in Structural Biology* 3, 564-571 (1993).

Several sources of V-gene segments can be used for phage display. Clackson *et al.*, *Nature* 352, 624-628 (1991) isolated a diverse array of anti-
30 oxazolone antibodies from a small random combinatorial library of V-genes derived from the spleens of immunized mice. A repertoire of V-genes from unimmunized human donors can be constructed and antibodies to a diverse array of antigens (including self-antigens) can be isolated essentially following

the techniques described by Marks *et al.*, *J. Mol. Biol.* 222, 581-597 (1991), or Griffith *et al.*, *EMBO J.* 12, 725-734 (1993). In a natural immune response, antibody genes accumulate mutations at a high rate (somatic hypermutation). Some of the changes introduced will confer higher affinity, and B cells

5 displaying high-affinity surface immunoglobulin are preferentially replicated and differentiated during subsequent antigen challenge. This natural process can be mimicked by employing the technique known as "chain shuffling" (Marks *et al.*, *Bio/Technol.* 10, 779-783). In this method, the affinity of "primary" human antibodies obtained by phage display can be improved by

10 sequentially replacing the heavy and light chain V-region genes with repertoires of naturally occurring variants (repertoires) of V-domain genes obtained from unimmunized donors. This technique allows the production of antibodies and antibody fragments with affinities in the nM range. A strategy for making very large phage antibody repertoires has been described by

15 Waterhouse *et al.*, *Nucl. Acids Res.* 21, 2265-2266 (1993).

For further information concerning the production of monoclonal antibodies see also Goding, J.W., *Monoclonal Antibodies: Principles and Practice*, 3rd Edition, Academic Press, Inc., London, San Diego, 1996; Liddell and Weeks: *Antibody Technology: A Comprehensive Overview*, Bios

20 Scientific Publishers: Oxford, UK, 1995; Breitling and Dubel: *Recombinant Antibodies*, John Wiley & Sons, New York, 1999; and Phage Display: A Laboratory Manual, Barbas *et al.*, editors, Cold Springs Harbor Laboratory, Cold Spring Harbor, 2001.

The inventors have made the unexpected discovery that novel

25 antibodies with distinct characteristics (*e.g.*, CDR regions, density of binding, and the like) can be generated from a CD20^{-/-} mammal. Accordingly, in one representative embodiment, the invention provides a method of producing a monoclonal antibody that specifically binds to CD20, comprising: (a) immunizing a CD20^{-/-} mammal (*e.g.*, a mouse) with CD20 or an antigenically

30 effective fragment thereof under conditions sufficient to elicit an antibody response; (b) harvesting antibody producing cells (*e.g.*, B cells) from the mammal; (c) fusing the antibody producing cells with immortalized cells (*e.g.*, myeloma cells) in culture to form monoclonal antibody-producing hybridoma cells; (d) culturing the hybridoma cells under conditions sufficient for

production of monoclonal antibodies; and (e) recovering monoclonal antibodies that specifically bind to CD20 from the culture. The method can optionally include isolation of a hybridoma cell line that produces an anti-CD20 mAb.

In another embodiment, the invention provides a method of producing a monoclonal antibody that specifically binds to CD20, comprising: (a) immunizing a CD20^{-/-} mammal with CD20 or an antigenically effective fragment thereof under conditions sufficient to elicit an antibody response; (b) harvesting a cell that produces an antibody that specifically binds to CD20 from the mammal; (c) isolating an immunoglobulin coding gene from the antibody-producing cell; (d) introducing the immunoglobulin coding gene into a different cell to produce a transformed cell; (e) culturing the transformed cell under conditions sufficient for transcription and translation of the immunoglobulin gene and production of a monoclonal antibody; and (e) recovering from the culture monoclonal antibodies that specifically bind to CD20. In particular embodiments, both heavy chain and light chain genes are isolated from the antibody producing cell or from different antibody producing cells and are introduced into the transformed cell(s). The transformed cell can be any suitable cell, for example, a mammalian cell or cell line such as CHO or BHK cells.

Also provided by the invention are hybridoma cells, hybridoma cell lines, and hybridoma cell cultures that produce the mAbs of the invention, as described above. Exemplary hybridoma cell lines of the invention include hybridoma HB20-1, HB20-2, HB20-3, HB20-4, HB20-5, HB20-6, HB20-25, MB20-1, MB20-2, MB20-3, MB20-6, MB20-7, MB20-8, MB20-10, MB20-11, MB20-13, MB20-14, MB20-16 and MB20-18.

Hybridoma cell lines HB20-3, HB20-4, HB20-25, MB20-1, MB20-11 and MB20-18 were deposited with the American Type Culture Collection (ATCC) in Manassas, VA, USA in accordance with the Budapest Treaty on May 5, 2004, and assigned ATCC Accession Nos.

The invention also provides nucleic acids encoding the mAbs, antigen-binding fragments, antibody heavy chains and/or antibody light chains or

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portions thereof (e.g., variable regions, CDR regions) of the invention. The nucleic acid can be DNA, RNA or chimeras thereof, single stranded or double-stranded, and can be fully or partially synthetic or naturally occurring. The nucleic acids can comprise modified nucleotides or nucleotide analogs.

- 5 Further, the nucleic acid can be from any species of origin, including mammalian species such as human, non-human primate, mouse, rat, rabbit, cattle, goat, sheep, horse, pig, dog, cat, *etc.*

In particular embodiments, the nucleic acid is an isolated nucleic acid. As used herein, an "isolated" nucleic acid means a nucleic acid separated or
10 substantially free from at least some of the other components of the naturally occurring organism, such as for example, the cell structural components or other polypeptides or nucleic acids commonly found associated with the nucleic acid.

The invention also provides vectors, including expression vectors and
15 gene delivery vectors, comprising the nucleic acids of the invention. Suitable vectors include bacterial expression vectors, fungal expression vectors, mammalian vectors, yeast expression vectors and plant expression vectors. Exemplary vectors include bacterial artificial chromosomes, cosmids, yeast artificial chromosomes, phage, plasmids, lipid vectors and viral vectors (e.g.,
20 adenovirus, adeno-associated virus, retrovirus, baculovirus, and the like).

Expression vectors can be designed for expression of polypeptides in prokaryotic or eukaryotic cells. For example, polypeptides can be expressed in bacterial cells such as *E. coli*, yeast cells, insect cells (e.g., in the baculovirus expression system) or mammalian cells. Some suitable host cells
25 are discussed further in Goeddel, Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, Calif. (1990). Examples of vectors for expression in yeast *S. cerevisiae* include pYepSecl (Baldari et al., (1987) *EMBO J.* 6:229-234), pMFa (Kurjan and Herskowitz, (1982) *Cell* 30:933-943), pJRY88 (Schultz et al., (1987) *Gene* 54:113-123), and pYES2
30 (Invitrogen Corporation, San Diego, Calif.). Baculovirus vectors available for expression of nucleic acids to produce proteins in cultured insect cells (e.g., Sf 9 cells) include the pAc series (Smith et al., (1983) *Mol. Cell. Biol.* 3:2156-2165) and the pVL series (Lucklow, V.A., and Summers, M.d. (1989) *Virology* 170:31-39).

Examples of mammalian expression vectors include pCDM8 (Seed, (1987) *Nature* 329:840) and pMT2PC (Kaufman et al. (1987), *EMBO J.* 6:187-195).

5 The vector generally comprises an expression control element (e.g., a promoter) operably associated with the nucleic acids of the invention. It will be appreciated that a variety of expression control elements can be used depending on the level and tissue-specific expression desired. Further, the promoter can be constitutive or inducible (e.g., the metallothionein promoter or a hormone inducible promoter). The expression control element can be
10 native or foreign to the host cell and can be a natural or a synthetic sequence. The promoter is generally chosen so that it will function in the target cell(s) of interest. The nucleic acids can further be associated with other appropriate expression control sequences, e.g., transcription/translation control signals and polyadenylation signals. Viral regulatory elements are often employed in
15 mammalian cells. For example, commonly used promoters in mammalian expression vectors are derived from polyoma, adenovirus 2, cytomegalovirus and Simian Virus 40.

Moreover, specific initiation signals are generally required for efficient translation of inserted protein coding sequences. These translational control
20 sequences, which can include the ATG initiation codon and adjacent sequences, can be of a variety of origins, both natural and synthetic.

Further provided are host cells (e.g., yeast, bacterial, mammalian, insect, plant or fungal cells) comprising the isolated nucleic acids and vectors of the invention. The cell can be transiently or stably transformed with the
25 nucleic acid or vector of the invention. In particular embodiments, the nucleic acid is stably incorporated into the genome of the host cell. Further, the cell can be cultured (*i.e.*, isolated) or can be a cell *in situ* in a living organism.

Methods of Use.

30 The antibodies, antigen-binding fragments, nucleic acids and pharmaceutical compositions of the invention can be used in a number of research, diagnostic and/or therapeutic applications. To illustrate, the antibodies and antigen-binding fragments of the invention specifically bind to CD20, which is a B cell specific marker. Accordingly, these reagents find use

in methods of identifying B cells, methods of studying CD20 function as well as methods for immunoaffinity purification of CD20 or B cells. Methods of isolating B cells can be used for laboratory research, therapeutic or diagnostic methods. For example, tissue or cells can be removed from a subject having
5 a B cell malignancy, the B cells purified away with the antibodies or antigen-binding fragments of the invention, and the B cell depleted tissue or cells re-introduced into the subject. Further, the antibodies, antigen-binding fragments and compositions of the invention can be used for diagnostic purposes, for example, to identify lymphomas. The methods of the invention
10 also provide for B cell specific delivery of molecules using antibodies or antigen-binding fragments conjugated with therapeutic agents (as described above). Further, the invention also provides therapeutic methods of depleting B cells, for example, for the treatment of B cell disorders such as B cell malignancies and autoimmune diseases.

15 In one particular embodiment, the invention provides a method of depleting B cells in an animal subject (*e.g.*, a mammalian subject) comprising administering a mAb, antigen-binding fragment or pharmaceutical composition of the invention to the mammalian subject in an amount effective to deplete B cells. By "amount effective to deplete B cells" it is meant an amount effective
20 to achieve a reduction (*i.e.*, depletion) in B cells of at least about 25%, 35%, 50%, 60%, 75%, 80%, 85%, 90%, 95%, 98% or more. In some embodiments, there will be no, or essentially no, detectable B cells. Methods of detecting B cells and measuring B cell depletion are known in the art (see, *e.g.*, Examples 9-12, 14 and 17). In representative embodiments, at least about 25%, 35%,
25 50%, 60%, 75%, 80%, 85%, 90%, 95%, 98% or more depletion is achieved in peripheral circulating and/or tissue (*e.g.*, spleen, lymph node) B cells. Those skilled in the art will understand that for clinical applications, peripheral circulating B cells are measured/monitored, which is generally less invasive than methods of evaluating B cell depletion in tissues.

30 The invention further provides methods of treating a B cell disorder comprising administering to an animal (*e.g.*, mammalian) subject having a B cell disorder a treatment-effective amount of one or more monoclonal antibodies, antigen-binding fragments or pharmaceutical formulations of the

invention. In particular embodiments, the B cell disorder is a B cell malignancy or an autoimmune disease.

The term "B cell malignancy" and grammatical variants thereof, are used in the broadest sense to refer to malignancies or neoplasms of B cells that typically arise in lymphoid tissues, such as bone marrow or lymph nodes, but may also arise in non-lymphoid tissues, such as thyroid, gastrointestinal tract, salivary gland and conjunctiva. The treatment methods of the present invention specifically concern CD20-positive B cell malignancies including, without limitation, B-cell subtype of non-Hodgkin's lymphoma (NHL), Burkitt's lymphoma, multiple myeloma, chronic lymphocytic leukemia, hairy cell leukemia, Waldenstrom's Macroglobulinemia, and prolymphocytic leukemia. B-cell subtype Non-Hodgkin's Lymphoma is a term that is used to encompass a large group (over 29 types) of lymphomas caused by malignant B cell lymphocytes, and represents a large subset of the known types of lymphoma including but not limited to low grade/follicular NHL, small lymphocytic (SL) NHL, intermediate grade/follicular NHL, intermediate grade diffuse NHL, high grade immunoblastic NHL, high grade lymphoblastic NHL, high grade small non-cleaved cell NHL and bulky disease NHL.

Autoimmune disorders are caused in part by a breakdown in self-tolerance leading to subsequent immune responses against self, including the production of autoantibodies and deposition of immunoglobulin in affected tissues. Autoantibodies form immune complexes that promote complement and Fc-receptor mediated tissue inflammation and destruction. Most autoimmune diseases result from, or are aggravated by, the production of antibodies reactive with normal body tissues. Since B lymphocytes are the source of autoantibodies, they afford a rational target for treatment of these types of immune-mediated diseases. B lymphocytes can also present antigen and regulate the development of effector T lymphocytes.

More than 80 autoimmune diseases have been identified. Autoimmune diseases, their etiology and treatment are discussed extensively in the Autoimmune Diseases Research Plan published by the Autoimmune Diseases Coordinating Committee of the National Institutes of Health. Representative autoimmune diseases that can be treated according to the present invention include, but are not limited to immune complex disorders

such as those that result in glomerulonephritis, Goodspature's syndrome, necrotizing vasculitis, lymphadenitis, peri-arteritis nodosa and systemic lupus erythematosus. Other illustrative autoimmune diseases include but are not limited to rheumatoid arthritis, psoriatic arthritis, systemic lupus erythematosus, 5 psoriasis, ulcerative colitis, systemic sclerosis, dermatomyositis/polymyositis, anti-phospholipid antibody syndrome, scleroderma, perphigus vulgaris, ANCA-associated vasculitis (e.g., Wegener's granulomatosis, microscopic polyangiitis), urveitis, Sjögren's syndrome, Crohn's disease, Reiter's syndrome, ankylosing spondylitis, Lyme arthritis, Guillain-Barre syndrome, 10 Hashimoto's thyroiditis, and cardiomyopathy. Other diseases associated with antibody production that can be treated according to the present invention include, but are not limited to multiple sclerosis, atopic dermatitis, thrombocytopenic purpura, agranulocytosis, autoimmune hemolytic anemias, immune reactions against foreign antigens such as fetal A-B-O blood groups 15 during pregnancy, myasthenia gravis, Type I diabetes, Graves' disease, and allergic responses.

The methods of the invention may be used to treat any other disorder or condition in which B cells or antibodies are implicated including, for example, transplant rejection.

20 A "treatment effective" amount is an amount of an anti-CD20 antibody or antigen-binding fragment sufficient to produce some improvement or amelioration in the subject's condition or to prevent or delay relapse or recurrence of the condition.

Subjects can be monitored by standard techniques known in the art to 25 follow clinical indicia of B-cell malignancy or the particular autoimmune disease. For example, in the case of B-cell malignancy, tumor regression (e.g. tumor size in the case of solid tumors), the phenotype of circulating B-cells or of biopsied tissues using anti-CD20 antibodies can be monitored.

Those skilled in the art will appreciate that dosages can be selected 30 based on a number of factors including the age, sex, species and condition of the subject, the desired degree of depletion, the disease to be treated and/or the particular antibody or antigen-binding fragment being used and can be determined by one of skill in the art. For example, non-Hodgkin's lymphoma patients or patients with autoimmune disease may receive from about 0.0005

to about 1500 mg/m²/week, specifically from about 0.001 to about 150 mg/m²/week, more specifically from about 0.25 to about 75 mg/m²/week, more specifically from about 2.5 to about 50 mg/m²/week of an anti-CD20 antibody as described herein.

5 In embodiments of the invention, the antibodies and antigen-binding fragments bind to B cells at a greater density than conventional anti-CD20 antibodies and, thus, can result in a more efficient (*i.e.*, at lower dosage) depletion of B cells (as defined above). Alternatively, or additionally, more efficient depletion may be a result of the particular epitope with which the
10 antibody reacts. In exemplary embodiments, dosages of the antibody or antigen-binding fragment (optionally in a pharmaceutically acceptable carrier as part of a pharmaceutical composition) are at least about 0.0005, 0.001, 0.05, 0.075, 0.1, 0.25, 0.375, 0.5, 1, 2.5, 5, 10, 20, 37.5, 50 or 100 mg/m² and/or less than about 200, 175, 150, 125, 100, 75, 60, 50, 37.5, 20, 15, 10,
15 5, 2.5, 1, 0.5, 0.375, 0.1, 0.075 or 0.01 mg/m². In other illustrative embodiments, the dosage is between about 0.0005 to about 200 mg/m², between about 0.001 and 150 mg/m², between about 0.075 and 125 mg/m², between about 0.375 and 100 mg/m², between about 2.5 and 75 mg/m², between about 10 and 75 mg/m², and between about 20 and 50 mg/m².

20 In some embodiments of the methods of this invention, mAbs, antigen-binding fragments and/or compositions of this invention can be administered at a dose lower than about 375 mg/m²; at a dose lower than about 37.5 mg/m²; at a dose lower than about 0.375 mg/m²; and/or at a dose between about 0.075 mg/m² and about 125 mg/m².

25 The specified dosage can result in B cell depletion (as described above) for a period of at least about 3, 5, 7, 10, 14, 20, 30, 45, 60, 75, 90, 120, 150 or 180 days or longer.

 In representative embodiments of the invention, a dosage of about 125 mg/m² or less of an antibody or antigen-binding fragment results in B cell
30 depletion (as described above) for a period of at least about 7, 14, 21, 30, 45, 60 days, 90 or 120 days. In another representative embodiment, a dosage of about 37.5 mg/m² or less depletes B cells for a period of at least about 7, 14, 21, 30, 45, 60, 90 or 120 days. In still other embodiments, a dosage of about 0.375 mg/m² or less results in depletion of B cells for at least about 7, 14, 21,

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30, 45 or 60 days. In another embodiment, a dosage of about 0.075 mg/m² or less results in depletion of B cells for a period of at least about 7, 14, 21, 30, 45 or 60 days. In yet other embodiments, a dosage of about 0.01 mg/m², 0.005 mg/m² or even 0.001 mg/m² or less results in depletion of B cells for at least about 3, 5, 7, 10, 14, 21 or 30 days. According to these embodiments, the dosage can be administered by any suitable route (as described below), but is optionally administered by a subcutaneous route.

As another aspect, the invention provides the discovery that B cell depletion and/or treatment of B cell disorders can be achieved at lower dosages of antibody or antibody fragments than employed in currently available methods. Thus, in another embodiment, the invention provides a method of depleting B cells and/or treating a B cell disorder, comprising administering to an animal subject (e.g., a mammalian subject), an effective amount of a mAb or antigen-binding fragment thereof that specifically binds to CD20, wherein a dosage of about 200, 175, 150, 125, 100, 75, 60, 50, 37.5, 20, 10, 5, 2.5, 1, 0.5, 0.375, 0.25, 0.1, 0.075, 0.05, 0.001, 0.0005 mg/m² or less results in a depletion of B cells (circulating and/or tissue B cells) of 25%, 35%, 50%, 60%, 75%, 80%, 85%, 90%, 95%, 98% or more for a period at least about 3, 5, 7, 10, 14, 21, 30, 45, 60, 75, 90, 120, 150 or 180 days or longer. In representative embodiments, a dosage of about 125 mg/m² or 75 mg/m² or less results in at least about 50%, 75% 85% or 90% depletion of B cells for at least about 7, 14, 21, 30, 60, 75, 90, 120, 150 or 180 days. In other embodiments, a dosage of about 50, 37.5 or 10 mg/m² results in at least about a 50%, 75% 85% or 90% depletion of B cells for at least about 7, 14, 21, 30, 60, 75, 90, 120 or 180 days. In still other embodiments, a dosage of about 0.375 or 0.1 mg/m² results in at least about a 50%, 75%, 85% or 90% depletion of B cells for at least about 7, 14, 21, 30, 60, 75 or 90 days. In further embodiments, a dosage of about 0.075, 0.01, 0.001, or 0.0005 mg/m² results in at least about a 50%, 75%, 85% or 90% depletion of B cells for at least about 7, 14, 21, 30 or 60 days. According to these embodiments, the dosage can be administered by any suitable route (as described below), but is optionally administered by a subcutaneous route.

According to this embodiment, the antibody or antigen-binding fragment is an antibody or antigen-binding fragment as described herein

(including functionally equivalent antibodies and antigen-binding fragments). In other particular embodiments, the antibody or antigen-binding fragment binds to CD20 or B cells at a higher density as compared with conventional antibodies (as described above).

5 The antibodies, antigen-binding fragments and pharmaceutical compositions of the invention can be used in combination with other therapeutic agents or regimes. For example, in the case of B-cell malignancies, such regimes or therapies include chemotherapy, radioimmunotherapy (RIT), chemotherapy and external beam radiation (combined modality therapy, CMT), or
10 combined modality radioimmunotherapy (CMRIT) alone or in combination, etc. Thus, the anti-CD20 antibodies and antibody fragments of the present invention can be combined with CHOP (Cyclophosphamide-Hydroxydoxorubicin-Oncovin (vincristine)-Prednisolone), the most common chemotherapy regimen for treating non-Hodgkin's lymphoma. In addition, the anti-CD20 antibodies herein may be
15 administered in combination with other antibodies, including anti-CD19, anti-CD22 (as described, for example, in U.S. Patent No. 5,484,892, U.S. patent publication number 2004/0001828 of U.S. application serial number 10/371,797, U.S. patent publication number 2003/0202975 of U.S. application serial number 10/372,481 and U.S. provisional application serial number 60/420,472, for their
20 teachings of CD22 antigens and anti-CD22 antibodies), and other anti-CD20 antibodies, such as RituxanTM (C2B8; Rituximab; IDEC Pharmaceuticals).

 Thus, in some embodiments, the present invention provides a method of depleting B cells in a mammalian subject, comprising administering a mAb and/or antigen-binding fragment thereof of this invention and further comprising
25 administering one or more additional antibodies and/or antigen binding fragments thereof to the subject. In some embodiments, the additional antibody can be an anti-CD22, an anti-CD19 antibody or both antibodies. The additional antibody or antibodies and/or antigen-binding fragment(s) thereof can be administered in any sequence relative to the administration of the antibody or antigen-binding
30 fragment of this invention.

For example, the additional antibody or antibodies and/or antigen-binding fragment(s) can be administered before, concurrently with, and/or after administration of the antibody and/or antigen-binding fragment of the invention to the subject. The additional antibody or antibodies and/or antigen
5 fragment(s) can be present in the same pharmaceutical composition as the antibody and/or antigen-binding fragment of the invention, and/or present in a different pharmaceutical composition. The dose and mode of administration of the antibody and/or antigen-binding fragment of this invention and the dose of the additional antibody or antibodies and/or antigen-binding fragment(s)
10 can be the same or different, in accordance with any of the teachings of dosage amounts and modes of administration as provided in this application and as are well known in the art.

In one particular embodiment, the subject is administered a compound that enhances monocyte or macrophage function (*e.g.*, at least about 25%,
15 50%, 75%, 85%, 90%, 9% or more) in addition to an antibody of the invention. Such compounds are known in the art and include, without limitation, cytokines such as interleukins (*e.g.*, IL-12), and interferons (*e.g.*, alpha or gamma interferon). The compound that enhances monocyte or macrophage function or enhancement can be formulated in the same pharmaceutical
20 composition as the antibody or antigen-binding fragment. When administered separately, the antibody/fragment and the compound can be administered concurrently (within a period of hours of each other), can be administered during the same course of therapy, or can be administered sequentially (*i.e.*, the patient first receives a course of the antibody/fragment treatment and then
25 a course of the compound that enhances macrophage/monocyte function or vice versa).

This embodiment of the invention can be practiced with the antibodies and antibody fragments of the invention or with other antibodies known in the art and is particularly suitable for subjects that are resistant to anti-CD20
30 monoclonal antibody therapy (for example, therapy with existing antibodies such as C2B8), subjects that are currently being or have previously been treated with chemotherapy, subjects that have had a relapse in a B cell disorder, subjects that are immunocompromised, or subjects that otherwise have an impairment in macrophage or monocyte function. The inventors have

discovered that antibody-dependent cytotoxicity (ADCC) primarily mediated by monocytes plays a more important role than previously recognized in B cell depletion. The prevalence of patients that are resistant to anti-CD20 therapy or have a relapse in a B cell disorder may be attributable, at least in part, to an impairment in macrophage or monocyte function. Thus, the invention provides methods of enhancing ADCC and/or macrophage and/or monocyte function to be used in conjunction with the methods of administering anti-CD20 antibodies and antigen-binding fragments.

Subjects according to the present invention can be a human subject, although the invention can also be practiced for veterinary purposes, to treat non-human mammals and avians. Non-limiting examples of mammalian subjects on which the diagnostic and therapeutic methods of the invention can be practiced include mice, rats, guinea pigs, pigs, goats, sheep, non-human primates, horses, dogs, cats, cattle, rabbits and humans. Avians include chickens, turkeys, quail, geese and ducks.

The antibody compositions of the invention can be administered using any mode of administration including, but not limited to, inhalation (e.g., via an aerosol), buccal (e.g., sub-lingual), topical (i.e., both skin and mucosal surfaces, including airway surfaces), intrathecal, intraarticular, intraplural, intracerebral, intravenous, intra-arterial, intraperitoneal, oral, intralymphatic, intramuscular, intradermal, subcutaneous, transdermal, intranasal, rectal or vaginal administration and can be delivered by peristaltic means or in the form of a depot, although the most suitable route in any given case will depend, as is well known in the art, on such factors as the species, age, gender and overall condition of the subject, the nature and severity of the condition being treated and/or on the nature of the particular composition (i.e., dosage, formulation) that is being administered.. In particular embodiments, the route of administration is via bolus or continuous infusion over a period of time, once or twice a week. In other particular embodiments, the route of administration is by subcutaneous injection, optionally once or twice weekly.

Suitable regimes for administration are variable with the subject and condition being treated, but are typified by an initial administration followed by repeated doses at one or more intervals by a subsequent injection or other administration. The intervals can be as short as a few hours, or as long as

one or more weeks between doses. Alternatively, continuous intravenous infusion can be employed to maintain effective concentrations in the blood.

The antibodies disclosed herein can also be used for *in vitro* procedures. The antibodies selectively bind CD20, which is expressed on B lymphocytes, and during specific phases of B lymphocyte development. As such, the antibodies of the present invention can be used to specifically deplete B lymphocytes from a mixed sample of cells, e.g. whole blood. Either the enriched or depleted B lymphocyte fractions can then be used as needed experimentally without risk of interference or interaction by other cell types.

Methods for utilizing the antibodies disclosed herein for isolating B lymphocytes from mixed cell populations *in vitro* are well known in the art. As non-limiting examples, FACS, panning and magnetic separation techniques can be used with the antibodies disclosed herein to separate B lymphocytes from mixed cell populations.

The antibodies disclosed herein can also be used to differentiate developmental subpopulations of B lymphocytes from each other. CD20 is not expressed on pro-B lymphocytes. Some expression can be found in pre-B lymphocytes. Immature, T₁ and T₂ transitional B lymphocytes express higher amounts of CD20. Mature B lymphocytes express lower levels of CD20. This information, in combination with the techniques discussed above, or others known to one of skill in the art, is useful for determining what stage of development a study population of B lymphocytes is undergoing.

Pharmaceutical Compositions.

Also provided are pharmaceutical compositions comprising the antibodies or antibody fragments of the invention. Pharmaceutical compositions of the present invention contain a pharmaceutically acceptable carrier together with one or more of the antibodies or antibody fragments described herein, dissolved or dispersed therein as an active ingredient.

As used herein, the terms "pharmaceutically acceptable" in reference to compositions, carriers, diluents and reagents, indicates that the materials are capable of administration to or upon an animal without the production of undesirable physiological effects or toxicity.

Formulation of pharmaceutical compositions is well known in the art of pharmaceutical chemistry. See, e.g. Remington's Pharmaceutical Sciences, (15th Edition, Mack Publishing Company, Easton, Pa. (1975), particularly Chapter 87, by Blaug, Seymour). Pharmaceutical compositions include
5 without limitation powders, pastes, ointments, jelly, waxes, oils, lipids, anhydrous absorption bases, oil-in-water or water-in-oil emulsions, emulsions carbowax (polyethylene glycols of a variety of molecular weights), semi-solid gels, and semi-solid mixtures containing carbowax. A typical dosage form is a sterile, isotonic, water-based solution suitable for administration by parenteral
10 (e.g., intravenous or subcutaneous) route. The concentration of the antibodies or antibody fragments of the invention in the pharmaceutical formulations can vary widely, e.g., from less than about 0.01%, 0.1%, 0.5%, 1% or 2% to as much as 5%, 10%, 20% or 50% or more by weight, and will be selected primarily by fluid volumes, viscosities, etc., in accordance with the
15 particular mode of administration selected.

The pharmaceutical compositions of the invention can also be administered via liposomes. Liposomes include emulsions, foams, micelles, insoluble monolayers, liquid crystals, phospholipid dispersions, lamellar layers and the like. In these preparations the composition of the invention to be
20 delivered is incorporated as part of a liposome, alone or in conjunction with a molecule, which binds to a desired target, such as an antibody, or with other therapeutic or immunogenic compositions. Liposomes for use in the invention are formed from standard vesicle-forming lipids, which generally include neutral and negatively charged phospholipids and a sterol, such as
25 cholesterol. The selection of lipids is generally guided by consideration of, e.g., liposome size, acid lability and stability of the liposomes in the blood stream. A variety of methods are available for preparing liposomes, as described in, e.g., Szoka et al. *Ann. Rev. Biophys. Bioeng.* 9:467 (1980), U.S. Pat. Nos. 4,235,871, 4,501,728, 4,837,028, and 5,019,369.

30 The preparation of a pharmaceutical composition that contains active ingredients dissolved or dispersed therein is well understood in the art. Typically such compositions are prepared as liquid solutions or suspension; however, solid forms suitable for solution, or suspensions, in liquid prior to use can also be prepared. The preparation can also be emulsified.

The active ingredient can be mixed with excipients that are pharmaceutically acceptable and compatible with the active ingredient and in amounts suitable for use in the methods described herein. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol or the like and combinations thereof. In addition, if desired, the composition can contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents and the like which enhance the effectiveness of the active ingredient.

The pharmaceutical composition can include pharmaceutically acceptable salts of the components therein. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the polypeptide) that are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, tartaric, mandelic and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine and the like.

Exemplary liquid carriers are sterile aqueous solutions that contain no materials in addition to the active ingredients and water, or contain a buffer such as sodium phosphate at physiological pH value, physiological saline or both, such as phosphate-buffered saline. Still further, aqueous carriers can contain more than one buffer salt, as well as salts such as sodium and potassium chlorides, dextrose, polyethylene glycol and other solutes.

Liquid compositions can also contain liquid phases in addition to and to the exclusion of water. Exemplary of such additional liquid phases are glycerin, vegetable oils such as cottonseed oil, and water-oil emulsions.

Having described the present invention, the same will be explained in greater detail in the following examples, which are included herein for illustration purposes only, and which are not intended to be limiting to the invention.

EXAMPLE 1

Materials and Methods

Generation of CD20^{-/-} Mice. DNAs encoding the 3' end of the *Cd20* gene were isolated from a 129/Sv strain mouse DNA phage library, mapped, and sequenced to identify intron/exon boundaries (**Figure 1A** and **Figure 1B**) (Tedder, et al. (1989) *J. Immunol.* 142:2560). Gene-targeting used a pBluescript SK-based vector (p594) containing a *Pst*I (exon 5) through *Eco*RV (exon 6, ~1.8 kb) DNA fragment downstream of the pMC1-HSV gene. An ~10 kb *Kpn*I DNA fragment was inserted downstream of the neomycin resistance (*Neo^r*) marker (**Figure 1C**). The plasmid was linearized using a unique *Sal*I restriction site and transfected into 129 strain-derived ES cells that were selected for using G418 according to standard methods (Koller and Smithies (1989) *Proc. Natl. Acad. Sci. USA* 86:8932). Six of 115 *Neo*-resistant ES cell colonies carried the targeted allele (**Figure 1D**). Appropriate targeting was further verified by Southern analysis of DNA digested with *Bam*HI (>12 kb fragment reduced to a 6.5 kb band), *Kpn*I (7.2 kb became 5.5 kb), and *Ssp*I (5.6 kb became 7.0 kb) using the same probe. Cells of one ES cell clone generated 80-100% chimeric male offspring that were crossed with C57BL/6 mice for ≥7 generations. Heterozygous offspring were crossed to generate homozygous CD20^{-/-} and wild-type littermates (**Figure 1E**). In most cases, results obtained using wild-type littermates of CD20^{-/-} mice and (C57BL/6 x 129)_{F1} mice were identical, therefore the results were pooled. Spleen and peritoneal cavity subset analysis was carried out using 3-10 littermates pairs at various ages so only comparisons between wild-type and CD20^{-/-} mice are valid. Mice were housed in a specific-pathogen-free barrier-facility and used at 2-3 months of age.

Knockout Mice. FcγRI^{-/-} and FcγRIII^{-/-} mice are as described (Bruhns, et al. (2003) *Immunity* 18:573-581). C57BL/6, FcγRII^{-/-} (B6.129S-Fcgr2tm1Rav), FcγRIII^{-/-} (C57BL/6-Fcgr3tm1Sjv), Beige (C57BL/6-*Lyst*^{bg/bg}), Perforin^{-/-} (C57BL/6-Pfptm1Sdz), CSF1^{op} (*Csf1*^{op}), and nude (C57BL/6-*Hfh1*^{nu}) mice were from The Jackson Laboratory (Bar Harbor, ME). FcR common γ chain (FcRγ)-deficient mice (FcRγ^{-/-}, B6.129P2-*Fcerg1*^{tm1}) were from Taconic Farms (Germantown, New York). C1q^{-/-} mice as described (Botto, et al. (1998) *Nat. Genet.* 19:56-59) were provided by Garnett Kelsoe (Duke University) with the

permission of Mark Walport (Imperial College, London, UK), LAT^{-/-} mice were from Weiguo Zhang (Duke University) as described (Zhang, et al. (1999) *Immunity* 10:323-332), and C3^{-/-} and C4^{-/-} mice were from Michael Carroll (Center for Blood Research, Boston, MA) as described (Wessels, et al. (1995) *Proc. Natl. Acad. Sci. USA* 92:11490-11494). Macrophage-deficient mice were generated by tail vein injections of clodronate encapsulated liposomes (0.1 mL/10 gram body weight; Sigma Chemical Co., St. Louis, MO) on day -2, 1 and 4 using standard methods (Van Rooijen and Sanders (1994) *J. Immunol. Methods* 174:83-93). All mice were housed in a specific pathogen-free barrier facility and first used at 2-3 months of age.

Immunofluorescence Analysis. Single-cell leukocyte suspensions were stained on ice using predetermined optimal concentrations of each antibody for 20-60 minutes using well-established methods (Zhou, et al. (1994) *Mol. Cell. Biol.* 14:3884). Cells with the forward and side light scatter properties of lymphocytes were analyzed on FACScan or FACScalibur flow cytometers (Becton Dickinson, San Jose, CA). Background staining was determined using unreactive control monoclonal antibodies (Caltag Laboratories, Burlingame, CA) with gates positioned to exclude ≥98% of the cells. Antibodies used included: CD19 monoclonal antibody (MB19-1) (Tedder, et al. (1988) *Mol. Immunol.* 25:1321; Tedder and Schlossman (1988) *J. Biol. Chem.* 263:10009; Valentine, et al. (1987) *Proc. Natl. Acad. Sci. U.S.A.* 84:8085); B220 monoclonal antibody (RA3-6B2) (DNAX Corp., Palo Alto, CA); Thy1.2 (Caltag Laboratories, Burlingame, CA); antibodies reactive with IgM, I-A, CD5, CD11b, CD23 and CD43 (BD PharMingen, Franklin Lakes, NJ); and anti-mouse IgG3, IgM and IgD antibodies (Southern Biotechnology Associates Inc., Birmingham, AL).

Antibodies. HB20-1 through HB20-6 monoclonal antibodies were generated in BALB/c mice immunized with a mouse pre-B cell line that was transfected with cDNAs encoding human CD20 using standard methods (Steeber, et al. (1997) *J. Immunol.* 159:952-963). The HB20-25 mouse anti-human CD20 monoclonal antibody was generated in CD20^{-/-} mice on a C57Bl/6 x 129 genetic background that had been immunized with the mouse

pre-B cell line 300.19 transfected with a cDNA encoding human CD20, using methods similar to those previously described (Steeber, et al. (1997) *supra*). All MB20 monoclonal antibodies were generated in CD20^{-/-} mice on a C57Bl/6 x 129 genetic background as described above.

5 Hybridomas producing CD20-specific mouse monoclonal antibodies were generated by the fusion of NS-1 myeloma cells with spleen cells from CD20^{-/-} mice immunized with murine CD20-green fluorescent protein (GFP) transfected 300.19 cells (Kearney, et al. (1979) *J. Immunol.* 123:1548). The anti-CD20 monoclonal antibodies MB20-1, -2 and -14 were of the IgG1
10 isotype; MB20-6, -11, and -16 were IgG2a; MB20-7, -8, -10 and -18 were IgG2b; and MB20-3 and -13 were IgG3 monoclonal antibodies. Chinese hamster ovary (CHO) cells and the 300.19 pre-B cell line expressing mouse CD20 fused with GFP were generated by transfecting each cell line with cDNA encoding the fused proteins (Tedder, et al. (1988) *J. Immunol.*
15 141:4388). Transfected cells were isolated by fluorescence-based cell sorting based on GFP expression.

Anti-CD20 monoclonal antibodies 1F5 (Shan, et al. (1999) *J. Immunol.* 162:6589-6595), B9E9 (Schultz, et al. (2000) *Cancer Res.* 60:6663-6669), and 1H4 (Haisma, et al. (1998) *Blood* 92:184-190) were obtained through the
20 Fifth International Workshop and Conference on Human Leukocyte Differentiation Antigens (Boston, MA; November 3-7, 1993). The B1 anti-CD20 monoclonal antibody (Stashenko, et al. (1980) *J. Immunol.* 125:1678) from Beckman-Coulter (Miami, FL) was used as purified monoclonal antibody or as diluted ascites fluid.

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Intracellular Ca²⁺ Measurements. Changes in [Ca²⁺]_i levels were monitored by flow cytometry using standard methods (Shan, et al. (1998) *Blood* 91:1644) after treating the cells with goat F(ab')₂ anti-IgM antibody (5-40 µg/mL; Cappel/ICN Pharmaceuticals, Inc., Aurora, OH), anti-mouse CD19
30 monoclonal antibody (MB19-1; 40 µg/mL), thapsigargin (1 µM; Sigma, St. Louis, MO), or ionomycin (2.67 µg/mL; CALBIOCHEM® Biosciences, Inc., La Jolla, CA). In some cases, EGTA (5 mM final) was added to the cell suspension, followed by the agents described above.

B Cell Activation Assays. Spleen B cells were purified (>93% B220⁺) by removing T cells with Thy1.2 antibody-coated magnetic beads (DYNAL[®] Inc., Lake Success, NY). For signal transduction studies, B cells were incubated (2 x 10⁷/mL) in RPMI 1640 medium containing 5% fetal calf serum at 37°C for 5 minutes before adding F(ab')₂ anti-mouse IgM antibody fragments (40 µg/mL). After adding cold saline containing 400 µM EDTA and 100 µM Na orthovanadate, the cells were detergent-lysed using well-established methods (Bradbury, et al. (1992) *J. Immunol.* 149:2841; Fujimoto, et al. (1999) *J. Immunol.* 162:7088). For CD20 structural studies, B cells were surface-biotinylated with EZ-LINK[™] Sulfo-NHS-Biotin (0.5 mg/mL; Pierce, Rockford, IL), then detergent-lysed. Cell lysates were precleared with IgG1 monoclonal antibody (1 µg) and 50 µL of a 50% suspension of Protein G-SEPHAROSE[™] (Amersham Biosciences, Piscataway, NJ), with proteins immunoprecipitated using 2 µg of monoclonal antibody and Protein G-SEPHAROSE[™]. The beads were washed twice with high- and low-salt RIPA buffers, twice with phosphate-buffered saline (PBS), boiled in sample buffer (with or without 10% 2-mercaptoethanol), electrophoresed, and transferred to nitrocellulose membranes. Blots of whole cell lysates were probed with MB20-1 monoclonal antibody, peroxidase-conjugated 4G10 antibody (Upstate Biotechnology, Lake Placid, NY) or with anti-phospho-CD19 (Y513), -PLCγ (Y783), -Syk (Y525/Y526), -BTK (Y223), -Src family kinase antibodies (Cell Signaling Technology, Inc., Beverly, MA), or anti-active MAPK antibody (PROMEGA[®], Madison, WI). The membranes were stripped and reprobed with a rabbit polyclonal anti-SHP-1 antibody (Upstate Biotechnology), or anti-Lyn (lyn-44), anti-Fyn (Fyn3) and anti-ERK2 (C-14) antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Biotinylated proteins or antibodies were detected using streptavidin-conjugated horseradish peroxidase (Southern Biotechnology Assoc., Birmingham, AL) and an enhanced chemiluminescence kit (ECL[™]; Pierce, Rockland, IL).

For studies of CD20 phosphorylation, primary B cells (10⁷/mL) were cultured with lipopolysaccharide (LPS) (*E. coli* serotype 0111:B4, 10 µg/mL, Sigma, St. Louis, MO) for 48 hours. Primary B cells and cell lines were then cultured in phosphate-free media for 1 hour, cultured in medium containing

200 $\mu\text{Ci/mL}$ [^{32}P]orthophosphate (PerkinElmer, Boston, MA) for 90 minutes, washed, lysed, immunoprecipitated and separated by SDS-PAGE, with autoradiography conducted in accordance with standard methods (Kansas and Tedder (1991) *J. Immunol.* 147:4094; Leveille, et al. (1999) *Eur. J. Immunol.* 29:65).

Functional Assays. Spleen B cell proliferation was measured by standard methods of [^3H]thymidine incorporation (Engel, et al. (1995) *Immunity* 3:39). Eight-week old mice were immunized with 2,4-dinitrophenol-conjugated keyhole limpet hemocyanin (100 μg , DNP-KLH; CALBIOCHEM[®]-Novabiochem, La Jolla, CA) or were immunized twice with (4-hydroxy-3-nitrophenyl acetyl) conjugated to chicken gammaglobulin (50 μg , NP₁₈-CGG) precipitated in alum according to well-known methods (Jacob, et al. (1991) *J. Exp. Med.* 173:1165). Serum DNP- and NP-specific antibody levels were measured by ELISA (Engel, et al. (1995) *Immunity* 3:39; Takahashi, et al. (1998) *J. Exp. Med.* 187:885), with the relative affinity/avidity of antibody responses assessed using standard methods (Takahashi, et al. (1998) *J. Exp. Med.* 187:885).

Immunotherapy. Sterile anti-mouse CD20 and isotype control monoclonal antibodies (0.5-250 μg) in 200 μL PBS were injected through lateral tail veins. All experiments used 250 μg of monoclonal antibody unless indicated otherwise. Blood and spleens were collected 1 hour and 2, 4, 7, 28, 48, 50, 52, 54, 56 or 58 days after treatment. Blood leukocyte numbers were quantified by hemocytometer following red cell lysis, with B220⁺ B cell frequencies determined by immunofluorescence staining with flow cytometry analysis. Antibody doses in humans and mice (Table 7) were compared using the Oncology Tool Dose Calculator.

C Assays. WT mouse spleen B cells were purified (>93% B220⁺) by T cell removal using Thy-1.2 monoclonal antibody-coated magnetic beads (DYNAL[®], Lake Success, NY). Quantification of C-mediated B cell killing *in*

vitro was according to standard methods (Gazzano-Santoro, et al. (1997) *J. Immunol. Methods* 202:163-171). Spleen B cells were incubated with each anti-CD20 monoclonal antibody (0.5 µg/mL) and baby rabbit C (diluted 50-fold; GIBCO-BRL, Grand Island, NY) for 2 hours at 37°C. PBS was added to
 5 each tube with incubation for 4 hours at 37°C, before the cells were washed, stained with propidium iodide (PI) and anti-B220 monoclonal antibody, with propidium iodide exclusion determined by flow cytometry analysis.

Heavy and Light Chain Gene Utilization. Cytoplasmic RNA was
 10 extracted from $1-10 \times 10^5$ hybridoma cells using the RNEASY® Mini Kit (QIAGEN®, Chatsworth, CA). First-strand cDNA was synthesized from cytoplasmic RNA using oligo-dT primers (dT₁₈) and a SUPERScript™ Kit (Gibco BRL, Gaithersburg, MD). One µL of cDNA solution was used as template for PCR amplification of V_H genes. PCR reactions were carried out in
 15 a 100-µL volume of a reaction mixture composed of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 200 µM dNTP (Perkin Elmer, Foster City, CA), 50 pmol of each primer, and 5 U of Taq DNA polymerase (ISC Bioexpress, Kaysville, UT). Amplification was for 30 cycles (94°C for 1 minute, 58°C for 1 minute, 72°C for 1 minute; Thermocycler, Perkin Elmer). V_H genes were
 20 amplified using a promiscuous sense 5' V_H primer (MsV_HE; 5' GGG AAT TCG AGG TGC AGC TGC AGG AGT CTG G 3'; SEQ ID NO:110) well-known in the art (Kantor, et al. (1996) *J. Immunol.* 158:1175-1186) and antisense primers complementary to the C_µ coding region (primer C_µ-in; 5' GAG GGG GAA GAC ATT TGG GAA GGA CTG 3'; SEQ ID NO:111), the C_γ region
 25 (primer C_γ1; 5' GAG TTC CAG GTC ACT GTC ACT GGC 3'; SEQ ID NO:112) or the C_α region (primer C_α; 5' GTG AAT TCA GGC GGC CGC TAA 3'; SEQ ID NO:113). Light chain cDNA was amplified using a sense V_k primer (Table 1) and a C_k antisense primer (5' ACT GGA TGG TGG GAA GAT G 3'; SEQ ID NO:114). Amplified PCR products were purified from agarose gels using the
 30 QIAQUICK® gel purification kit (QIAGEN®) and were directly sequenced in both directions using an ABI 377 PRISM® DNA sequencer after amplification using the Perkin Elmer Dye Terminator Sequencing system with AMPLITAQ® DNA polymerase and the same primers for initial PCR amplification. All V_H

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and light chain regions were completely sequenced on both the sense and anti-sense DNA strands (Figure 14 and Figure 17).

TABLE 1

Antibody	Ig Isotype	V _H Chain	D _H Chain	J _H Chain	HC Identity
1F5	γ2a	1S121*01	L16	J2	
B9E9	γ2a	1S121*01	Q52	J1	
1H4	γ1/γ2a/γ2b	1S121*01	L16	J1	
2H7	γ2b	1S121*01	SP2	J1	
2B8	γ1	1S121*01	L16	J1	
Leu-16 [†]	γ1				
HB20-1(=2=6)	M	1S121*01	L16	2	
HB20-3	G2b	1S121*01	L16	4	
HB20-4	G2b	1S121*01	L16	4	
HB20-5	M	1S121*01	L16	2	
HB20-25	G2a	1S121*01	L16	4	
MB20-1	G1	5S11*02	Q52	4	1=13
MB20-2	G1	1S59*01	Q52	3	
MB20-3	G3				
MB20-6	G2a				
MB20-7	G2b	1S59*01	Q52	3	
MB20-8	G2b	1S59*01	Q52	3	#
MB20-10	G2b	1S59*01	Q52	3	#
MB20-11	G2a	1S59*01		4	
MB20-13	G3	5S11*02	Q52	4	1=13
MB20-14	G1	1S59*01	Q52	3	
MB20-16	G2a	1S59*01	SP2	2	
MB20-18	G2b	1S59*01	Q52	3	

Antibody	V _L Chain	D _L Chain	J _L Chain	LC Identity	Family (HL)
1F5	4-72*01	5*01			AA
B9E9	4-72*01	5*01			AA
1H4	4-72*01	5*01			AA
2H7	4-72*01	5*01			BA
2B8	4-72*01	1*02			AA
Leu-16 [†]		1*02			AA
HB20-1(=2=6)	6-15*01	4*01		VK7	AE
HB20-3	12-41*02	1*01		VK8	CC
HB20-4	12-41*02	1*01		VK8	CC
HB20-5	6-15*01	4*01		VK7	DE
HB20-25	12-46*01	1*01		VK8	CC
MB20-1	8-27*01	4*01		VK7	GG
MB20-2	4-91*01	5*01	2=14	VK5	EB
MB20-3	4-91*01	5*01		VK5	-B

MB20-6					
MB20-7	4-91*01	5*01		VK5	EB
MB20-8	4-91*01	5*01	#	VK5	EB
MB20-10	4-91*01	5*01	#	VK5	EB
MB20-11				VK5	F-
MB20-13	4-91*01	5*01		VK5	GB
MB20-14	4-91*01	5*01	2=14	VK5	EB
MB20-16					F-
MB20-18	6-32*01	5*01		VK7	EF

[†] Leu-16 is also known as L27.

8 and 10 are identical in amino acid sequence and differ by only one base pair in the heavy chain and three base pairs in the light chain.

- 5 CDR sequences for heavy and light chain regions of anti-human and anti-mouse CD20 monoclonal antibodies are listed in Table 2 and Table 3, respectively.

TABLE 2

Antibody	CDR1	CDR2	CDR3
HB20-1,2,6	SYNMH SEQ ID NO:57	AIYPGNGDTSYNQKFKG SEQ ID NO:65	WDYYGSSYVGFFDY SEQ ID NO:75
HB20-03	NYNMH SEQ ID NO:58	AIYPENGDTSYNQKFKG SEQ ID NO:66	FYYYGSYYGAMDY SEQ ID NO:76
HB20-04	NYNMH SEQ ID NO:58	AIYPENGDTSYNQRFKG SEQ ID NO:67	FYYYGSYYGALDY SEQ ID NO:77
HB20-05	SYNMH SEQ ID NO:57	AIYPGNGDTSYNQKFKG SEQ ID NO:65	WDYYGSSYVGFLTT SEQ ID NO:78
HB20-25	NYNLH SEQ ID NO:59	AIYPGNGGETSYNQKFKG SEQ ID NO:68	FYYYGSSYGAMDY SEQ ID NO:79
MB20-1,13	DYGMA SEQ ID NO:60	FISNLAYSIIYADTVTG SEQ ID NO:69	TGYIALFDY SEQ ID NO:80
MB20-02	DYYIK SEQ ID NO:61	DINPNNGDTIYNQKFKG SEQ ID NO:70	ERFAY SEQ ID NO:81
MB20-07	DYYMK SEQ ID NO:62	DINPNNGDTTYNQKFEG SEQ ID NO:71	ERFAY SEQ ID NO:81
MB20-8,10	DYYMK SEQ ID NO:62	DINPNNGDIIYNQKFEG SEQ ID NO:72	ERFAY SEQ ID NO:81
MB20-11	DYNMH SEQ ID NO:63	YIAPYNGGTTYNQKFKG SEQ ID NO:73	ALDY SEQ ID NO:82
MB20-14	DYYIK SEQ ID NO:61	DINPNNGDTIYNQKFKG SEQ ID NO:70	ERFAY SEQ ID NO:81
MB20-16	DYNLH SEQ ID NO:64	YINPNNGGATYNQKFTG SEQ ID NO:74	IYDGY SEQ ID NO:83
MB20-18	DYYMK SEQ ID NO:62	DINPNNGDIIYNQKFEG SEQ ID NO:72	ERFAY SEQ ID NO:81

TABLE 3

Antibody	CDR1	CDR2	CDR3
HB20-1,2,6	KASQNVGTNVA SEQ ID NO:84	SASYRNS SEQ ID NO:94	QQYNSSPFT SEQ ID NO:101

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HB20-03	RASGNIHNYLA SEQ ID NO:85	NAKTLAD SEQ ID NO:95	QHFWSTPWT SEQ ID NO:102
HB20-04	RASGSIHNYLA SEQ ID NO:86	NAKTLAD SEQ ID NO:95	QHFWSSIPWT SEQ ID NO:103
HB20-05	KASQNVGTNVA SEQ ID NO:84	SASYRYS SEQ ID NO:96	QQYNSSPFT SEQ ID NO:101
HB20-25	RASENIYSNLA SEQ ID NO:87	AATNLAD SEQ ID NO:97	QHFWGIPWT SEQ ID NO:104
MB20-01	KSSQSVLYSSKRKNYLA SEQ ID NO:88	WASTRES SEQ ID NO:98	HQYLSSFT SEQ ID NO:105
MB20-2,14	SVSSSIRSNYLH SEQ ID NO:89	RTSNLAS SEQ ID NO:99	QQGSSIPLT SEQ ID NO:106
MB20-03	SASSSISSNYLH SEQ ID NO:90	RTSNLAS SEQ ID NO:99	QQGSSIPLT SEQ ID NO:106
MB20-07	SVSSSIRSNYLH SEQ ID NO:89	RTSNLAS SEQ ID NO:99	QQGSSLPLT SEQ ID NO:107
MB20-8,10	SVSSNIRSNYLH SEQ ID NO:91	RTSNLAS SEQ ID NO:99	QQGSSIPLT SEQ ID NO:106
MB20-13	SASSSITSNYLH SEQ ID NO:92	RTSNLAS SEQ ID NO:99	QQGSSKTLT SEQ ID NO:108
MB20-18	KASQTVTNDLA SEQ ID NO:93	YASNRYT SEQ ID NO:100	QQDYSSPLT SEQ ID NO:109

Antibody Sequence Alignments. The heavy and light chain sequences from known hybridomas producing anti-CD20 monoclonal antibodies were: 1F5 (Shan, et al. (1999) *J. Immunol.* 162:6589-6595), B9E9 (Schultz, et al. (2000) *Cancer Res.* 60:6663-6669), 2H7 (U.S. Patent No. 6,120,767), 2B8 (U.S. Patent No. 5,843,439), 1H4 (Haisma, et al. (1998) *Blood* 92:184-190), Leu-16 (Wu, et al. (2001) *Protein Eng.* 14:1025-1033).

Statistical analysis. All data are shown as means $\pm$ SEM. The Student's *t*-test was used to determine the significance of differences between population means.

EXAMPLE 2

Generation of CD20^{-/-} mice

The targeting vector replaced exons encoding part of the second extracellular loop, the fourth transmembrane domain, and the large carboxyl-terminal cytoplasmic domain of CD20 with a neomycin resistance gene (**Figure 1A-1D**). Mice homozygous for *Cd20* gene disruption were obtained at the expected Mendelian frequency by crossing heterozygous offspring of founder mice generated using targeted ES cells. Southern blot and PCR analysis of genomic DNA from homozygous offspring further verified

appropriate *Cd20* gene targeting and the genomic deletion of exons 6-8 (Figure 1E and Figure 1F). Wild-type CD20 mRNA was absent in CD20^{-/-} mice as confirmed by PCR amplification of cDNA generated from splenocytes of CD20^{-/-} mice (Figure 1G). A fused *CD20-Neo^r* gene transcript was
 5 detected at low levels in CD20^{-/-} mice by PCR, which translated into an aberrant CD20 peptide truncated at amino acid 157 that was fused with an 88 amino acid peptide encoded by the *Neo^r* gene promoter sequence. Absence of cell-surface CD20 protein expression in CD20^{-/-} mice was verified using a
 10 panel of twelve mouse anti-mouse CD20 monoclonal antibodies that were reactive with 300.19 and CHO cells transfected with CD20-GFP cDNA, but not with untransfected cells (Figure 1H). These monoclonal antibodies reacted with cell-surface CD20 epitopes expressed by CD19⁺ splenocytes from wild-type mice, but not from CD20^{-/-} mice (Figure 1I). Therefore, the targeted *Cd20* gene mutation abrogated cell-surface CD20 expression.

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EXAMPLE 3

B Cell Development in CD20^{-/-} Mice

CD20^{-/-} mice thrived and reproduced as well as their wild-type littermates over nine years of observation and did not present any obvious
 20 anatomical or morphological abnormalities, or susceptibility to infections during the first year of life. CD20^{-/-} mice had normal frequencies of IgM⁻ B220^{lo} pro/pre-B cells, IgM⁺ B220^{lo} immature B cells and IgM⁺ B220^{hi} mature B cells (Figure 1J, Table 4) and normal numbers of AA4.1⁺ or heat stable antigen (HSA)^{hi} B220^{lo} immature/transitional B cells in their bone marrow.
 25 Numbers of blood, spleen and lymph node IgM⁺ B220⁺ B cells were not significantly different between CD20^{-/-} mice and their wild-type littermates (Table 4).

TABLE 4

		% of B lymphocytes		B cell numbers (x10 ⁻⁶) ^b		IgM levels in CD20 ^{-/-} mice
Tissue	Phenotype	Wild-type	CD20 ^{-/-}	Wild-type	CD20 ^{-/-}	% of wild-type

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Bone Marrow	IgM ⁻ B220 ^{lo}	28 ± 3	27 ± 3			
	IgM ⁺ B220 ^{lo}	14 ± 2	11 ± 1			62 ± 3**
	IgM ⁺ B220 ^{hi}	13 ± 1	17 ± 2			93 ± 7
Blood ^c	IgM ⁺ B220 ⁺	63 ± 1	67 ± 2	3.0 ± 0.3	3.7 ± 0.4	69 ± 11*
Spleen	IgM ⁺ B220 ⁺	46 ± 4	50 ± 4	50 ± 8	66 ± 6	78 ± 6**
	IgM ^{hi} B220 ^{lo}	4 ± 2	1 ± 1*	2.0 ± 0.3	0.8 ± 0.3*	
	CD21 ^{lo} HSA ^{hi}	17 ± 1	22 ± 2*	7.4 ± 0.8	11.2 ± 1.4	
	CD21 ^{hi} HSA ^{int}	14 ± 2	9 ± 1*	6.1 ± 1.2	4.5 ± 0.4	
	CD1d ^{hi} CD21 ⁺	5 ± 1	4 ± 1	2.6 ± 0.6	1.8 ± 0.3	
Lymph Node ^d	IgM ⁺ B220 ⁺	21 ± 4	20 ± 1	1.0 ± 0.2	1.4 ± 0.3	88 ± 10
Peritoneum	IgM ⁺ B220 ⁺	73 ± 3	63 ± 5	1.1 ± 0.2	1.5 ± 0.2	84 ± 6
	CD5 ⁺ B220 ^{lo}	45 ± 3	16 ± 5**	0.8 ± 0.1	0.4 ± 0.1**	
	CD11b ⁺ CD5 ⁻ B220 ^{lo}	12 ± 1	12 ± 1	0.3 ± 0.1	0.3 ± 0.2	
	CD5 ⁻ B220 ^{hi}	28 ± 1	55 ± 3**	0.5 ± 0.1	1.1 ± 0.2*	

^a Values represent mean (± SEM) numbers or percentages of lymphocytes (based on side and forward light scatter properties) expressing the indicated cell surface markers from 3-10 wild-type and CD20^{-/-} 2-month-old littermates.

5 ^b B cell numbers were calculated based on total numbers of cells harvested from each tissue.

^c Values indicate numbers of cells/mL.

^d Values for pairs of inguinal lymph nodes.

* Sample means were significantly different from wild-type littermates, $p < 0.05$; ** $p < 0.01$.

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15 B cell IgM expression was significantly lower in CD20^{-/-} mice relative to immature and mature B cells of wild-type littermates (**Table 4, Figure 1J**). In addition, there was an ~50% reduction in numbers of IgM^{hi} B220^{lo} B cells in the spleens of CD20^{-/-} littermates. Decreased numbers of IgM^{hi} B220^{lo} B cells may reflect reduced IgM expression by most B cells, but was not attributable to a loss in spleen marginal zone B cells since the number of cells with a CD1d^{hi}CD21⁺ phenotype was not significantly different between CD20^{-/-} and wild-type littermates (**Table 4**). Likewise, numbers of transitional T1 (CD21^{lo}HSA^{hi}) and T2 (CD21^{hi}HSA^{int}) B cells, which represent recent

emigrants from the bone marrow, were not reduced (**Table 4**). Rather, the frequency and number of T1 cells was usually higher in CD20^{-/-} mice, similar to the increase in frequency of mature IgM⁺B220^{hi} B cells observed in bone marrow of CD20^{-/-} mice. Decreased numbers of IgM^{hi} B220^{lo} B cells may be attributable in part to a reduction in spleen B1 cells since there was a 64% decrease in the number of CD5⁺ B220^{lo} B1a cells within the peritoneal cavity of CD20^{-/-} mice. The overall number of IgM⁺ B220⁺ B cells in the peritoneum of CD20^{-/-} and wild-type littermates were similar due to an increase in the number of CD5⁻ B220^{hi} B cells (**Table 4, Figure 1J**). The number of B1b B cells (CD11b⁺ CD5⁻ B220^{lo}) was similar in CD20^{-/-} and wild-type littermates (**Table 4**). There were no obvious differences in the size (light scatter properties) of CD20^{-/-} B cells isolated from bone marrow, blood, lymph nodes or spleen when compared with B cells from wild-type littermates. An immunohistochemical analysis of spleen tissue sections revealed an otherwise normal architecture and organization of B220⁺ B cells. Therefore, with the exception of decreased IgM expression, a reduction in the IgM^{hi} B220^{lo} B cell subset in the spleen, and low numbers of B1 cells within the peritoneal cavity, CD20 expression was not an obligate requirement for B cell development and tissue localization.

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EXAMPLE 4

CD20^{-/-} B cell function

The proliferative response of purified CD20^{-/-} B cells to surface IgM ligation was comparable to wild-type B cells over a range of antibody concentrations (1-40 µg/mL; **Figure 1K**). Proliferation was also normal when the B cells were activated by LPS (**Figure 1K**) over a range of concentrations (0.1-10 µg/mL) or using IL-4 (10-100 U/mL) plus anti-IgM antibody at a suboptimal (5 µg/mL) concentration. Thus, CD20 loss had no detectable effect on mitogen-induced proliferation. Normal levels of all Ig isotypes were found in sera from CD20^{-/-} mice (**Figure 1L**). CD20^{-/-} mice also generated primary and secondary antibody responses of all isotypes that were similar to those observed in wild-type littermates following immunization with a T cell-dependent antigen, DNP-KLH (**Figure 1M**). In addition, CD20^{-/-} mice and

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their wild-type littermates generated equivalent primary and secondary IgM and IgG1 anti-NP antibody responses following immunization with NP-CGG (5 mice for each group). Moreover, the affinities of primary and secondary IgG1 anti-NP antibody responses generated in CD20^{-/-} mice were similar to those generated in their wild-type littermates. Therefore, CD20 function was not required for T-B cell interactions, isotype switching or affinity maturation during the generation of humoral immune responses.

EXAMPLE 5

10 CD20 Expression During B cell Development

Using the panel of mouse anti-mouse CD20 monoclonal antibodies, two mouse pre-B cell lines (300.19 and 38B9) and two T cell lines (BW5147 and BL4) failed to express CD20 cell surface protein, while the 70Z pre-B line, A20 and AJ9 mature B cell lines and NS-1 plasmacytoma line were CD20⁺ (Figure 1H and Figure 2A). Similarly, CD20 was only expressed by subsets of B220⁺ cells in the bone marrow (Figure 2B); 30±3% of B220^{lo} lymphocytes were CD20⁺, while all B220^{hi} B cells were CD20⁺ (n=6 mice). A similar fraction of CD19⁺ B cells in the bone marrow were CD20⁺ (51±2%, n=6). Consistent with this, CD43⁺ B220⁺ pro-B cells did not express CD20, while 10±1% (n=3) of CD43⁻ IgM⁻ B220^{lo} pre-B cells expressed CD20 at low densities (Figure 2G). All CD20⁺ pre-B cells (CD43⁻ IgM⁻ B220^{lo}) were small based on their light scatter properties, indicating that CD20 expression was primarily initiated at or near the time of heavy chain expression. Consistent with this, the majority of immature IgM⁺ B220^{lo} B cells expressed CD20 (76±9%, n=3; fraction I, Figure 2G). A subpopulation of immature IgM^{hi} B220⁺ (fraction II, Figure 2G) or CD19^{lo} B cells in the bone marrow expressed CD20 at 277±53% (n=3) higher densities than mature B220^{hi} (fraction III, Figure 2G) or CD19^{hi} B cells (Figure 2B). Thus, CD20 is first expressed during the small pre-B cell to immature B cell transition, with CD20 expression increasing with maturation and then decreasing with entry into the mature B220^{hi} pool of recirculating B cells.

In the spleen, blood, peripheral lymph nodes and peritoneal cavity, the vast majority of IgM⁺ or B220⁺ B cells expressed CD20 (Figure 2C-2F). A

small subpopulation of CD20^{hi} B220^{lo} cells was observed among blood (9±1%, n=3) and spleen (7±2%, n=3) B cells (**Figure 2C** and **Figure 2E**). The CD20^{hi} B220^{lo} B cells in the spleen were predominantly transitional T1 and T2 B cells (**Figure 2H**), and are likely to represent recent emigrants from the bone marrow. T1 cells (CD21^{lo} HSA^{hi}) expressed CD20 at 139±23% (n=3) higher densities than mature B cells, while T2 cells (CD21^{hi} HSA^{hi}) expressed CD20 at 58±11% (n=3) higher densities. T1 cells (CD21⁻ CD23⁻ IgM^{hi}) and marginal zone B cells (CD21⁺ CD23⁻ IgM^{hi}) (Loder, et al. (1999) *J. Exp. Med.* 190:75) also expressed CD20 at levels higher than the majority of spleen B cells (**Figure 2I**). Small numbers of CD20⁻ peripheral B cells were observed in some mice, but this number was typically <2% of B220⁺ cells. In the peritoneal cavity, CD20 was expressed similarly by both CD5⁺ and CD5⁻ B cells (**Figure 2F**). CD20 was not expressed at detectable levels by other subpopulations of leukocytes in any of the tissues examined. Thus, mouse CD20 was expressed exclusively by B cells with expression initiated late during small pre-B cell maturation.

EXAMPLE 6

Structural Characteristics of CD20

Mouse and human CD20 were compared by precipitating these molecules from surface-labeled B cell lines using the MB20-1 monoclonal antibody reactive with mouse CD20 and the PB4 monoclonal antibody reactive with a cytoplasmic epitope of human CD20. Mouse CD20 migrated faster than human CD20 under non-reducing conditions, but also migrated as at least two distinct molecular species with M_r of 33,000 and 35,000 (**Figure 3A**). Under reducing conditions, mouse CD20 migrated as at least two equally represented molecular species with M_r of 40,000 and 42,000 (**Figure 3A**). Multiple cell-surface molecules coprecipitated with mouse CD20, as occurs with human CD20 (Tedder, et al. (1988) *Molec. Immunol.* 25:1321; Deans, et al. (1993) *J. Immunol.* 151:4494). The PB4 monoclonal antibody coprecipitates molecules associated with human CD20 better than monoclonal antibodies that react with CD20 extracellular domains. Coprecipitation of CD20-associated molecules in mouse was not due to

monoclonal antibody cross-reactivity since the MB20-1 monoclonal antibody only reacted with mouse CD20 in western blot analysis and CD20 or other proteins were not precipitated from lysates of CD20^{-/-} B cells (**Figure 3B**). Unexpectedly, mouse CD20 was not a dominant phosphoprotein in resting
 5 primary mouse B cells, in anti-IgM antibody- or LPS-activated B cells, or B cell lines, even after phorbol myristyl acetate (PMA) treatment (**Figure 3C**), as it is in human B cells (Tedder and Schlossman (1988) *J. Biol. Chem.* 263:10009; Genot, et al. (1993) *J. Immunol.* 151:71). Furthermore, PMA-induced phosphorylation of CD20 in LPS-blasts or B cell lines did not lead to a
 10 significant shift in CD20 protein M_r from the faster species to the slower species as characterizes human CD20 (Tedder and Schlossman (1988) *J. Biol. Chem.* 263:10009; Valentine, et al. (1987) *Proc. Natl. Acad. Sci. U.S.A.* 84:8085). Thus, mouse and human CD20 share many structural features, with several distinct characteristics.

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EXAMPLE 7

Reduced [Ca²⁺]_i Responses in CD20^{-/-} B Cells

Despite normal B cell development in CD20^{-/-} mice, splenic B220⁺ B cells from CD20^{-/-} mice generated reduced [Ca²⁺]_i responses following IgM
 20 ligation with optimal (40 µg/mL; **Figure 4A**) and suboptimal concentrations (5 µg/mL) of anti-IgM antibodies when compared with wild-type B cells. The kinetics of the immediate [Ca²⁺]_i response was not altered in CD20^{-/-} B cells. However, the magnitude of the maximal [Ca²⁺]_i increase was 34±4% lower (p<0.001, n=9) in CD20^{-/-} B cells, with the level of the sustained increase
 25 observed at later time points reduced similarly. Chelation of extracellular Ca²⁺ with EGTA reduced the kinetics and magnitude of the [Ca²⁺]_i increase observed following IgM crosslinking on CD20^{-/-} and wild-type B cells. The maximal magnitude of the [Ca²⁺]_i response in the presence of EGTA was 38±7% lower (p<0.002, n=7) in CD20^{-/-} B cells relative to wild-type B cells.
 30 CD19-induced [Ca²⁺]_i responses were significantly lower (70±4%, p<0.001, n=5) for CD20^{-/-} B cells relative to wild-type B cells (**Figure 4B**). Lower [Ca²⁺]_i responses did not result from decreased CD19 expression by

CD20^{-/-} B cells (**Figure 4D**). Chelation of extracellular Ca²⁺ with EGTA mostly eliminated CD19-induced [Ca²⁺]_i responses in both wild-type and CD20^{-/-} B cells. Reduced [Ca²⁺]_i responses following IgM- or CD19-ligation by CD20^{-/-} B cells were not likely to result from differences in internal Ca²⁺ stores or extracellular Ca²⁺ concentrations since thapsigargin- and ionomycin-induced [Ca²⁺]_i responses were slightly higher on average in CD20^{-/-} B cells than in wild-type B cells (**Figure 4C**). The decrease in [Ca²⁺]_i responses in CD20^{-/-} B cells were also unlikely to result from differences in genetic backgrounds. CD20^{-/-} mice and their wild-type littermates were generated from 129 strain ES cells, but were backcrossed with C57BL/6 mice for at least seven generations. In control experiments, IgM-induced and CD19-induced [Ca²⁺]_i responses were similar, if not identical for C57BL/6, (C57BL/6 x 129)_{F1} and 129 B cells (n=4). Therefore, reduced [Ca²⁺]_i responses in CD20^{-/-} mice were likely to result from the absence of CD20 function, rather than background differences. Since [Ca²⁺]_i responses observed following CD19 cross-linking were primarily dependent on transmembrane Ca²⁺ flux and CD19-induced [Ca²⁺]_i responses were significantly perturbed in CD20^{-/-} mice, CD20 function may be particularly important for transmembrane Ca²⁺ transport.

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EXAMPLE 8

Signal Transduction in CD20^{-/-} B Cells

The effect of CD20 loss on B cell transmembrane signal transduction was evaluated by assessing total cellular protein tyrosine phosphorylation in purified B cells following IgM ligation. Overall levels of tyrosine phosphorylation were similar in resting splenic B cells from CD20^{-/-} and wild-type littermates, although some variation was observed between B cells from individual mice in individual experiments (**Figure 5A**). Protein tyrosine phosphorylation after IgM ligation was also similar in B cells from CD20^{-/-} and wild-type littermates. Phosphorylation of individual signaling molecules downstream of IgM, including Lyn and other Src kinases, PLCγ, CD19, BTK, and MAP kinase, was also similar in B cells from CD20^{-/-} and wild-type

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littermates (**Figure 5B**). Thus, CD20-deficiency was unlikely to significantly alter basal or IgM-induced transmembrane signaling.

EXAMPLE 9

5 Anti-CD20 Monoclonal Antibody Depletion of B cells *in vivo*

Twelve mouse anti-mouse CD20 monoclonal antibodies, with representatives of each IgG isotype, were assessed for their ability to bind B cells and deplete them *in vivo*. Each monoclonal antibody reacted uniformly with CD19⁺ primary B cells *in vitro* with characteristic mean fluorescence
 10 intensities that were independent of monoclonal antibody isotype (**Figure 6A**). When monoclonal antibody reactivity with primary B cells was assessed over a range of monoclonal antibody concentrations, most monoclonal antibodies reached saturating levels of staining when used at concentrations between 1-10 µg/mL (**Figure 6B**). On average, 50%-maximal log monoclonal antibody
 15 staining was achieved at monoclonal antibody concentrations of ~0.5 µg/mL (arrows, **Figure 6B**). When all monoclonal antibodies were used at 0.5 µg/mL, each monoclonal antibody reacted uniformly with CD19⁺ primary B cells with characteristic low to high mean fluorescence intensities (**Figure 6C, Table 5**). Similar results were obtained using a mouse CD20 cDNA-transfected pre-B
 20 cell line with anti-mouse Ig secondary antibody. Based on this analysis, the MB20-1 monoclonal antibody represented monoclonal antibodies with the lowest relative affinity/avidity, while the MB20-18 monoclonal antibody reacted strongly with B cells and stained B cells at the highest levels of all 12 anti-CD20 monoclonal antibodies (**Table 5**). Thus, each monoclonal antibody
 25 reacted specifically with B cells and displayed reasonable binding characteristics as assessed by flow cytometry.

TABLE 5

Isotype	Ab	B cell reactivity ^a	% <i>in vivo</i> depletion ^b	
			Blood	Spleen
IgG1	MB20-1	69	95±3	93±3
	MB20-2	209	88±1	67±8

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	MB20-14	166	94±4	77±8
IgG2a	MB20-6	96	99±1	93±5
	MB20-11	158	98±1	92±2
	MB20-16	170	99±1	95±0
IgG2b	MB20-7	525	82±9	36±36
	MB20-8	240	94±1	3±18
	MB20-10	317	91±1	3±9
	MB20-18	729	96±1	74±3
IgG3	MB20-3	47	1±1	1±3
	MB20-13	603	18±1	3±1

^a Values represent the mean linear fluorescence intensity for immunofluorescence staining of spleen CD19⁺ B cells with 0.5 µg/mL of each MB20 monoclonal antibody (**Figure 6C**). Splenocyte staining was visualized using isotype-specific secondary antibodies. Control staining was ≤6 in all cases.

^b Values (±SEM) indicate the % of B220⁺ B cells depleted from blood or spleen 7 days after monoclonal antibody treatment ($n \geq 3$) compared with isotype-matched control monoclonal antibodies.

Each anti-mouse CD20 monoclonal antibody was given to mice at 250 µg/mouse, a single dose equivalent to a dose ~10-fold lower (**Table 7**) than the 375 mg/m² dose primarily given four times for anti-CD20 therapy in humans (Press, et al. (2001) *Hematology*:221-240; Kaminski, et al. (1993) *N. Engl. J. Med.* 329:459-465; Weiner (1999) *Semin. Oncol.* 26:43-51; Onrust, et al. (1999) *Drugs* 58:79-88; McLaughlin, et al. (1998) *Oncology* 12:1763-1769). Under these conditions, multiple monoclonal antibodies had potent and long-lasting effects on peripheral B cell numbers, while other monoclonal antibodies had heterogeneous *in vivo* effects (**Figure 7**). The effectiveness of monoclonal antibody-induced B cell depletion from the circulation by day 2 and spleen by day 7 correlated closely with monoclonal antibody isotype (**Table 5, Figure 7A and Figure 7B**), with IgG2a>IgG1>IgG2b>IgG3. MB20-11 and other IgG2a monoclonal antibodies (MB20-6 and -16) depleted >95% of blood B cells and ~93% of splenic B cells. The few remaining peripheral B cells primarily represented phenotypically immature cells emerging from the bone marrow. The MB20-11 monoclonal antibody depleted significant

numbers of circulating B cells when given as a single dose as low as 0.5 $\mu\text{g}/\text{mouse}$, while significant depletion of spleen B cells by d 7 required a 5-fold higher mAb dose of 2.5 $\mu\text{g}/\text{mouse}$ (**Figure 7C**). Equally striking was the finding that a single injection of MB20-11 monoclonal antibody depleted circulating B cells within 1 hour of monoclonal antibody treatment, with a durable effect for ~57 days before B cells began to repopulate the circulation and spleen (**Figure 7D**). By contrast, none of the monoclonal antibodies had significant effects when given to $\text{CD20}^{-/-}$ mice and isotype-control monoclonal antibodies given under identical conditions did not affect B cell numbers (**Figure 7**). Likewise, circulating and tissue Thy1.2^{+} T cell numbers were unchanged in anti-CD20 monoclonal antibody-treated mice (**Figure 7A**), consistent with B cell-restricted CD20 expression.

EXAMPLE 10

15 Role for Fc γ R in B Cell Depletion

The role of the innate immune system in B cell depletion by anti-CD20 monoclonal antibody treatment was assessed using Fc γ R-deficient mice (Takai, et al. (1994) *Cell* 76:519-529). Mouse effector cells express three different Fc γ R classes for IgG, the high-affinity Fc γ RI (CD64), and the low-affinity Fc γ RII (CD32) and Fc γ RIII (CD16) molecules (Ravetch and Clynes (1998) *Ann. Rev. Immunol.* 16:421-432). Fc γ RI and Fc γ RIII are hetero-oligomeric complexes in which the respective ligand-binding γ chains associate with a common γ chain (Fc γ R γ). Fc γ R γ chain expression is required for Fc γ R assembly and for Fc γ R triggering of effector functions, including phagocytosis by macrophages and cytotoxicity by NK cells (Takai, et al. (1994) *Cell* 76:519-529). High-affinity Fc γ RI preferentially binds monomeric IgG2a > IgG2b > IgG3/IgG1, while the two low-affinity receptors bind polymeric IgGs of different isotypes (Fossati-Jimack, et al. (2000) *J. Exp. Med.* 191:1293-1302). Fc γ RIII binds IgG2a>IgG1>IgG2b>> IgG3 (Fossati-Jimack, et al. (2000) *J. Exp. Med.* 191:1293-1302).

In contrast to almost complete B cell depletion in wild-type mice (**Figure 7**), MB20-11 monoclonal antibody treatment reduced circulating B cell numbers by only 20-35% in Fc γ R $^{-/-}$ mice over 4 days (**Figure 8A**), with no effect from day 7 to 18. Moreover, MB20-11 monoclonal antibody treatment

actually increased spleen B cell numbers in FcR γ ^{-/-} mice compared with control monoclonal antibody-treated littermates (**Figure 8B** and **Figure 8C**), predominantly due to increased numbers of immature B cells. An isotype-matched control monoclonal antibody had no significant effect in FcR γ ^{-/-} mice.

5 In Fc γ RI^{-/-} mice, the MB20-11 monoclonal antibody induced an initial decrease in B cell numbers at 1 hour, but incomplete depletion of circulating B cells on day 2. MB20-11 monoclonal antibody treatment only partially depleted B cells in Fc γ RI^{-/-} mice with 21% of spleen B cells persisting at day 7 compared to control monoclonal antibody-treated littermates. By contrast, the MB20-11

10 monoclonal antibody depleted circulating and tissue B cells by $\geq 95\%$ in wild-type, Fc γ RII^{-/-} and Fc γ RIII^{-/-} mice by day 7. Identical results to those observed herein were obtained using two independent Fc γ RIII^{-/-} mouse lines (Bruhns, et al. (2003) *Immunity* 18:573-581; Hazenbos, et al. (1996) *Immunity* 5:181-188). B cell depletion by the IgG1 MB20-1 and IgG2b MB20-18 monoclonal

15 antibodies was similarly affected by FcR γ chain-deficiency. Circulating B cells were not significantly reduced by MB20-1 monoclonal antibody treatment of FcR γ ^{-/-} mice, while circulating B cells were depleted in wild-type mice (**Figure 8D**). Likewise, spleen B cells were not significantly reduced by MB20-1 monoclonal antibody treatment of FcR γ ^{-/-} mice, while spleen B cell numbers

20 were reduced by 93% in wild-type mice. Circulating B cells were significantly reduced by MB20-18 monoclonal antibody treatment of FcR γ ^{-/-} mice, but not to the same extent as occurred in wild-type mice (**Figure 8E**). However, spleen B cells were not significantly reduced by MB20-18 monoclonal antibody treatment of FcR γ ^{-/-} mice, while spleen B cell numbers were reduced

25 by 74% in wild-type mice. Thus, anti-CD20 monoclonal antibody therapy primarily depleted B cells through pathways that require FcR γ chain expression.

EXAMPLE 11

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The Role of C in B cell Depletion

Since C activation is considered a major mechanism for B cell depletion during anti-CD20 monoclonal antibody therapy, the role of C in B cell depletion by anti-CD20 monoclonal antibody treatment was assessed using C-deficient (Wessels, et al. (1995) *Proc. Natl. Acad. Sci. USA* 92:11490-

11494) and C1q-deficient (Zhang, et al. (1999) *Immunity* 10:323-332) mice. The C-activating ability of each anti-CD20 monoclonal antibody was first assessed *in vitro*. In the presence of C, most of the anti-CD20 monoclonal antibodies induced significant B cell lysis as indicated by propidium iodide uptake relative to isotype-matched control monoclonal antibodies, although cytotoxic capability varied between antibodies (**Figure 9A**). Without C, none of the anti-CD20 monoclonal antibodies induced B cell PI-uptake or apoptosis during these *in vitro* assays. The MB20-18 monoclonal antibody initiated the most potent C-dependent lysis of B cells, although the MB20-11 monoclonal antibody was also effective in inducing significant C-mediated B cell lysis *in vitro*. However, the ability of each monoclonal antibody to induce C-dependent B cell killing *in vitro* did not correlate with the ability of each monoclonal antibody to deplete B cells *in vivo* (**Figure 7B**). Moreover, the MB20-11 monoclonal antibody effectively cleared all blood and >90% of spleen B cells in C3^{-/-} and C4^{-/-} mice (**Figure 9B**). This observation was not monoclonal antibody isotype-specific since the MB20-1 and MB20-18 monoclonal antibodies effectively depleted blood and spleen B cells to similar extents in both C3^{-/-} and wild-type mice (**Figure 9C** and **Figure 9D**). Thus, anti-CD20 monoclonal antibody therapy primarily depletes B cells through FcγR-dependent and C3-, C4- and C1q-independent mechanisms.

EXAMPLE 12

Monocytes Mediate B Cell Depletion *in vivo*

Since the depleting ability of anti-CD20 monoclonal antibody treatment correlated directly with monoclonal antibody isotype and FcγR expression, the contributions of NK cells, T cells, and macrophages to FcγR-mediated B cell depletion was determined. Mice rendered macrophage-deficient by treatment with liposome-encapsulated clodronate did not significantly deplete circulating B cells by 1 hour after MB20-11 monoclonal antibody treatment, and had normal numbers of circulating B cells for up to 7 days (**Figure 10A**). Similarly, spleen B cell numbers in clodronate-treated mice were only decreased by 39% on day 7 relative to control monoclonal antibody-treated littermates (**Figure 10B**). Mice with tissue-specific losses in macrophage subpopulations (Cecchini, et al. (1994) *Development* 120:1357-1372) due to CSF-1 deficiency

(CSF-1^{op}) were also slow to clear circulating B cells after MB20-11 monoclonal antibody treatment and only depleted 84% of phenotypically mature spleen B cells by day 7 (**Figure 10**). By contrast, athymic nude and LAT^{-/-} mice that lack functional T cells (Zhang, et al. (1999) *Immunity* 10:323-332) depleted >96% of blood and spleen B cells. Likewise, anti-CD20 monoclonal antibody treatment removed ~95% of circulating and spleen B cells in beige and perforin^{-/-} mice (**Figure 10**) with defective NK cell function (Kagi, et al. (1994) *Nature* 369:31-37). These findings indicate that both CSF-1-dependent and -independent macrophage subsets are the major effector cells for depletion of CD20⁺ B cells *in vivo*, and essentially exclude T cell-, NK cell-, and perforin-dependent mechanisms.

EXAMPLE 13

Monoclonal Antibody Sequence Analysis

CD20 is unique among most B lymphocyte cell surface molecules in that only a relatively small portion of the molecule is expressed on the cell surface, estimated to be approximately 42 amino acids. Thus, most anti-CD20 monoclonal antibodies predominantly block the binding of other anti-CD20 monoclonal antibodies due to spatial constraints. While this has left the impression that most anti-CD20 monoclonal antibodies bind to similar, if not identical regions or epitopes on the CD20 protein, this has not been shown. Moreover, interactions between protein antigens and the monoclonal antibodies that bind to specific epitopes on these antigens are complex and are almost unique to each monoclonal antibody and its specific amino acid sequence. This level of complexity in antigen and antibody interactions contributes to the generation of a diverse antibody repertoire to most foreign antigens. However, a limit on anti-CD20 antibody diversity is imposed by the fact that mice also express CD20 as a self antigen. Thus, under normal circumstances, mice will not generate antibodies reactive with antigenic determinants present on human CD20 that are also shared by mouse CD20, since these monoclonal antibodies would be autoreactive. It is therefore possible that anti-CD20 monoclonal antibodies generated in a normal mouse bind to a limited number of defined epitopes that are present on, and unique to, human CD20.

In contrast to the diverse repertoire of antibodies that can be generated against most protein antigens, inbred strains of mice often respond to haptens or structurally simple antigens by producing remarkably homogenous antibodies (Blier and Bothwell (1988) *Immunol. Rev.* 105:27-43). One of the

5 best examples of restricted humoral responses is that of C57BL/6 (*Igh^b*) mice to the (4-hydroxy-3-nitrophenyl)acetyl (NP) hapten (Imanishi and Mäkelä (1975) *J. Exp. Med.* 141:840-854). C57BL/6 mice immunized with NP coupled to protein carriers generate serum antibodies that bear the uncommon $\lambda 1$ light

10 antibody or $\lambda 1^+$ B cells (Imanishi and Mäkelä (1975) *supra*; Mäkelä and Karjalainen (1977) *Immunol. Rev.* 34:119-138; Reth, et al. (1978) *Eur. J. Immunol.* 8:393-400; Reth, et al. (1979) *Eur. J. Immunol.* 9:1004-1013; Karjalainen, et al. (1980) *J. Immunol.* 125:313-317; Weiss and Rajewsky (1990) *J. Exp. Med.* 172:1681-1689; Jacob, et al. (1991) *J. Exp. Med.*

15 173:1165-1175; Cumano and Rajewsky (1986) *EMBO J.* 5:2459-2466). Early in the anti-NP response (days 4-8 post-immunization) a large proportion of antigen-activated $\lambda 1^+$ B cells express various D gene segments in combination with select members of the large J558 (V1) family of V_H genes, including V186.2 (V1S2), C1H4, CH10, V23 (V1S4), 24.8, V102 (V1S7), and

20 V583.5 (Jacob and Kelsoe (1992) *J. Exp. Med.* 176:679-687; Bothwell, et al. (1981) *Cell* 24:625-637; Allen, et al. (1988) *EMBO J.* 7:1995-2001; Jacob, et al. (1993) *J. Exp. Med.* 178:1293-1307). By day 10 after immunization, the majority of $\lambda 1^+$ B cells express V1S2-to-DFL16.1 gene rearrangements that encode a tyrosine-rich CDR3 region with a YYGS (SEQ ID NO:115)

25 consensus amino acid motif (Weiss and Rajewsky (1990) *supra*; Bothwell, et al. (1981) *supra*; Jacob, et al. (1993) *supra*; Cumano and Rajewsky (1985) *Eur. J. Immunol.* 15:512-520; McHeyzer-Williams, et al. (1993) *J. Exp. Med.* 178:295-305). The V1S.2-to-DFL16.1 heavy chain rearrangement paired with the $\lambda 1$ light chain is referred to as the canonical anti-NP B cell antigen

30 receptor (Reth, et al. (1978) *supra*; Reth, et al. (1979) *supra*), which dominates the primary and secondary humoral immune responses of C57BL/6 mice to NP. Thus, the use of these specific antibody gene segments is considered to predict the antigen specificity of the monoclonal antibody.

The homogeneity of the anti-NP antibody response in *Igh^b* mice (Imanishi and Mäkelä (1975) *supra*) is mirrored in the response of BALB/c mice to phosphorylcholine (Crews, et al. (1981) *Cell* 25:59-66); antibodies produced against *p*-azophenylarsonate in strain A mice (Pawlak, et al. (1973) *J. Exp. Med.* 137:22-31); the 2-phenyloxazolone response in BALB/c and DBA/2 mice (Mäkelä, et al. (1978) *J. Exp. Med.* 148:1644-1656); and the response of BALB/c mice to poly(Glu⁶⁰-Ala³⁰-Tyr¹⁰) (Thèze and Sommé (1979) *Eur. J. Immunol.* 9:294-301). The cause of low genetic variance in these antibody responses remains obscure. Linkage of restricted antibody responses to single V gene segments (Siekevitz, et al. (1983) *Eur. J. Immunol.* 13:123-132) or *Igh* alleles (Siekevitz, et al. (1982) *Eur. J. Immunol.* 12:1023-1032) suggests an occasional, single-best solution to antigen-complementarity that results in expansion of a few B cell clones that bear homologous V(D)J rearrangements. In this case, strain-specific differences in the repertoire of germline V_H genes would regulate the antibody response to structurally similar molecules. Alternatively, restricted antibody responses may be circumscribed by self-tolerance (Manser, et al. (1987) *Immunol. Rev.* 96:141-162; Hande, et al. (1998) *Immunity* 8:189-198) or depend on lymphocyte clones that express V(D)J rearrangements that are robustly tolerant of mutational change (Manser, et al. (1984) *Science* 226:1283-1288). Regardless of the mechanism, antibody responses to defined structures can be homogenous and reflect a limited response within the antibody repertoire, which may also reflect the fact that antibodies are binding the same target antigen through similar, if not identical molecular interactions that are mediated by specific conserved amino acids within the variable regions of antibodies. While monoclonal antibody interactions with target antigens are primarily mediated by amino acids within complementarity-determining regions (CDR) of antibody molecules, framework amino acids are also critical to antigen-binding activity. Thus, structurally similar antibodies are likely to bind to the same antigens or region of a target molecule, while structurally dissimilar antibodies with different V regions are likely to interact with different regions of antigens through different molecular interactions.

Since structurally similar antibodies that bind to the same region of a target antigen are more likely to bind the same molecular site on the target antigen, the amino acid sequences of published anti-human CD20 monoclonal antibodies was examined. The heavy and light chain V regions of the 1F5 (Shan, et al. (1999) *supra*), B9E9 (Schultz, et al. (2000) *supra*), 2H7 (U.S. Patent No. 6,120,767), 2B8 (U.S. Patent No. 5,843,439), 1H4 (Haisma, et al. (1998) *supra*), and Leu-16 (Wu, et al. (2001) *supra*) monoclonal antibodies are homologous in amino acid sequence (**Figure 11**). This level of conservation reflects the fact that each of these monoclonal antibodies is also similar at the nucleotide level. The heavy chains of these anti-CD20 monoclonal antibodies are generated through similar combinations of V(D)J gene segments with the V regions derived from the V1S121*01 gene segment, D regions derived from L16, Q52 or SP2 gene segments, and J regions derived from either J1 or J2 gene segments (**Table 1**). Similarly, the light chains were generated from either V4-72*01 gene segments, with J regions from either the J1*02 or J5*01 gene segments. The level of homogeneity among known anti-human CD20 monoclonal antibodies suggests that each of these monoclonal antibodies is binding to similar, or identical sites on human CD20.

The level of amino acid sequence homology among known anti-CD20 monoclonal antibodies is highlighted by comparisons with a larger panel of anti-human CD20 and anti-mouse CD20 monoclonal antibodies. For comparisons, the known anti-CD20 monoclonal antibodies were first contrasted with a second group of anti-human CD20 monoclonal antibodies (HB20-03, -04 and -25) with homogeneous nucleotide and amino acid sequences. Comparative similarities between heavy chain and light chain V regions was visualized using UPGMA trees (unweighted pair group method using arithmetic averages) as shown in **Figure 12**. In these diagrams, horizontal distances between tree branch points is a measure of sequence relatedness. For example, the heavy and light chains of the known mouse anti-human CD20 monoclonal antibodies were more similar to each other than the sequences of the HB20-03, -04 and -25 monoclonal antibodies, which were most similar to each other. Among light chain V region sequences, the 1H4 and B9E9 sequences were most similar, but were also quite similar to the

sequences for the 2H7, 1F5 and 2B8 monoclonal antibody sequences. Similarly, the HB20-3 and HB20-4 monoclonal antibodies had quite similar amino acid sequences, which were relatively less similar with the corresponding sequence of the HB20-25 monoclonal antibody. However, the
5 light chain sequences of the HB20-3, -4 and -25 monoclonal antibodies were even more distinct from the sequences of the known anti-CD20 monoclonal antibodies. When sequence homologies of paired heavy and light chains between each monoclonal antibody were compared, the level of sequence homology between the known anti-CD20 monoclonal antibodies and the
10 HB20-3, -4 and -25 monoclonal antibodies were quite distinct (**Figure 12**). This indicates that these two groups of monoclonal antibodies are distinct and likely to bind to human CD20 through different molecular interactions or at distinct sites on the CD20 protein. It is also contemplated that the HB20-3, -4 and -25 monoclonal antibodies would have shared biological properties that
15 are distinct from the shared properties of the known anti-CD20 monoclonal antibodies.

The level of amino acid sequence homology among known anti-CD20 monoclonal antibodies was further shown by comparisons with a larger panel of anti-human CD20 and anti-mouse CD20 monoclonal antibodies. Generally,
20 the 2B8, B9E9, 1F5, 1H4, and Leu-16 heavy chains were similar in sequence with heavy chains of the HB20-01, -02 and -06 monoclonal antibodies (**Figure 13** and **Figure 15**). Based on sequence similarities, these heavy chain V^HJ segments have been designated as group A sequences (**Figure 13**). The amino acid sequence of the 2H7 monoclonal antibody was similar, but
25 divergent from the other group A heavy chains so this heavy chain was designated to represent group B heavy chains. HB20-03, -04 and -25 were structurally similar and were designated as group C heavy chains. The HB20-05 monoclonal antibody heavy chain was also structurally distinct and was designated to represent group D heavy chains. Many of the anti-mouse CD20
30 monoclonal antibodies shared structurally similar heavy chains, MB20-08, -10, -18, -07, -02 and -14, and were designated as group E heavy chains. The MB20-11 and -16 monoclonal antibody heavy chains were sufficiently distinct to represent group F. The MB20-01 and -13 monoclonal antibodies used very diverse heavy chains that were structurally distinct from all other anti-CD20

monoclonal antibodies and were designated as group G heavy chains. These different groups of heavy chain amino acid sequences correlated closely with utilization of different V(D)J gene segments for generation of each of the anti-CD20 heavy chains (**Table 1**). Thus, the known anti-CD20 monoclonal antibody heavy chains were structurally distinct from the majority of the anti-CD20 monoclonal antibody heavy chains disclosed herein.

The striking level of amino acid sequence homology among known anti-CD20 monoclonal antibodies was further highlighted by comparisons of light chain utilization among a panel of anti-human CD20 and anti-mouse CD20 monoclonal antibodies. The 2B8, B9E9, 1F5, 1H4, Leu-16 and 2H7 light chains were quite similar in sequence, but were distinct from the light chains used by other anti-CD20 monoclonal antibodies (**Figure 16** and **Figure 18**). Based on sequence similarities, these light chains were designated as group A sequences (**Figure 16**). The amino acid sequence of multiple anti-mouse CD20 monoclonal antibody light chains was most similar, but divergent from the group A light chains, so these light chains were designated as group B. HB20-03, -04 and -25 were structurally distinct and were designated as group C light chains. The HB20-01, -02 and -06 monoclonal antibodies used similar light chains that were designated as group E. MB20-18 and MB20-01 used structurally distinct light chains and were therefore designated as groups F and G, respectively. These different groups of light chain amino acid sequences correlated closely with utilization of different VJ gene segments for generation of each of the anti-CD20 monoclonal antibodies (**Table 1**). Thus, the known anti-CD20 monoclonal antibody light chains were structurally distinct from those used by the anti-CD20 monoclonal antibodies disclosed herein.

An analysis of amino acid sequences of paired heavy and light chains further verified that different anti-CD20 monoclonal antibodies fell into structurally distinct groups and would therefore bind human or mouse CD20 through different molecular interactions. The 2B8, B9E9, 1F5, 1H4 and Leu-16 monoclonal antibodies used structurally similar heavy and light chains, designated as AA, respectively (**Figure 19, Table 1**). Since the 2H7 monoclonal antibody heavy chain was structurally different from the heavy chain used by other known anti-CD20 monoclonal antibodies but was paired

with a similar light chain, this monoclonal antibody was grouped as a BA monoclonal antibody. The group CC monoclonal antibodies, HB20-03, -04 and -25, represent a unique class of structurally distinct anti-CD20 monoclonal antibodies. Similarly, the utilization of unique heavy and light chains and the combinatorial diversity achieved by using different pairs of heavy and light chains allowed each of the other anti-CD20 monoclonal antibodies to be categorized as structurally distinct from the known anti-CD20 monoclonal antibodies (**Figure 19**).

10

EXAMPLE 14

Anti-CD20 Monoclonal Antibody Binding Density and B Cell Depletion

CD20 expression is quite heterogeneous in different lymphoma types, as well as among cells of an individual tumor sample, which may affect anti-CD20 monoclonal antibody therapeutic outcome (Smith (2003) *Oncogene* 22:7359-7368). Typically, chronic lymphocytic leukemia cells and small lymphocytic lymphoma cells express CD20 at low levels, with corresponding lower Rituximab response rates than follicular lymphoma cells expressing CD20 at higher levels (McLaughlin, et al. (1998) *J. Clin. Oncol.* 16:2825-2833). Thus, CD20 expression density may be an important factor influencing anti-CD20 therapeutic efficacy since CD20 density dictates the number of anti-CD20 monoclonal antibodies that are able to bind B cells and target them for depletion. To assess whether CD20 expression density affects therapeutic efficacy and to determine the extent that density changes affect B cell depletion, heterozygous CD20^{+/-} mice were treated with the MB20-11 monoclonal antibody at high (250 µg) and low (10 µg) doses. B cells from CD20^{+/-} mice express CD20 at half the density found in wild-type littermates. At high anti-CD20 monoclonal antibody doses, wild-type or haplo-insufficient CD20 expression had no detectable influence on circulating or spleen B cell depletion by day 7, with 93-97% of B cells cleared from the spleen (**Figure 20A and Figure 20B**). By contrast, a low dose of anti-CD20 monoclonal antibody effectively depleted 93-98% of circulating and spleen B cells from wild-type mice, but only removed 30-41% of circulating or spleen B cells from CD20^{+/-} mice by day 7 (**Figure 20A and Figure 20B**). Thus, the effectiveness of anti-CD20 monoclonal antibody treatment was significantly altered by only

30

a 50% decrease in B cell expression of CD20. Moreover, the density of anti-CD20 monoclonal antibody binding to the surface of target B cells was critically influenced by cell surface CD20 density, particularly when lower monoclonal antibody concentrations were present.

5

EXAMPLE 15

MB20-11 Monoclonal Antibody Binding and Cell Surface CD20 Expression

Cell surface CD20 expression density is an important factor for anti-CD20 therapeutic efficacy (Smith (2003) *supra*). Therefore, attempts have been made to upregulate CD20 expression during therapy to try to enhance anti-CD20 monoclonal antibody efficacy *in vivo*, such as treating patients with G- or GM-CSF. However, mechanisms other than CD20 upregulation are likely to account for the enhanced effects that have been observed (Ravetch and Lanier (2000) *Leukemia* 16:693-699; van der Kolk, et al. (2002) *Leukemia* 16:693-699). It has been demonstrated herein that MB20-11 monoclonal antibody is exceptionally effective in depleting mouse B cells *in vivo* (**Figures 7-10**). Although the *in vivo* effectiveness of the MB20-11 monoclonal antibody appears to result, in part, from the fact that it is of the IgG2a isotype, other factors are also likely to contribute therapeutic efficacy. Therefore, the effect of MB20-11 monoclonal antibody binding on cell surface CD20 expression by spleen B cells was assessed *in vitro*. Unexpectedly, binding of the MB20-11 monoclonal antibody to cultured B cells at 37°C induced binding of more MB20-11 monoclonal antibodies over time compared with cells kept on ice or incubated with other MB20 monoclonal antibodies of similar or different isotypes (**Figure 21**; data not shown). On average, MB20-11 monoclonal antibody binding increased by $97 \pm 29\%$ ($n=4$, $p<0.05$) over a time period of 30 minutes to 8 hours (**Figure 21**). Increased MB20-11 monoclonal antibody binding with time did not appear to be related to low monoclonal antibody affinity since the MB20-11 monoclonal antibody reached saturating levels of staining at monoclonal antibody concentrations similar to other MB20 monoclonal antibodies that did not induce increased CD20 expression on the cell surface (**Figure 6**). Since other anti-mouse CD20 monoclonal antibodies did not display this property (**Figure 21**, data not shown), the MB20-11

monoclonal antibody may bind to a unique region or epitope on CD20.
 Binding of the MB20-11 monoclonal antibody to CD20 may either induce
 increased cell surface CD20 expression on B cells or induce conformational
 changes in cell surface CD20 molecules that expose nascent MB20-11
 5 monoclonal antibody binding sites.

EXAMPLE 16

Binding Densities of HB20-3, -4 and -25 Monoclonal Antibodies

Monoclonal antibody binding density is critical for optimal anti-CD20
 10 monoclonal antibody mediated B cell depletion *in vivo* (**Figure 20**) indicating
 that monoclonal antibodies that bind to B cells at higher densities may be
 more therapeutically effective. The MB20-18 monoclonal antibody bound to
 the surface of B cells at the highest density of the MB20 group of monoclonal
 antibodies, particularly when monoclonal antibody concentrations were
 15 limiting (**Figure 6**). This may have contributed to the particular effectiveness
 of the MB20-18 monoclonal antibody among other IgG2b anti-CD20
 monoclonal antibodies for B cell depletion *in vivo* (**Figure 7B**). Based on the
 significant differences in their amino acid sequences, it is contemplated that
 the HB20-3, -4 and -25 monoclonal antibodies in group CC (**Table 1**) have
 20 shared biological properties that are distinct from the shared properties of the
 known anti-CD20 monoclonal antibodies (**Figure 12**). Among these
 differences, the HB20-3, -4 and -25 monoclonal antibodies bound to primary
 B cells and B lymphoma cell lines expressing CD20 at significantly higher
 levels than the known anti-CD20 monoclonal antibodies in indirect
 25 immunofluorescence staining assays (**Figure 22**). On average, the HB20-3, -4
 and -25 monoclonal antibodies bound to human blood B cells at 3.7-fold
 higher levels than the 1F5, B9E9 and 1H4 monoclonal antibodies (**Table 6**).
 Similarly, the HB20-3, -4 and -25 group of monoclonal antibodies bound to B
 cell lines, Raji, BJAB and DHL-4 at 4.5-, 3.1- and 4.3-fold higher levels,
 30 respectively, than the 1F5, B9E9 and 1H4 group of monoclonal antibodies. It
 was consistently observed that the B1 monoclonal antibody, which was the
 first described anti-CD20 monoclonal antibody (Stashenko, et al. (1980) *J.*
Immunol. 125:1678), stains B cells and characteristically binds to B cells at
 high density when compared with other published anti-CD20 monoclonal

antibodies (**Table 6**). Nonetheless, the HB20-3, -4 and -25 group of monoclonal antibodies reacted with primary B cells and B cell lines at higher levels than the B1 monoclonal antibody (**Table 6**). Specifically, the HB20-3 monoclonal antibody bound to primary B cells, Raji cells, BJAB cells and DHL-4 cells at 69%, 130%, 71%, and 57% higher levels, respectively, than the B1 monoclonal antibody. Thus, a unique characteristic of group CC monoclonal antibodies is their uncharacteristically high binding activity for cell surface CD20 in comparison with other known anti-CD20 monoclonal antibodies.

In addition to binding at a higher density as well as reacting with primary B cells and B cell lines at higher levels than the 1F5 monoclonal antibody, HB20-3, HB20-4 and HB20-25 share a common amino acid motif in the heavy chain CDR3 and CDR1 regions and in the light chain CDR3 region. The common motif is apparent in Figure 15 for the heavy chain and Figure 18 for the light chain. For example, the heavy chain CDR3 region comprises the amino acid sequence motif of FYXYXXX¹YGAX²XXY, wherein X can be any amino acid, and wherein X¹ can be any amino acid and is preferably a Y or an S, and wherein X² can be any amino acid and is preferably an M or an L and wherein F is a Phenylalanine, Y is a Tyrosine, G is a Glycine, A is an Alanine, M is a methionine, L is a Leucine and S is a Serine.

The heavy chain CDR1 region comprises the amino acid sequence motif of NXXXX wherein X can be any amino acid and N is an Asparagine.

Further, the light chain CDR3 region comprises the amino sequence motif of XHFWXX³XWX, wherein X can be any amino acid sequence, H is Histidine, F is a Phenylalanine, W is a Tryptophan and X³ can be any amino acid and is preferably a T or an I, wherein T is Threonine and I is Isoleucine.

TABLE 6

Antibody	Cell Source			
	Blood B Cells	Raji	BJAB	DHL4
Control	1	3	10	1
HB20-3	469	2216	1198	765
HB20-4	397	1579	1095	578
HB20-25	227	1347	849	514

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1F5	104	455	386	169
B9E9	112	438	377	162
1H4-2a	75	244	240	98
B1	277	944	700	486

Reactivity of human blood lymphocytes and the Raji, BJAB or DHL-4 B lymphoblastoid cell lines with anti-CD20 monoclonal antibodies at saturating concentrations or with secondary antibody alone (control). The anti-CD20 monoclonal antibodies were used at concentrations that were determined to be saturating and that gave optimal staining. 1F5, B9E9 and 1H4 (IgG2a) monoclonal antibodies were used as ascites fluid diluted 1:200. HB20-3, -4 and -25 monoclonal antibodies were used as tissue culture supernatant fluid. B1 monoclonal antibody was used at either 10 µg/mL of purified monoclonal antibody or as tissue culture supernatant fluid. In all cases, monoclonal antibody staining was visualized using PE-conjugated isotype-specific secondary antibodies with flow cytometry analysis. Values represent the mean linear fluorescence intensity of staining for each B cell population as determined by flow cytometry analysis. Results are representative of those obtained in ≥3 experiments.

EXAMPLE 17

Therapeutic Effectiveness of Anti-CD20 Monoclonal Antibody

Since the MB20-11 monoclonal antibody given at 2.5 µg doses i.v. effectively depleted circulating and tissue B cells (**Figure 7C**), it was determined whether similar small doses of anti-CD20 monoclonal antibody given subcutaneously (s.c.) depleted B cells to an equivalent extent. The vast majority of circulating and tissue B cells were depleted in mice given anti-CD20 monoclonal antibodies as 5 µg doses either i.v. or s.c. (**Figure 23A and Figure 23B**). Rituximab is normally given to humans i.v. at 375 mg/m² doses, which would correspond to a dose of 2,500 µg/mouse (**Table 7**). Effective B cell depletion in mice was obtained by giving a single 5-10 µg dose of MB20-11 monoclonal antibody s.c., which would be equivalent to 250- to 500-fold lower an amount of antibody than the amount of rituximab that is currently given i.v. to patients. Based on the current mouse findings, an anti-CD20 monoclonal antibody that was therapeutically comparable to the MB20-11 monoclonal antibody could effectively deplete both circulating and tissue B cells when given as 1.3-2.6 mg s.c. injections to humans.

TABLE 7

Mouse $\mu\text{g}/\text{mouse}$	Mouse* mg/kg	Human [#] mg/kg	Human Dose [#] mg	Human [#] mg/m^2
0.5	0.025	0.0020	0.128	0.075
1	0.05	0.0039	0.257	0.15
2.5	0.125	0.0099	0.641	0.375
5	0.25	0.0197	1.28	0.75
10	0.5	0.039	2.57	1.5
25	1.25	0.099	6.41	3.75
50	2.5	0.197	12.8	7.5
100	5	0.395	25.7	15
250	12.5	0.987	64.1	37.5
2500	124	9.9	641	375

* Assume weight of 0.02 kg.

Assume weight of 65 kg, 1.71 m^2 for body surface area.

5 Resource: Dose Calculator

The foregoing is illustrative of the present invention, and is not to be construed as limiting thereof. The invention is defined by the following claims, with equivalents of the claims to be included therein.

Claims:

1. A monoclonal antibody (mAb) or antigen-binding fragment thereof that specifically binds to CD20, wherein the mAb or antigen-binding fragment thereof comprises a heavy chain CDR1 region having the amino acid sequence of SEQ ID NO:59, a heavy CDR2 region having the amino acid sequence of SEQ ID NO:68, and a heavy chain CDR3 region having the amino acid sequence of SEQ ID NO:79; and a light chain CDR1 region having the amino acid sequence of SEQ ID NO:87, a light chain CDR2 region having the amino acid sequence of SEQ ID NO:97, and a light chain CDR3 region having the amino acid sequence of SEQ ID NO:104, and wherein the density of binding of the mAb or antigen-binding fragment to Raji cells is higher than the density of binding of the mAb 1F5 to Raji cells.
2. The mAb or antigen-binding fragment of claim 1, wherein the mAb or antigen-binding fragment specifically binds to human CD20.
3. The mAb or antigen-binding fragment of claim 1, which is a humanized mAb or antigen-binding fragment.
4. A pharmaceutical composition comprising the mAb or antigen binding fragment of claim 1 in a pharmaceutically acceptable carrier.
5. The antigen-binding fragment of a mAb of claim 1, wherein the antigen binding fragment is a F(ab')₂, Fab', Fab, or F_v fragment.
6. The mAb or antigen-binding fragment of claim 1, wherein said mAb is an IgG2a, IgG1, or IgG2b isotype molecule.
7. An isolated nucleic acid comprising a nucleotide sequence encoding a heavy chain comprising heavy chain CDR1, CDR2 and CDR3 and encoding a light chain comprising a light chain CDR1, CDR2 and CDR3 of an antibody of claim 1.
8. A vector comprising the isolated nucleic acid of claim 7.
9. A cell comprising the isolated nucleic acid of claim 7 or the vector of claim 8.

10. Use of the mAb or antigen-binding fragment of claim 1 for treating a B lymphocyte disorder in a mammalian subject.
11. The use of claim 10, wherein the B lymphocyte disorder is a B lymphocyte malignancy or an autoimmune disorder.
12. The use of claim 10, wherein the mammalian subject is a human.
13. The use of claim 10, wherein said mAb or antigen-binding fragment is for use in a pharmaceutical composition, wherein the pharmaceutical composition further comprises a pharmaceutically acceptable carrier.
14. The use of claim 10, wherein the mammalian subject is resistant to an anti-CD20 mAb therapy, wherein the anti-CD20 mAb associated with the therapy is different than the antibody of claim 1.
15. The use of claim 10, wherein the mammalian subject is resistant to therapy with mAb Rituximab.
16. The use of claim 10, further comprising the use of chemotherapy for treatment of the mammalian subject.
17. The use of claim 10, wherein the mammalian subject has had a relapse in a B lymphocyte disorder.
18. Use of the mAb or antigen-binding fragment of claim 1 for preparation of a medicament for treatment of a B lymphocyte disorder in a mammalian subject.
19. The use of claim 18, wherein the B lymphocyte disorder is a B lymphocyte malignancy or an autoimmune disorder.
20. The use of claim 18, wherein the mammalian subject is a human.
21. The use of claim 18, wherein said mAb or antigen-binding fragment is suitable for administration in a pharmaceutical composition, wherein the pharmaceutical composition further comprises a pharmaceutically acceptable carrier.

22. The use of claim 18, wherein the mammalian subject is resistant to anti-CD20 mAb therapy, wherein the anti-CD20 mAb associated with the therapy is different than the antibody of claim 1.
23. The use of claim 18, wherein the mammalian subject is resistant to therapy with mAb Rituximab.
24. The use of claim 18, further comprising the use of chemotherapy for treatment of the mammalian subject.
25. The use of claim 18, wherein the mammalian subject has had a relapse in a B lymphocyte disorder.

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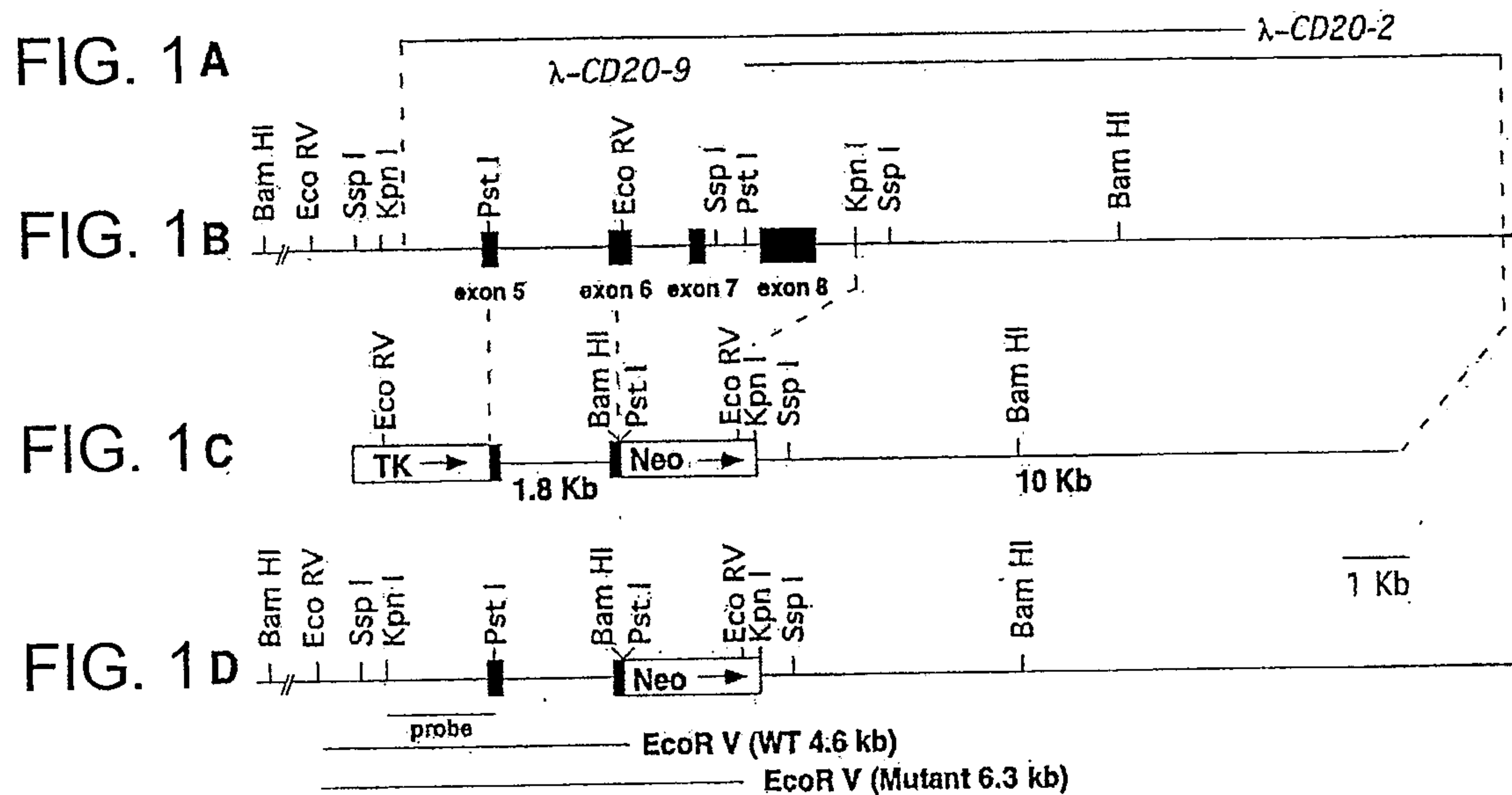


FIG. 1E

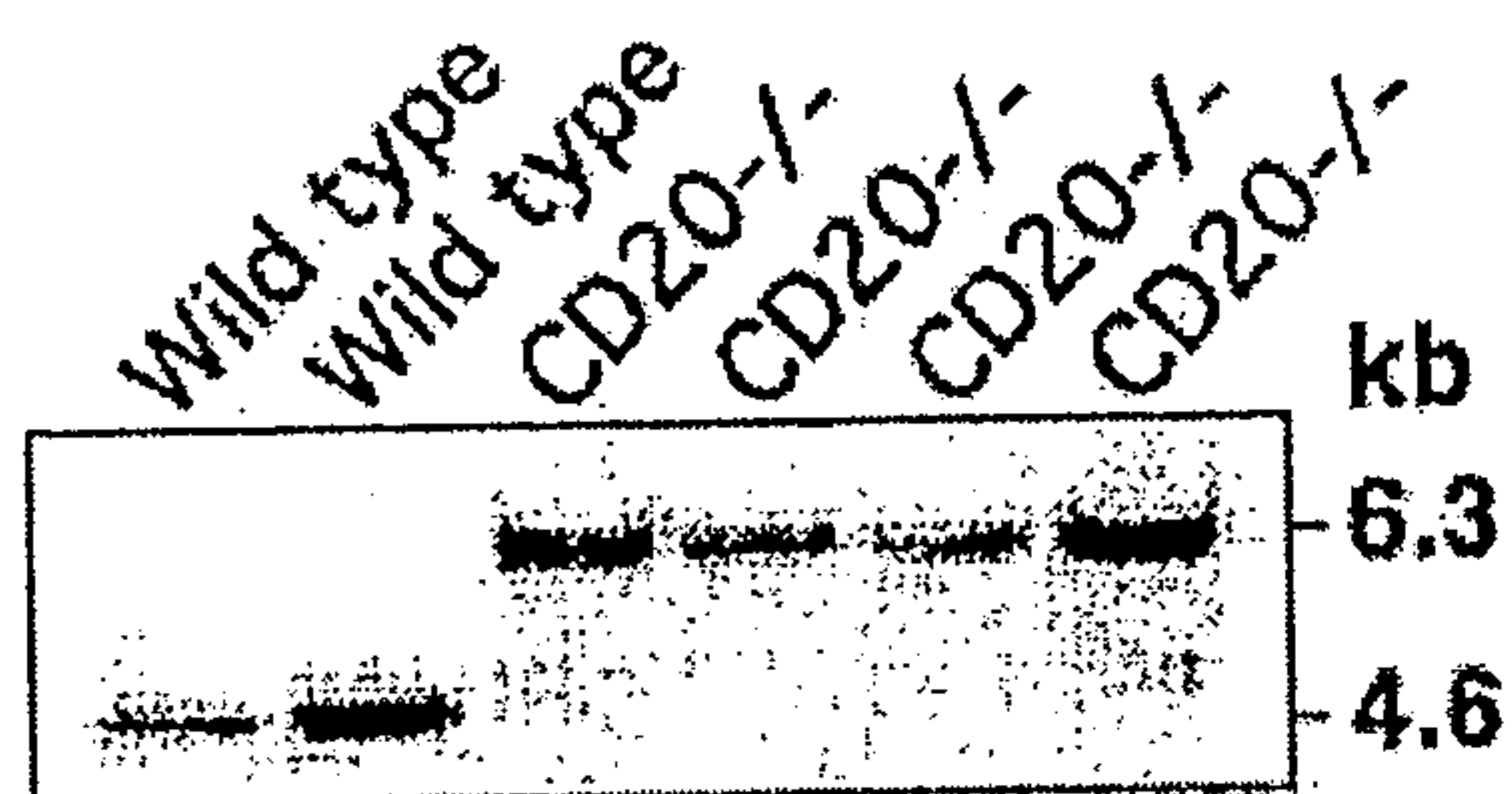


FIG. 1F

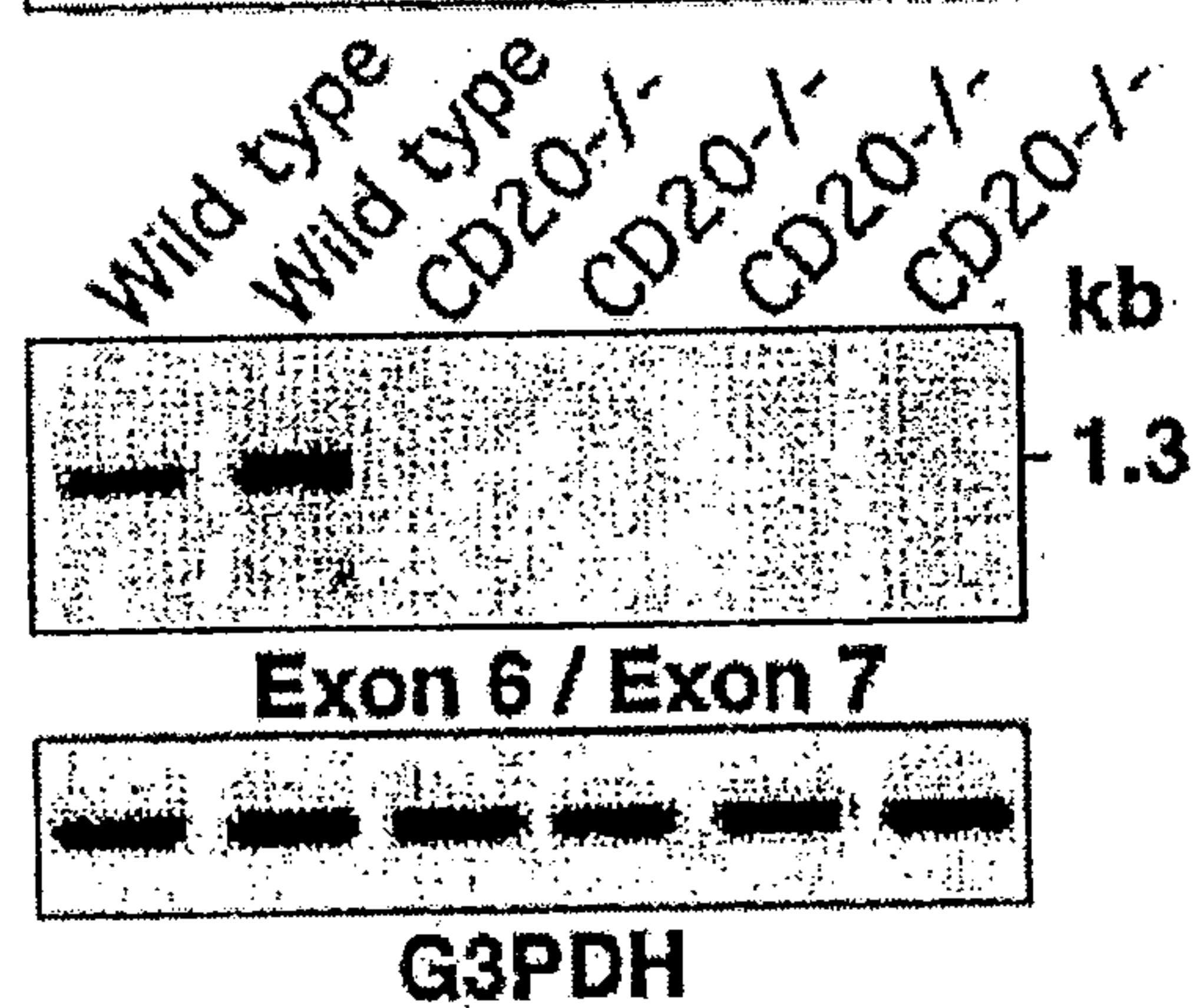
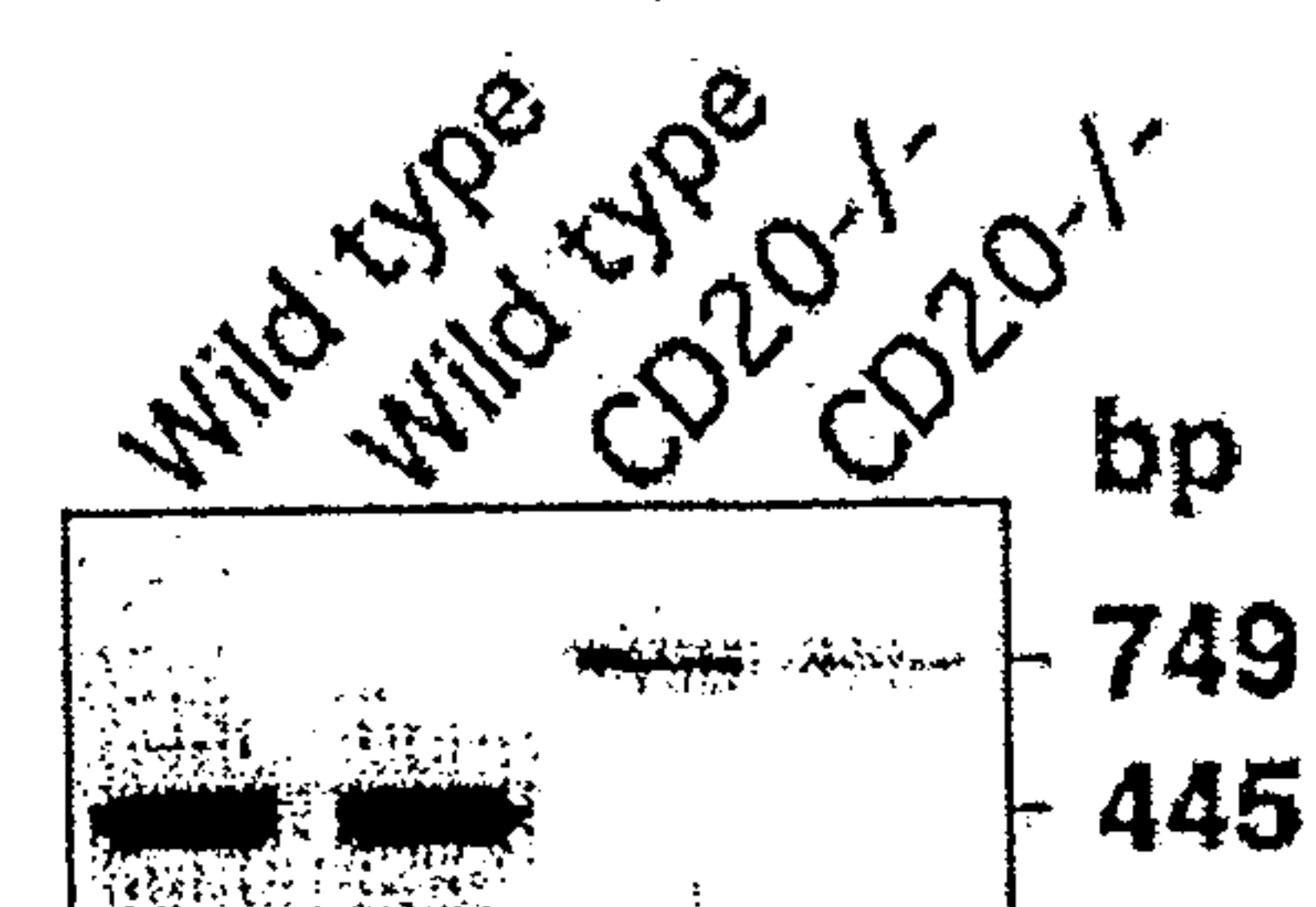


FIG. 1G



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FIG. 1H

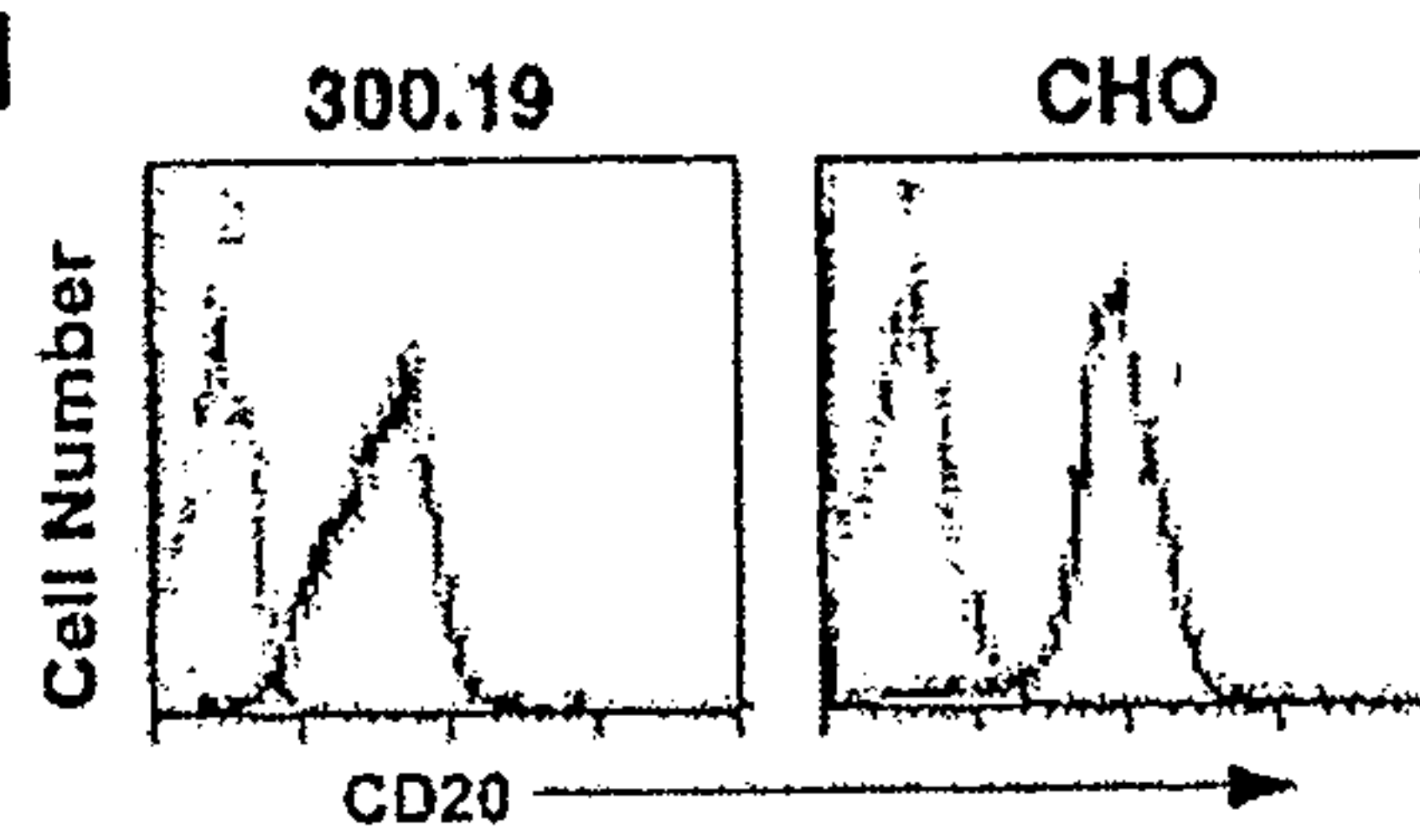


FIG. 1I

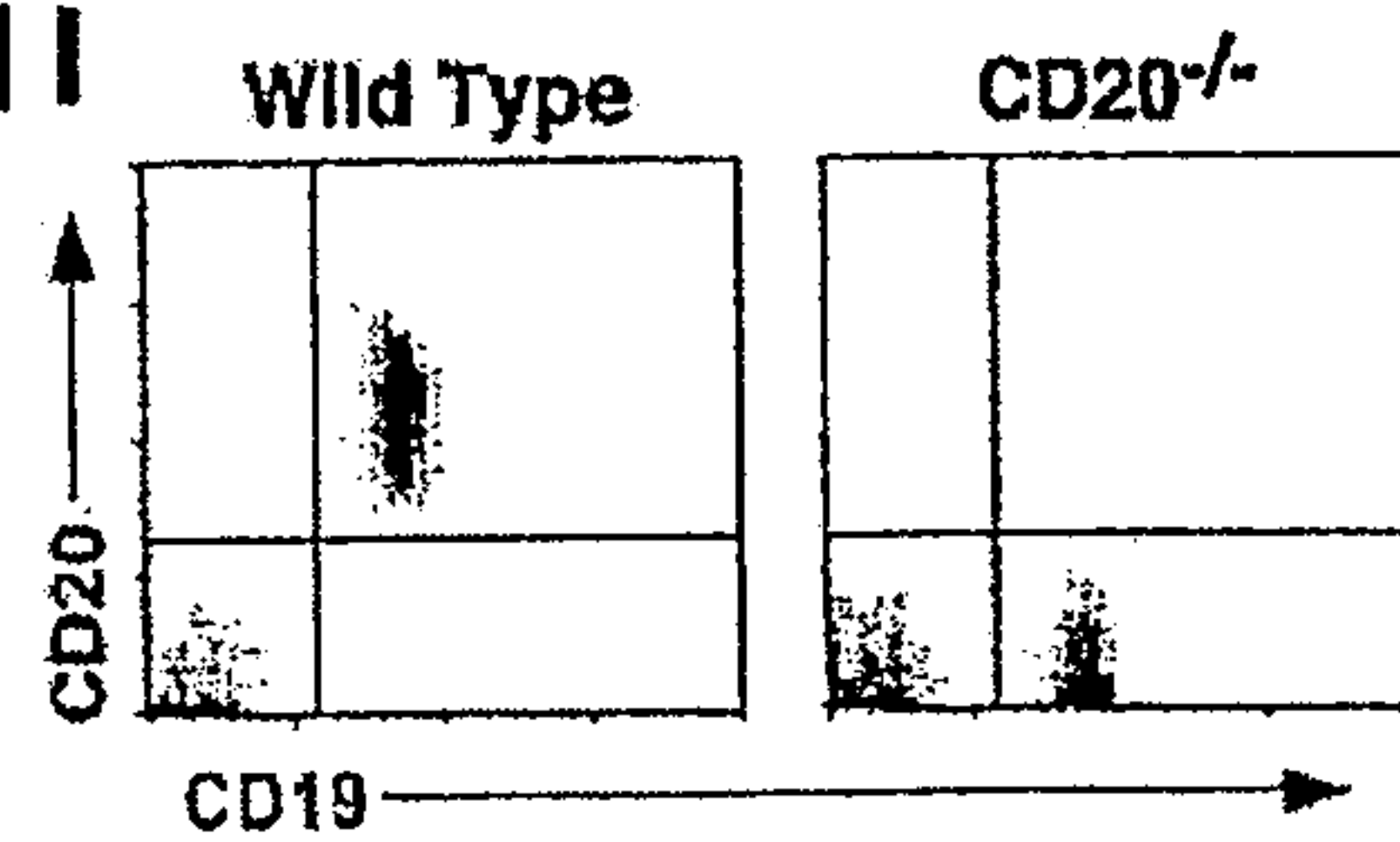
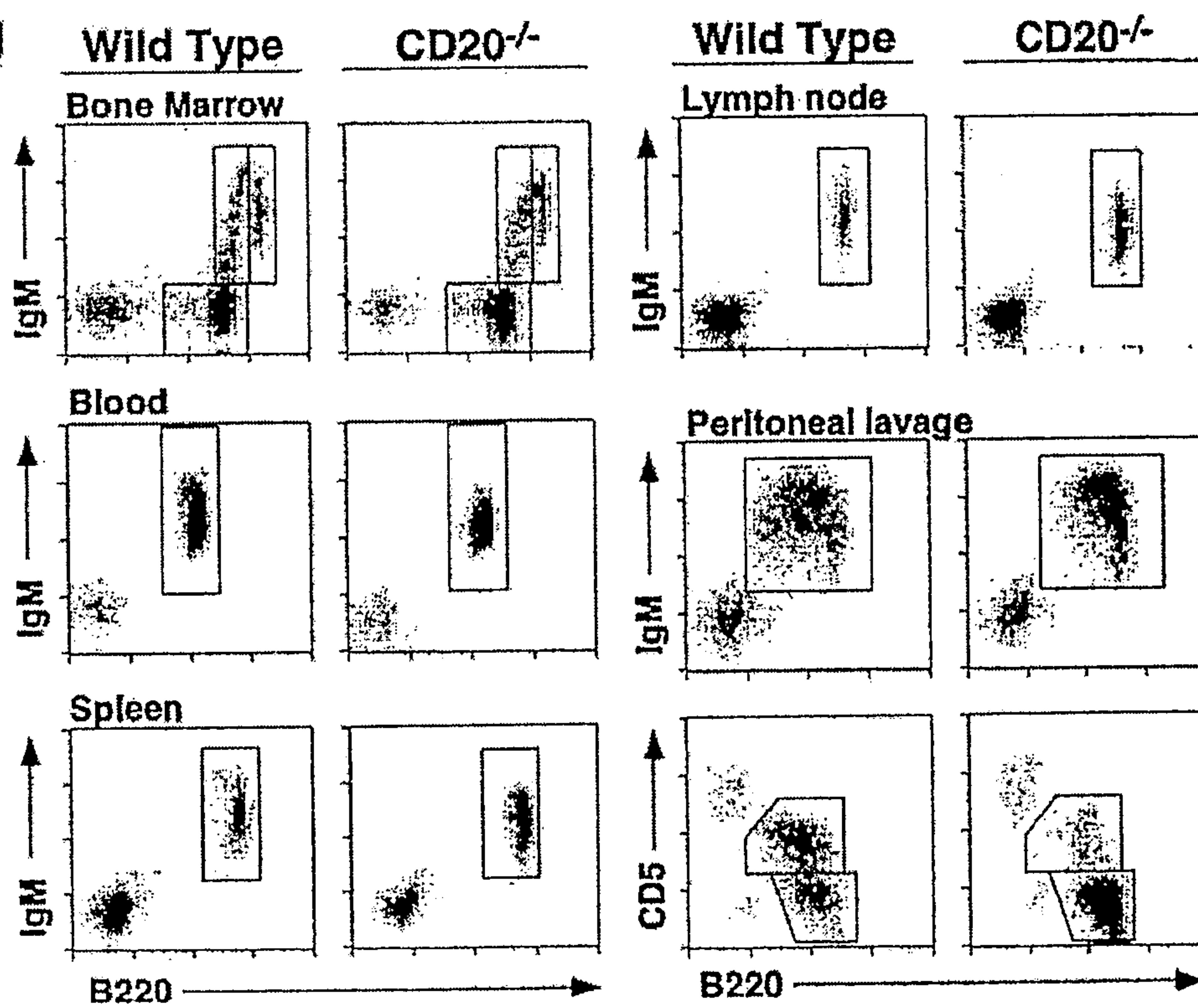


FIG. 1J



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FIG. 1K

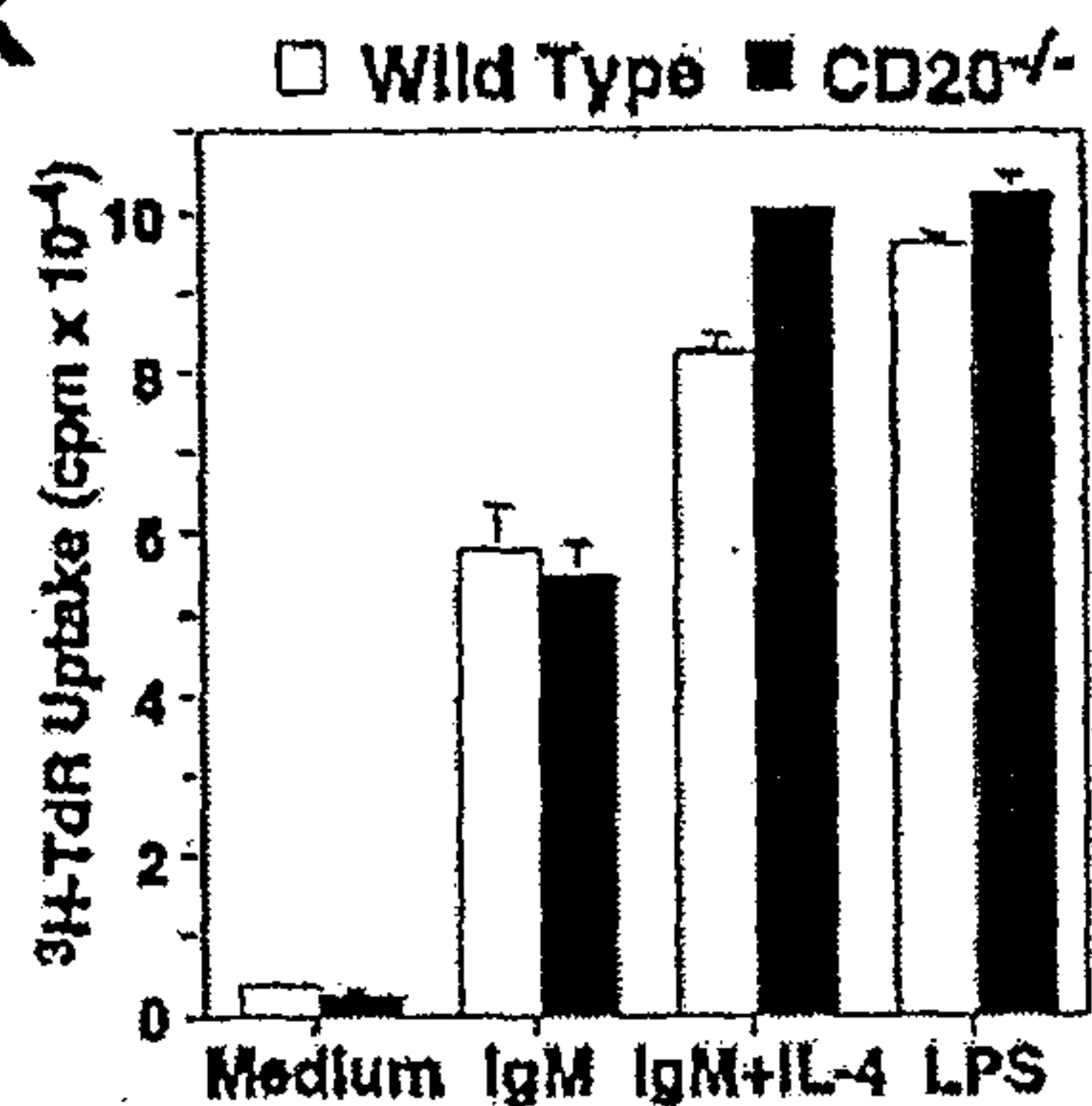


FIG. 1L

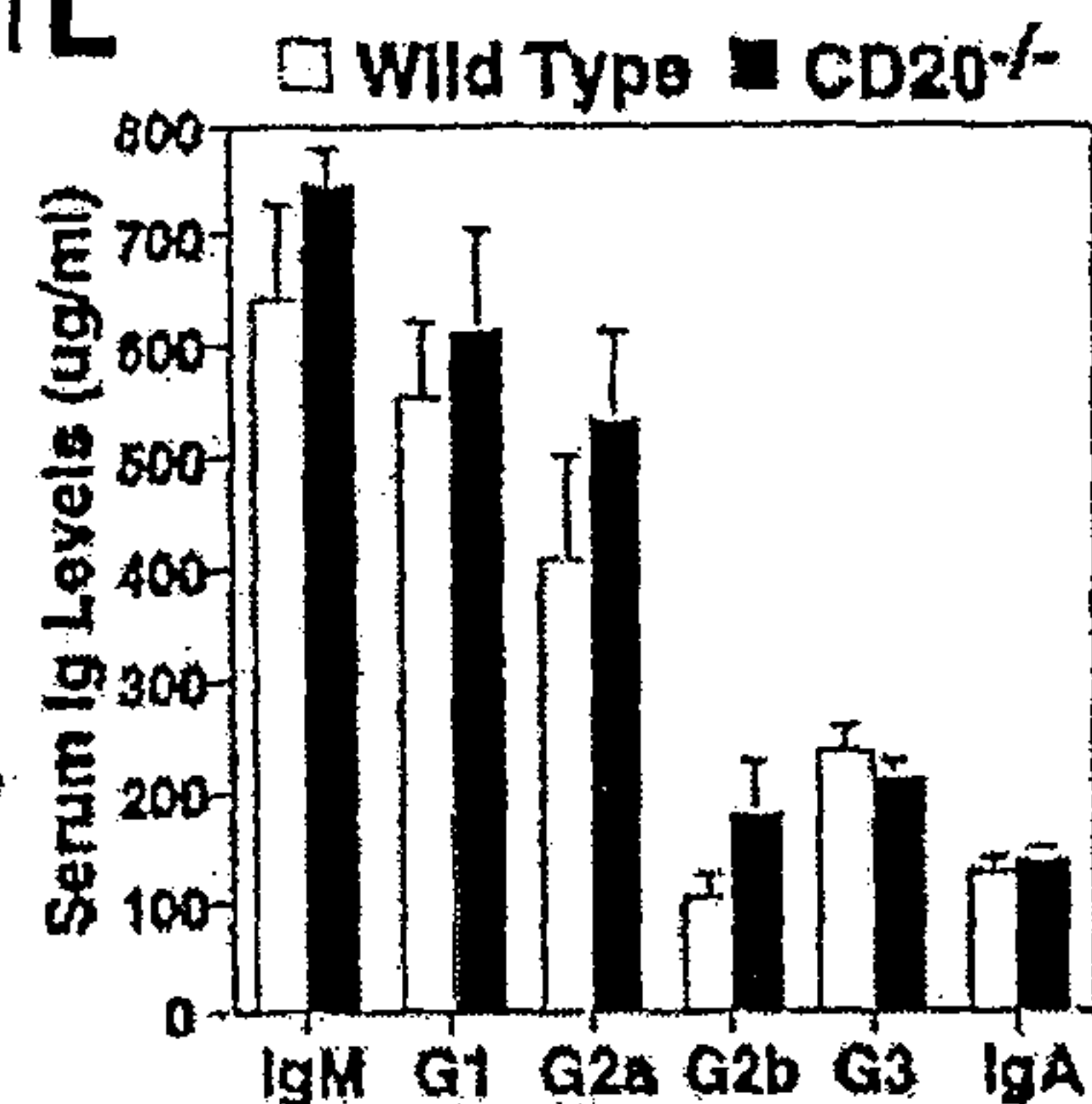
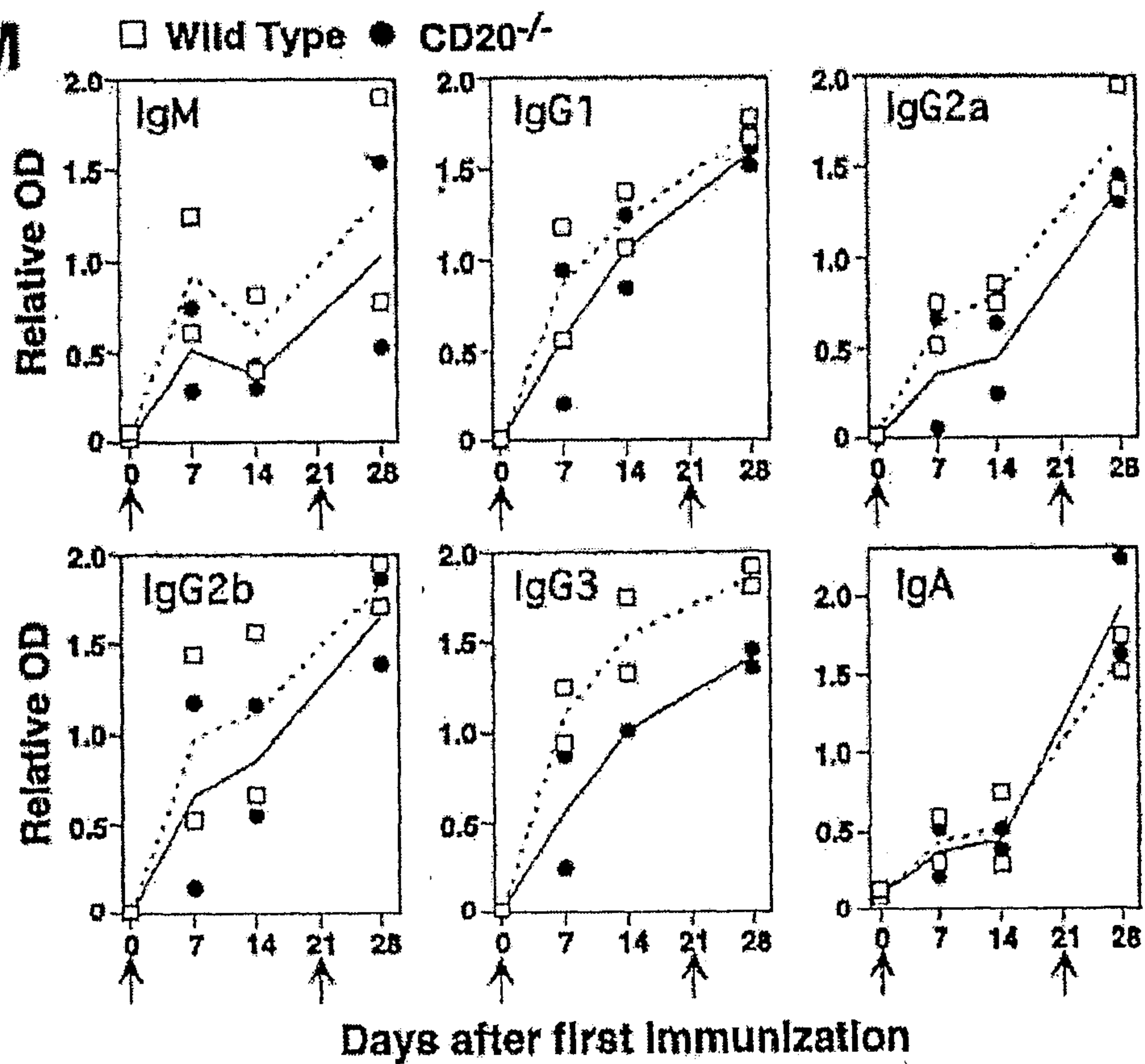
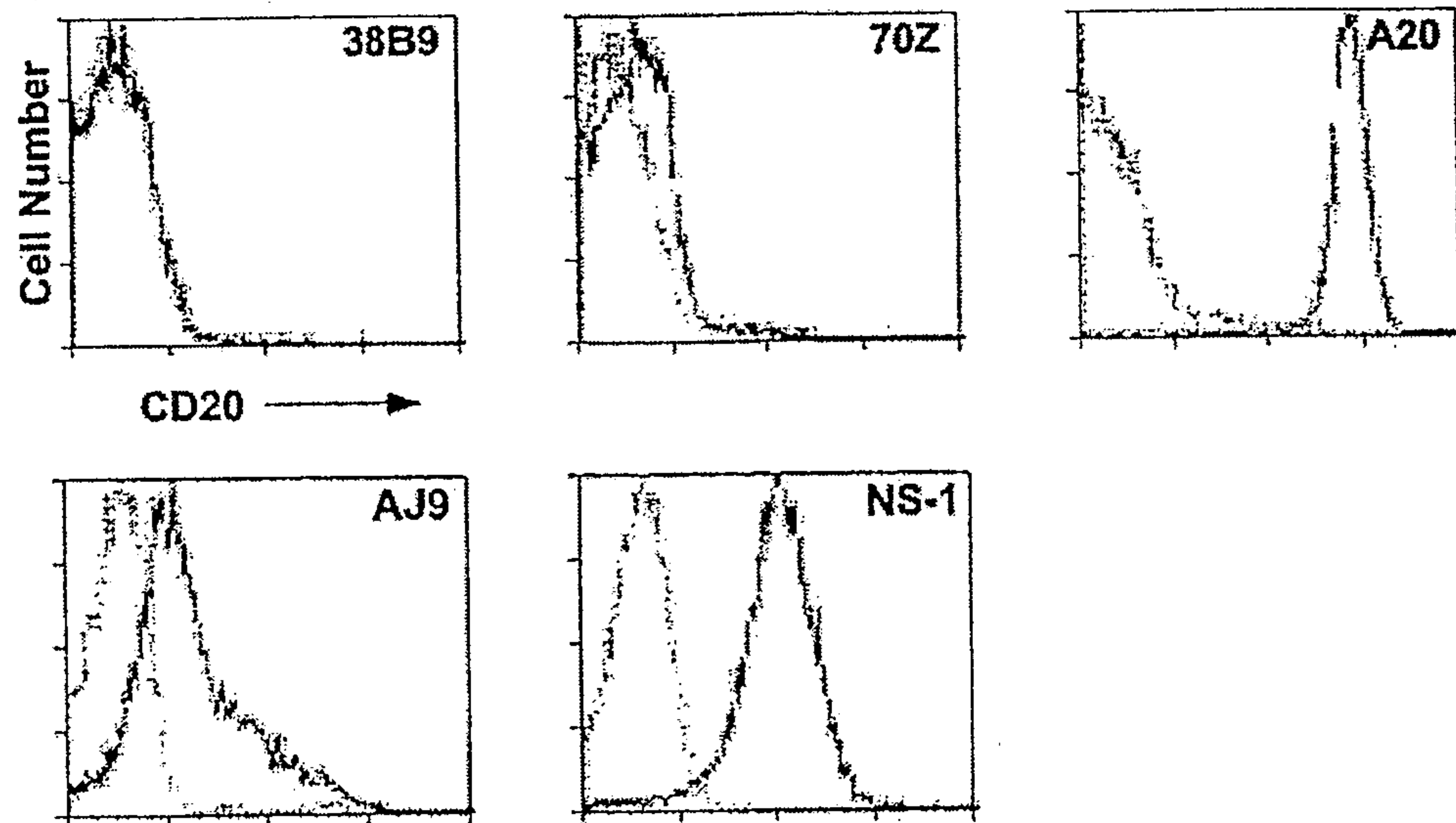
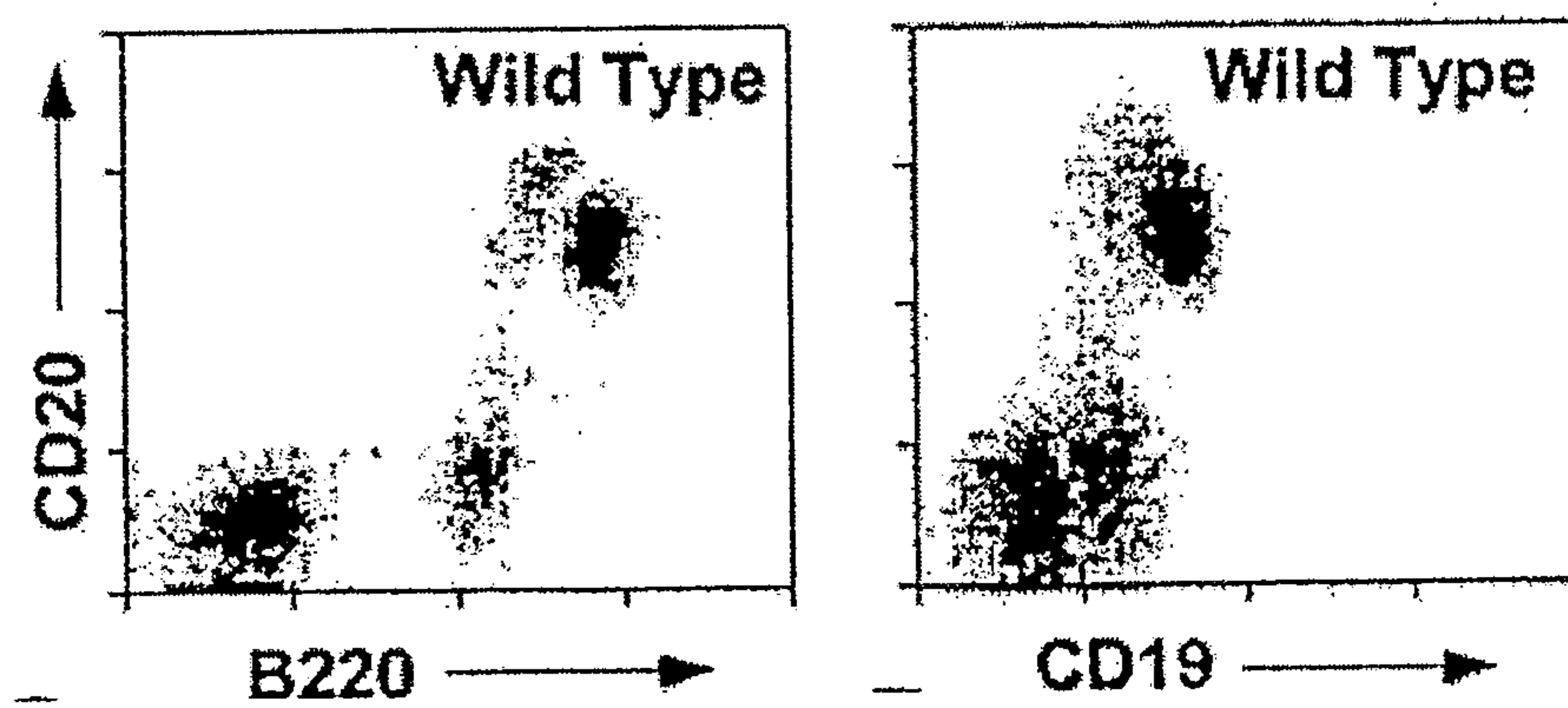


FIG. 1M



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FIG. 2A Cell Lines**FIG. 2B** Bone Marrow

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FIG. 2C Blood

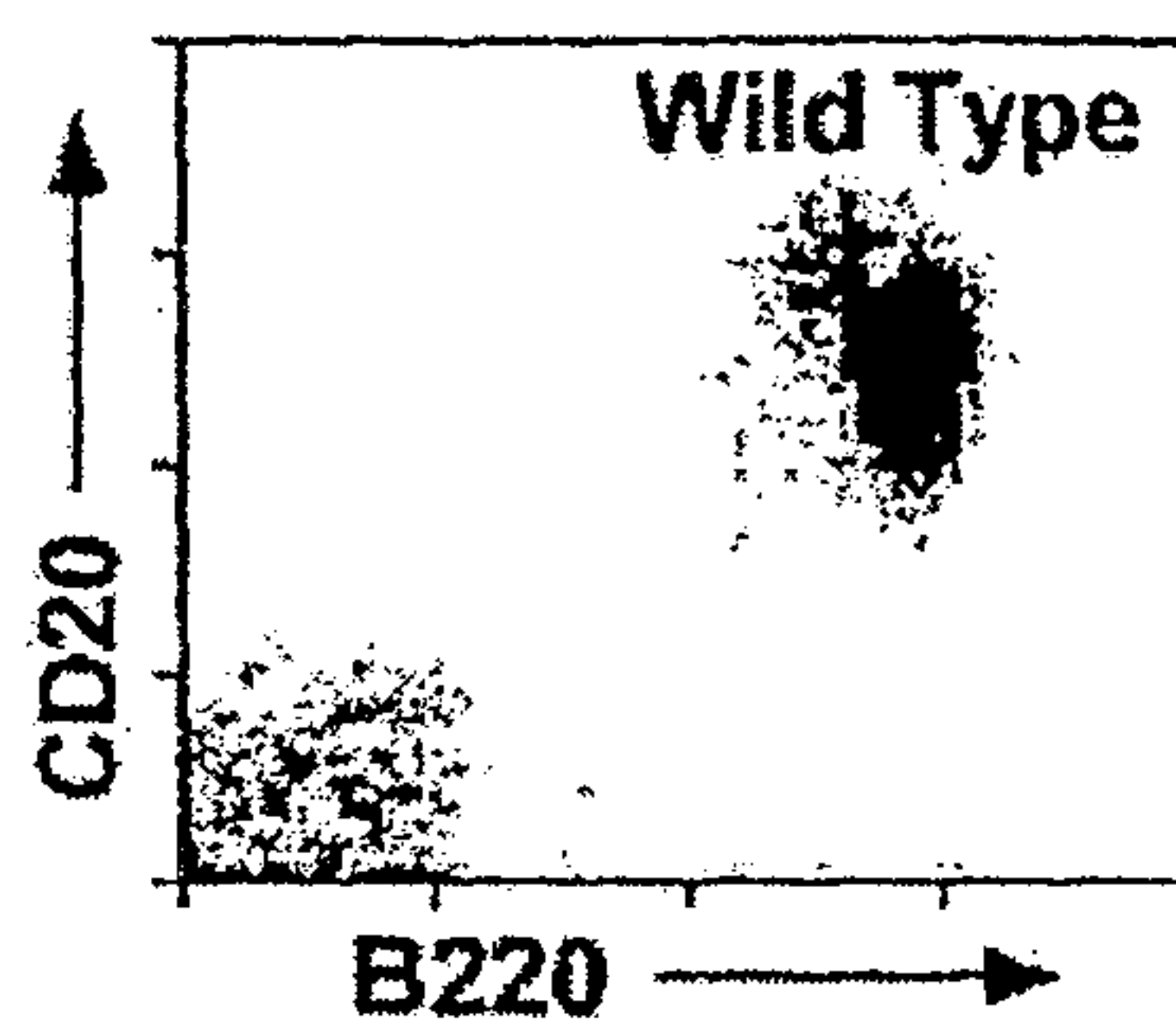


FIG. 2D PLN

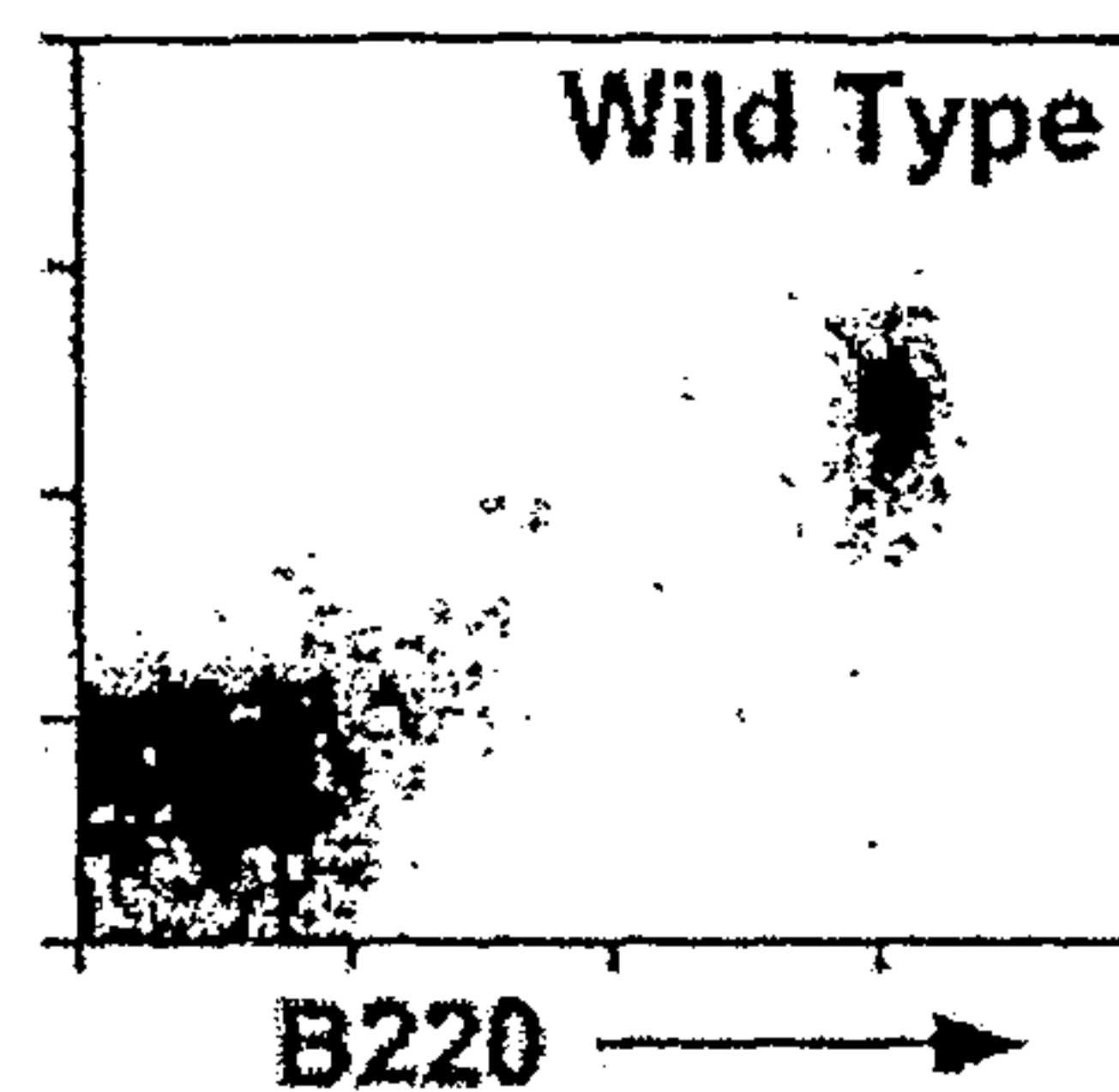


FIG. 2E Spleen

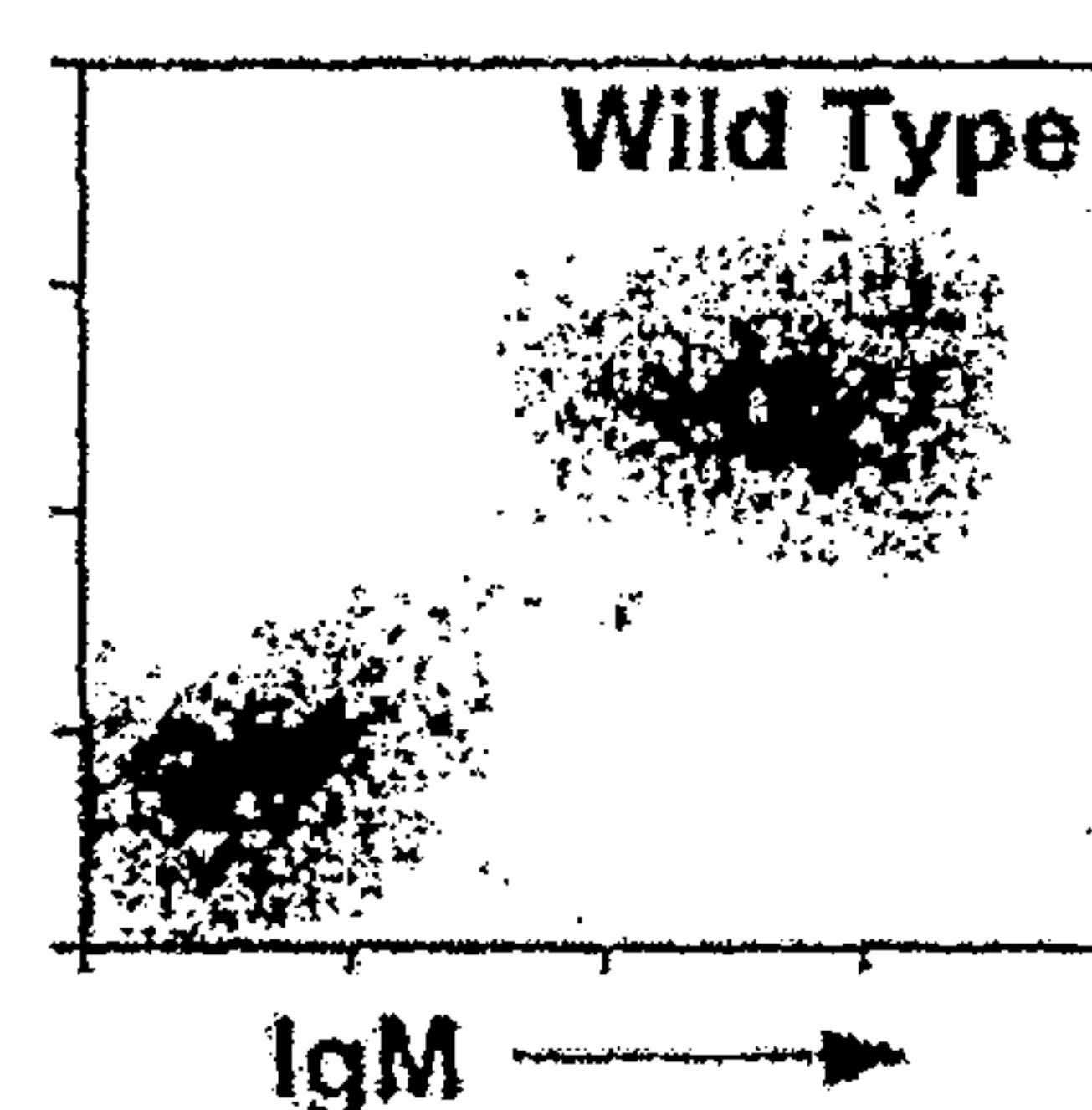
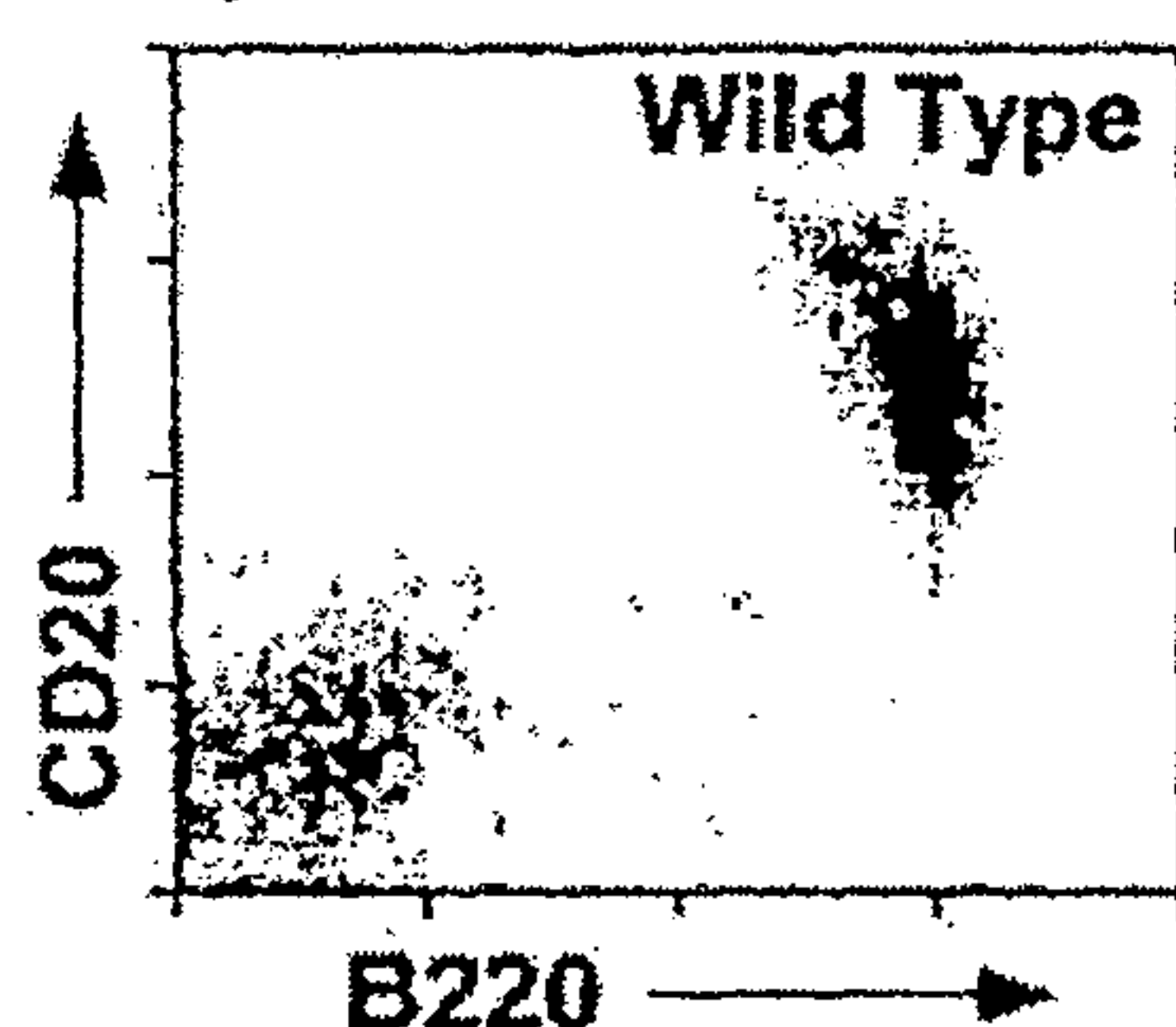


FIG. 2F Peritoneum

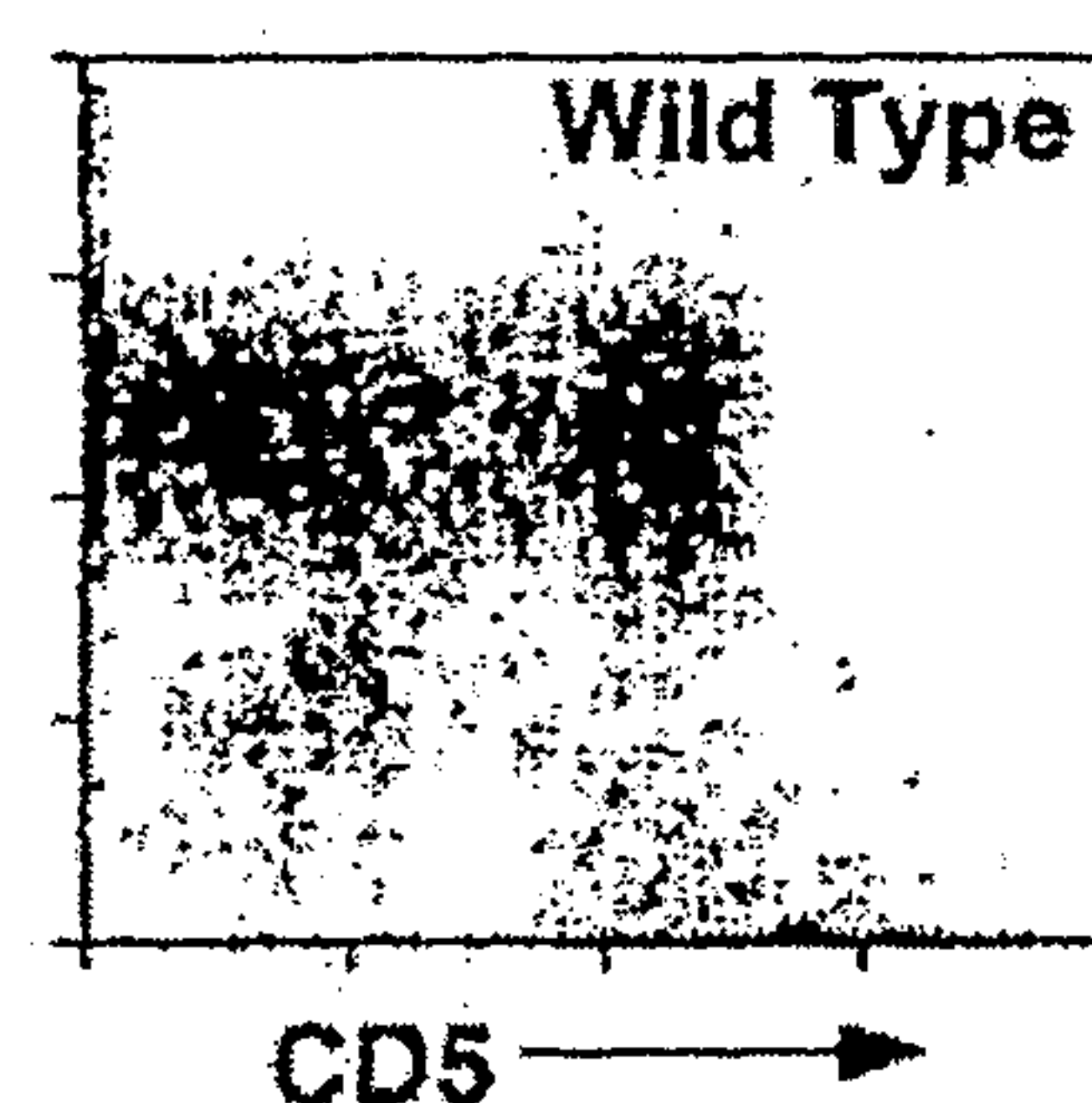
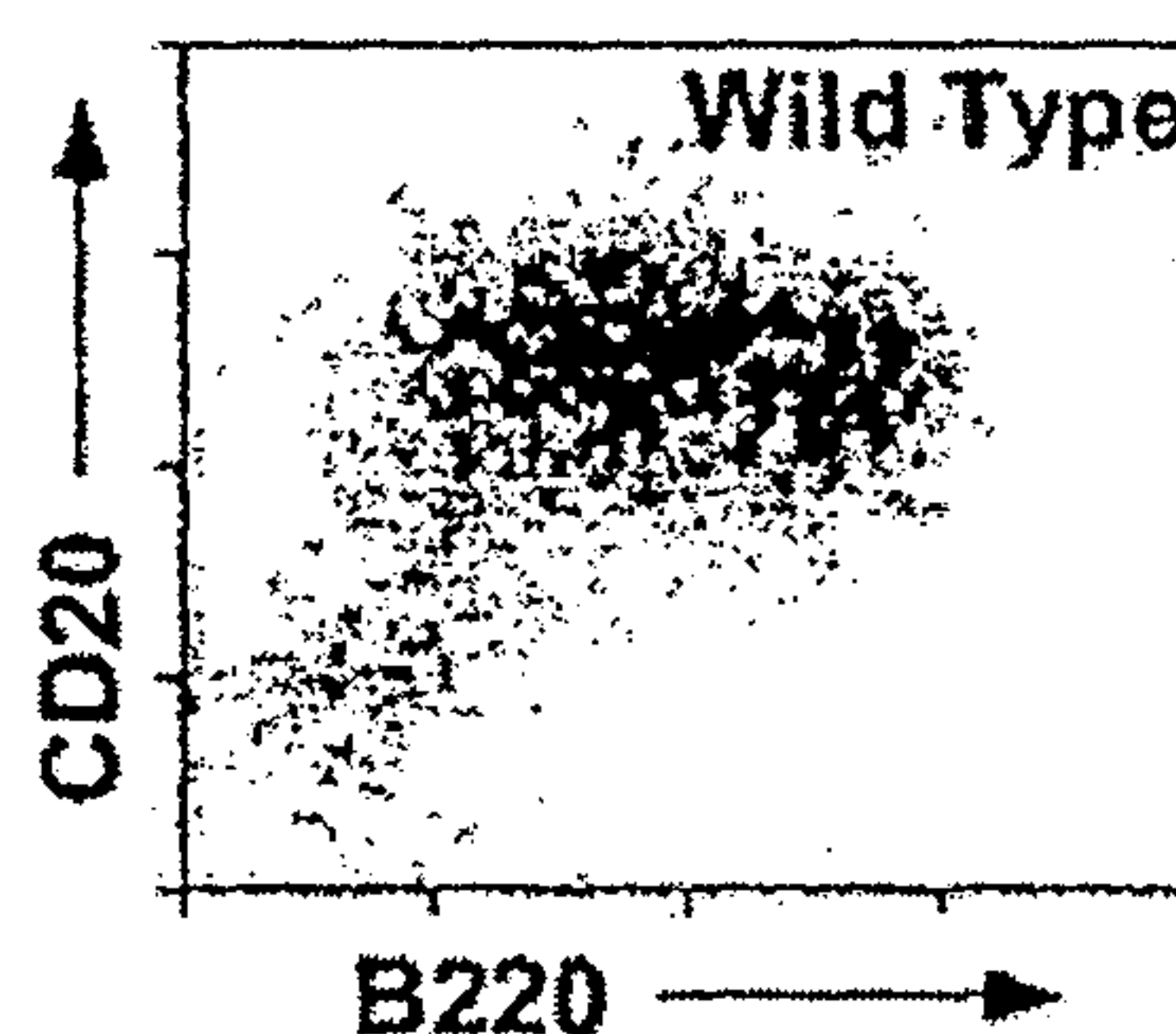
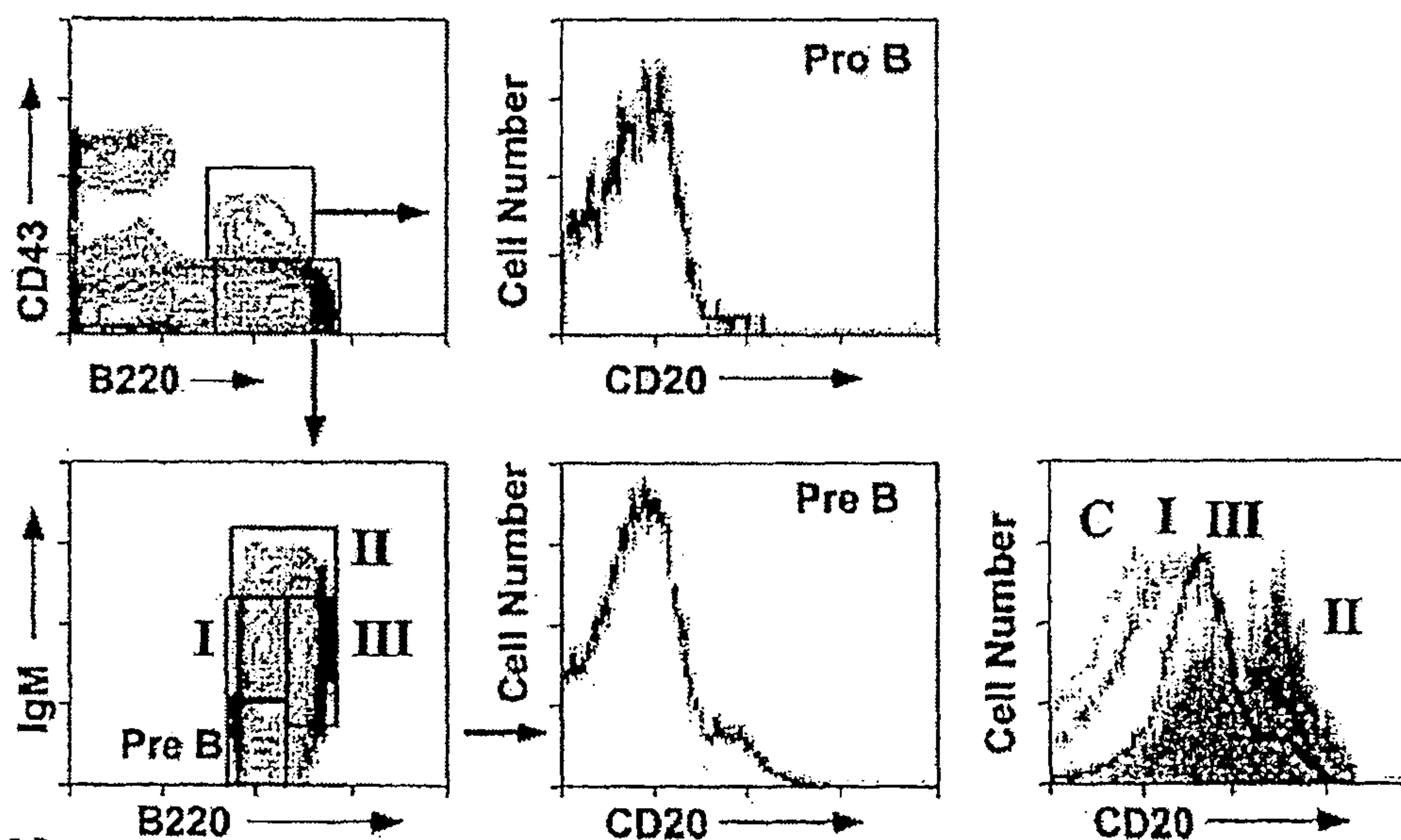


FIG. 2G Bone Marrow



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FIG. 2H Spleen

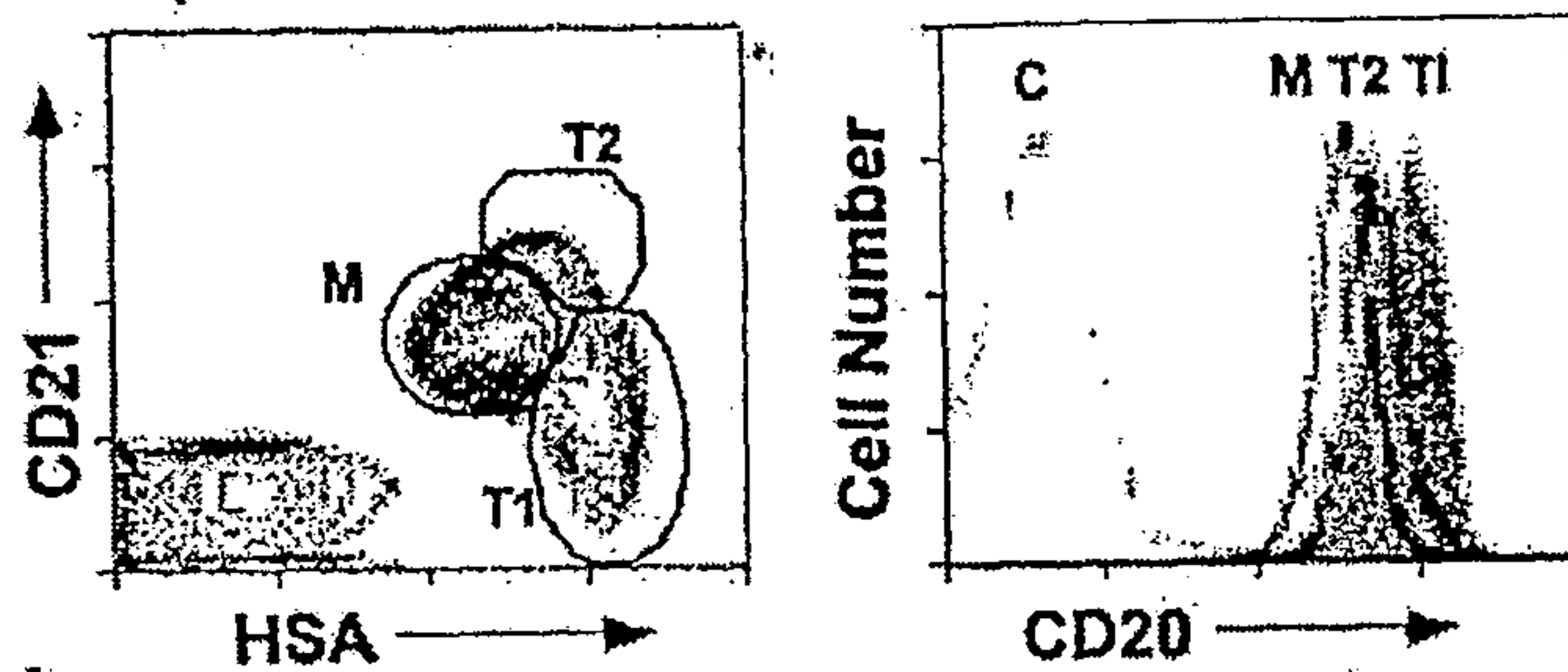


FIG. 2I Spleen

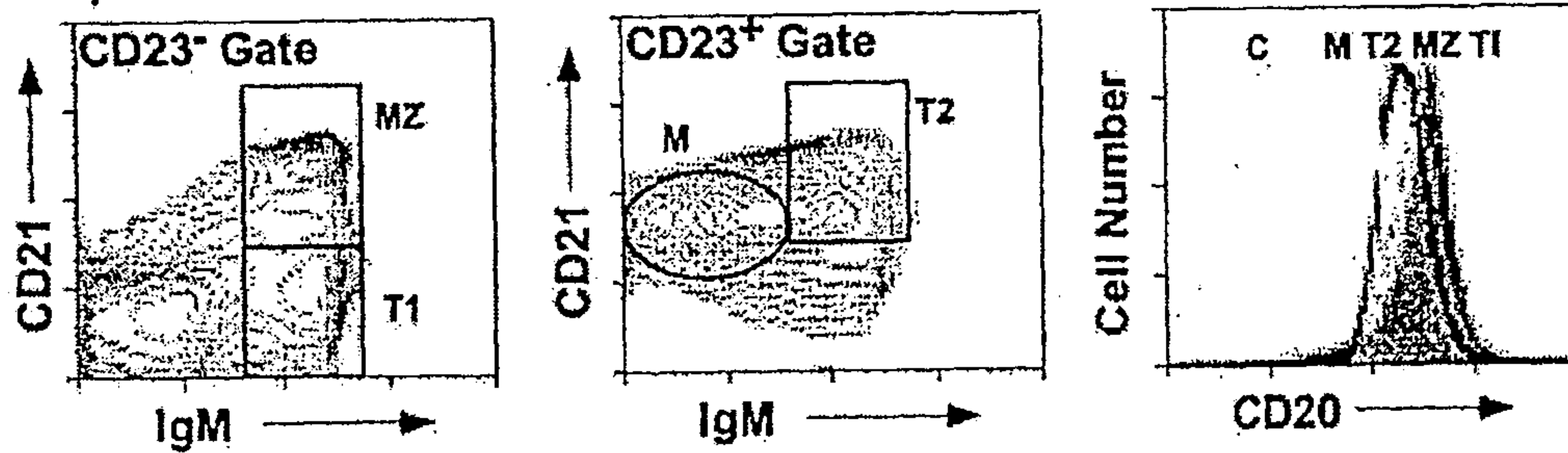


FIG. 3A

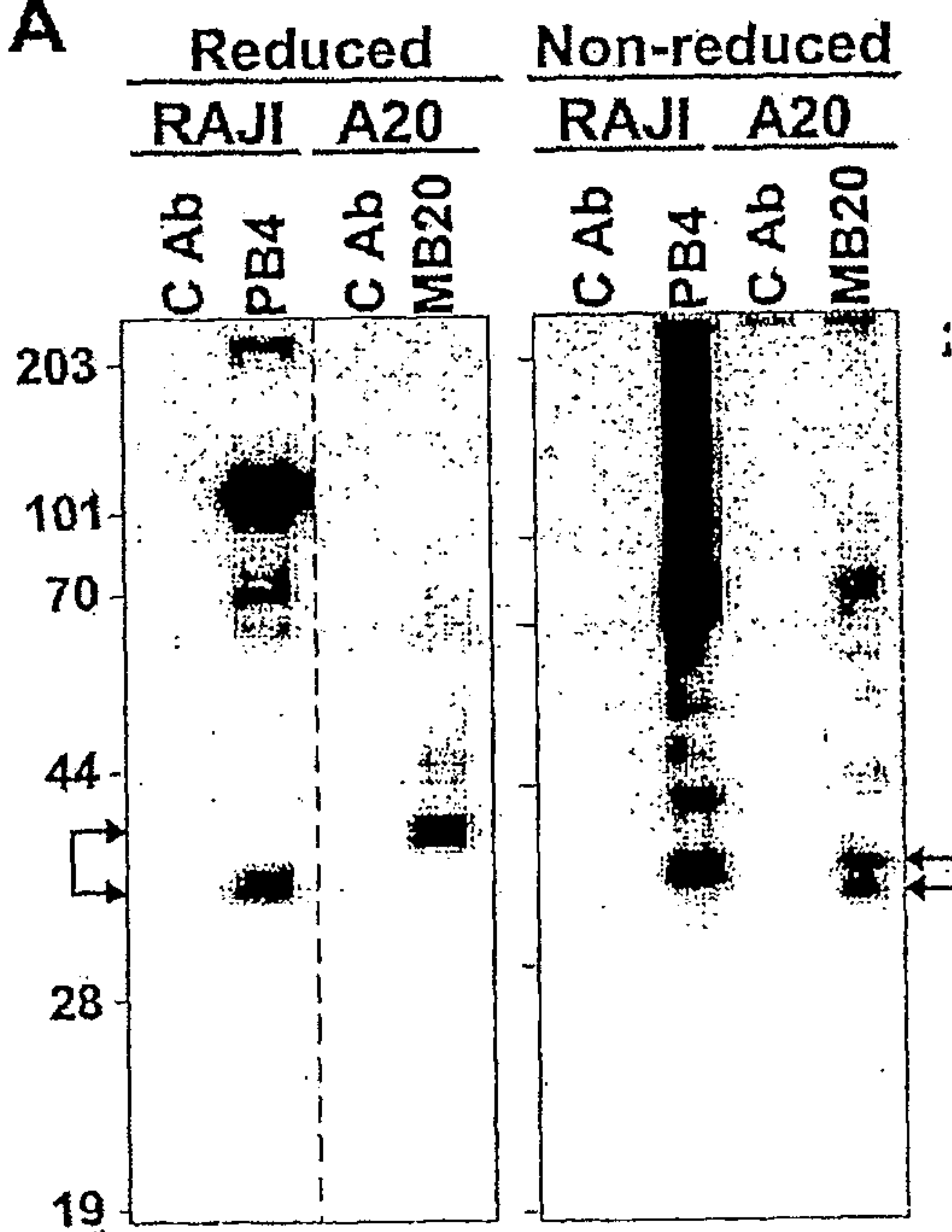
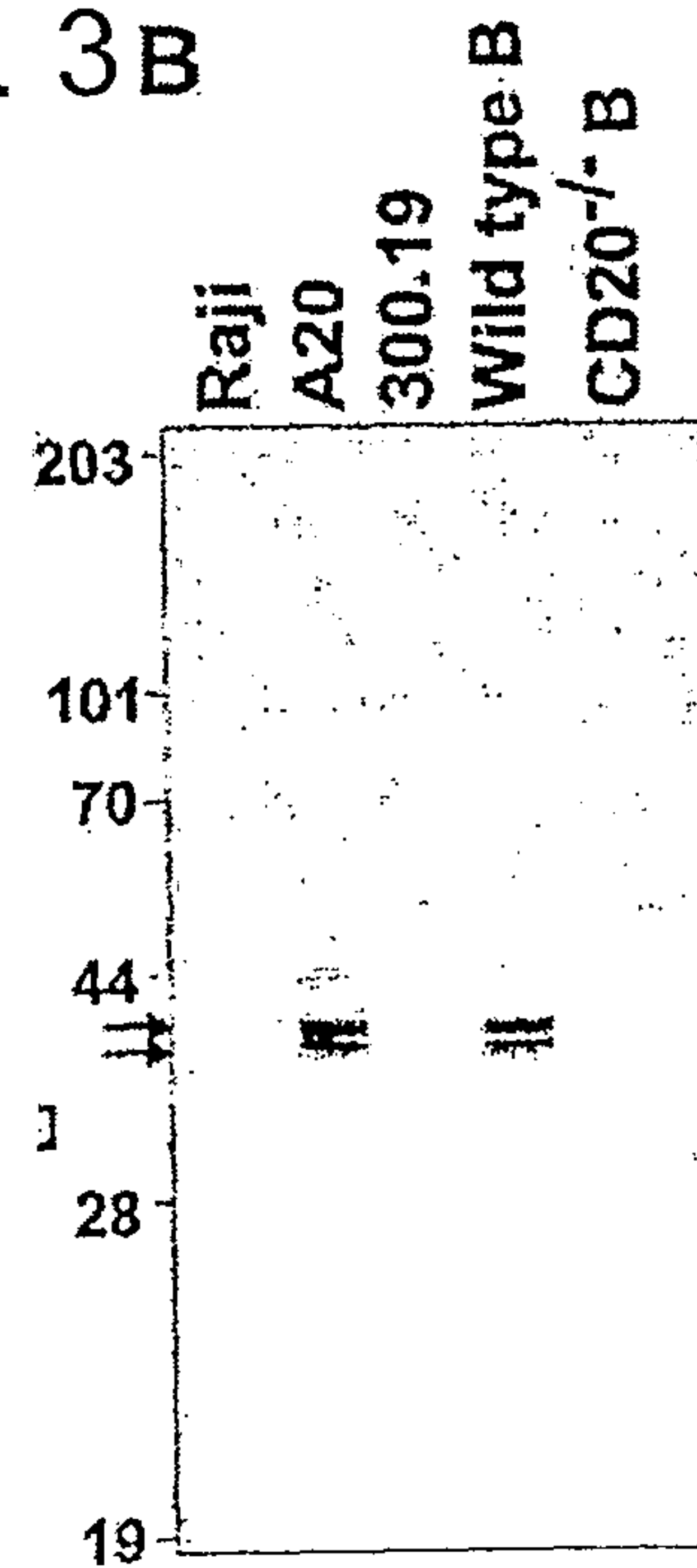


FIG. 3B



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FIG. 3C

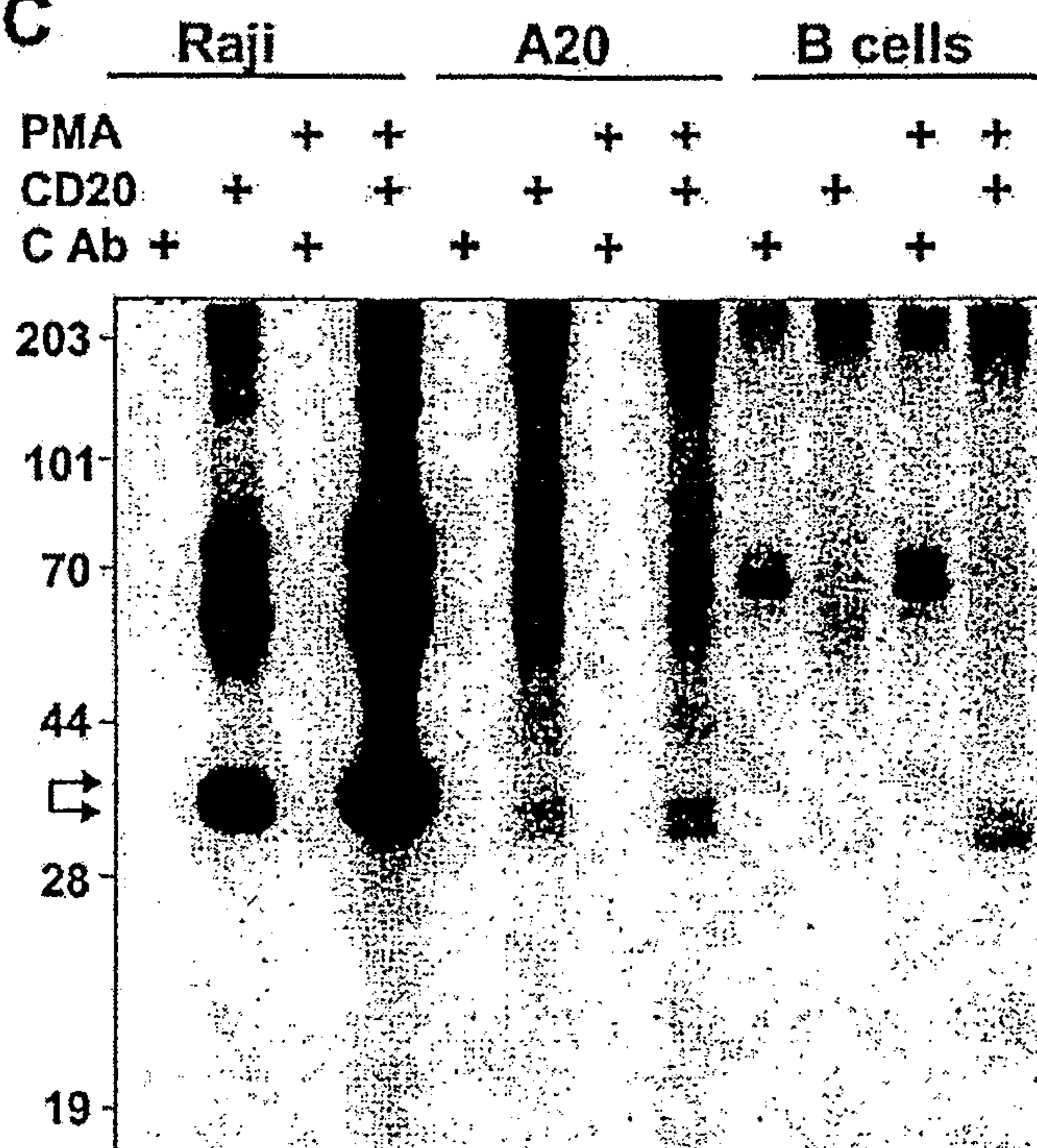


FIG. 4A

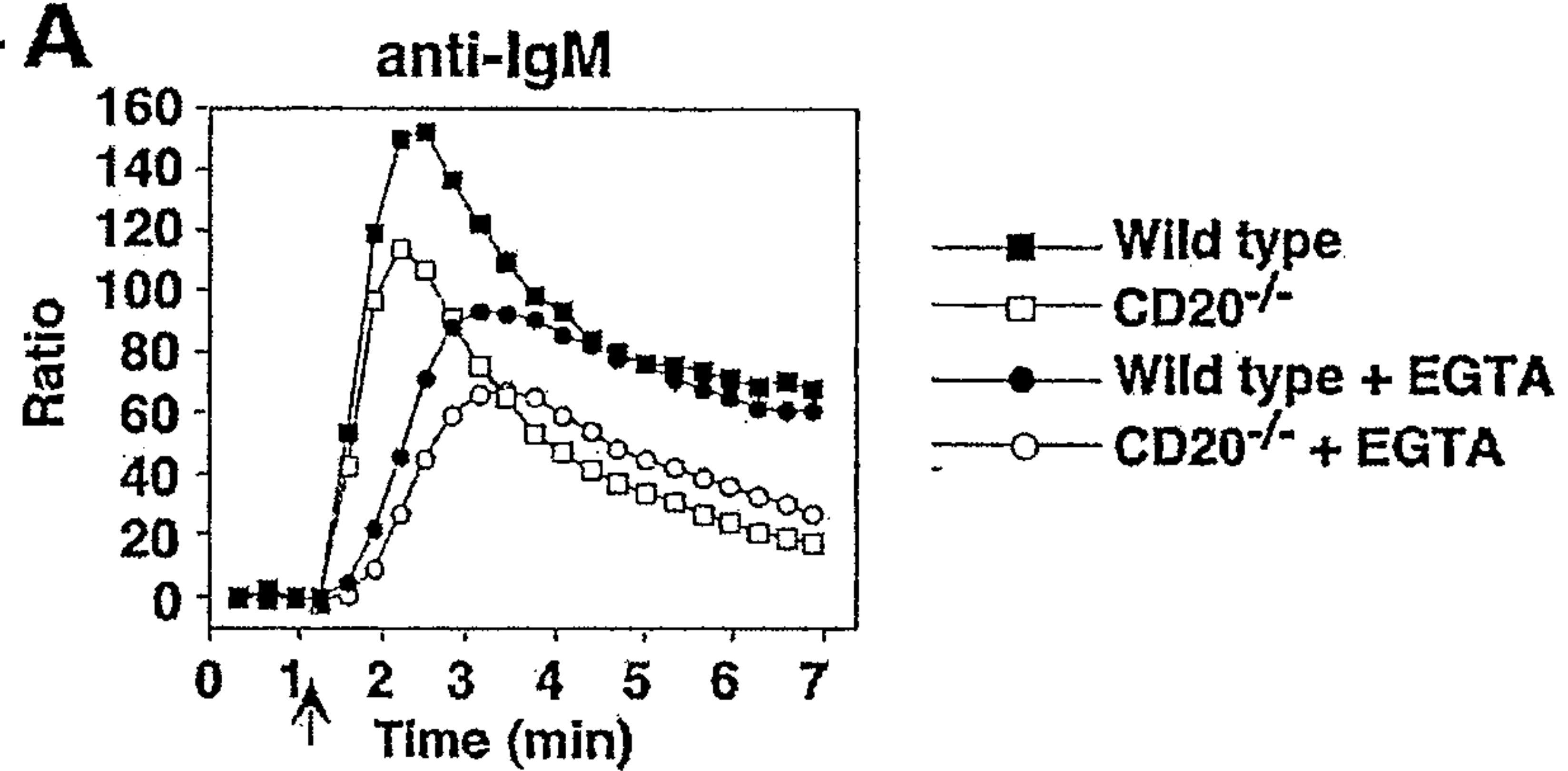
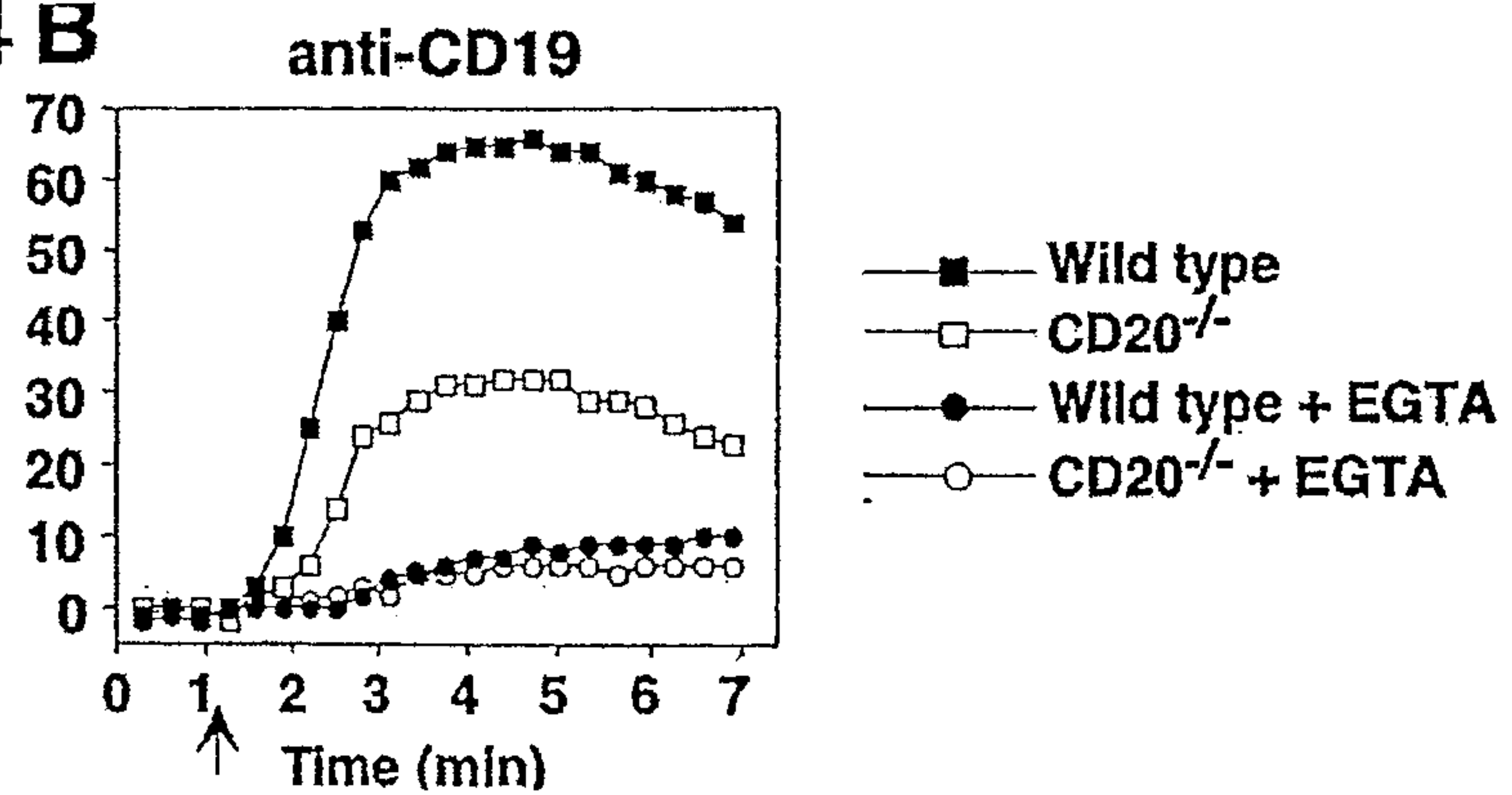


FIG. 4B



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FIG. 4C

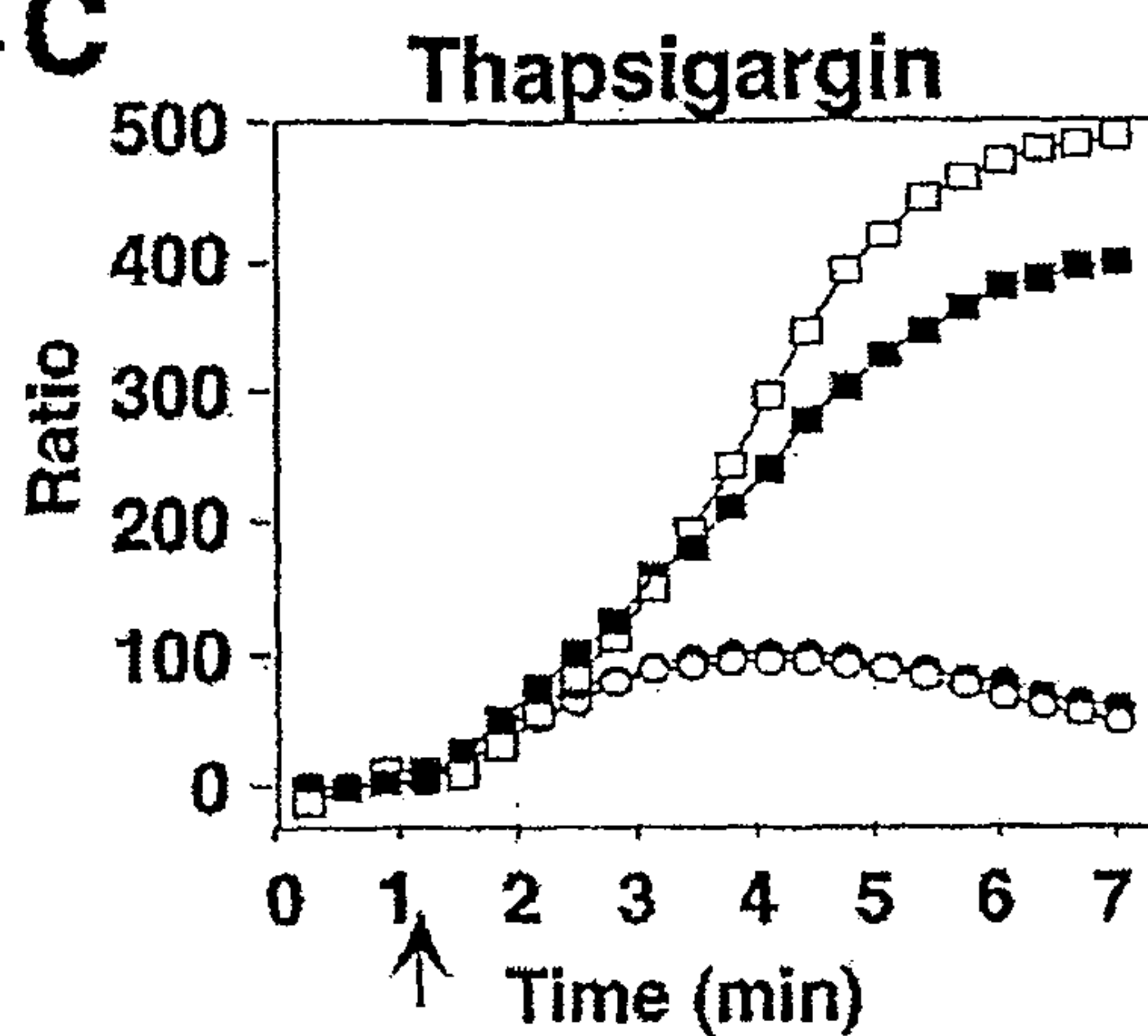


FIG. 4D

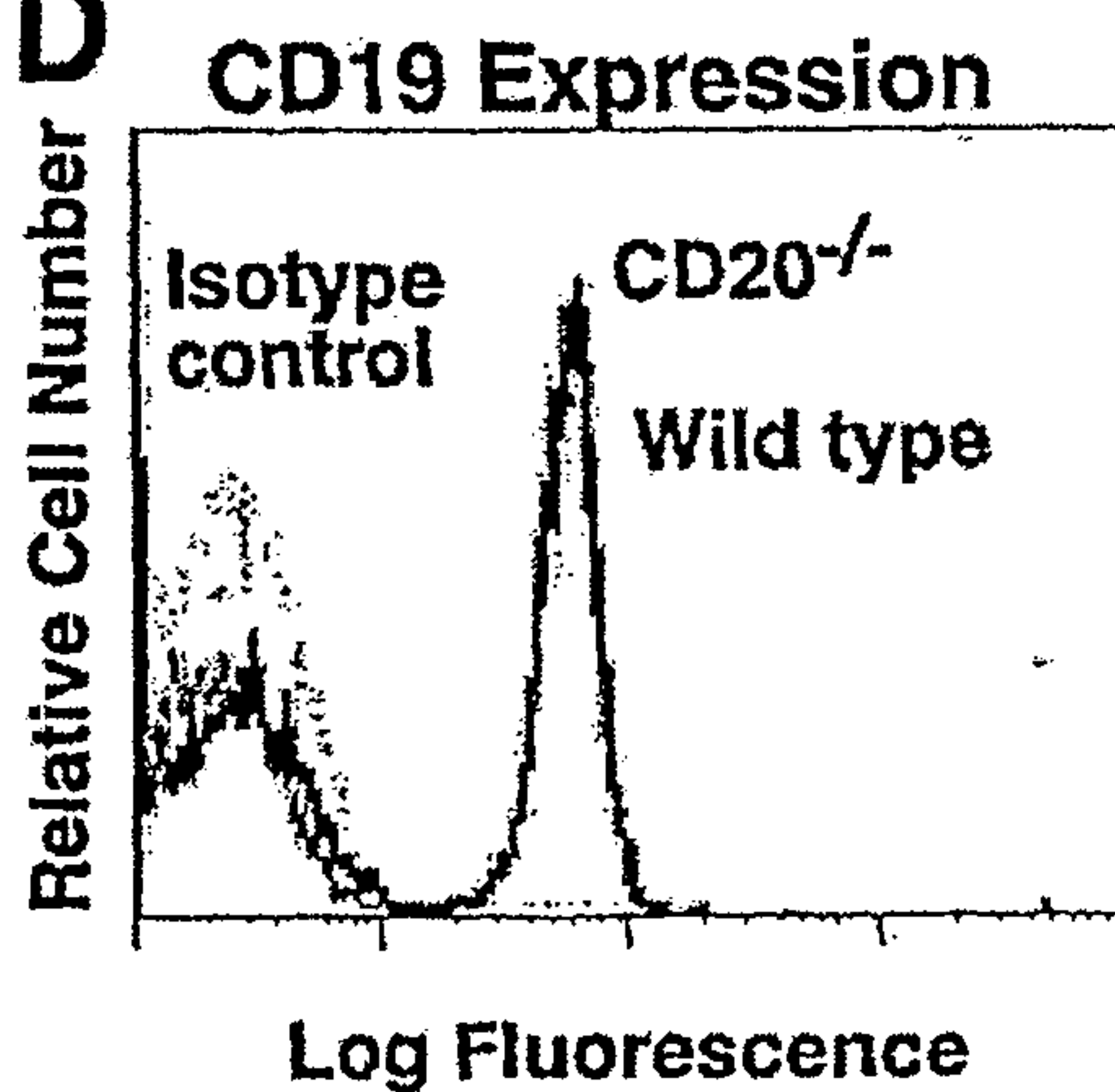
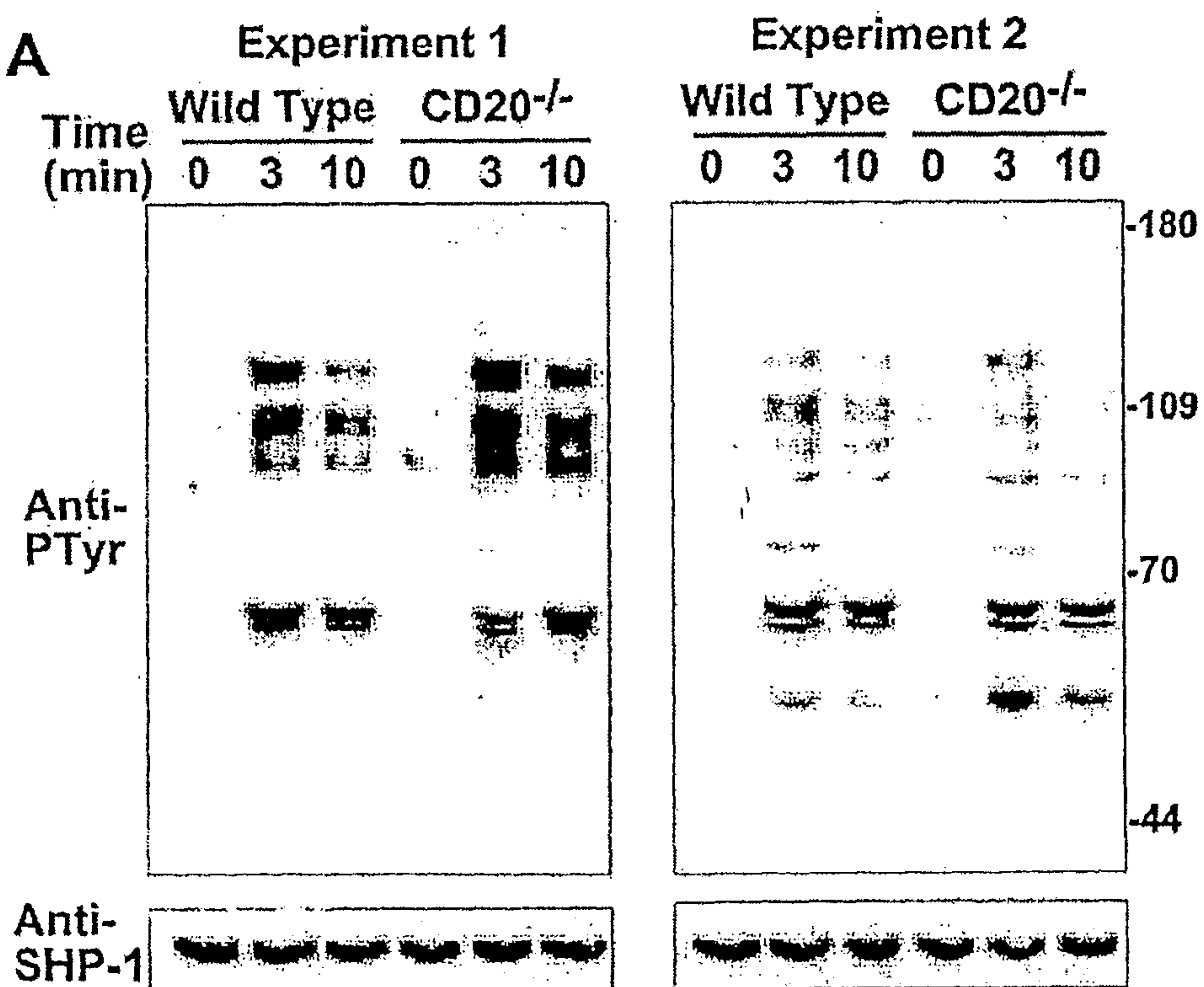
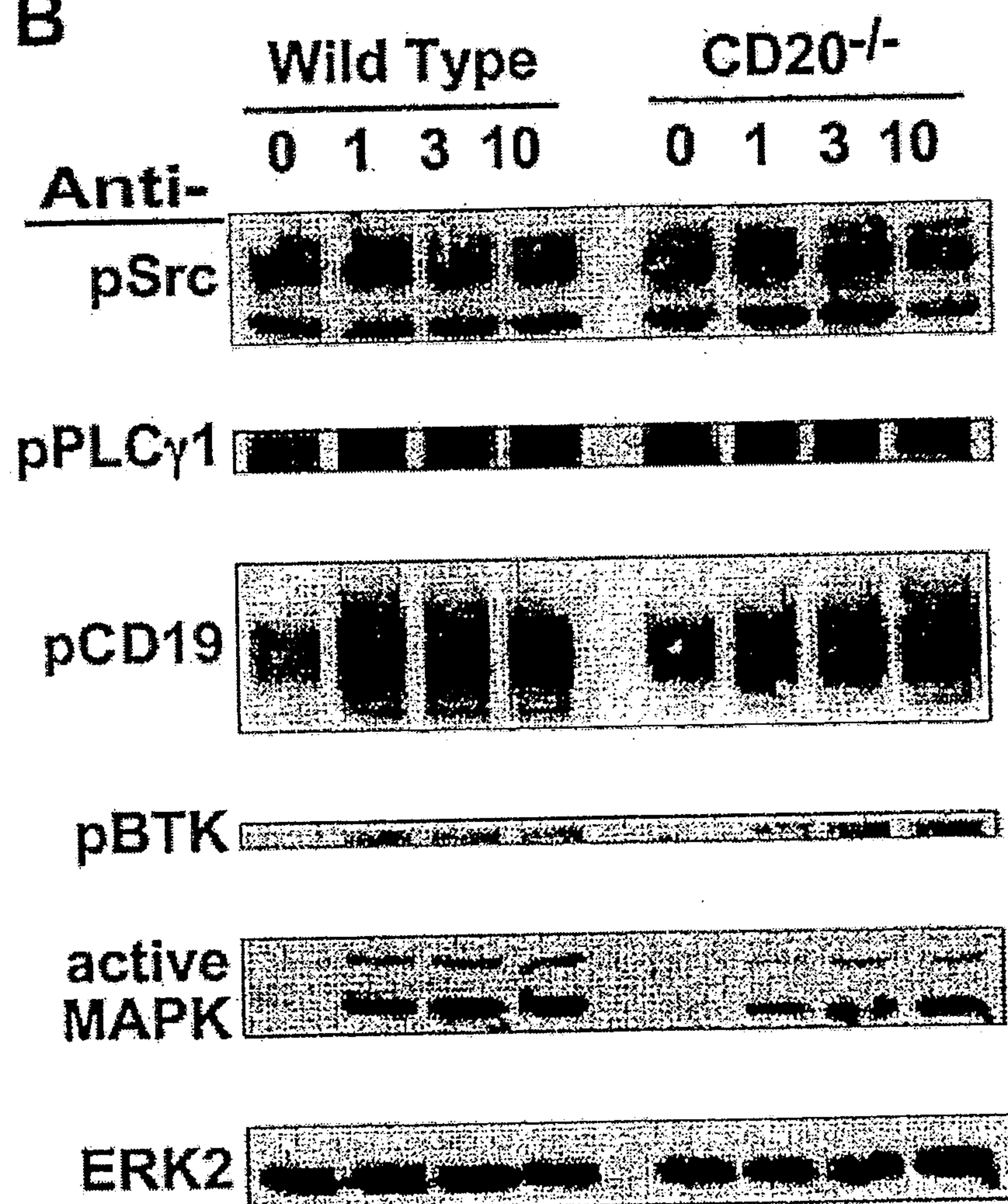


FIG. 5A

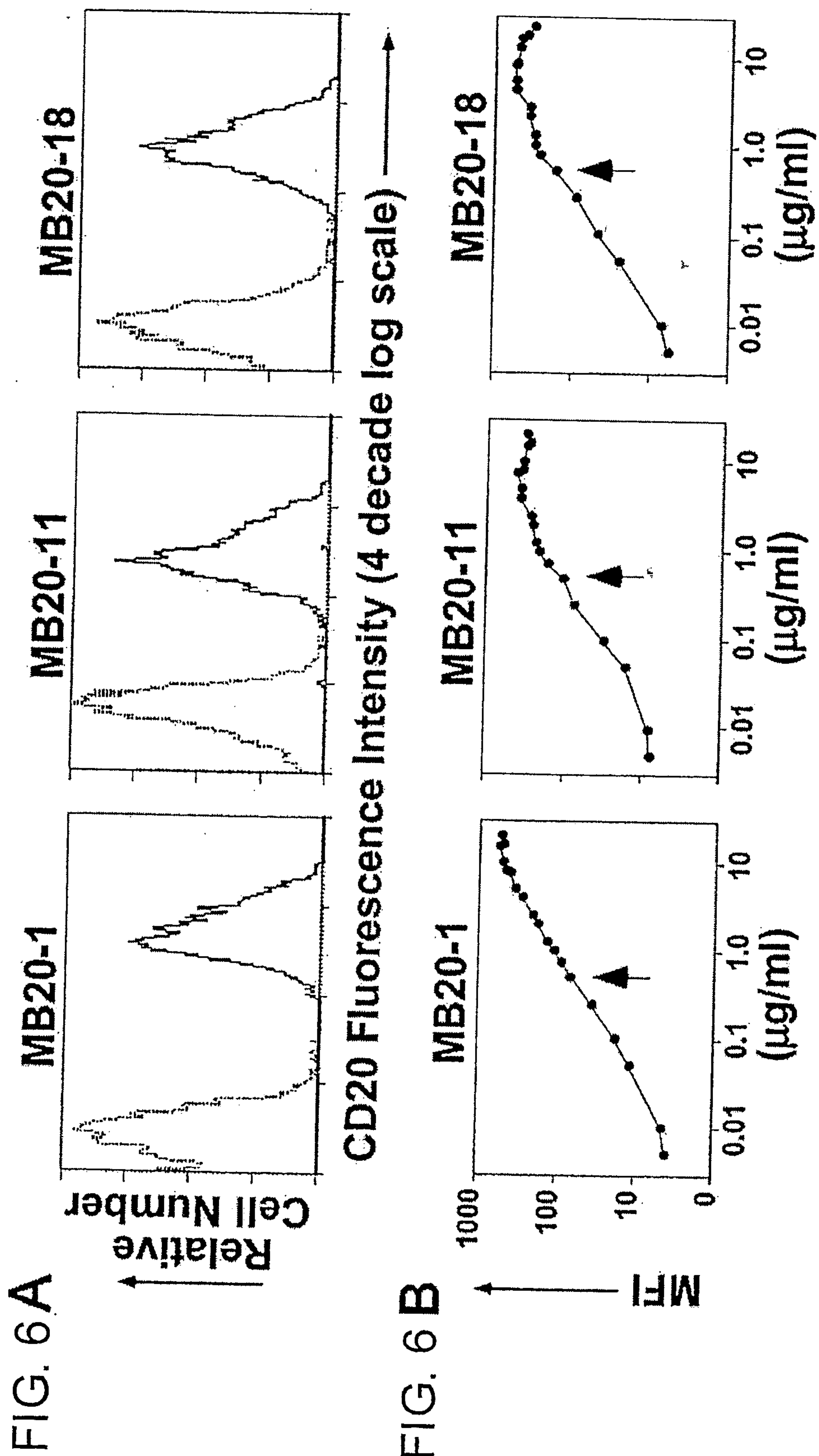


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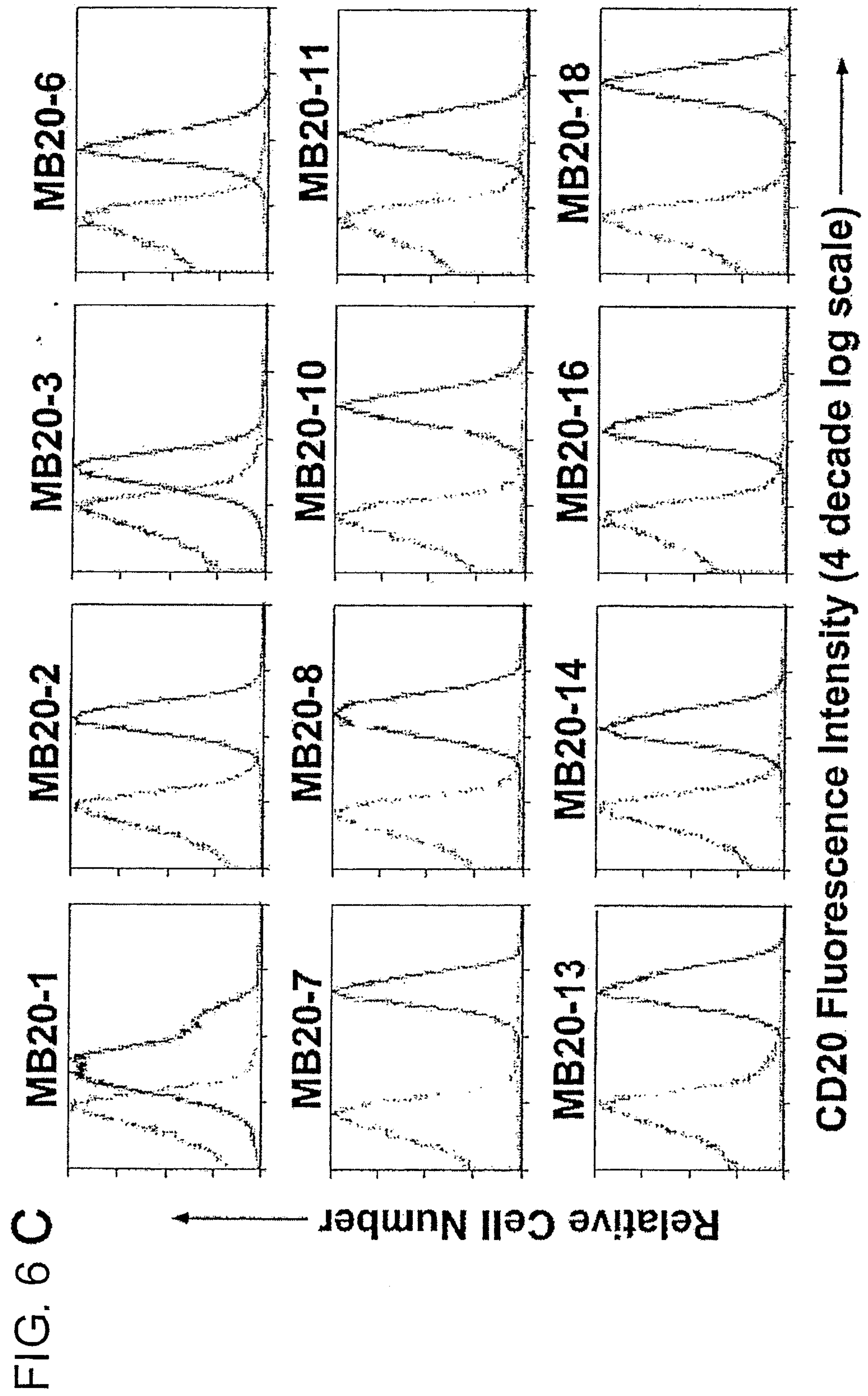
FIG. 5 B



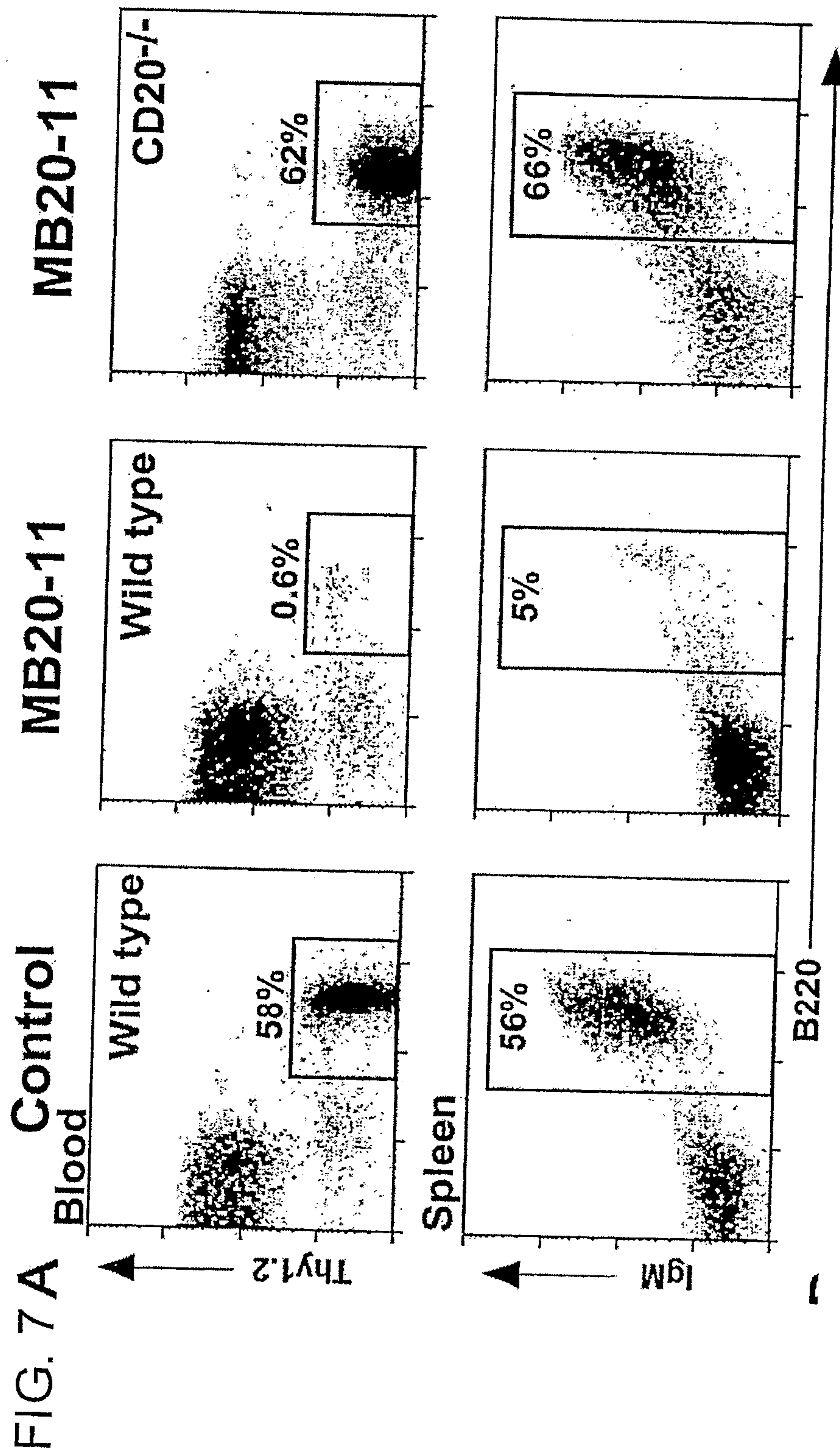
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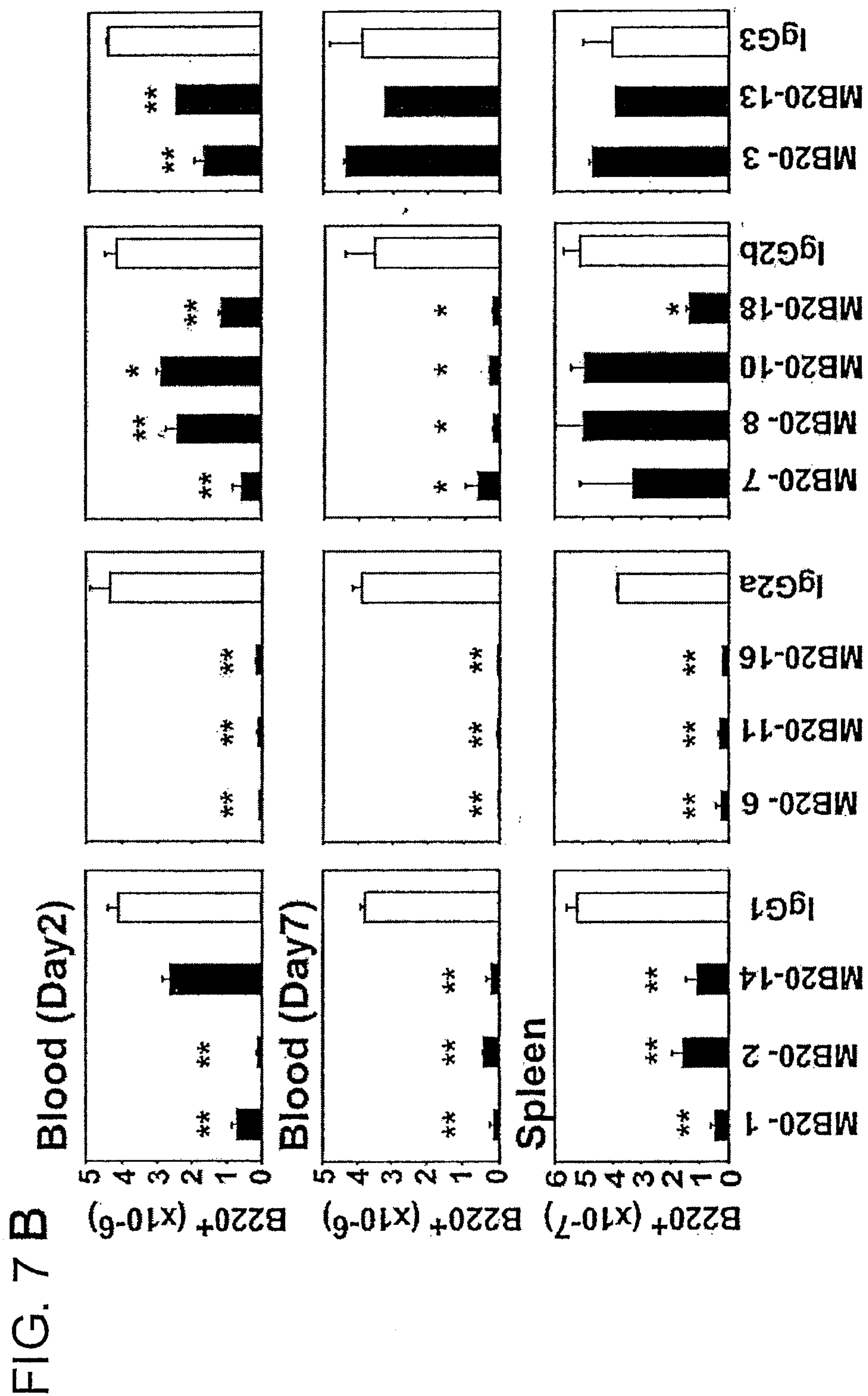
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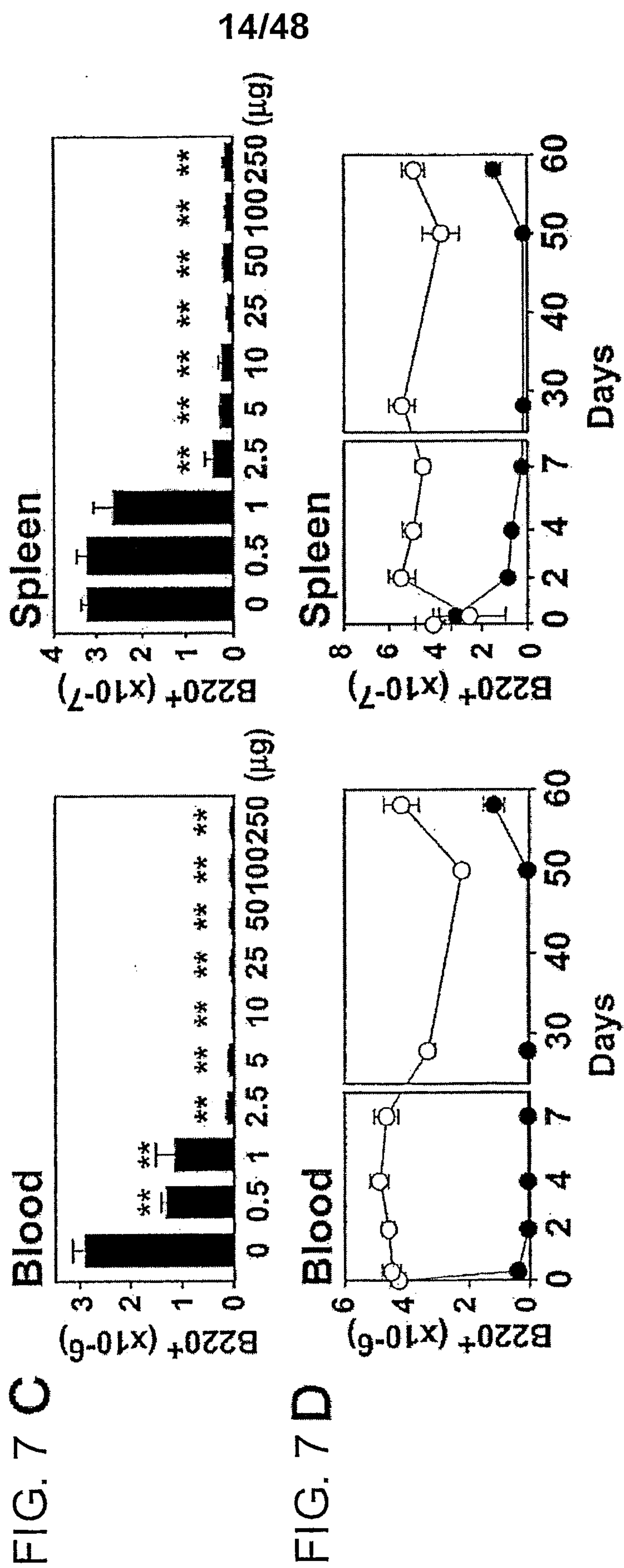


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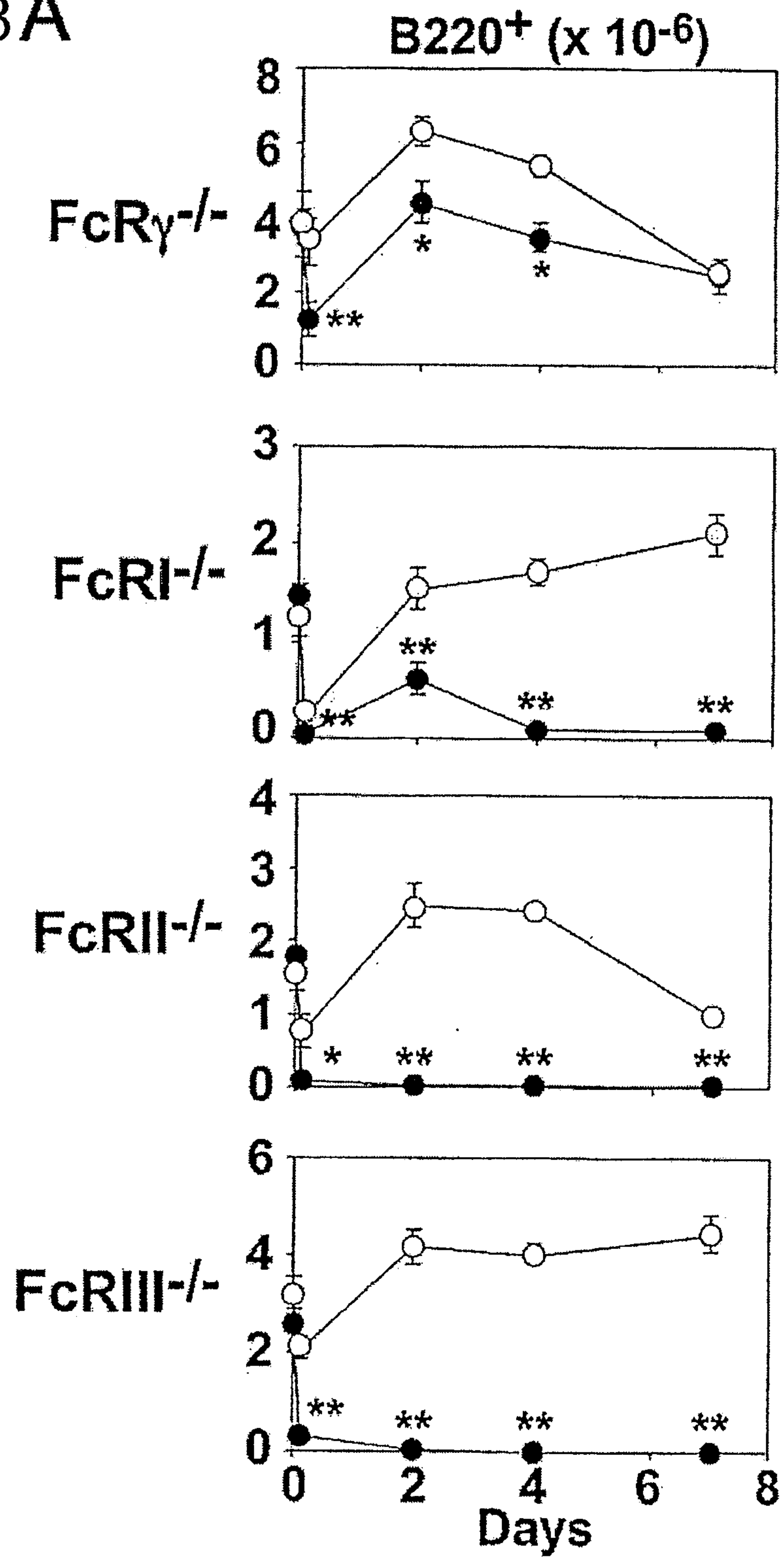




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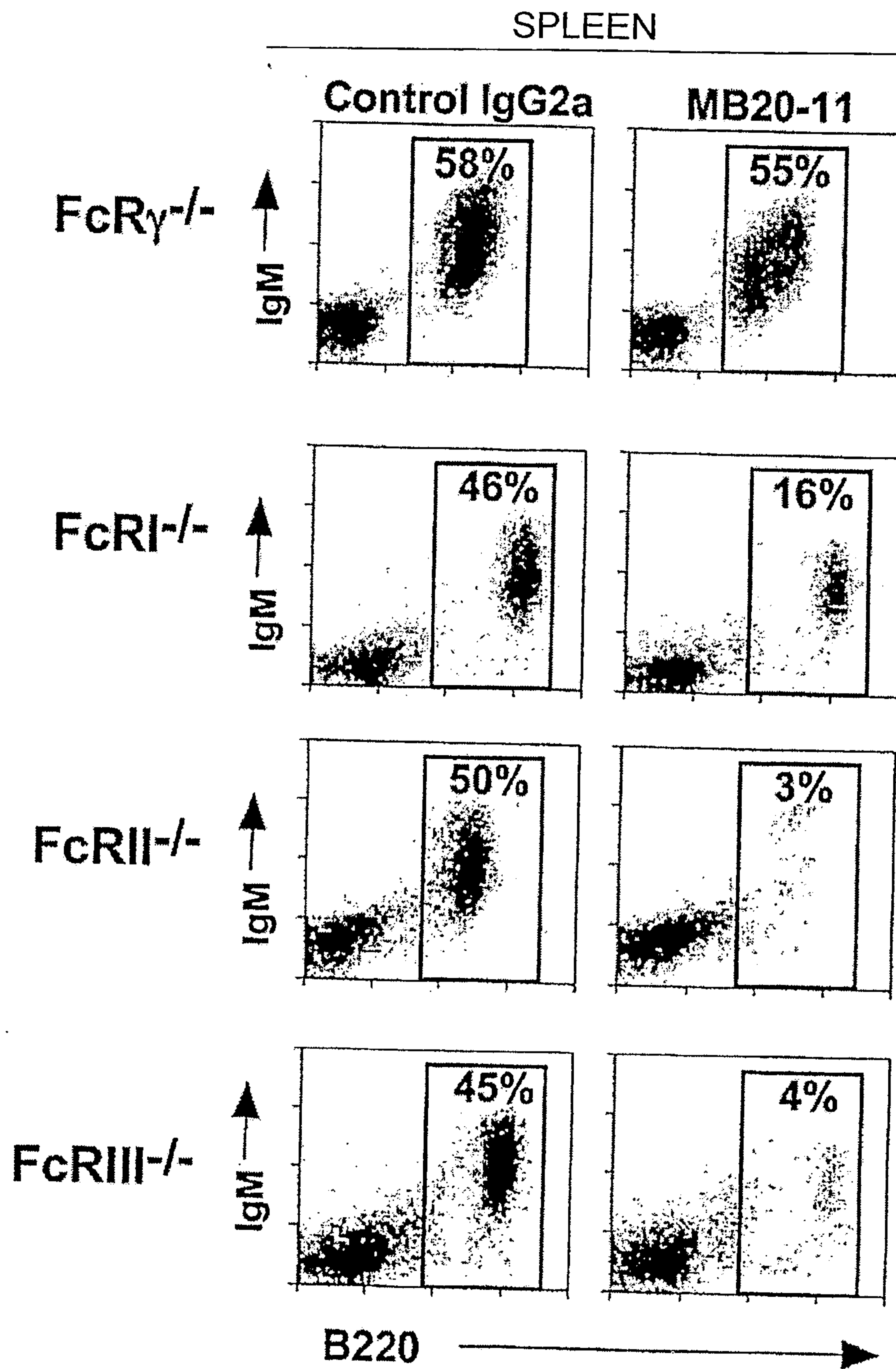
Blood

FIG. 8A



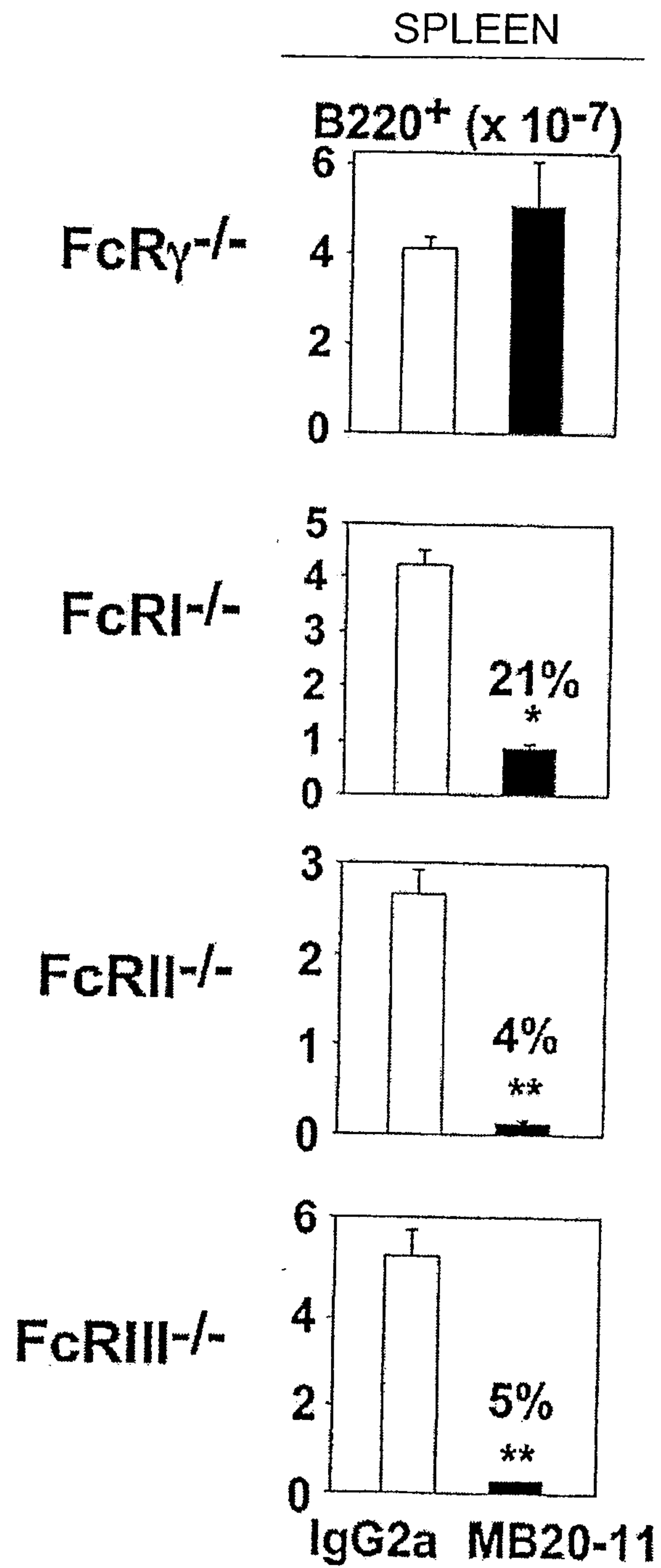
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FIG. 8B

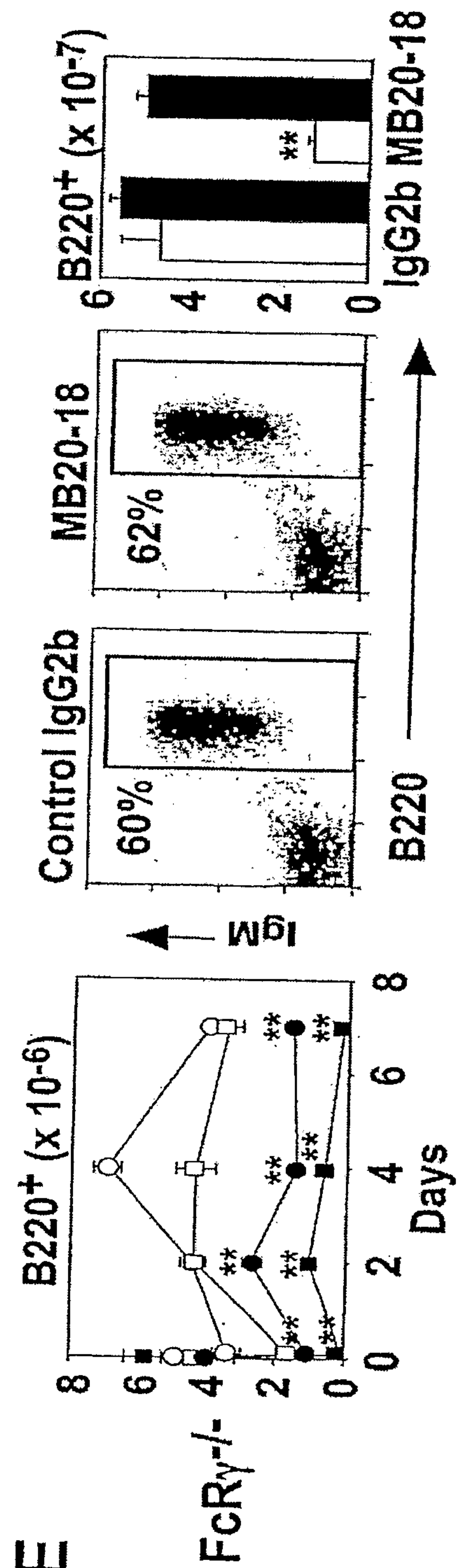
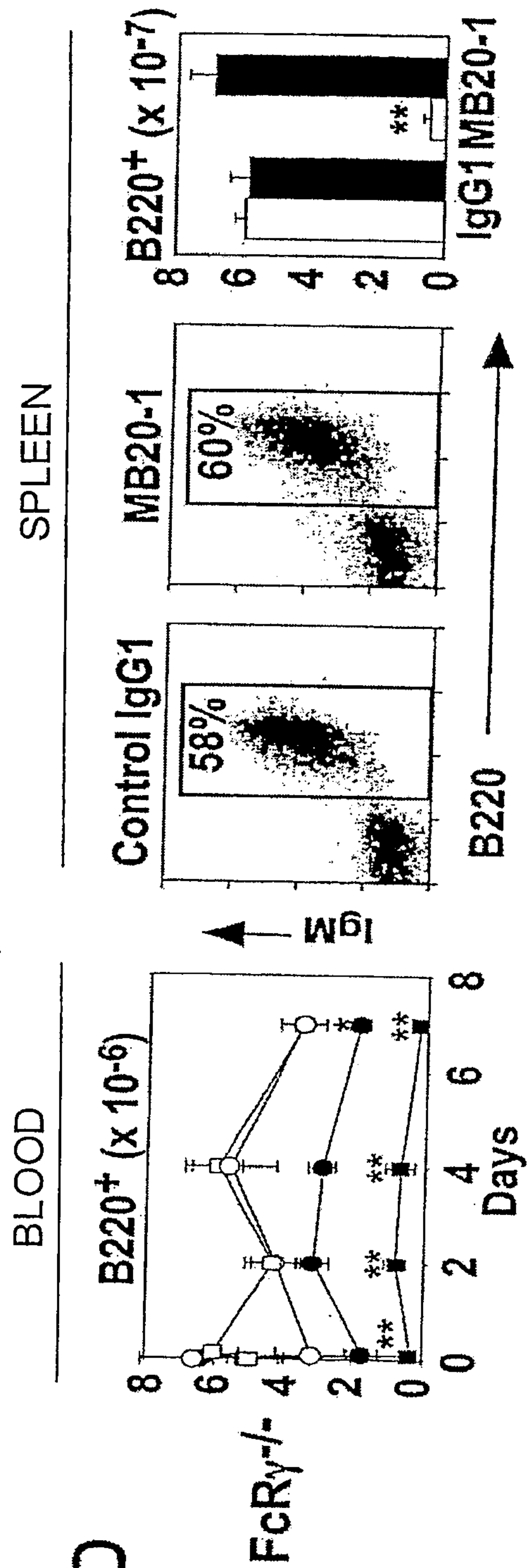


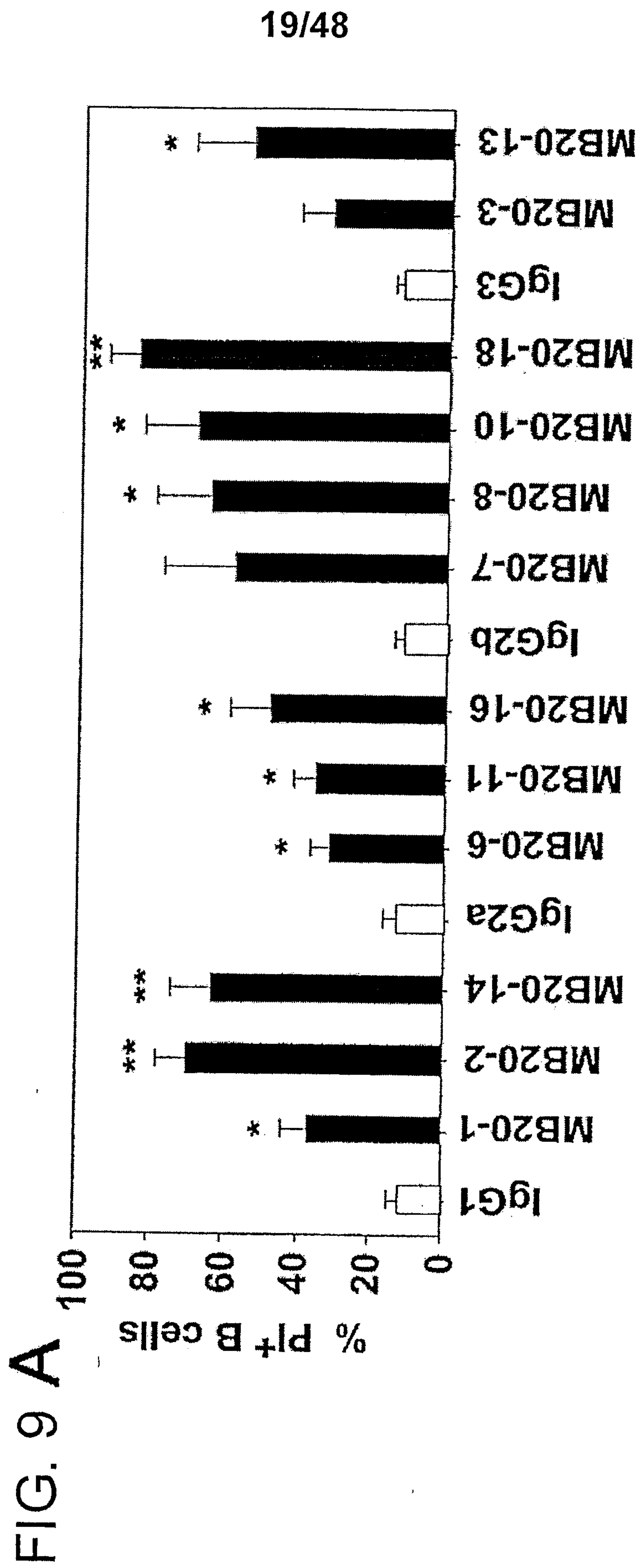
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FIG. 8C

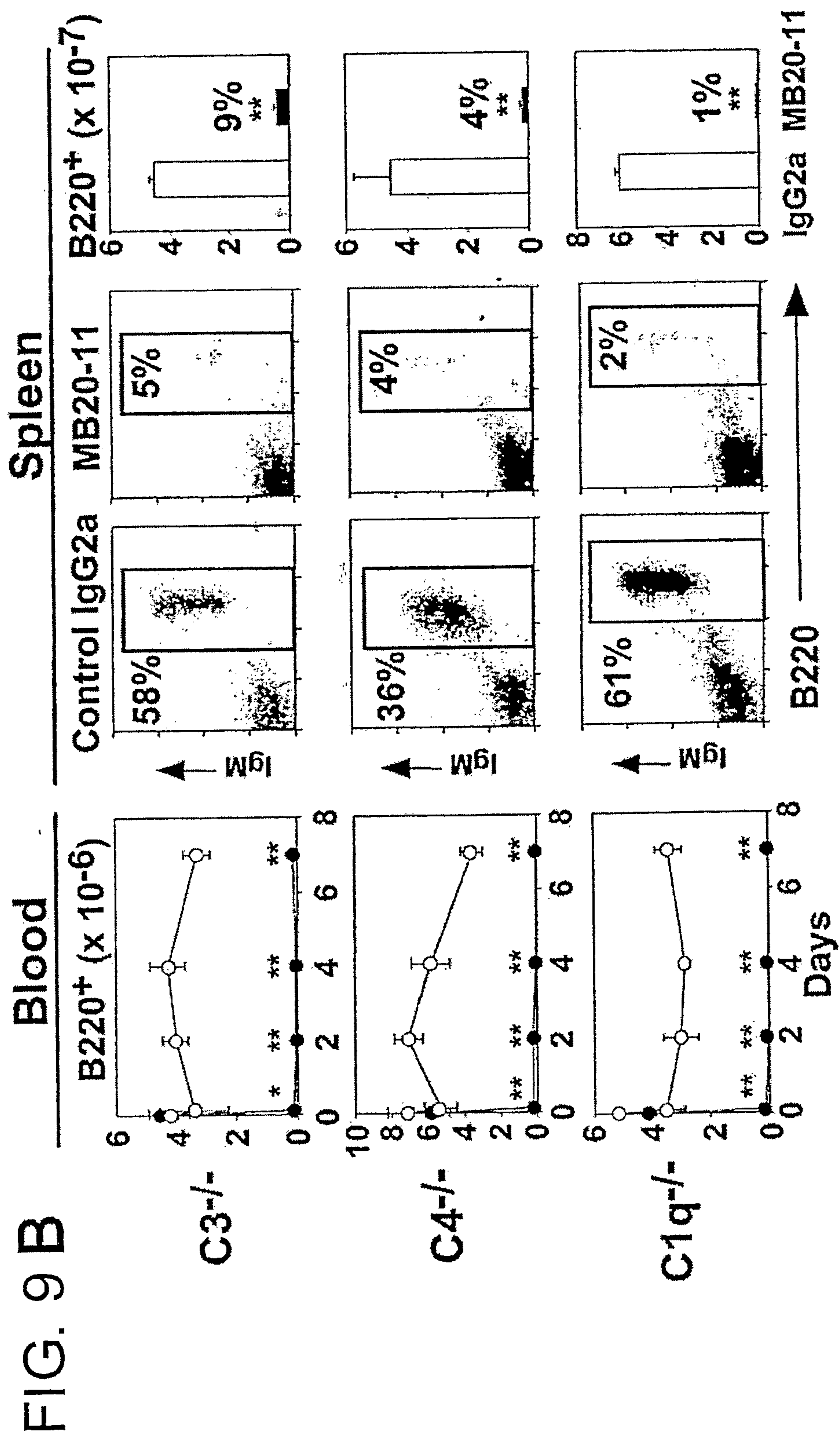


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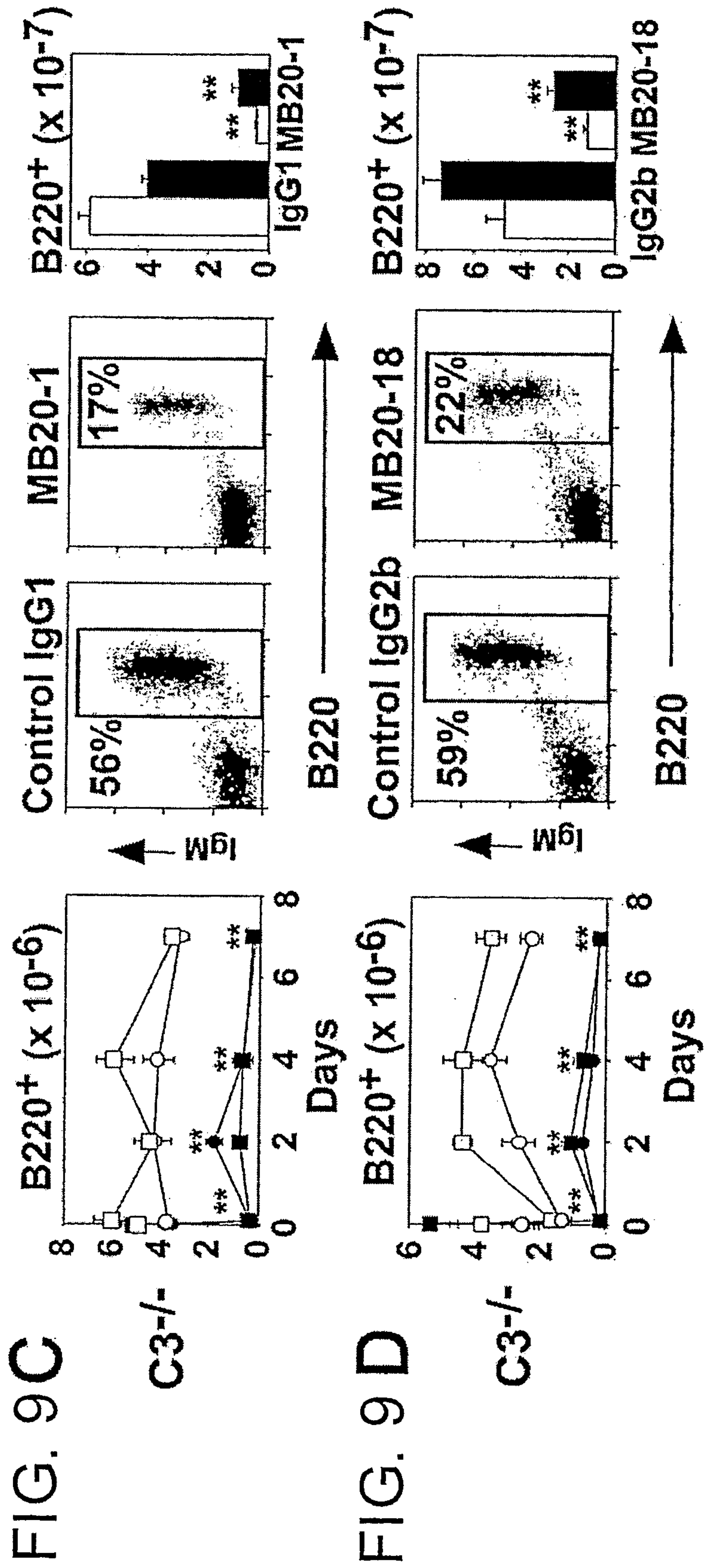




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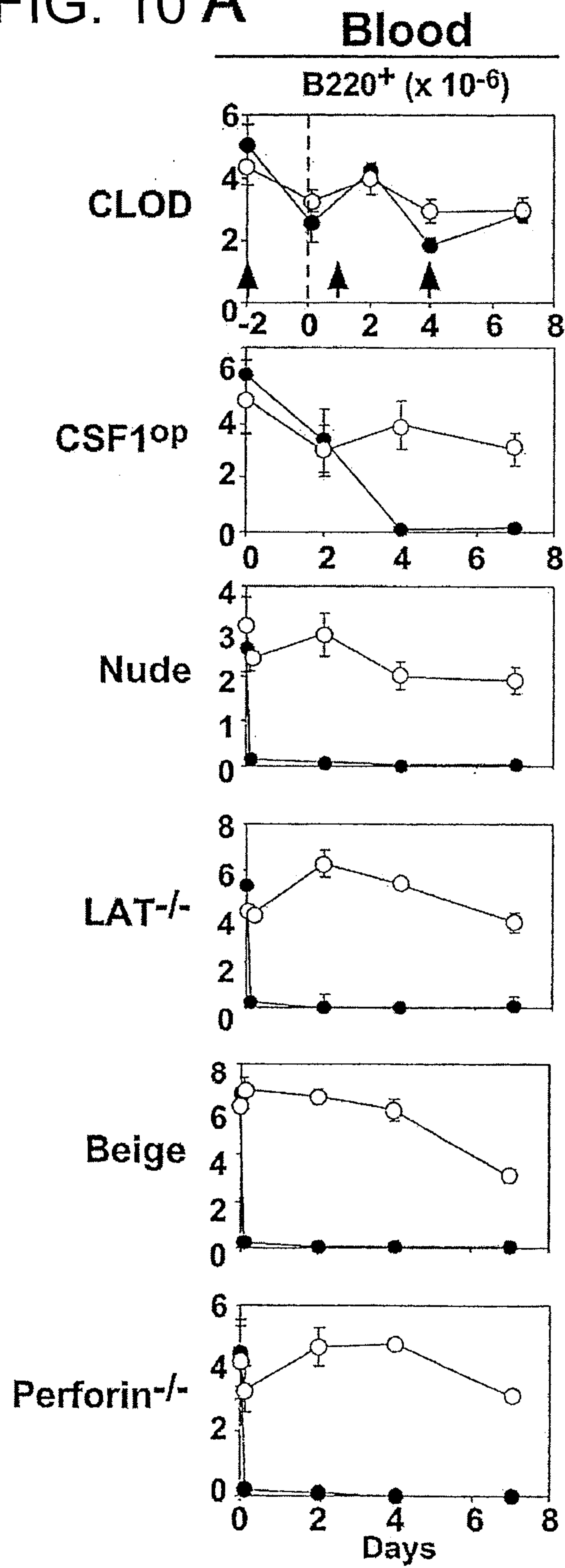


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FIG. 10 A



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FIG. 10 B

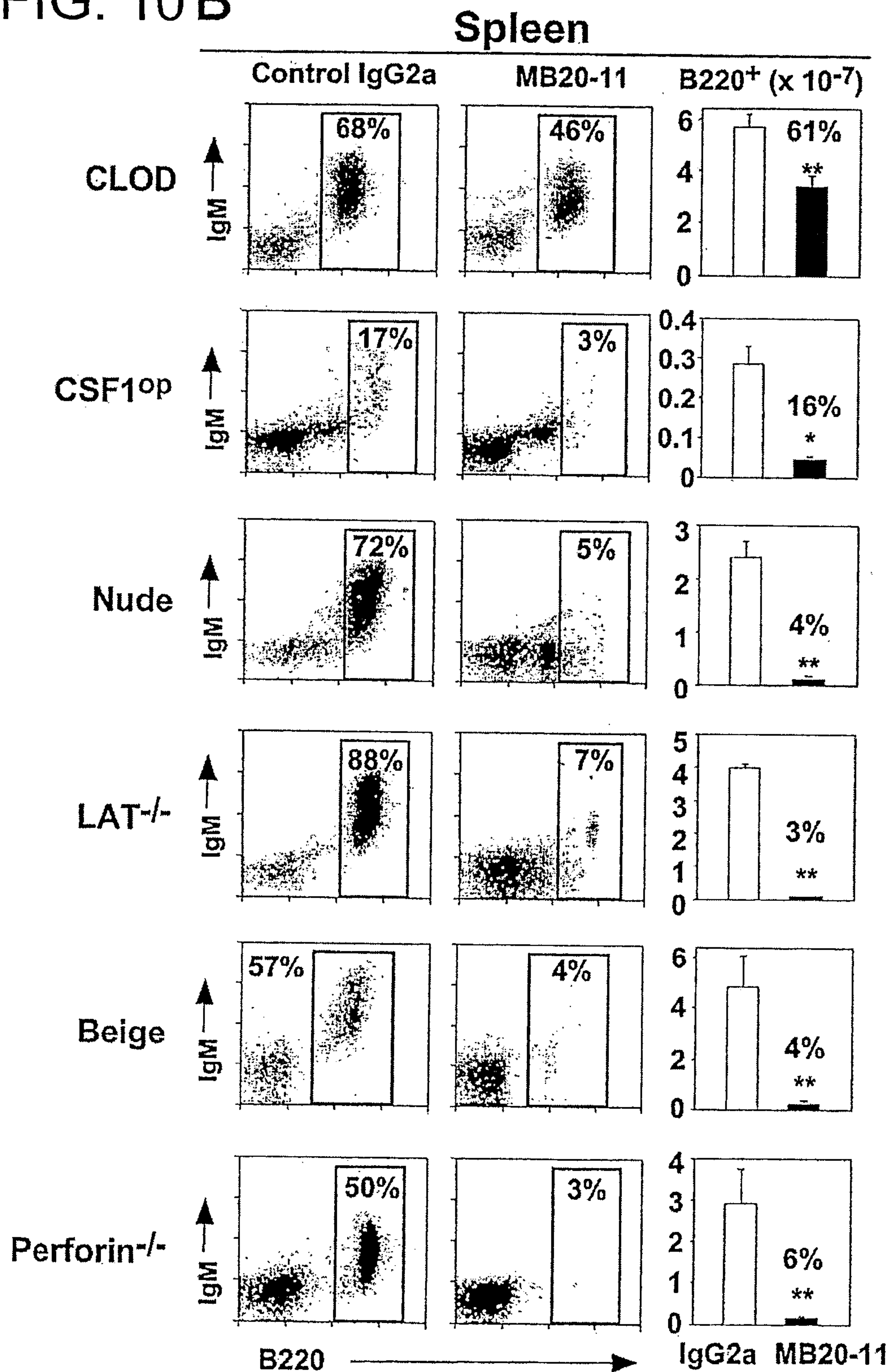


FIG. 11A

Residue 1	10	20	30	CDR1	40	50	CDR2	60	70	80	90	CDR3
Consen.	QVQLQQ	GAE LVKPGASVKM	SCKASGYTFT	SYNMHWVKQT	PGQGLEWIGAI	YPGNGDTSY	NQKFKG	KATL	TADKSSSTAY	MQLSSTLSED	SAVIYCAR	S. YY... YW. F- DVTWGAGTTVT VS.
2B8		P.....										. T .. GG-D. Y. . N. A
1F5		R. P.....										. H . GSN-. VDYF . Y . Q... L. . .S
1H4		P.....					F.....					. N .. GSS. VM. F S
2H7		AY. . S.....										VV .. SNS. . Y. . . T..... ..-
B9E9		V. S.....										AQ LRPN-. Y.-
Leu-16	E.....	S.....										N .. GSS. F. S

D Regions J Sequences

V_H Sequences

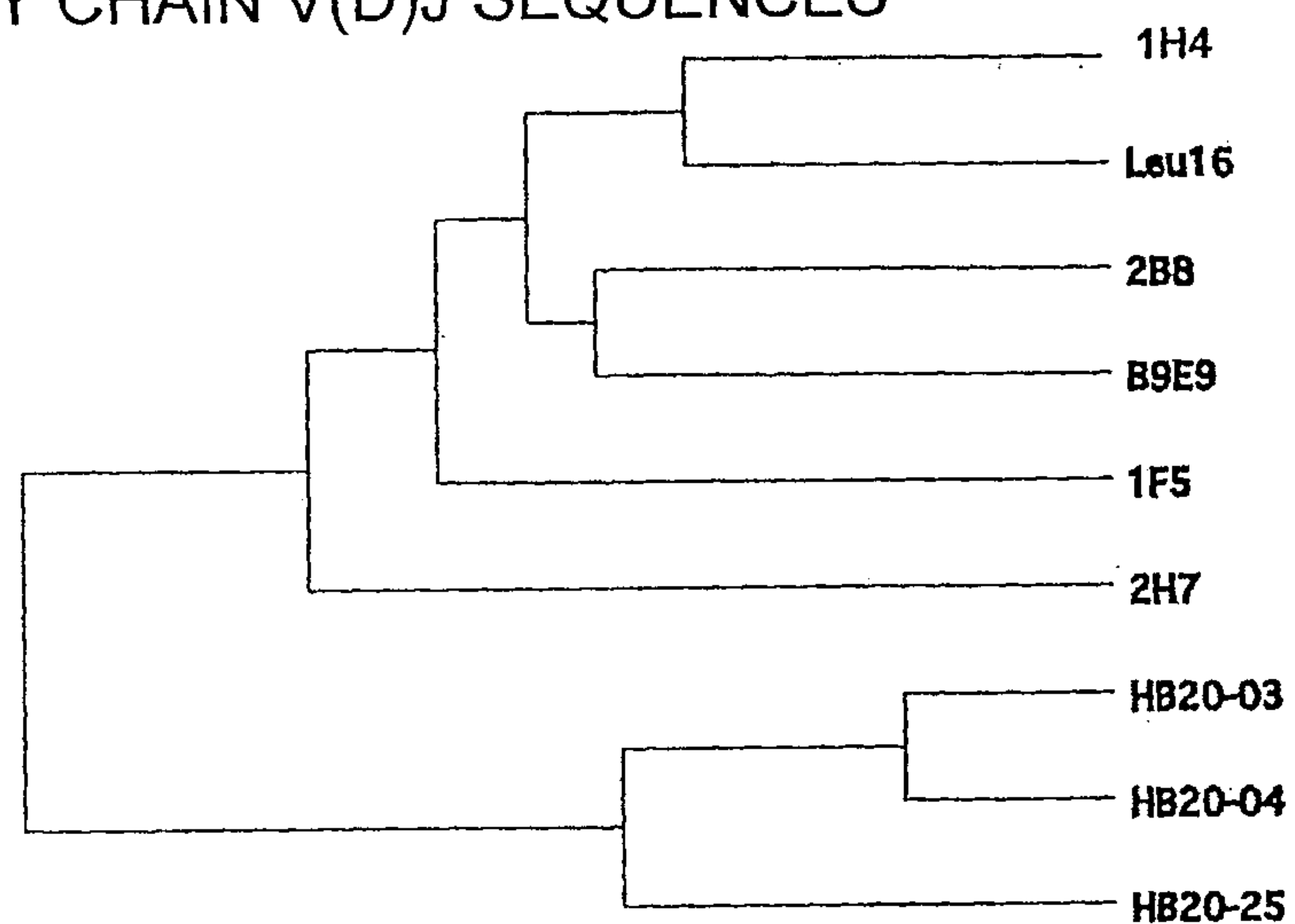
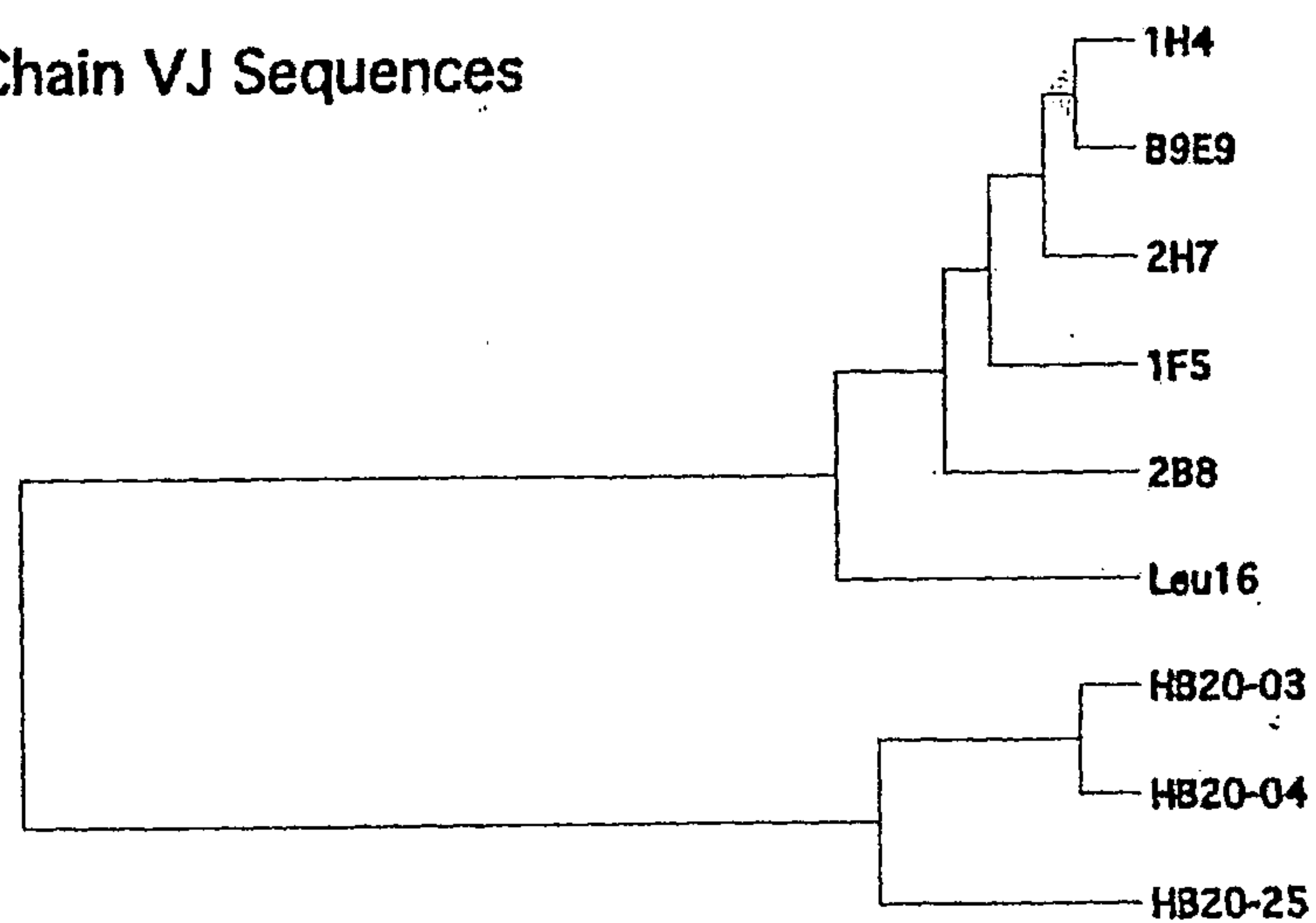
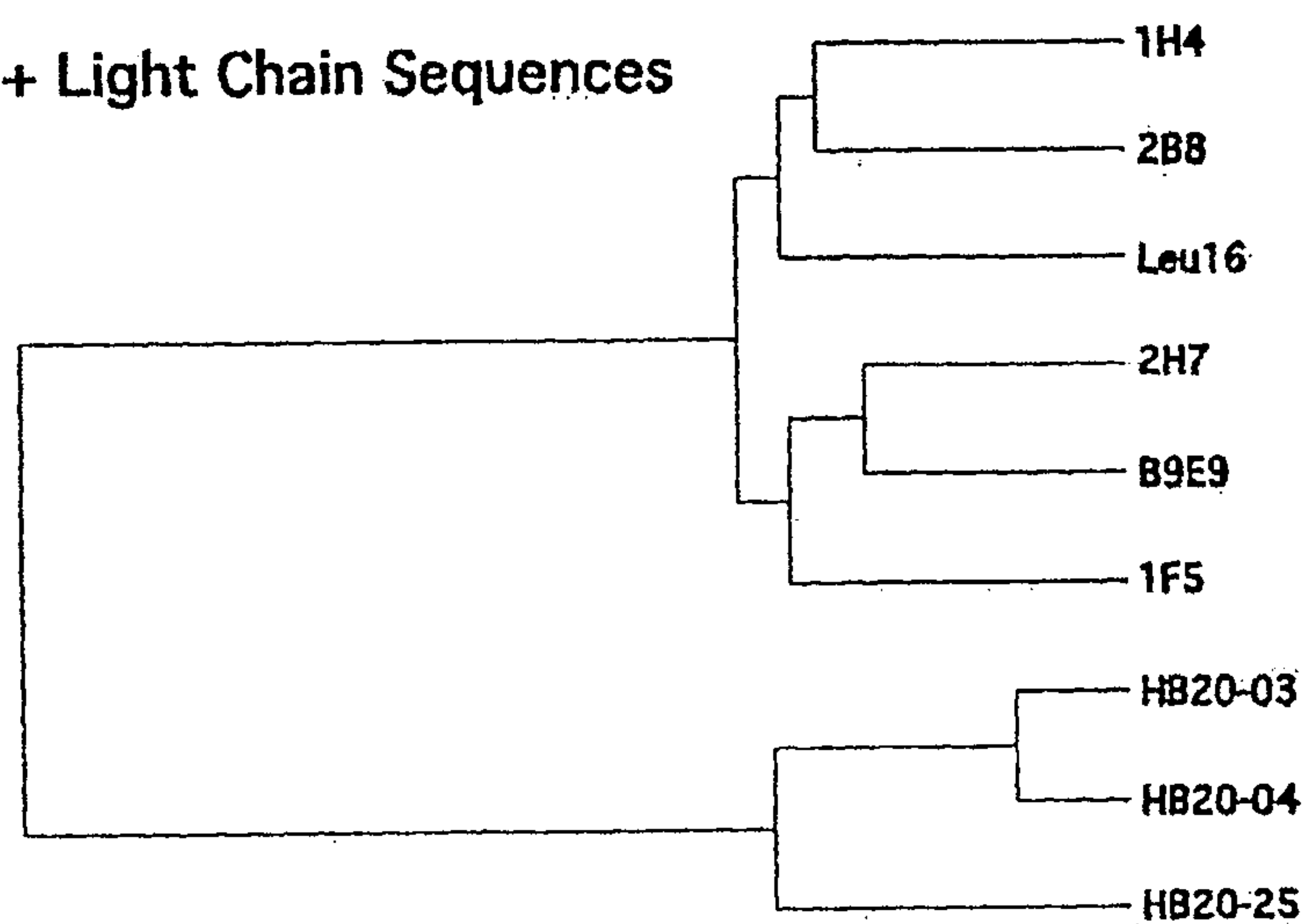
FIG. 11B

Residue 1	10	20	CDR1	35	40	50	CDR2	60	70	80	90	CDR3
Consen	---QIVLSQ	SPAILLSAPG	EKVTMTCSR	SSVSIMHWYQ	QKPGSSPKPW	IYATSNLASE	VPAKFSGSGS	GTSYSLTISR	VEAEDAATYY	CDQW. SNP	PT	FGAGTKLEIK
2B8												
1F5												
1H4												
2H7												
B9E9												
Leu-16												

V_K Sequences

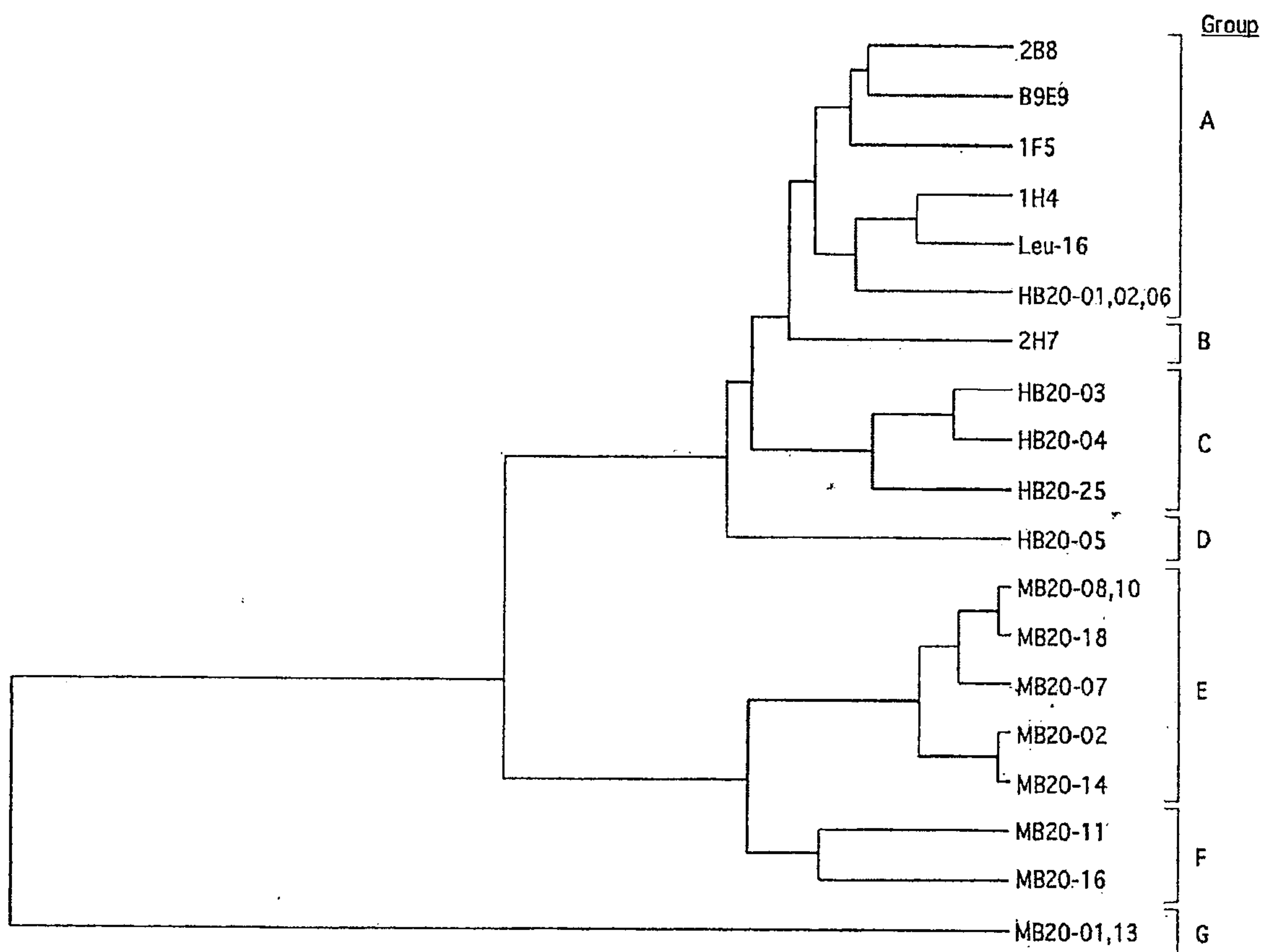
J Regions

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FIG. 12**HEAVY CHAIN V(D)J SEQUENCES****Light Chain VJ Sequences****Heavy + Light Chain Sequences**

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FIG. 13



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FIG. 14A

```
1      10      20
E V Q L Q E S G A E L V K P G A S V K M
GAG GTG CAG CTG CAG GAG TCT GGG GCT GAG CTG GTG AAG CCT GGG GCC TCA GTG AAG ATG 60

21      30      40
S C K A S G Y T F T S Y N M H W V K K T
TCC TGC AAG GCT TCT GGC TAC ACA TTT ACC AGT TAC AAT ATG CAC TGG GTA AAG AAG ACA 120

41      50      60
P G Q G L E W I G A I Y P G N G D T S Y
CCT GGA CAG GGC CTG GAA TGG ATT GGA GCT ATT TAT CCA GGA AAT GGT GAT ACT TCC TAC 180

61      70      80
N Q K F K G K A T L T A D K S S S T A Y
AAT CAG AAG TTC AAA GGC AAG GCC ACA TTG ACT GCA GAC AAA TCC TCC AGC ACA GCC TAC 240

81      90      100
M Q L S S L T S E D S A V Y Y C T R W D
ATG CAG CTC AGC AGC CTG ACA TCT GAG GAC TCT GCG GTC TAT TAC TGT ACA AGA TGG GAT 300

101      110      120
Y Y G S S Y V G F F D Y W G Q G T T L T
TAC TAC GGT AGT AGC TAC GTT G gg Ttt TTT GAC TAC TGG GGC CAA GGC ACC ACT CTC ACA 360

121
V S S (SEQ ID NO:1)
GTC TCC TCA 369 (SEQ ID NO:2)
```

FIG. 14B

```
1      10      20
E V Q L Q E S G A E L V K P G A S V K M
GAG GTG CAG CTG CAG GAG TCT GGG GCT GAG CTG GTG AAG CCT GGG GCC TCA GTG AAG ATG 60

21      30      40
S C K A S G F T F T N Y N M H W L K Q T
TCC TGC AAG GCT TCT GGC TTC ACA TTT ACC AAT TAC AAT ATG CAC TGG TTA AAG CAG ACG 120

41      50      60
P G Q G L E W I G A I Y P E N G D T S Y
CCT GGA CAG GGC CTG GAA TGG ATT GGA GCT ATT TAT CCA GAA AAT GGT GAT ACT TCC TAC 180

61      70      80
N Q K F K G K A T L T A D K A S S T A Y
AAT CAG AAA TTT AAA GGC AAG GCC ACA TTG ACT GCA GAC AAA GCC TCC AGC ACA GCC TAC 240

81      90      100
M H L S S L T S E D S A V Y F C A R F Y
ATG CAC CTC AGC AGC CTG ACA TCT GAG GAC TCT GCG GTC TAT TTC TGT GCA AGA TTT TAT 300

101      110      120
Y Y G S Y Y G A M D Y W G Q G T S V T V
TAC TAC GGT AGT T AT TAC ggT GCT ATG GAC TAC TGG GGT CAA GGA ACC TCA GTC ACC GTC 360

121
S S (SEQ ID NO:3)
TCC TCA 366 (SEQ ID NO:4)
```

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FIG. 14C

```
1      10      20
E  V  Q  L  Q  E  S  G  A  E  L  V  K  P  G  A  S  V  K  M
GAG GTG CAG CTG CAG GAG TCT GGG GCT GAG CTG GTG AAG CCT GGG GCC TCA GTG AAG ATG 60

21      30      40
S  C  K  A  S  G  F  T  F  T  N  Y  N  M  H  W  V  K  Q  T
TCC TGC AAG GCT TCT GGC TTC ACA TTT ACC AAT TAC AAT ATG CAC TGG GTA AAG CAG ACG 120

41      50      60
P  G  Q  G  L  E  W  I  G  A  I  Y  P  E  N  G  D  T  S  Y
CCT GGA CAG GGC CTG GAA TGG ATT GGA GCT ATT TAT CCA GAA AAT GGT GAT ACT TCC TAC 180

61      70      80
N  Q  R  F  K  G  K  A  T  L  T  A  D  K  S  S  S  T  A  Y
AAT CAG AGG TTC AAA GGC AAG GCC ACA TTG ACT GCA GAC AAA TCC TCC AGC ACA GCC TAC 240

81      90      100
M  H  L  S  S  L  T  S  E  D  T  A  V  Y  F  C  A  R  F  Y
ATG CAC CTC AGC AGC CTG ACA TCT GAG GAC ACT GCG GTC TAT TTC TGT GCA AGA TTT TAT 300

101     110     120
Y  Y  G  S  Y  Y  G  A  L  D  Y  W  G  Q  G  T  S  V  T  V
TAT TAC GGT AGT TAT TAC ggT GCT ttG GAC TAC TGG GGT CAA GGA ACC TGA GTC ACC GTC 360

121
S  S      (SEQ ID NO:5)
TCC TCA 366 (SEQ ID NO:6)
```

FIG. 14D

```
1      10      20
E  V  Q  L  Q  E  S  G  A  E  L  V  K  P  G  A  S  V  K  M
GAG GTG CAG CTG CAG GAG TCT GGG GCT GAG CTG GTG AAG CCT GGG GCC TCA GTG AAG ATG 60

21      30      40
S  C  K  A  S  G  Y  T  F  I  S  Y  N  M  H  W  V  K  Q  K
TCC TGC AAG GCT TCT GGC TAC ACA TTT ATT AGT TAC AAT ATG CAC TGG GTA AAG CAG AAA 120

41      50      60
P  G  Q  G  L  E  W  I  G  A  I  Y  P  G  N  G  D  T  S  Y
CCT GGA CAG GGC CTG GAA TGG ATT GGA GCT ATT TAT CCA GGA AAT GGT GAT ACT TCC TAC 180

61      70      80
N  Q  K  F  K  G  K  A  T  L  T  A  D  K  S  S  S  T  A  Y
AAT CAG AAG TTC AAA GGC AAG GCC ACA TTG ACT GCA GAC AAA TCC TCC AGC ACA GCC TAC 240

81      90      100
M  Q  L  S  S  L  T  S  E  D  S  A  V  Y  Y  C  A  R  W  D
ATG CAG CTC AGC AGC CTG ACA TCT GAG GAC TCT GCG GTC TAT TAC TGT GCA AGA TGG GAT 300

101     110     120
Y  Y  G  S  S  Y  V  G  F  L  T  T  G  A  K  A  P  L  V  T
TAC TAC GGT AGT AGC TAC GTT ggg ttT TTG ACT ACT GGG GCC AAG GCA CCA CTC GTC ACA 360

121
V  S  S      (SEQ ID NO:7)
GTC TCC TCA 369 (SEQ ID NO:8)
```

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FIG. 14E

```
1      10      20
E  V  Q  L  Q  E  S  G  A  E  L  V  K  P  G  A  S  V  K  M
GAG GTG CAG CTG CAG GAG TCT GGG GCT GAG CTG GTG AAG CCT GGG GCC TCA GTG AAG ATG 60

21      30      40
S  C  K  A  S  G  F  R  F  T  N  Y  N  L  H  W  V  K  Q  T
TCC TGC AAG GCT TCT GGC TTC AGA TTT ACC AAT TAC AAT TTG CAC TGG GTA AAA CAG ACA 120

41      50      60
P  G  Q  G  L  E  W  I  G  A  I  Y  P  G  N  G  E  T  S  Y
CCT GGA CAG GGC CTG GAA TGG ATT GGA GCT ATT TAT CCA GGA AAT GGT GAA ACT TCC TAC 180

61      70      80
N  Q  K  F  K  G  K  A  T  L  T  A  D  K  S  S  S  T  A  Y
AAT CAG AAG TTC AAA GGC AAG GCC ACA TTG ACT GCA GAC AAA TCC TCC AGT ACA GCC TAC 240

81      90      100
M  Q  L  R  S  L  T  S  G  D  S  A  V  Y  Y  C  A  R  F  Y
ATG CAG CTC AGA AGC CTG ACA TCT GGG GAC TCT GCG GTC TAT TAC TGT GCA AGA TTT TAT 300

101      110      120
Y  Y  G  S  S  Y  G  A  M  D  Y  W  G  Q  G  T  S  V  T  V
TAC TAC GGT AGT A gc TAC ggT GCT ATG GAC TAC TGG GGT CAA GGA ACC TCA GTC ACC GTC 360

121
S  S      (SEQ ID NO:9)
TCC TCA 366 (SEQ ID NO:10)
```

FIG. 14F

```
1      10      20
E  V  Q  L  Q  E  S  G  G  G  L  V  Q  P  G  G  S  L  K  L
GAG GTG CAG CTG CAG GAG TCT GGG GGA GGC TTA GTG CAG CCT GGA GGG TCC CTG AAA CTC 60

21      30      40
S  C  A  A  S  G  F  T  F  S  D  Y  G  M  A  W  V  R  Q  A
TCC TGT GCA GCC TCT GGA TTC ACT TTC AGT GAC TAC GGA ATG GCG TGG GTT CGA CAG GCT 120

41      50      60
P  R  K  G  P  E  W  V  A  F  I  S  N  L  A  Y  S  I  Y  Y
CCA AGG AAG GGG CCT GAG TGG GTA GCA TTC ATT AGT AAT TTG GCA TAT AGT ATC TAC TAT 180

61      70      80
A  D  T  V  T  G  R  F  T  I  S  R  E  N  A  K  N  T  L  Y
GCA GAC ACT GTG ACG GGC CGA TTC ACC ATC TCT AGA GAG AAT GCC AAG AAC ACC CTG TAC 240

81      90      100
L  E  M  S  S  L  R  S  E  D  T  A  M  Y  F  C  T  R  T  G
CTG GAA ATG AGC AGT CTG AGG TCT GAG GAC ACG GCC ATG TAT TTC TGT ACA AGA ACT G gg 300

101      110
Y  Y  A  L  D  Y  W  G  Q  G  T  S  V  T  V  S  S      (SEQ ID NO:11)
TAC TAT GCT tTG GAC TAC TGG GGT CAA GGA ACC TCA GTC ACC GTC TCC TCA 351 (SEQ ID NO:12)
```

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FIG. 14G

1 10 20
E V Q L Q E S G P E L V K P G A S V K I
GAG GTG CAG CTG CAG GAG TCT GGA CCT GAG CTG GTG AAG CCT GGG GCT TCA GTG AAG ATA 60

21 30 40
S C K A S G Y M F T D Y Y I K W V K Q S
TCC TGT AAG GCT TCT GGA TAC ATG TTC ACT GAC TAC TAT ATA AAG TGG GTG AAG CAG AGC 120

41 50 60
H G K S L E W I G D I N P N N G D T I Y
CAT GGA AAG AGT CTT GAG TGG ATT GGA GAT ATT AAT CCT AAT AAT GGT GAT ACT ATC TAC 180

61 70 80
N Q K F K G K A T L T V D K S S N T A Y
AAC CAG AAG TTC AAG GGC AAG GCC ACA TTG ACT GTA GAC AAG TCC TCC AAC ACA GCC TAC 240

81 90 100
M D L R S L T S E D S A V Y Y C A R | E R
ATG GAC CTC CGC AGC CTG ACA TCT GAG GAC TCT GCA GTC TAT TAC TGT GCA AGA | G | ag cGG 300

101 110
F A Y W G Q G T L V T V S A (SEQ ID NO:13)
TTT GCT TAC TGG GGC CAA GGG ACT CTG GTC ACT GTC TCT GCA 342 (SEQ ID NO:14)

FIG. 14H

1 10 20
E V Q L Q E S G P D L V K P G A S V K I
GAG GTG CAG CTG CAG GAG TCT GGA CCT GAC CTG GTG AAG CCT GGG GCT TCA GTG AAG ATA 60

21 30 40
S C K A S G Y M F T D Y Y M K W V K Q S
TCC TGT AAG GCT TCT GGA TAC ATG TTC ACT GAC TAC TAC ATG AAG TGG GTG AAG CAG AGC 120

41 50 60
H G K S L E W I G D I N P N N G D T T Y
CAT GGA AAG AGC CTT GAG TGG ATA GGG GAT ATT AAT CCT AAC AAT GGT GAT ACT ACC TAC 180

61 70 80
N Q K F E G K A T L T V D K S S S T A Y
AAC CAG AAG TTC GAG GGC AAG GCC ACA TTG ACT GTA GAC AAG TCC TCC AGC ACG GCC TAC 240

81 90 100
M E L R S L T S E D S A V Y Y C A R | E R
ATG GAG CTT CGC AGC CTG ACA TCT GAG GAC TCT GCA GTC TAT TAC TGT GCA AGA | G | aa cGG 300

101 110
F A Y W G Q G T L V T V S A (SEQ ID NO:15)
TTT GCT TAC TGG GGC CAA GGG ACT CTG GTC ACT GTC TCT GCA 342 (SEQ ID NO:16)

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FIG. 14I

```
1      10      20
E  V  Q  L  Q  E  S  G  P  D  L  V  K  P  G  A  S  V  K  I
GAG GTG CAG CTG CAG GAG TCT GGA CCT GAC CTG GTG AAG CCT GGG GCT TCA GTG AAG ATA 60

21      30      40
S  C  K  A  S  G  Y  T  F  T  D  Y  Y  M  K  W  V  K  Q  S
TCC TGT AAG GCT TCT GGA TAC ACG TTC ACT GAC TAC TAC ATG AAG TGG GTG AAG CAG AGC 120

41      50      60
H  G  K  S  L  D  W  I  G  D  I  N  P  N  N  G  D  I  I  Y
CAT GGA AAG AGC CTT GAC TGG ATA GGG GAT ATT AAT CCT AAC AAT GGT GAT ATT ATT TAC 180

61      70      80
N  Q  K  F  E  G  K  A  T  L  T  V  D  K  S  S  S  T  A  Y
AAC CAG AAG TTC GAG GGC AAG GCC ACA TTG ACT GTA GAC AAG TCC TCC AGC ACG GCC TAC 240

81      90      100
M  E  L  R  S  L  T  S  E  D  S  A  V  Y  Y  C  A  R  |  |  E  R
ATG GAG CTT CGC AGC CTG ACA TCT GAG GAC TCT GCA GTC TAT TAC TGT GCA AGA |G|aa cGG 300

101      110
F  A  Y  W  G  Q  G  T  L  V  T  V  S  A      (SEQ ID NO:17)
TTT GCT TAC TGG GGC CAA GGG ACT CTG GTC ACT GTC TCT GCA 342 (SEQ ID NO:18)
```

FIG. 14J

```
1      10      20
E  V  Q  L  Q  E  S  G  P  D  L  V  K  P  G  A  S  V  K  I
GAG GTG CAG CTG CAG GAG TCT GGA CCT GAC CTG GTG AAG CCT GGG GCT TCA GTG AAG ATA 60

21      30      40
S  C  K  A  S  G  Y  T  F  T  D  Y  Y  M  K  W  V  K  Q  S
TCC TGT AAG GCT TCT GGA TAC ACG TTC ACT GAC TAC TAC ATG AAG TGG GTG AAG CAG AGC 120

41      50      60
H  G  K  S  L  D  W  I  G  D  I  N  P  N  N  G  D  I  I  Y
CAT GGA AAG AGC CTT GAC TGG ATA GGG GAT ATT AAT CCT AAC AAT GGT GAT ATT ATT TAC 180

61      70      80
N  Q  K  F  E  G  K  A  T  L  T  V  D  K  S  S  S  T  A  Y
AAC CAG AAG TTC GAG GGC AAG GCC ACA TTG ACT GTG GAC AAG TCC TCC AGC ACG GCC TAC 240

81      90      100
M  E  L  R  S  L  T  S  E  D  S  A  V  Y  Y  C  A  R  |  |  E  R
ATG GAG CTT CGT AGT CTG ACA TCT GAG GAC TCT GCA GTC TAT TAC TGT GCA AGA |G|aa cGG 300

101      110
F  A  Y  W  G  Q  G  T  L  V  T  V  S  A      (SEQ ID NO:19)
TTT GCT TAC TGG GGC CAA GGG ACT CTG GTC ACT GTC TCT GCA 342 (SEQ ID NO:20)
```

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FIG. 14K

```
1      10      20
E  V  Q  L  Q  E  S  G  P  E  L  V  K  P  G  A  S  V  K  M
GAG GTG CAG CTG CAG GAG TCT GGA CCT GAG CTG GTG AAG CCT GGG GCT TCA GTG AAG ATG 60

21      30      40
S  C  K  A  S  G  Y  K  I  T  D  Y  N  M  H  W  V  K  Q  S
TCC TGC AAG GCT TCT GGA TAT AAA ATC ACT GAC TAC AAC ATG CAC TGG GTG AAG CAG AGT 120

41      50      60
H  G  K  S  L  E  W  I  G  Y  I  A  P  Y  N  G  G  T  T  Y
CAT GGA AAG AGC CTT GAG TGG ATT GGA TAC ATT GCC CCT TAC AAT GGT GGT ACT ACC TAC 180

61      70      80
N  Q  K  F  K  G  K  A  T  L  T  V  N  K  S  S  S  T  A  Y
AAC CAG AAG TTC AAG GGC AAG GCC ACA TTG ACT GTA AAC AAG TCC TCC AGC ACA GCC TAT 240

81      90      100
M  E  L  R  S  L  T  S  E  D  S  A  V  Y  Y  C  A  G  A  L
ATG GAG CTC CGC AGT CTG ACA TCG GAG GAT TCT GCA GTC TAT TAT TGT GCA GGT GCT tTG 300

101      110
D  Y  W  G  Q  G  T  S  V  T  V  S  S      (SEQ ID NO:21)
GAC TAT TGG GGT CAA GGA ACC TCA GTC ACC GTC TCC TCT 339 (SEQ ID NO:22)
```

FIG. 14L

```
1      10      20
E  V  Q  L  Q  E  S  G  P  E  L  V  K  P  G  A  S  V  K  I
GAG GTG CAG CTG CAG GAG TCT GGA CCT GAG CTG GTG AAG CCT GGG GCT TCA GTG AAG ATA 60

21      30      40
S  C  K  A  S  G  Y  M  F  T  D  Y  Y  I  K  W  V  K  Q  S
TCC TGT AAG GCT TCT GGA TAC ATG TTC ACT GAC TAC TAT ATA AAG TGG GTG AAG CAG AGC 120

41      50      60
H  G  K  S  L  E  W  I  G  D  I  N  P  N  N  G  D  T  I  Y
CAT GGA AAG AGT CTT GAG TGG ATT GGA GAT ATT AAT CCT AAT AAT GGT GAT ACT ATC TAC 180

61      70      80
N  Q  K  F  K  G  K  A  T  L  T  V  D  K  S  S  N  T  A  Y
AAC CAG AAG TTC AAG GGC AAG GCC ACA TTG ACT GTA GAC AAG TCC TCC AAC ACA GCC TAC 240

81      90      100
M  D  L  R  I  L  T  S  E  D  S  A  V  Y  Y  C  A  R  E  R
ATG GAC CTC CGC ATC CTG ACA TCA GAG GAC TCT GCA GTC TAT TAC TGT GCA AGA G ag cGG 300

101      110
F  A  Y  W  G  Q  G  T  L  V  T  V  S  A      (SEQ ID NO:23)
TTT GCT TAC TGG GGC CAA GGG ACT CTG GTC ACT GTC TCT GCA 342 (SEQ ID NO:24)
```

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FIG. 14M

```
1      10      20
E V Q L Q E S G P E L V K P G A S V K M
GAG GTG CAG CTG CAG GAG TCT GGA CCT GAG CTG GTG AAG CCT GGG GCT TCA GTG AAA ATG 60

21      30      40
S C K A S G Y T F T D Y N L H W V K Q S
TCC TGC AAG GCT TCT GGA TAC ACA TTC ACT GAC TAC AAC TTG CAC TGG GTG AAG CAG AGC 120

41      50      60
H G Q S L E W I G Y I N P N N G G A T Y
CAT GGA CAG AGC CTT GAG TGG ATT GGA TAT ATT AAC CCT AAC AAT GGT GGT GCT ACA TAC 180

61      70      80
N Q K F T G K A T L T V N R S S S T A Y
AAT CAG AAG TTC ACT GGC AAG GCC ACA TTG ACT GTA AAC AGG TCC TCC AGC ACA GCC TAC 240

81      90      100
M E L R S L T S D D S A V Y Y C A E I Y
ATG GAG CTC CGC AGC CTG ACA TCG GAC GAT TCT GCA GTC TAT TAC TGT GCA GAA ATC T At 300

101      110
D G Y Y W G Q G T T L T V S S (SEQ ID NO:25)
gAt ggT tAC TAC TGG GGC CAA GGC ACC ACT CTC ACA GTC TCC TCA 345 (SEQ ID NO:26)
```

FIG. 14N

```
1      10      20
E V Q L Q E S G L D L V K P G A S V K I
GAG GTG CAG CTG CAG GAG TCT GGA CTT GAC CTG GTG AAG CCT GGG GCT TCA GTG AAG ATA 60

21      30      40
S C K A S G Y T F T D Y Y M K W V K Q S
TCC TGT AAG GCT TCT GGA TAC ACG TTC ACT GAC TAC TAC ATG AAG TGG GTG AAA CAG AGC 120

41      50      60
H G K S L D W I G D I N P N N G D I I Y
CAT GGA AAG AGC CTT GAC TGG ATA GGG GAT ATT AAT CCT AAC AAT GGT GAT ATT ATT TAC 180

61      70      80
N Q K F E G K A T L T V D K S S S T A Y
AAC CAG AAG TTC GAG GGC AAG GCC ACA TTG ACT GTA GAC AAG TCC TCC AGC ACG GCC TAC 240

81      90      100
M E L R S L T S E D S A V Y Y C A R E R
ATG GAG CTT CGC AGC CTG ACA TCT GAG GAC TCT GCA GTC TAT TAC TGT GCA AGA G aa cGG 300

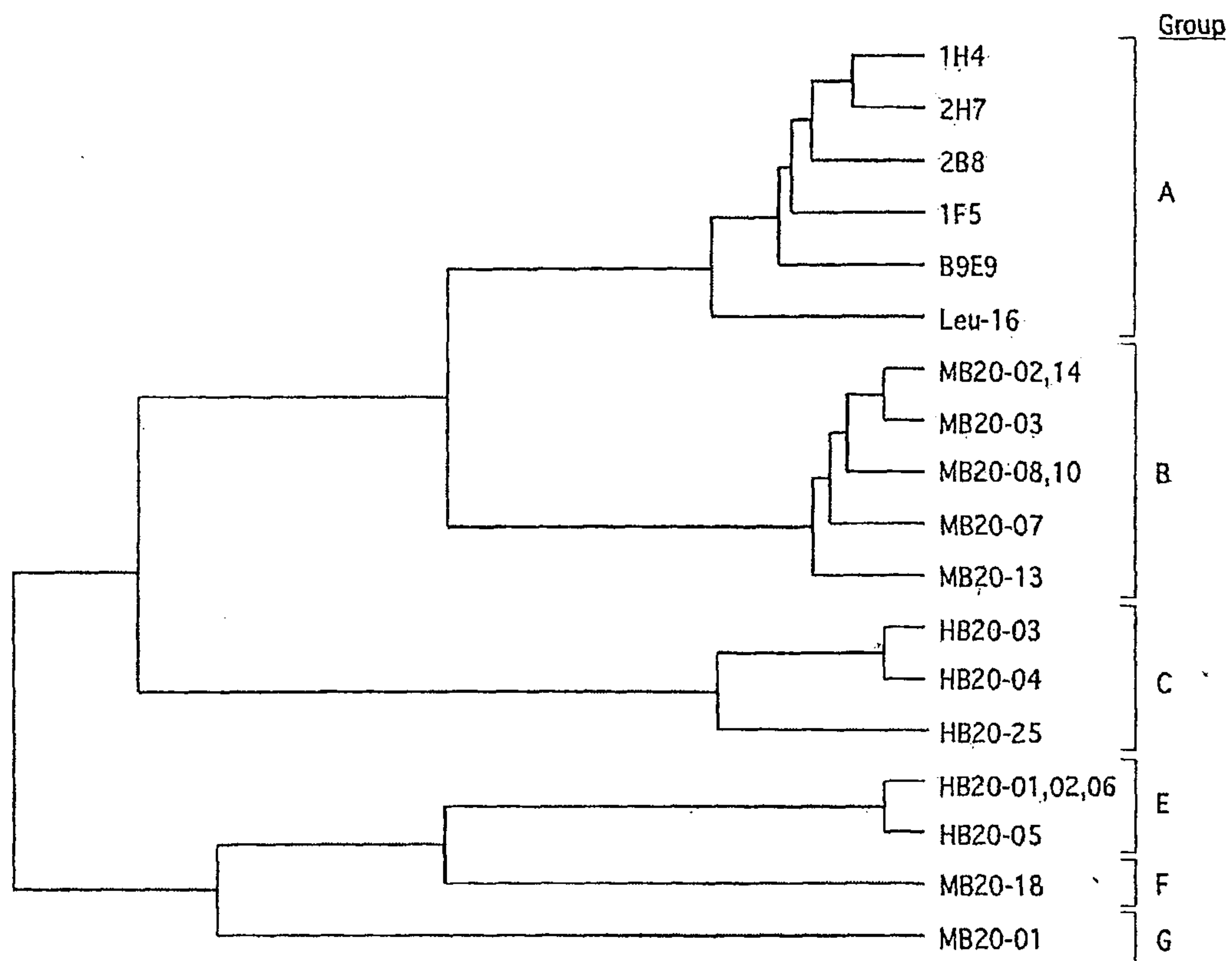
101      110
F A Y W G Q G T L V T V S A (SEQ ID NO:27)
TTT GCT TAC TGG GGC CAA GGG ACT CTG GTC ACT GTC TCT GCA 342 (SEQ ID NO:28)
```

FIG. 15

Residue	1	10	20	CDR1	40	50	CDR2	60	70	80	90	CDR3
Consen.	EVQLQESGAELVKPGASVKMSCKASGYTFTSYNMHWVKQTPGQGLEWIGAIYPGNGDTSYNQKFKGKATLTVDKSSSTAYMQLSSLTSEDSAVYFCAR											. Y. GS. . . . F-DVMG GTTIVTVSS
2B8	Q. . . . QP.								A.		Y.	ST. Y. G-DWY. . N. . . A. A
1F5	Q. . . . RQP.								A.		Y.	SH. GSN-YVDYF. Y. . . Q. . . L. . . .
B9E9	Q. . . . VQ.								A.		Y.	AQLRPN-YWY. A. -
Leu-16	 Q.								A.		D. Y. . . .	SN. Y. . SYWF. A.
1H4	Q. . . . QP.		T.	V.				F.	A.	V.	Y.	SN. Y. . SYVM. F. . . . A.
HB20-1, 2, 6					K.				A.		Y. T. . . .	WD. Y. . SYVG. F. Y. . Q. . . L. . . .
2H7	QAY. . Q. R.											VV. YSNSYWY. T. -
HB20-03				F.	L.				A. A. . . . H.			FY. Y. . YYGAM. Y. . . Q. . S. . . .
HB20-04				F.				R.	A. H.		T.	FY. Y. . YYGAL. Y. . . Q. . S. . . .
HB20-25				FR. . N. L.					A. R.		G. Y. . . .	FY. Y. . SYGAM. Y. . . Q. . S. . . .
HB20-05				I.					A.		Y.	WD. Y. . SYVG. LTTGAKAPL.
MB20-11		P.		KI. . D.				Y. A Y. G. T.		N.	E. R. . . . Y. G. . . .	ALD. Y. . . Q. . S. . . .
MB20-16		P.		D. L.				Y. N. N. GAT. T. . . .		NR.	D. . . . Y. E. . . .	IYDG. Y-Y. . Q. . L. . . .
MB20-08, 10		PD.	I.	D. Y. K.				D. N. N. . II. E. . . .		E. R. . . .	Y. . . . Y. . .	ERFA. Y. . . Q. . L. . . . A
MB20-18		LD.	I.	D. Y. K.				D. N. N. . II. E. . . .		E. R. . . .	Y. . . . Y. . .	ERFA. Y. . . Q. . L. . . . A
MB20-07		PD.	I.	D. Y. K.				D. N. N. . T. E. . . .		E. R. . . .	Y. . . . Y. . .	ERFA. Y. . . Q. . L. . . . A
MB20-02		P.	I.	D. YIK.				D. N. N. . I.		N.	D. R. . . . Y. . .	ERFA. Y. . . Q. . L. . . . A
MB20-14		P.	I.	D. YIK.				D. N. N. . I.		N.	D. RI. . . . Y. . .	ERFA. Y. . . Q. . L. . . . A
MB20-01, 13		GG. . Q. . G. L. L. . A. . . F. . SD. G. A. . R. A. RK. P. . VAF. SNLAYSIY. ADTVT. RF. ISRENAKN. L. LEM. . R. . T. M. . T. .										TG. Y. AL. . Y. . Q. . S.

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FIG. 16



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FIG. 17A

```
1      10      20
M  G  I  K  M  E  S  Q  T  Q  V  F  V  Y  M  L  L  W  L  S
ATG GGC ATC AAG ATG GAG TCA CAG ACC CAG GTC TTT GTA TAC ATG TTG CTG TGG TTG TCT 60

21      30      40
G  V  D  G  D  I  V  M  T  Q  S  Q  K  F  M  S  T  S  V  G
GGT GTT GAT GGA GAC ATT GTG ATG ACC CAG TCT CAA AAA TTC ATG TCA ACA TCA GTT GGA 120

41      50      60
D  R  V  S  V  T  C  K  A  S  Q  N  V  G  T  N  V  A  W  Y
GAC AGG GTC AGC GTC ACC TGC AAG GCC AGT CAG AAT GTG GGT ACT AAT GTA GCC TGG TAT 180

61      70      80
Q  Q  K  L  G  Q  S  P  K  P  L  I  Y  S  A  S  Y  R  N  S
CAA CAG AAA CTA GGG CAA TCT CCT AAA CCA CTG ATT TAT TCG GCA TCC TAC CGG AAC AGT 240

81      90      100
G  V  P  D  R  F  T  G  S  G  S  G  T  D  F  T  L  T  I  S
GGA GTC CCT GAT CGC TTC ACA GGC AGT GGA TCT GGG ACA GAT TTC ACT CTC ACC ATC AGC 300

101     110     120
N  V  Q  S  E  D  L  A  E  Y  F  C  Q  Q  Y  N  S  S  P  F
AAT GTG CAG TCT GAA GAC TTG GCA GAG TAT TTC TGT CAG CAA TAT AAC AGT TCT CCA TTC 360

121     130
T  F  G  S  G  T  K  L  E  I  K  R  A  D  A  A  P  T  V (SEQ ID NO:29)
ACG TTC GGC TCG GGG ACA AAG TTG GAA ATA AAA CGG GCT GAT GCT GCA CCA ACT GTA TC 419
                                                                    (SEQ ID NO:30)
```

FIG. 17B

```
1      10      20
M  W  G  S  V  F  N  F  S  I  V  G  A  R  C  D  I  Q  M  T
ATG TGG GGA TCT GTT TTC AAT TTT TCA ATT GTA GGT GCC AGA TGT GAC ATC CAG ATG ACT 60

21      30      40
Q  S  P  A  S  L  S  A  S  V  G  E  T  V  T  I  T  C  R  A
CAG TCT CCA GCC TCC CTA TCT GCA TCT GTG GGG GAA ACT GTC ACC ATC ACA TGT CGA GCA 120

41      50      60
S  G  N  I  H  N  Y  L  A  W  Y  Q  Q  K  Q  G  K  S  P  Q
AGT GGG AAT ATT CAC AAT TAT TTA GCA TGG TAT CAG CAG AAA CAG GGA AAA TCT CCT CAA 180

61      70      80
L  L  V  Y  N  A  K  T  L  A  D  G  V  P  S  R  F  S  G  S
CTC CTG GTC TAT AAT GCA AAA ACC TTA GCA GAT GGT GTG CCA TCA AGG TTC AGT GGC AGT 240

81      90      100
G  S  G  T  Q  F  S  L  K  I  N  S  L  Q  P  E  D  F  G  S
GGA TCA GGA ACA CAA TTT TCT CTC AAG ATC AAC AGC CTG CAG CCT GAA GAT TTT GGG AGT 300

101     110     120
Y  Y  C  Q  H  F  W  S  T  P  W  T  F  G  G  G  T  K  L  E
TAT TAC TGT CAA CAT TTT TGG AGT ACG CCG TGG ACG TTC GGT GGA GGC ACC AAG CTG GAA 360

121     130
I  K  R  A  D  A  A  P  T  V (SEQ ID NO:31)
ATC AAA CGG GCT GAT GCT GCA CCA ACT GTA TC 392 (SEQ ID NO:32)
```

FIG. 17C

FIG. 17D

121 130
T F G S G T K L E I K R A D A A P T V (SEQ ID NO:35)
ACG TTC GGC TCG GGG ACA AAG TTG GAA ATA AAA CGG GCT GAT GCT GCA CCA ACT GTA TC 419
(SEQ ID NO:36)

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FIG. 17E

	1	M	W	G	S	V	F	N	F	S	I	V	D	A	R	C	D	I	Q	M	T	
		ATG	TGG	GGA	TCT	GTT	TTC	AAT	TTT	TCA	ATT	GTA	GAT	GCC	AGA	TGT	GAC	ATC	CAG	ATG	ACT	60
	21											30									40	
		Q	S	P	A	S	L	S	V	S	V	G	E	T	V	T	I	T	C	R	A	
		CAG	TCT	CCA	GCC	TCC	CTG	TCT	GTA	TCT	GTG	GGA	GAA	ACT	GTC	ACC	ATC	ACA	TGT	CGA	GCA	120
	41											50									60	
		S	E	N	I	Y	S	N	L	A	W	Y	Q	Q	K	Q	G	K	S	P	Q	
		AGT	GAA	AAT	ATT	TAC	AGT	AAT	TTA	GCA	TGG	TAT	CAG	CAG	AAA	CAG	GGA	AAA	TCT	CCT	CAG	180
	61											70									80	
		L	L	V	Y	A	A	T	N	L	A	D	G	V	P	S	R	F	S	G	S	
		CTC	CTG	GTC	TAT	GCT	GCA	ACA	AAC	TTA	GCA	GAT	GGT	GTG	CCA	TCA	AGG	TTC	AGT	GGC	AGT	240
	81											90									100	
		G	S	G	T	Q	Y	S	L	K	I	N	S	L	Q	S	E	D	F	G	S	
		GGA	TCA	GGC	ACA	CAG	TAT	TCC	CTC	AAG	ATC	AAC	AGC	CTG	CAG	TCT	GAA	GAT	TTT	GGG	AGT	300
	101											110									120	
		Y	Y	C	Q	H	F	W	G	I	P	W	T	F	G	G	G	T	K	L	E	
		TAT	TAC	TGT	CAA	CAT	TTT	TGG	GGT	ATT	CCG	TGG	ACG	TTC	GGT	GGA	GGC	ACC	AAG	CTG	GAA	360
	121											130										
		I	K	R	A	D	A	A	P	T	V				(SEQ ID NO:37)							
		ATC	AAA	CGG	GCT	GAT	GCT	GCA	CCA	ACT	GTA	TC	392	(SEQ ID NO:38)								

FIG. 17F

[illegible]

FIG. 17G

1	M	D	L	Q	V	Q	I	I	S	F	10	L	L	I	S	V	T	V	I	V	20	
	ATG	GAT	TTA	CAG	GTG	CAG	ATT	ATC	AGC	TTC		CTG	CTA	ATC	AGT	GTC	ACA	GTC	ATA	GTG	TCT	60
21	N	G	E	I	V	L	T	Q	S	P	30	T	T	M	A	A	S	P	G	E	K	40
	AAT	GGA	GAA	ATT	GTG	CTC	ACC	CAG	TCT	CCA		ACC	ACC	ATG	GCT	GCA	TCT	CCC	GGG	GAG	AAG	120
41	I	T	I	T	C	S	V	S	S	S	50	I	R	S	N	Y	L	H	W	Y	Q	60
	ATC	ACT	ATC	ACC	TGC	AGT	GTC	AGC	TCA	AGT		ATA	AGG	TCC	AAT	TAT	TTA	CAT	TGG	TAT	CAG	180
61	Q	K	P	G	F	S	P	K	L	L	70	I	Y	R	T	S	N	L	A	S	G	80
	CAG	AAG	CCA	GGA	TTC	TCC	CCT	AAA	CTC	TTG		ATT	TAT	AGG	ACA	TCC	AAT	CTG	GCT	TCT	GGA	240
81	V	P	A	R	F	S	G	S	G	S	90	G	T	S	Y	S	L	T	V	A	T	100
	GTC	CCA	GCT	CGC	TTC	AGT	GGC	AGT	GGG	TCT		GGG	ACC	TCT	TAC	TCT	CTC	ACA	GTT	GCC	ACC	300
101	M	E	A	E	D	V	A	T	Y	Y	110	C	Q	Q	G	S	S	I	P	L	T	120
	ATG	GAG	GCT	GAA	GAT	GTT	GCC	ACT	TAC	TAC		TGC	CAG	CAG	GGT	AGT	AGT	ATA	CCG	CTC	ACG	360
121	F	G	A	G	T	K	L	E	L	K	130	R	A	D	A	A	P	T	V			(SEQ ID NO:41)
	TTC	GGT	GCT	GGG	ACC	AAG	CTG	GAG	CTG	AAA		CGG	GCT	GAT	GCT	GCA	CCA	ACT	GTA	TC	416	(SEQ ID NO:42)

FIG. 17H

	1	M	D	L	Q	V	Q	I	I	S	F	10		L	L	I	S	V	T	V	I	V	?	20	
		ATG	GAT	TTA	CAG	GTG	CAG	ATT	ATC	AGC	TTC		CTG	CTA	ATC	AGT	GTC	ACA	GTC	ATA	GTG	TNT		60	
	21	N	G	E	I	V	L	T	Q	S	P	30		T	T	M	A	A	S	P	G	E	K	40	
		AAT	GGA	GAA	ATT	GTG	CTC	ACC	CAG	TCT	CCA		ACC	ACC	ATG	GCT	GCA	TCT	CCC	GGG	GAG	AAG		120	
	41	I	T	I	T	C	S	A	S	S	S	50		I	S	S	N	Y	L	H	W	Y	Q	60	
		ATC	ACT	ATC	ACA	TGC	AGT	GCC	AGC	TCA	AGT		ATA	AGT	TCC	AAT	TAT	TTG	CAT	TGG	TAT	CAG		180	
	61	Q	K	P	G	F	S	P	K	L	L	70		I	Y	R	T	S	N	L	A	S	G	80	
		CAG	AAG	CCA	GGA	TTC	TCC	CCT	AAA	CTC	TTG		ATT	TAC	AGG	ACA	TCC	AAT	CTG	GCT	TCT	GGA		240	
	81	V	P	A	R	F	S	G	S	G	S	90		G	T	S	Y	T	L	T	V	A	T	100	
		GTC	CCA	GCT	CGC	TTC	AGT	GGC	AGT	GGG	TCT		GGG	ACC	TCT	TAC	ACT	CTC	ACA	GTC	GCC	ACC		300	
	101	M	E	A	E	D	V	A	T	Y	Y	110		C	Q	Q	G	S	S	I	P	L	T	120	
		ATG	GAG	GCT	GAA	GAT	GTT	GCC	ACT	TAC	TAC		TGC	CAG	CAG	GGT	AGT	AGT	ATA	CCG	CTC	ACG		360	
	121	F	G	A	G	T	K	L	E	L	K	130		R	A	D	A	A	P	T	V	(SEQ ID NO:43)			
		TTC	GGT	GCT	GGG	ACC	AAG	CTG	GAG	CTG	AAA		CGG	GCT	GAT	GCT	GCA	CCA	ACT	GTA	TC	416			
		(SEQ ID NO:44)																							

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FIG. 17I

```
1      10      20
M D L Q V Q I I S F L L I S V T V S N G
ATG GAT TTA CAG GTG CAG ATT ATC AGC TTC CTG CTA ATC AGT GTC ACA GTG TCT AAT GGA 60

21      30      40
E L V L T Q S P T T K A A S P G E K I T
GAA CTT GTG CTC ACC CAG TCT CCA ACC ACC AAG GCT GCA TCT CCC GGG GAG AAG ATC ACT 120

41      50      60
I T C S V S S S I R S N Y L H W Y Q Q R
ATC ACC TGC AGT GTC AGC TCA AGT ATA CGT TCC AAT TAC TTG CAT TGG TAT CAG CAG AGG 180

61      70      80
P G F S P K L L I Y R T S N L A S G V P
CCA GGA TTC TCC CCT AAG CTC TTG ATT TAT AGG ACA TCC AAT CTG GCT TCT GGA GTC CCA 240

81      90      100
A R F S G S G S G T S Y S L T I G T M E
GCT CGC TTC AGT GGC AGT GGG TCT GGG ACC TCT TAC TCT CTC ACA ATT GGC ACC ATG GAG 300

101     110     120
A E D V A T Y Y C Q Q G S S L P L T F G
GCT GAA GAT GTT GCC ACT TAC TAC TGC CAG CAG GGT AGT AGT TTA CCG CTC ACG TTC GGT 360

121     130
A G T K L E L K R A D A A P T V (SEQ ID NO:45)
GCT GGG ACC AAG CTG GAG CTG AAA CGG GCT GAT GCT GCA CCA ACT GTA TC 410 (SEQ ID NO:46)
```

FIG. 17J

```
1      10      20
M D L Q V Q I I S F L L I S V T V S N G
ATG GAT TTA CAG GTG CAG ATT ATC AGC TTC CTG CTA ATC AGT GTC ACA GTG TCT AAT GGA 60

21      30      40
E I V L T Q S P T T M A A S P G E K I T
GAA ATT GTG CTC ACC CAG TCT CCA ACC ACC ATG GCT GCA TCT CCC GGG GAG AAG ATC ACT 120

41      50      60
I T C S V S S N I R S N Y L H W Y Q Q K
ATC ACC TGT AGT GTC AGT TCA AAT ATA CGT TCC AAT TAC TTG CAT TGG TAT CAG CAG AAG 180

61      70      80
P G F S P K L L I Y R T S N L A S G V P
CCA GGA TTC TCC CCT AAA CTC TTG ATT TAT AGG ACA TCC AAT CTG GCT TCT GGA GTC CCA 240

81      90      100
A R F S G S G S G T S Y S L T I G T M K
GCT CGC TTC AGT GGC AGT GGG TCT GGG ACC TCT TAC TCT CTC ACA ATT GGC ACC ATG AAG 300

101     110     120
A E D V A T Y Y C Q Q G S S I P L T F G
GCT GAA GAT GTT GCC ACT TAC TAC TGC CAG CAG GGT AGT AGT ATA CCG CTC ACG TTC GGT 360

121     130
A G T K L E L K R A D A A P T V (SEQ ID NO:47)
GCT GGG ACC AAG CTG GAG CTG AAA CGG GCT GAT GCT GCA CCA ACT GTA TC 410 (SEQ ID NO:48)
```

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FIG. 17K

1	M	D	L	Q	V	Q	I	I	S	F	10	L	L	I	S	V	T	V	S	N	G	20
	ATG	GAT	TTA	CAG	GTG	CAG	ATT	ATC	AGC	TTC		CTG	CTA	ATC	AGT	GTC	ACA	GTG	TCT	AAT	GGA	60
21											30											40
	E	I	V	L	T	Q	S	P	T	T		M	A	A	S	P	G	E	K	I	T	
	GAA	ATT	GTG	CTC	ACC	CAG	TCT	CCA	ACC	ACC		ATG	GCT	GCA	TCT	CCC	GGG	GAG	AAG	ATC	ACT	120
41											50											60
	I	T	C	S	V	S	S	N	I	R		S	N	Y	L	H	W	Y	Q	Q	K	
	ATC	ACC	TGT	AGT	GTC	AGT	TCA	AAT	ATC	CGT		TCC	AAT	TAC	TTG	CAT	TGG	TAT	CAG	CAG	AAG	180
61											70											80
	P	G	F	S	P	K	L	L	I	Y		R	T	S	N	L	A	S	G	V	P	
	CCA	GGA	TTC	TCC	CCT	AAA	CTC	TTG	ATT	TAT		AGG	ACA	TCC	AAT	CTG	GCT	TCT	GGA	GTC	CCA	240
81											90											100
	A	R	F	S	G	S	G	S	G	T		S	Y	S	L	T	I	G	T	M	K	
	GCT	CGC	TTC	AGT	GGC	AGT	GGG	TCT	GGG	ACC		TCT	TAC	TCT	CTC	ACA	ATT	GGC	ACC	ATG	AAG	300
101											110											120
	A	E	D	V	A	T	Y	Y	C	Q		Q	G	S	S	I	P	L	T	F	G	
	GCT	GAA	GAT	GTT	GCC	ACT	TAC	TAC	TGC	CAG		CAG	GGT	AGT	AGT	ATA	CCG	CTC	ACG	TTC	GGT	360
121											130											
	A	G	T	K	L	E	L	K	R	A		D	A	A	P	T	V					(SEQ ID NO:49)
	GCT	GGG	ACC	AAG	CTG	GAG	CTG	AAA	CGG	GCT		GAT	GCT	GCA	CCA	ACT	GTA	TC				410 (SEQ ID NO:50)

FIG. 17L

1	M	D	L	Q	V	Q	I	I	S	F	10	L	L	I	S	V	T	V	I	V	20	S
	ATG	GAT	TTA	CAG	GTG	CAG	ATT	ATC	AGC	TTC		CTG	CTA	ATC	AGT	GTC	ACA	GTC	ATA	GTG	TCT	60
21	N	G	E	I	V	L	A	Q	S	P	30	T	T	T	A	A	S	P	G	E	K	40
	AAT	GGA	GAA	ATT	GTG	CTC	GCC	CAG	TCT	CCA		ACC	ACC	ACG	GCT	GCA	TCT	CCC	GGG	GAG	AAG	120
41	I	T	I	T	C	S	A	S	S	S	50	I	T	S	N	Y	L	H	W	Y	Q	60
	ATC	ACT	ATC	ACC	TGC	AGT	GCC	AGC	TCA	AGT		ATA	ACT	TCC	AAT	TAC	TTG	CAT	TGG	TAT	CAG	180
61	Q	K	P	G	F	S	P	K	L	L	70	I	Y	R	T	S	N	L	A	S	G	80
	CAG	AAG	CCA	GGA	TTC	TCC	CCT	AAA	CTC	TTG		ATT	TAT	AGG	ACA	TCC	AAT	CTG	GCT	TCT	GGA	240
81	V	P	A	R	F	S	G	S	G	S	90	G	T	S	Y	S	L	T	I	G	T	100
	GTC	CCA	GCT	CGC	TTC	AGT	GGC	AGT	GGA	TCT		GGG	ACC	TCT	TAC	TCT	CTC	ACA	ATT	GGC	ACC	300
101	M	E	A	E	D	V	A	T	Y	Y	110	C	Q	Q	G	S	S	K	T	L	T	120
	ATG	GAG	GCT	GAA	GAT	GTT	GCC	ACT	TAC	TAC		TGC	CAG	CAG	GGT	AGT	AGT	AAA	ACA	CTC	ACG	360
121	F	G	A	G	T	K	L	E	L	K	130	R	A	D	A	A	P	T	V			
	TTC	GGT	GCT	GGG	ACC	AAG	CTG	GAG	tTG	AAA		CGG	GCT	GAT	GCT	GCA	CCA	ACT	GTA	TC		

(SEQ ID NO:51)
416

(SEQ ID NO:52)

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FIG. 17M

```
1      10      20
M D L Q V Q I I S F L L I S V T V I V S
ATG GAT TTA CAG GTG CAG ATT ATC AGC TTC CTG CTA ATC AGT GTC ACA GTC ATA GTG TCT 60

21      30      40
N G E I V L T Q S P T T M A A S P G E K
AAT GGA GAA ATT GTG CTC ACC CAG TCT CCA ACC ACC ATG GCT GCA TCT CCC GGG GAG AAG 120

41      50      60
I T I T C S V S S S I R S N Y L H W Y Q
ATC ACT ATC ACC TGC AGT GTC AGC TCA AGT ATA AGG TCC AAT TAT TTA CAT TGG TAT CAG 180

61      70      80
Q K P G F S P K L L I Y R T S N L A S G
CAG AAG CCA GGA TTC TCC CCT AAA CTC TTG ATT TAT AGG ACA TCC AAT CTG GCT TCT GGA 240

81      90      100
V P A R F S G S G S G T S Y S L T V A T
GTC CCA GCT CGC TTC AGT GGC AGT GGG TCT GGG ACC TCT TAC TCT CTC ACA GTT GCC ACC 300

101     110     120
M E A E D V A T Y Y C Q Q G S S I P L T
ATG GAG GCT GAA GAT GTT GCC ACT TAC TAC TGC CAG CAG GGT AGT AGT ATA CCG CTC ACG 360

121     130
F G A G T K L E L K R A D A A P T V (SEQ ID NO:53)
TTC GGT GCT GGG ACC AAG CTG GAG TTG AAA CGG GCT GAT GCT GCA CCA ACT GTA TC 416
(SEQ ID NO:54)
```

FIG. 17N

```
1      10      20
M G I K M E S Q T Q V F V F L L L C V S
ATG GGC ATC AAG ATG GAG TCA CAG ACC CAG GTC TTC GTA TTT CTA CTG CTC TGT GTG TCT 60

21      30      40
G A H G S I V M T Q T P K F L L V S T G
GGT GCT CAT GGG AGT ATT GTG ATG ACC CAG ACT CCC AAA TTC CTG CTT GTA TCA ACA GGA 120

41      50      60
D R V T I T C K A S Q T V T N D L A W Y
GAC AGG GTT ACC ATT ACC TGC AAG GCC AGT CAG ACT GTG ACT AAT GAT TTA GCT TGG TAC 180

61      70      80
Q Q K P G Q S P K L L I Y Y A S N R Y T
CAA CAG AAG CCA GGG CAG TCT CCT AAA CTG CTG ATA TAC TAT GCA TCC AAT CGC TAC ACT 240

81      90      100
G V P D R F T G S G Y G T D F T F T I N
GGA GTC CCT GAT CGC TTC ACT GGC AGT GGA TAT GGG ACG GAC TTC ACT TTC ACC ATC AAC 300

101     110     120
T V Q A E D L A V Y F C Q Q D Y S S P L
ACT GTG CAG GCT GAA GAC CTG GCA GTT TAT TTC TGT CAG CAG GAT TAT AGC TCT CCT CTC 360

121     120
T F G A G T K L E L K R A D A A P T V (SEQ ID NO:55)
ACG TTC GGT GCT GGG ACC AAG CTG GAA CTG AAA CGG GCT GAT GCT GCA CCA ACT GTA 417
(SEQ ID NO:56)
```

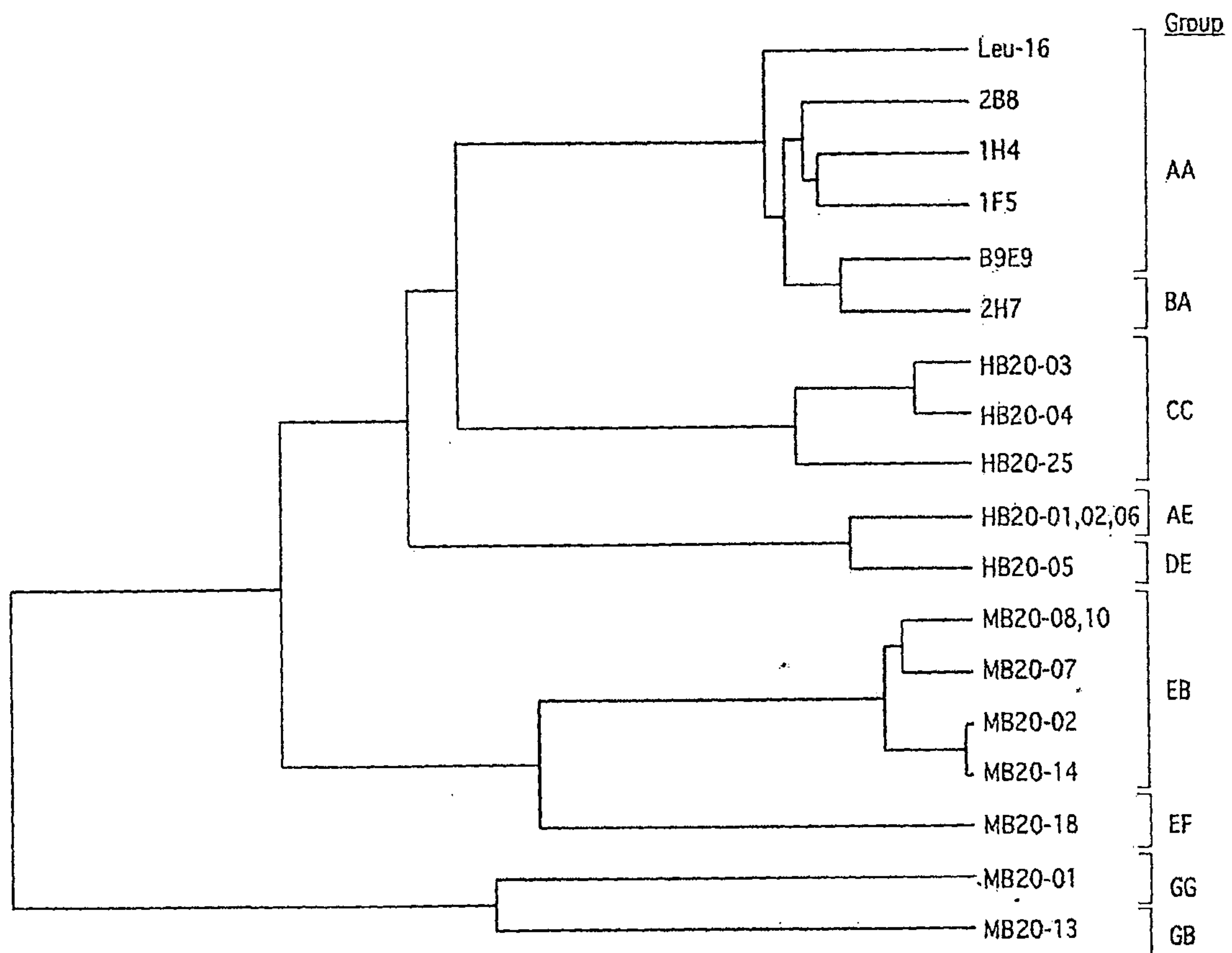
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FIG. 18

Residue	1	10	20	CDR1	35	40	50	CDR2	60	70	80	90	CDR3
Consen.	QIVLSQSPAL--LSASPGKVTMTCTCRASSSV-----SYMHVYQQKPGSSPKPWIVATSNLASGVPAFSGSGSGTSLTISRVEADAATYYCQW.SN P.TFGAGTKLEIK												
2H7								P.....				SF.....	P.....
B9E9	D.....											I.....	P.....
1H4		T.....										I.....	P.....
2B8					I.....	F.....			V.....			T.....	P.....
1F5				L.....	F.....							F.H.S.....	L.....
Leu-16	D..T.....				N.D.....	K.....				Y.....		SF.....	P.....
MB20-02,14	E..T..TT.MA.....	I.I.....	SV.....	IRS.....	N.L.....		F...LL.....	R.....		VATM.....	V.....	GS.I.....	L.....
MB20-03	E..T..TT.MA.....	I.I.....	S.....	ISS.....	N.L.....		F...LL.....	R.....		T.VATM.....	V.....	GS.I.....	L.....
MB20-08,10	E..T..TT.MA.....	I.I.....	SV.....	NIRS.....	N.L.....		F...LL.....	R.....		GTMK.....	V.....	GS.I.....	L.....
MB20-13	E..A..TT.TA.....	I.I.....	S.....	ITS.....	N.L.....		F...LL.....	R.....		GTM.....	V.....	GS.K.TL.....	TL.....
MB20-07	EL.T..TT.KA.....	I.I.....	SV.....	IRS.....	N.L.....		R.F...LL.....	R.....		GTM.....	V.....	GS.L.....	L.....
HB20-03	D.QMT.....S.....	V.T.I.....	..GNI.H.....	N.LA.....		Q.K..QLLV.....	NAKT.D.....		S.....	QF.K.NSLQP.....	FGS.....	HF.W.T.....	W.....
HB20-04	D.QMT.....S.....	V.T.I.....	..G.I.H.....	N.LA.....		L.K..QLLV.....	NAKT.D.....		S.....	QF.K.NSLQP.....	FGS.....	HF.W.I.....	W.....
HB20-25	D.QMT.....S.....	V.V.T.I.....	..ENI.Y.....	NLA.....		Q.K..QLLV.....	AT...D.....		S.....	Q...K.NSLQS.....	FGS.....	HF.WGI.....	W.....
HB20-01,02,06	D..MT..QKF..M.T.V.DR.SV.....	K.QN.....	GTNVA.....			L.Q...L.....	SA.YRN.....		D..T.....	DFT.....	N.QS..L.E.F.....	YN.S.....	F.....
HB20-05	D..MT..QKF..M.T.V.DR.SV.....	K.QN.....	GTNVA.....			Q...Q...AL.....	SA.YRY.....		D..T.....	DFT.....	N.QS..L.E.F.....	YN.S.....	F.....
MB20-18	S..MT.T.KFLV.T--.DR..I.....	K.QT.....	TNDLA.....			Q...Q...LL.....	YA.RYT.....		D..T...Y..DFTF.....	NT.Q...L.V.F.....		DY.S.....	L.....
MB20-01	N.MWT...SS...AV.A.....	R.....	KS.Q..LYSSKRKN.LA.....			K...K...TLL.....	WA.TRE.....		D..T.....	DFT...TS.Q...L.V.....		H.YL.S.....	-F.....

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FIG. 19



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FIG. 20 A. Blood

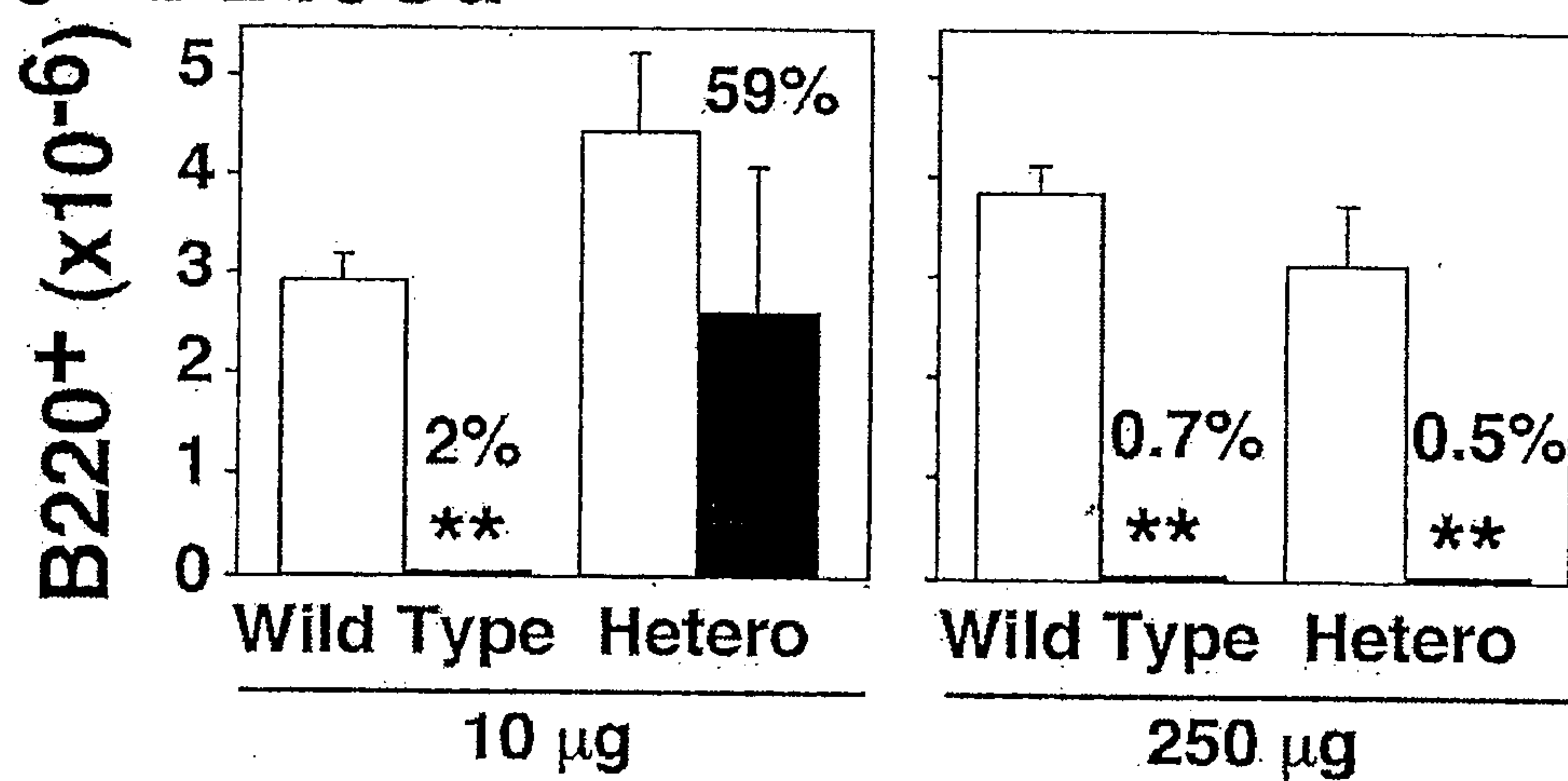
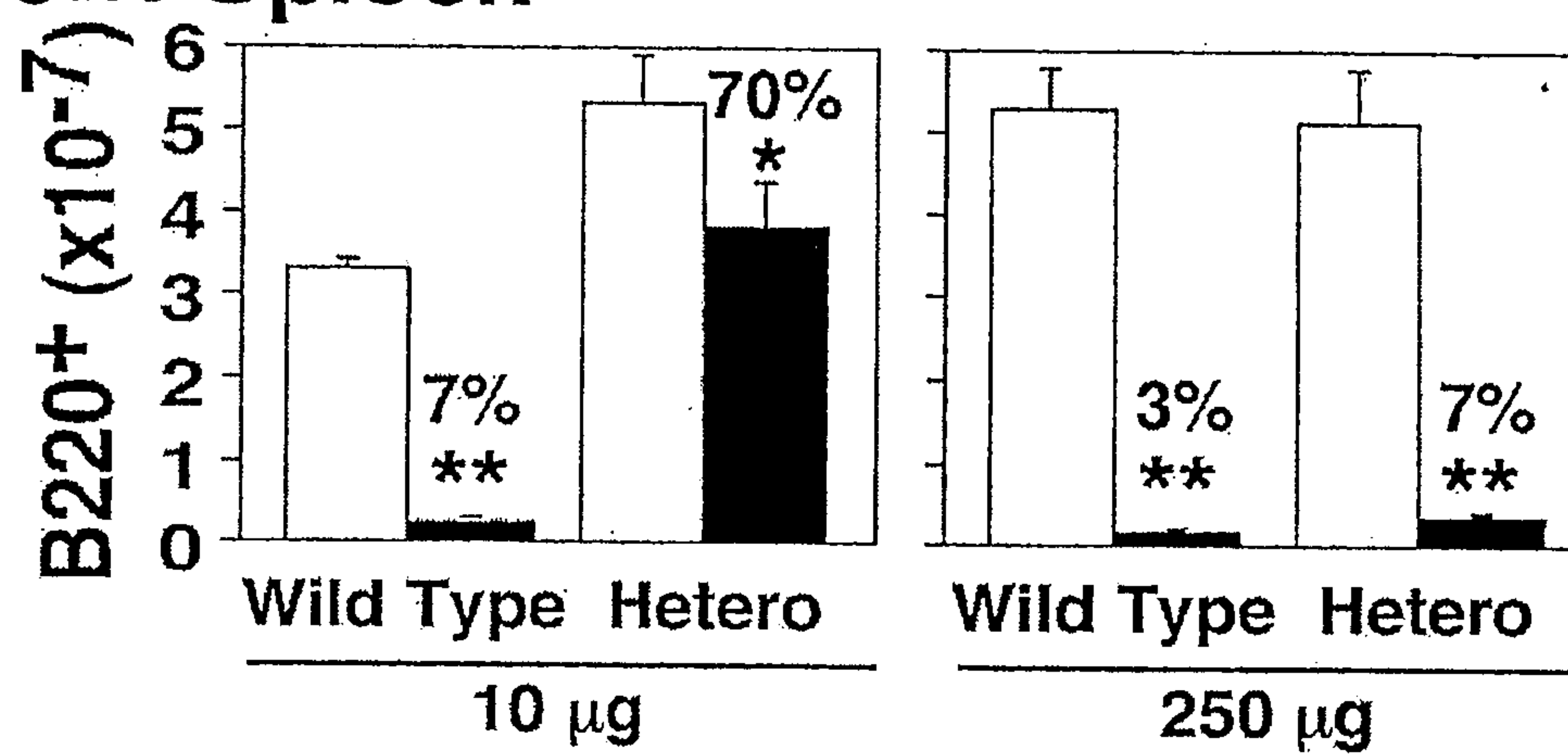


FIG. 20B. Spleen



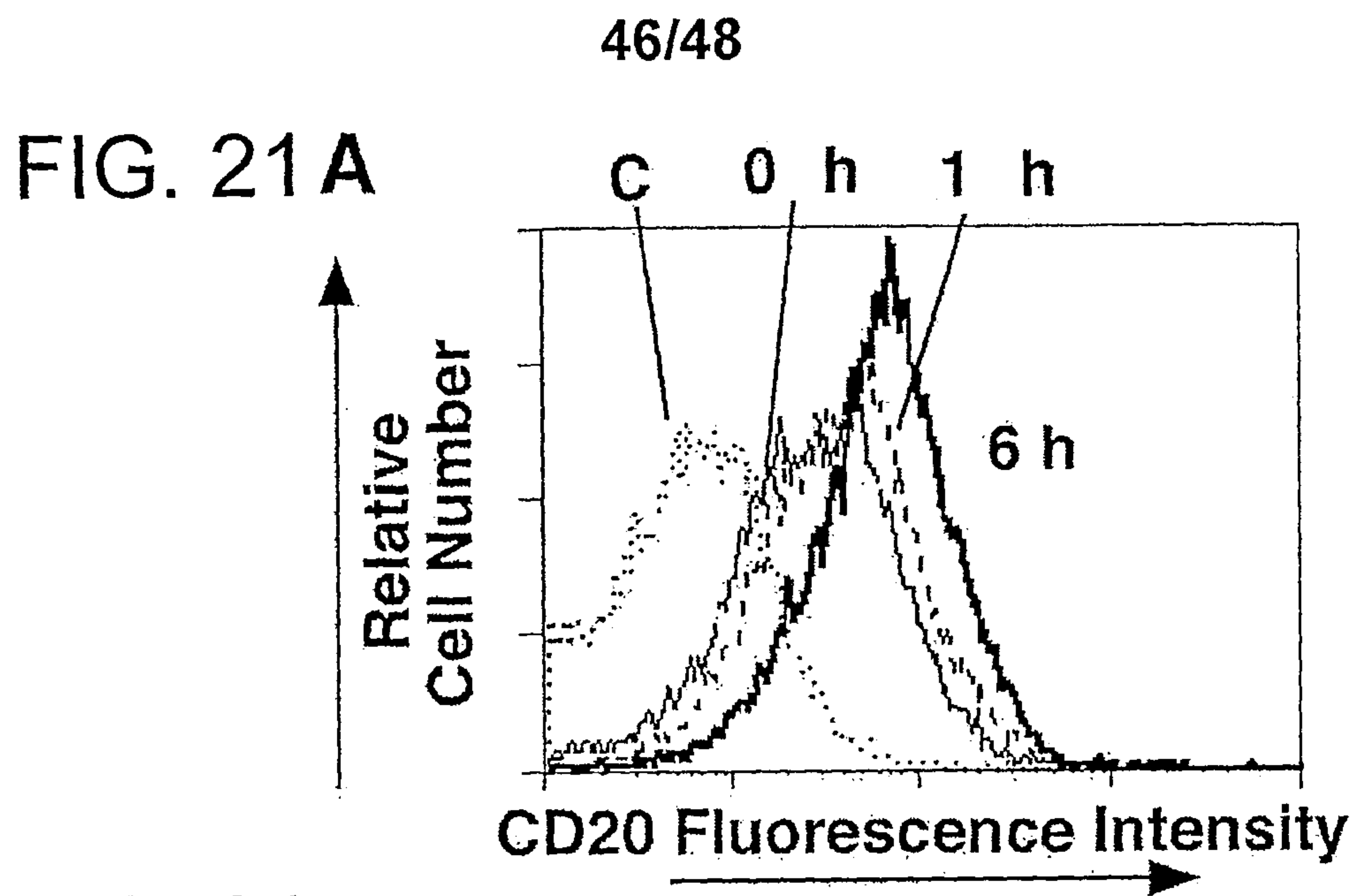
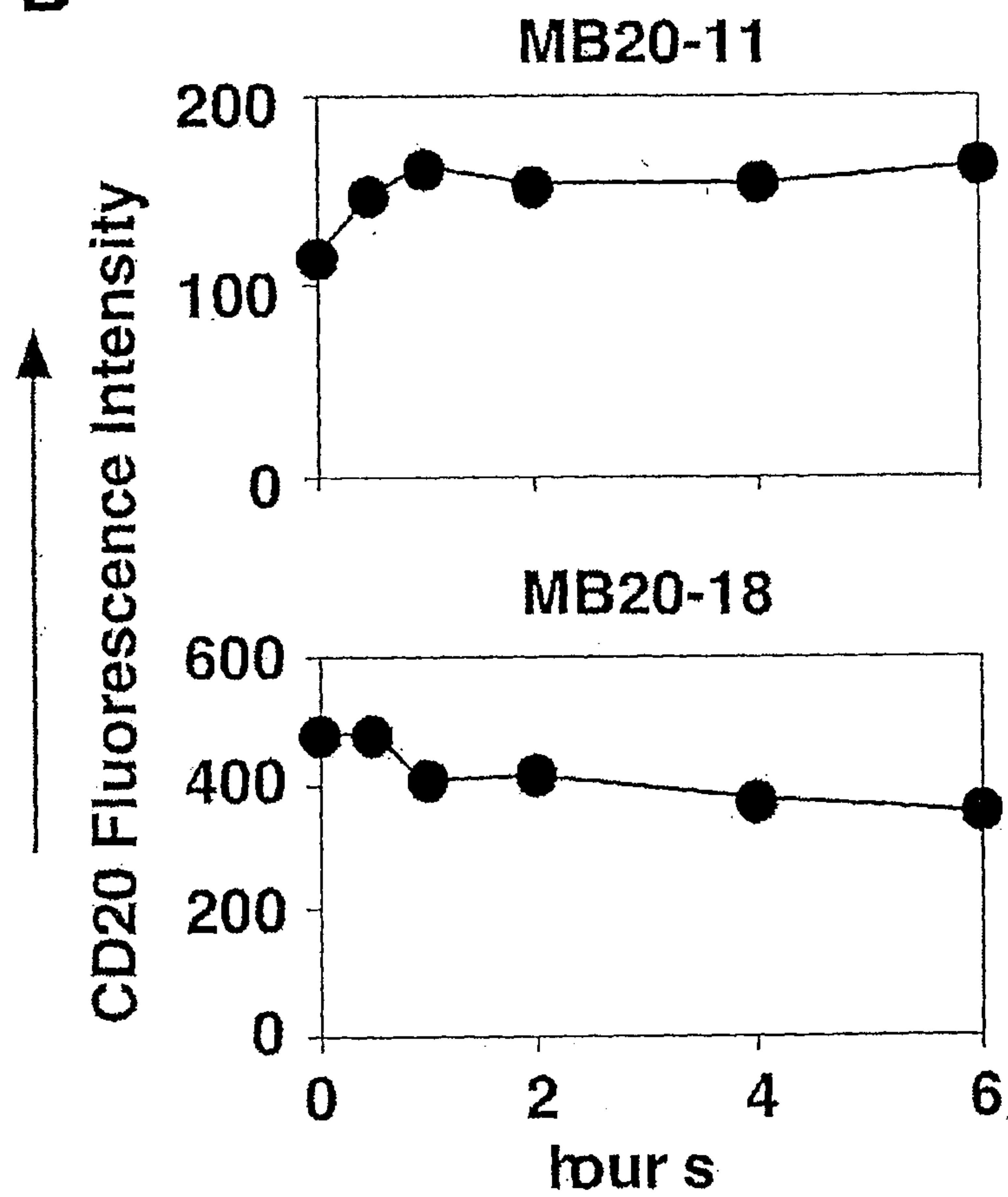


FIG. 21 B



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FIG. 22

A. Blood

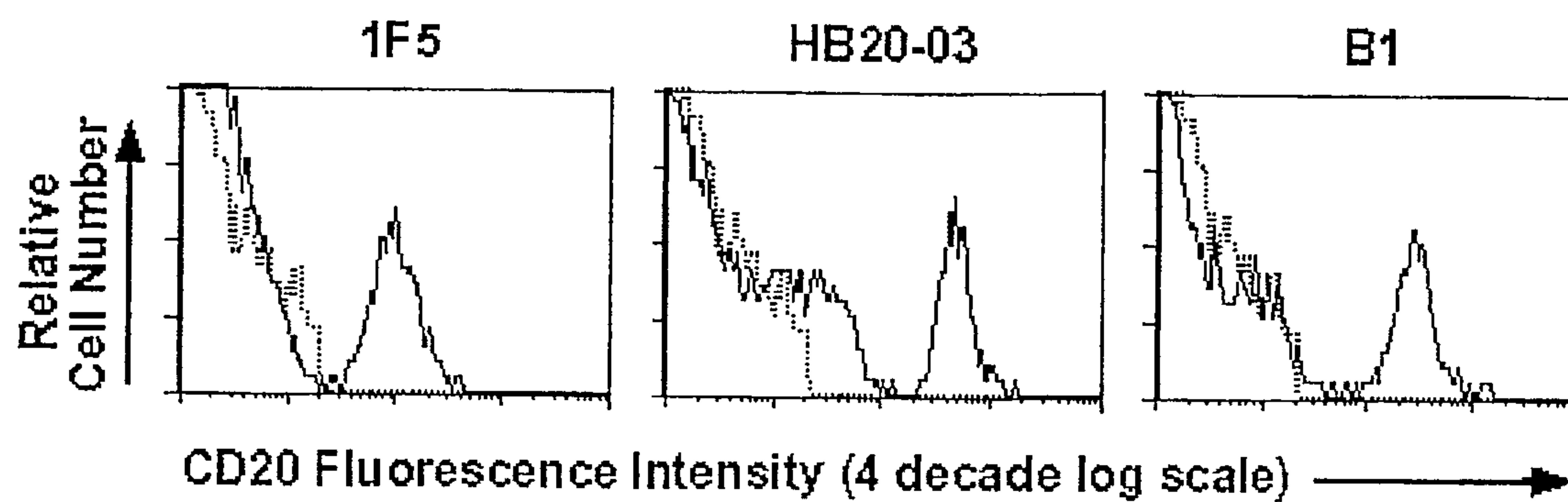
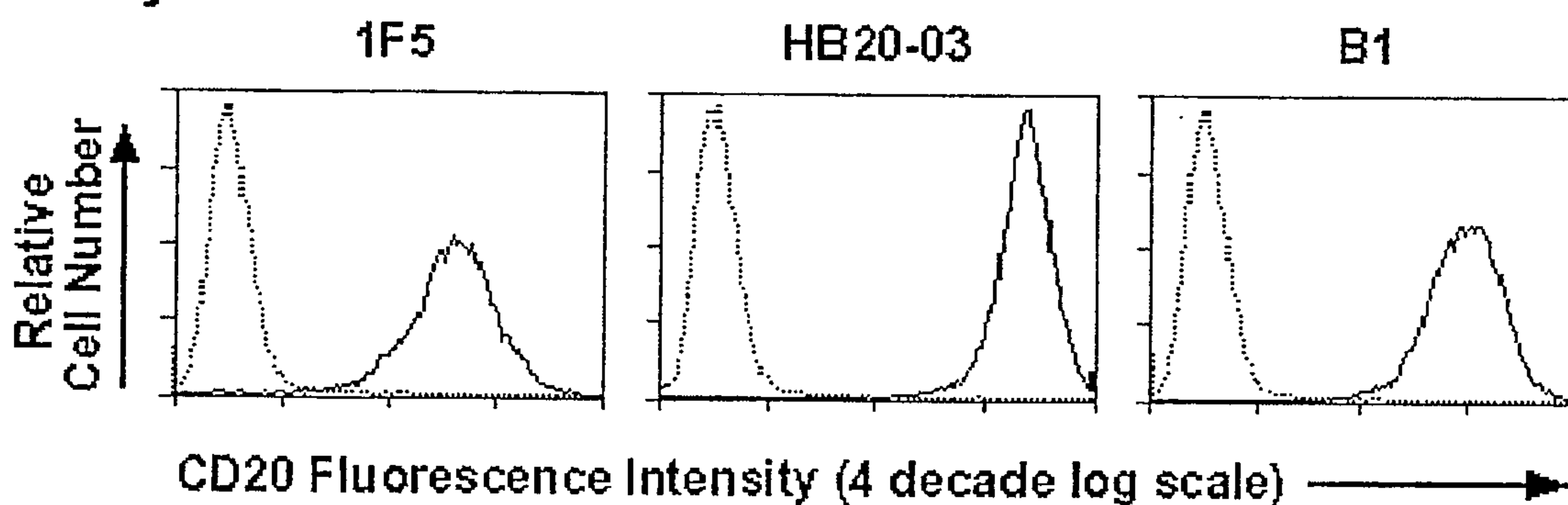


FIG. 22

B. Raji



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FIG. 23A

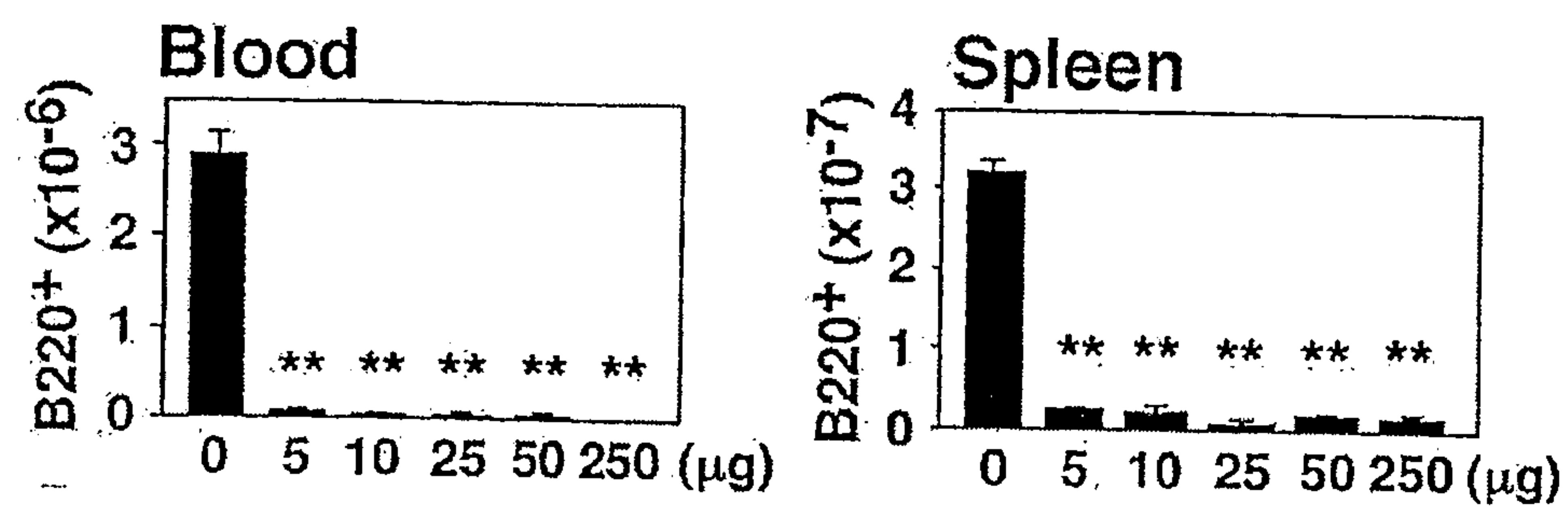


FIG. 23B

