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(54) Titre : UTILISATION DES INHIBITEURS OX-2 POUR LE TRAITEMENT DU CANCER

(54) Title: USE OF OX-2 INHIBITORS FOR THE TREATMENT OF CANCER

(57) **Abrégé/Abstract:**

Methods and compositions for regulating tumor growth are disclosed. For reducing tumor growth, agents that inhibit OX-2 are administered. Such methods are useful in treating cancer. For enhancing tumor growth, an OX-2 protein or a nucleic acid encoding an OX-2 protein is administered. Such methods are useful in studying cancer and/or tumor metastasis.

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(54) Title: METHODS AND COMPOSITIONS FOR MODULATING TUMOR GROWTH

(57) Abstract: Methods and compositions for regulating tumor growth are disclosed. For reducing tumor growth, agents that inhibit OX-2 are administered. Such methods are useful in treating cancer. For enhancing tumor growth, an OX-2 protein or a nucleic acid encoding an OX-2 protein is administered. Such methods are useful in studying cancer and/or tumor metastasis.



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Title: Use of OX-2 Inhibitors For The Treatment of Cancer**FIELD OF THE INVENTION**

The present invention relates to methods and compositions for modulating tumor growth. In particular, the invention includes the use of
5 inhibitors to the protein OX-2 to treat cancer.

BACKGROUND OF THE INVENTION

Interest in the field of tumor immunology has increased in the last several years with the growing evidence that it is possible to identify, and immunize animals for protection with, discrete tumor antigens (15-17). A
10 number of groups have reported on the use of DC coated with tumor antigen, or expressing tumor antigen (following transfection with cDNAs encoding the antigen), as immunogens to induce tumor rejection (18-22). An alternative approach has used tumor cells themselves, transfected with gene(s) such as those controlling cytokines or expression of costimulatory molecules
15 (CD80/CD86) on the cell surface, as an immunogen (11, 23-26). In an interesting model using as recipients animals receiving allogeneic bone marrow transplantation (BMT), which mimicks a therapy used for treatment of leukemia in man, Blazar et al reported that it was possible to promote a Graft-versus-Leukemia (GVL) effect, without unwanted Graft-versus-Host Disease
20 (GVHD), by pre-immunizing tumor transplanted mice with tumor cells transfected to express CD80, but not CD86 (27).

There is a need in the art to develop new immunotherapeutic approaches to treating cancer.

SUMMARY OF THE INVENTION

25 The present invention provides a method of inhibiting, preventing or reducing the growth of a tumor cell comprising administering an effective amount of an agent that inhibits OX-2 to a cell or animal in need thereof.

The inventors also includes a use of an agent that inhibits OX-2
30 to prepare a medicament to inhibit, reduce or prevent the growth of a tumor cell.

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The invention further includes pharmaceutical compositions containing an OX-2 inhibitor for use in inducing or augmenting an immune response to a tumor to inhibit tumor growth.

Other features and advantages of the present invention will
5 become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples while indicating preferred embodiments of the invention are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from
10 this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention will now be described in relation to the drawings in which:

Figure 1 is a graph showing Inhibition of EL4 or C1498 tumor
15 growth in C3H bone marrow reconstituted C57BL/6 mice. Groups of 6 BL/6 mice received 20×10^6 T-depleted BL/6 or C3H bone marrow cells 24hrs following cyclophosphamide treatment. 5×10^6 EL4 or 5×10^5 C1498 tumor cells were injected 28 days later into these mice, and control BL/6 or C3H mice. >85% of PBL from C3H reconstituted BL/6 were stained by FITC anti-H2K^k
20 mAb at this time.

Figure 2 is a graph showing EL4 tumor growth in BL/6 mice immunized twice, at 14 day intervals, with 5×10^6 EL4 cells transfected to express CD80 or CD86. 5×10^6 EL4 cells were injected as tumor challenge 10 days after the last immunization .

25 Figure 3 is a graph showing suppression of growth inhibition in C57BL/6 BMT recipients of EL4 or C1498 tumor cells (see Figure 19) following 4 weekly infusions of 100µg/mouse anti-CD4 or anti-CD8 mAb, beginning on the day of BMT (tumor cells were injected at 28 days post BMT). Data are shown for 6 mice/group.

30 Figure 4 is a graph showing inhibition of immunity to EL4 or C1498 tumor challenge following infusion of CD200Fc in C57BL/6 mice reconstituted with C3H bone marrow -see Figure 19 and text for more details. Cyclophosphamide treated BL/6 mice received bone marrow rescue with T-

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depleted C3H or BL/6 cells. 28 days later all mice, and groups of control normal C3H mice, received ip injection with 5×10^6 EL4 or 5×10^5 C1498 tumor cells. Bone marrow reconstituted mice received further iv infusion of normal mouse IgG or CD200Fc ($10 \mu\text{g}/\text{mouse}/\text{injection}$) 5 times at 2 day intervals
5 beginning on the day of tumor injection.

Figure 5 is a graph showing inhibition of immunity to EL4 tumor cells in EL4-CD80 immunized BL/6 mice using CD200Fc-see Figure 2 and text for details. Mice received iv infusion of control IgG or CD200Fc as described in Figure 4.

10 Figure 6 is a graph showing improved tumor immunity in EL4-CD86 or C1498-CD86 immunized C57BL/6 mice following infusion of anti-CD200 mAb. See legend to Figure 2 and text for more details. Where shown, groups of mice received iv infusion of anti-CD200, $100 \text{mg}/\text{mouse}$, on 3 occasions at 3 day intervals beginning on the day of tumor injection.

15 Figure 7 is a graph showing $\log_{10}$ relative concentrations of CD200 mRNAs compared with standardized control mRNA-see Materials and Methods for technique used for quantitation. All samples were first normalized for equivalent concentrations of GAPDH mRNA. Values shown represent arithmetic means $\pm \text{SD}$ for 3 individual samples for each time point. Mice were
20 preimmunized with CD80/CD86-transfected tumor cells as described in the text. *, $p < 0.02$ compared with untreated control, or mice immunized with EL4 or EL4-transfected with CD80.

Figure 8 is a graph showing increased inhibition of tumor immunity using infusion of CD200Fc with CD200^{r+} cells in C57BL/6 recipients
25 of C3H bone marrow -see text and Materials and Methods for more details. In this experiment some mice received not only CD200Fc with EL4 or C1498 tumor, but in addition a lymphocyte-depleted, LPS-stimulated, macrophage population stained ($>65\%$) with anti-CD200^r mAb (2F9).

Figure 9 is a graph showing combinations of CD200Fc and anti-
30 CD4 or anti-CD8 mAb produce increased suppression of tumor growth inhibition in C57BL/6 recipients of C3H BMT. Groups of 6 mice received weekly iv infusions of $100 \mu\text{g}$ anti-T cell mab or 5 iv infusions of $10 \mu\text{g}/\text{mouse}$

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CD200Fc, alone or in combination, beginning on the day of tumor injection (28 days post BMT).

Figure 10 is a graph showing effect of combined CD200Fc and anti-CD4 or anti-CD8 mAb on suppression of EL4 tumor growth inhibition in C57BL/6 recipients preimmunized with EL4-CD80 transfected cells (see Figure 20). Data are shown for groups of 6 mice/group. Weekly iv infusions of 100µg anti-T cell mab or 5 iv infusions of 10µg/mouse CD200Fc, alone or in combination, were begun on the day of tumor injection (10 days after the final immunization with EL4-CD80 cells).

Figures 11A and B are bar graphs showing the median number of lung nodules in mice receiving allogeneic blood by tail vein.

Figures 12A and B are bar graphs showing the number of lung nodules in the presence of anti-OX2 in mice receiving allogeneic blood by tail vein.

Figures 13A and B are bar graphs showing the number of lung nodules in the presence of anti-OX-2, DEC205 or anti-CD11c in mice receiving allogeneic blood by tail vein.

Figure 14 shows the cDNA sequence of rat (SEQ.ID.NO.:1), mouse (SEQ.ID.NO.:3) and human MRC OX-2 (SEQ.ID.NO.:5).

Figure 15 shows the deduced protein sequence of rat (SEQ.ID.NO.:2), mouse (SEQ.ID.NO.:4) and human MRC OX-2 (SEQ.ID.NO.:6) protein.

DETAILED DESCRIPTION OF THE INVENTION

(a) Preventing Tumor Growth

The present inventors have previously disclosed that OX-2 (or CD200) plays a role in the development of immune suppression or tolerance and may be useful in developing therapies for the prevention and treatment of transplant rejection, fetal loss, autoimmune disease or allergies (WO 99/24565).

The inventors have now shown that OX-2 promotes tumor cell growth and inhibiting OX-2 inhibits tumor cell growth.

Accordingly, in one aspect, the present invention provides a method of inhibiting tumor cell growth comprising administering an effective

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amount of an agent that inhibits OX-2 to an animal in need thereof. The invention also provides a use of an effective amount of an agent that inhibits OX-2 to inhibit tumor growth or to prepare a medicament to inhibit tumor cell growth.

5 The term "effective amount" as used herein means an amount effective, at dosages and for periods of time necessary to achieve the desired result (e.g. to inhibit tumor growth).

 The term "animal" includes all members of the animal kingdom and is preferably a mammal, more preferably a human.

10 The term "OX-2 protein" includes OX-2 or CD200 from any species or source and includes a full length OX-2 protein as well as fragments or portions of the protein. The term "OX-2" is also generally referred to as "CD200" due to a change in nomenclature. Both "OX-2" and "CD200" may be used interchangeably in the application.

15 The agent that inhibits OX-2 can be any agent that decreases the expression or activity of an OX-2 protein such that the immune suppression caused by OX-2 is reduced, inhibited and/or prevented. Such agents can be selected from agents that inhibit OX-2 activity (such as antibodies, OX-2 ligands, small molecules), agents that inhibit OX-2
20 expression (such as antisense molecules) or agents that inhibit the interaction of OX-2 with its receptor (such as soluble OX-2 receptor and antibodies that bind the OX-2 receptor).

 One of skill in the art can readily determine whether or not a particular agent is effective in inhibiting OX-2. For example, the agent can be
25 tested in *in vitro* assays to determine if the function or activity of OX-2 is inhibited. The agent can also be tested for its ability to induce an immune response using *in vitro* immune assays including, but not limited to, enhancing a cytotoxic T cell response; inducing interleukin-2 (IL-2) production; inducing IFN γ production; inducing a Th1 cytokine profile; inhibiting IL-4 production;
30 inhibiting TGF β production; inhibiting IL-10 production; inhibiting a Th2 cytokine profile and any other assay that would be known to one of skill in the art to be useful in detecting immune activation.

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One of skill in the art can determine whether a particular agent is useful in inhibiting tumor cell growth. As mentioned above, one can test the agent for its ability to induce an immune response using known in vitro assays. In addition, the agent can be tested in an animal model, for example
5 as described in Examples 1 and 2, wherein the agent is administered to an animal with cancer.

The term "inhibiting or reducing tumor cell growth" means that the agent that inhibits OX-2 causes an inhibition or reduction in the growth or metastasis of a tumor as compared to the growth observed in the absence of
10 the agent. The agent may also be used prophylactically to prevent the growth of tumor cells.

The tumor cell can be any type of cancer including, but not limited to, hematopoietic cell cancers (including leukemias and lymphomas), colon cancer, lung cancer, kidney cancer, pancreas cancer, endometrial
15 cancer, thyroid cancer, oral cancer, laryngeal cancer, hepatocellular cancer, bile duct cancer, squamous cell carcinoma, prostate cancer, breast cancer, cervical cancer, colorectal cancer, melanomas and any other tumors which are antigenic or weakly antigenic. This could include, for example, EBV-induced neoplasms, and neoplasms occurring in immunosuppressed patients,
20 e.g. transplant patients, AIDS patients, etc.

(i) Antibodies

In a preferred embodiment, the agent that inhibits OX-2 is an OX-2 specific antibody. The present inventors have prepared antibodies to OX-2 which are described in WO 99/24565. Antibodies to OX-2 may also be
25 obtained commercially or prepared using techniques known in the art such as those described by Kohler and Milstein, Nature 256, 495 (1975) and in U.S. Patent Nos. RE 32,011; 4,902,614; 4,543,439; and 4,411,993. (See also Monoclonal Antibodies, Hybridomas: A New Dimension in Biological Analyses, Plenum Press, Kennett, McKearn, and Bechtol (eds.), 1980, and
30 Antibodies: A Laboratory Manual, Harlow and Lane (eds.), Cold Spring Harbor Laboratory Press, 1988).

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Conventional methods can be used to prepare the antibodies. For example, by using the OX-2 protein, polyclonal antisera or monoclonal antibodies can be made using standard methods. A mammal, (e.g., a mouse, hamster, or rabbit) can be immunized with an immunogenic form of the OX 2
5 receptor which elicits an antibody response in the mammal. Techniques for conferring immunogenicity on a peptide include conjugation to carriers or other techniques well known in the art. For example, the peptide can be administered in the presence of adjuvant. The progress of immunization can be monitored by detection of antibody titers in plasma or serum. Standard
10 ELISA or other immunoassay procedures can be used with the immunogen as antigen to assess the levels of antibodies. Following immunization, antisera can be obtained and, if desired, polyclonal antibodies isolated from the sera.

To produce monoclonal antibodies, antibody producing cells
15 (lymphocytes) can be harvested from an immunized animal and fused with myeloma cells by standard somatic cell fusion procedures thus immortalizing these cells and yielding hybridoma cells. Such techniques are well known in the art, (e.g., the hybridoma technique originally developed by Kohler and Milstein (Nature 256, 495-497 (1975)) as well as other techniques such as the
20 human B-cell hybridoma technique (Kozbor et al., Immunol. Today 4, 72 (1983)); the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al. Monoclonal Antibodies in Cancer Therapy (1985) Allen R. Bliss, Inc., pages 77-96); and screening of combinatorial antibody libraries (Huse et al., Science 246, 1275 (1989)). Hybridoma cells can be screened
25 immunochemically for production of antibodies specifically reactive with the OX-2 receptor and the monoclonal antibodies can be isolated. Therefore, the invention also contemplates hybridoma cells secreting monoclonal antibodies with specificity for OX-2.

The term "antibody" as used herein is intended to include
30 fragments thereof which also specifically react with OX-2 or a peptide thereof. Antibodies can be fragmented using conventional techniques and the fragments screened for utility in the same manner as described above. For example, F(ab')₂ fragments can be generated by treating antibody with

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pepsin. The resulting F(ab')₂ fragment can be treated to reduce disulfide bridges to produce Fab' fragments.

Chimeric antibody derivatives, i.e., antibody molecules that combine a non-human animal variable region and a human constant region are also contemplated within the scope of the invention. Chimeric antibody molecules can include, for example, the antigen binding domain from an antibody of a mouse, rat, or other species, with human constant regions. Conventional methods may be used to make chimeric antibodies containing the immunoglobulin variable region which recognizes an OX-2 receptor (See, for example, Morrison et al., Proc. Natl Acad. Sci. U.S.A. 81,6851 (1985); Takeda et al., Nature 314, 452 (1985), Cabilly et al., U.S. Patent No. 4,816,567; Boss et al., U.S. Patent No. 4,816,397; Tanaguchi et al., European Patent Publication EP171496; European Patent Publication 0173494, United Kingdom patent GB 2177096B).

Monoclonal or chimeric antibodies specifically reactive with the OX-2 as described herein can be further humanized by producing human constant region chimeras, in which parts of the variable regions, particularly the conserved framework regions of the antigen-binding domain, are of human origin and only the hypervariable regions are of non human origin. Such immunoglobulin molecules may be made by techniques known in the art (e.g., Teng et al., Proc. Natl. Acad. Sci. U.S.A., 80, 7308-7312 (1983); Kozbor et al., Immunology Today, 4, 7279 (1983); Olsson et al., Meth. Enzymol., 92, 3-16 (1982); and PCT Publication WO 92/06193 or EP 0239400). Humanized antibodies can also be commercially produced (Scotgen Limited, 2 Holly Road, Twickenham, Middlesex, Great Britain.)

Specific antibodies, or antibody fragments reactive against OX-2 may also be generated by screening expression libraries encoding immunoglobulin genes, or portions thereof, expressed in bacteria with peptides produced from nucleic acid molecules of the present invention. For example, complete Fab fragments, VH regions and FV regions can be expressed in bacteria using phage expression libraries (See for example Ward et al., Nature 341, 544-546: (1989); Huse et al., Science 246, 1275-1281 (1989); and McCafferty et al. Nature 348, 552-554 (1990)).

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Accordingly, the present invention provides a method of inhibiting, preventing or reducing tumor cell growth comprising administering an effective amount of an antibody that inhibits OX-2 to an animal in need thereof.

5 (ii) Antisense Oligonucleotides

In another embodiment, the OX-2 inhibitor is an antisense oligonucleotide that inhibits the expression of OX-2. Antisense oligonucleotides that are complimentary to a nucleic acid sequence from an OX-2 gene can be used in the methods of the present invention to inhibit OX-
10 2. The present inventors have prepared antisense oligonucleotides to OX-2 which are described in WO 99/24565.

Accordingly, the present invention provides a method of inhibiting, preventing or reducing tumor cell growth comprising administering an effective amount of an antisense oligonucleotide that is complimentary to a
15 nucleic acid sequence from a OX-2 gene to an animal in need thereof.

The term "antisense oligonucleotide" as used herein means a nucleotide sequence that is complimentary to its target, the sense strand of messenger RNA that is translated into protein at the ribosomal level.

In one embodiment of the invention, the present invention
20 provides an antisense oligonucleotide that is complimentary to a nucleic acid molecule having a sequence as shown in Figure 14 (SEQ.ID.NO.:1, 3 or 5), wherein T can also be U, or a fragment thereof.

The term "oligonucleotide" refers to an oligomer or polymer of nucleotide or nucleoside monomers consisting of naturally occurring bases, sugars, and intersugar (backbone) linkages. The term also includes modified
25 or substituted oligomers comprising non-naturally occurring monomers or portions thereof, which function similarly. Such modified or substituted oligonucleotides may be preferred over naturally occurring forms because of properties such as enhanced cellular uptake, or increased stability in the presence of nucleases. The term also includes chimeric oligonucleotides
30 which contain two or more chemically distinct regions. For example, chimeric oligonucleotides may contain at least one region of modified nucleotides that confer beneficial properties (e.g. increased nuclease resistance, increased

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uptake into cells), or two or more oligonucleotides of the invention may be joined to form a chimeric oligonucleotide.

The antisense oligonucleotides of the present invention may be ribonucleic or deoxyribonucleic acids and may contain naturally occurring
5 bases including adenine, guanine, cytosine, thymidine and uracil. The oligonucleotides may also contain modified bases such as xanthine, hypoxanthine, 2-aminoadenine, 6-methyl, 2-propyl and other alkyl adenines, 5-halo uracil, 5-halo cytosine, 6-aza uracil, 6-aza cytosine and 6-aza thymine, pseudo uracil, 4-thiouracil, 8-halo adenine, 8-aminoadenine, 8-thiol adenine,
10 8-thiolalkyl adenines, 8-hydroxyl adenine and other 8-substituted adenines, 8-halo guanines, 8-amino guanine, 8-thiol guanine, 8-thiolalkyl guanines, 8-hydroxyl guanine and other 8-substituted guanines, other aza and deaza uracils, thymidines, cytosines, adenines, or guanines, 5-trifluoromethyl uracil and 5-trifluoro cytosine.

15 Other antisense oligonucleotides of the invention may contain modified phosphorous, oxygen heteroatoms in the phosphate backbone, short chain alkyl or cycloalkyl intersugar linkages or short chain heteroatomic or heterocyclic intersugar linkages. For example, the antisense oligonucleotides may contain phosphorothioates, phosphotriesters, methyl
20 phosphonates, and phosphorodithioates. In an embodiment of the invention there are phosphorothioate bonds links between the four to six 3'-terminus bases. In another embodiment phosphorothioate bonds link all the nucleotides.

The antisense oligonucleotides of the invention may also
25 comprise nucleotide analogs that may be better suited as therapeutic or experimental reagents. An example of an oligonucleotide analogue is a peptide nucleic acid (PNA) wherein the deoxyribose (or ribose) phosphate backbone in the DNA (or RNA), is replaced with a polyamide backbone which is similar to that found in peptides (P.E. Nielsen, et al Science 1991, 254,
30 1497). PNA analogues have been shown to be resistant to degradation by enzymes and to have extended lives *in vivo* and *in vitro*. PNAs also bind stronger to a complimentary DNA sequence due to the lack of charge repulsion between the PNA strand and the DNA strand. Other

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oligonucleotides may contain nucleotides containing polymer backbones, cyclic backbones, or acyclic backbones. For example, the nucleotides may have morpholino backbone structures (U.S. Pat. No. 5,034,506). Oligonucleotides may also contain groups such as reporter groups, a group
5 for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an antisense oligonucleotide. Antisense oligonucleotides may also have sugar mimetics.

The antisense nucleic acid molecules may be constructed using chemical synthesis and enzymatic ligation reactions using procedures known
10 in the art. The antisense nucleic acid molecules of the invention or a fragment thereof, may be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical stability of the duplex formed with mRNA or the native gene e.g. phosphorothioate
15 derivatives and acridine substituted nucleotides. The antisense sequences may be produced biologically using an expression vector introduced into cells in the form of a recombinant plasmid, phagemid or attenuated virus in which antisense sequences are produced under the control of a high efficiency regulatory region, the activity of which may be determined by the cell type into
20 which the vector is introduced.

(iii) Other OX-2 Inhibitors

In addition to antibodies and antisense molecules, other agents that inhibit OX-2 may also be used in the present invention.

Accordingly, the present invention also includes the isolation of
25 other ligands or molecules that can bind to OX-2 or the OX-2 receptor. Biological samples and commercially available libraries may be tested for proteins that bind to OX-2 or the OX-2 receptor. In addition, antibodies prepared to the OX-2 or the OX-2 receptor may be used to isolate other peptides with OX-2 or OX-2 receptor binding affinity. For example, labelled
30 antibodies may be used to probe phage displays libraries or biological samples.

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Conditions which permit the formation of protein complexes may be selected having regard to factors such as the nature and amounts of the substance and the protein.

The substance-protein complex, free substance or non-complexed proteins may be isolated by conventional isolation techniques, for example, salting out, chromatography, electrophoresis, gel filtration, fractionation, absorption, polyacrylamide gel electrophoresis, agglutination, or combinations thereof. To facilitate the assay of the components, the antibodies, proteins, or substances may be labelled with a detectable substance.

Once potential binding partners have been isolated, screening methods may be designed in order to determine if the molecules that bind to the OX-2 peptide or OX-2 receptor and are useful in the methods of the present invention.

Therefore, the invention also provides methods for identifying substances which are capable of binding to the OX-2. In particular, the methods may be used to identify substances which are capable of binding to and which suppress the effects of OX-2. Accordingly the invention provides a method of identifying substances which bind with OX-2, comprising the steps of:

(a) reacting OX-2 and a substance, under conditions which allow for formation of a complex, and

(b) assaying for complexes, for free substance, and for non-complexed OX-2.

Substances which can bind with the OX-2 of the invention may be identified by reacting OX-2 with a substance which potentially binds to the OX-2, and assaying for complexes, for free substance, or for non-complexed OX-2. Any assay system or testing method that detects protein-protein interactions may be used including co-immunoprecipitation, crosslinking and co-purification through gradients or chromatographic columns may be used. Additionally, x-ray crystallographic studies may be used as a means of evaluating interactions with substances and molecules. For example, purified recombinant molecules in a complex of the invention when crystallized in a

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suitable form are amenable to detection of intra-molecular interactions by x-ray crystallography. Spectroscopy may also be used to detect interactions and in particular, Q-TOF instrumentation may be used. Biological samples and commercially available libraries may be tested for OX-2-binding peptides.

5 In addition, antibodies prepared to the peptides of the invention may be used to isolate other peptides with OX-2 binding affinity. For example, labelled antibodies may be used to probe phage display libraries or biological samples. In this respect peptides of the invention may be developed using a biological expression system. The use of these systems allows the
10 production of large libraries of random peptide sequences and the screening of these libraries for peptide sequences that bind to particular proteins. Libraries may be produced by cloning synthetic DNA that encodes random peptide sequences into appropriate expression vectors. (see Christian et al. 1992, J. Mol. Biol. 227:711; Devlin et al., 1990 Science 249:404; Cwirla et al.
15 1990, Proc. Natl. Acad. Sci. USA, 87:6378). Libraries may also be constructed by concurrent synthesis of overlapping peptides (see U.S. Pat. No. 4,708,871).

It will be understood that the agonist and antagonist that can be assayed using the methods of the invention may act on one or more of the
20 binding sites on the protein or substance including agonist binding sites, competitive antagonist binding sites, non-competitive antagonist binding sites or allosteric sites.

The invention also makes it possible to screen for antagonists that inhibit the effects of an agonist of the interaction of OX-2 with a
25 substance which is capable of binding to OX-2. Thus, the invention may be used to assay for a substance that competes for the same binding site of OX-2. As such it will also be appreciated that intracellular substances which are capable of binding to OX-2 may be identified using the methods described herein.

30 The reagents suitable for applying the methods of the invention to evaluate substances and compounds that affect or modulate a OX-2 may be packaged into convenient kits providing the necessary materials packaged

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into suitable containers. The kits may also include suitable supports useful in performing the methods of the invention.

(b) Inducing Tumor Cell Growth

In another aspect, the present invention provides a method of
5 inducing tumor cell growth or metastasis comprising administering an
effective amount of an OX-2 protein or fragment thereof or a nucleic acid
sequence encoding an OX-2 protein or fragment thereof to an animal in need
of such treatment. The invention includes a use of an effective amount of an
OX-2 protein or fragment thereof or a nucleic acid sequence encoding an OX-
10 2 protein or fragment thereof to induce tumor cell growth or metastasis. The
method of inducing tumor growth can be used to study tumor cell growth or
metastasis. The method can also be used to develop an animal model to
study or test cancer therapies or chemotherapeutic agents. The method is
generally conducted on non-human animals.

15 The OX-2 protein or nucleic acid encoding the OX-2 protein for
use in the method can be obtained from any species or source. The nucleic
acid sequence and amino acid sequence of an OX-2 protein from human,
mouse and rat are shown in Figures 14 and 15 and in SEQ.ID.NOs.1-6.
Preferred fragments or portions of the OX-2 or CD200 protein are those that
20 are sufficient to suppress an immune response. Determining whether a
particular OX-2 or CD200 protein can suppress an immune response and
result in increased tumor growth can be assessed using known *in vitro*
immune assays including, but not limited to, inhibiting a mixed leucocyte
reaction; inhibiting a cytotoxic T cell response; inhibiting interleukin-2
25 production; inhibiting IFN γ production; inhibiting a Th1 cytokine profile;
inducing IL-4 production; inducing TGF β production; inducing IL-10
production; inducing a Th2 cytokine profile; and any other assay that would
be known to one of skill in the art to be useful in detecting immune
suppression.

30 The term "administering an OX-2 protein" includes both the
administration of the OX-2 protein as well as the administration of a nucleic
acid sequence encoding an OX-2 protein. In the latter case, the OX-2 protein
is produced *in vivo* in the animal.

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In a preferred embodiment, the OX-2 protein is prepared and administered as a soluble fusion protein. The fusion protein may contain the extracellular domain of OX-2 linked to an immunoglobulin (Ig) Fc Region. The OX-2 fusion may be prepared using techniques known in the art. Generally, a
5 DNA sequence encoding the extracellular domain of OX-2 is linked to a DNA sequence encoding the Fc of the Ig and expressed in an appropriate expression system where the OX-2 - FcIg fusion protein is produced.

The OX-2 or protein may be obtained from known sources or prepared using recombinant DNA techniques. The protein may have any of
10 the known published sequences for OX-2 or CD200. The sequences can be obtained from GenBank. The human sequence has accession no. M17226 X0523; the rat sequence has accession no. X01785; and the mouse sequence has accession no. AF029214. The nucleic acid and protein sequences of OX-2 (CD200) from human, mouse and rat are also shown in
15 SEQ.ID.Nos.: 1, 3 and 5 (nucleic acid) and SEQ.ID.Nos.:2, 4 and 6 (protein).

The OX-2 protein may also be modified to contain amino acid substitutions, insertions and/or deletions that do not alter the immunosuppressive properties of the protein. Conserved amino acid substitutions involve replacing one or more amino acids of the OX-2 amino
20 acid sequence with amino acids of similar charge, size, and/or hydrophobicity characteristics. When only conserved substitutions are made the resulting analog should be functionally equivalent to the OX-2 protein. Non-conserved substitutions involve replacing one or more amino acids of the OX-2 amino acid sequence with one or more amino acids which possess dissimilar
25 charge, size, and/or hydrophobicity characteristics.

The OX-2 protein may be modified to make it more therapeutically effective or suitable. For example, the OX-2 protein may be cyclized as cyclization allows a peptide to assume a more favourable conformation. Cyclization of the OX-2 peptides may be achieved using
30 techniques known in the art. In particular, disulphide bonds may be formed between two appropriately spaced components having free sulfhydryl groups. The bonds may be formed between side chains of amino acids, non-amino acid components or a combination of the two. In addition, the OX-2 protein or

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peptides of the present invention may be converted into pharmaceutical salts by reacting with inorganic acids including hydrochloric acid, sulphuric acid, hydrobromic acid, phosphoric acid, etc., or organic acids including formic acid, acetic acid, propionic acid, glycolic acid, lactic acid, pyruvic acid, oxalic acid, succinic acid, malic acid, tartaric acid, citric acid, benzoic acid, salicylic acid, benzenesulphonic acid, and tolunesulphonic acids.

Administration of an "effective amount" of the OX-2 protein and nucleic acid of the present invention is defined as an amount effective, at dosages and for periods of time necessary to achieve the desired result. The effective amount of the OX-2 protein or nucleic acid of the invention may vary according to factors such as the disease state, age, sex, and weight of the animal. Dosage regima may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation.

(c) Compositions

The invention also includes pharmaceutical compositions containing OX-2 proteins or nucleic acids for use in inducing tumor cell growth as well as pharmaceutical compositions containing an OX-2 inhibitor for use in preventing tumor cell growth.

Such pharmaceutical compositions can be for intralesional, intravenous, topical, rectal, parenteral, local, inhalant or subcutaneous, intradermal, intramuscular, intrathecal, transperitoneal, oral, and intracerebral use. The composition can be in liquid, solid or semisolid form, for example pills, tablets, creams, gelatin capsules, capsules, suppositories, soft gelatin capsules, gels, membranes, tubelets, solutions or suspensions.

The pharmaceutical compositions of the invention can be intended for administration to humans or animals. Dosages to be administered depend on individual needs, on the desired effect and on the chosen route of administration.

The pharmaceutical compositions can be prepared by per se known methods for the preparation of pharmaceutically acceptable compositions which can be administered to patients, and such that an

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effective quantity of the active substance is combined in a mixture with a pharmaceutically acceptable vehicle. Suitable vehicles are described, for example, in Remington's Pharmaceutical Sciences (Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pa., USA
5 1985).

On this basis, the pharmaceutical compositions include, albeit not exclusively, the active compound or substance in association with one or more pharmaceutically acceptable vehicles or diluents, and contained in buffered solutions with a suitable pH and iso-osmotic with the physiological
10 fluids. The pharmaceutical compositions may additionally contain other agents such as immunosuppressive drugs or antibodies to enhance immune tolerance or immunostimulatory agents to enhance the immune response.

In one aspect, the pharmaceutical composition for use in inhibiting tumor cell growth comprises an effective amount of a OX-2 inhibitor
15 in admixture with a pharmaceutically acceptable diluent or carrier. Such compositions may be administered as a vaccine either alone or in combination with other active agents.

In one embodiment, the pharmaceutical composition for use in inhibiting tumor cell growth comprises an effective amount of an antibody to
20 OX-2 in admixture with a pharmaceutically acceptable diluent or carrier. The antibodies may be delivered intravenously.

In another embodiment, the pharmaceutical composition for use in inhibiting tumor cell growth comprises an effective amount of an antisense oligonucleotide nucleic acid complimentary to a nucleic acid sequence from a
25 OX-2 gene in admixture with a pharmaceutically acceptable diluent or carrier. The oligonucleotide molecules may be administered as described below for the compositions containing OX-2 nucleic acid sequences.

When used in inhibiting tumor cell growth or in treating cancer the composition can additionally contain other agents such as other immune
30 stimulants (including cytokines and adjuvants) as well as chemotherapeutic agents.

In another embodiment, the pharmaceutical composition for use in inducing tumor cell growth comprises an effective amount of a OX-2 protein

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in admixture with a pharmaceutically acceptable diluent or carrier. The OX-2 protein is preferably prepared as an immunoadhesion molecule in soluble form which can be administered to the patient.

In another embodiment, the pharmaceutical composition for use
5 in inducing tumor cell growth comprises an effective amount of a nucleic acid molecule encoding a OX-2 protein in admixture with a pharmaceutically acceptable diluent or carrier.

The nucleic acid molecules of the invention encoding a OX-2 protein may be used in gene therapy to induce inducing tumor cell growth. In
10 addition antisense oligonucleotides to OX-2 may be used in gene therapy to prevent or inhibit tumor cell growth. Recombinant molecules comprising a nucleic acid sequence encoding a OX-2 protein, or fragment thereof, may be directly introduced into cells or tissues *in vivo* using delivery vehicles such as retroviral vectors, adenoviral vectors and DNA virus vectors. They may also
15 be introduced into cells *in vivo* using physical techniques such as microinjection and electroporation or chemical methods such as coprecipitation and incorporation of DNA into liposomes. Recombinant molecules may also be delivered in the form of an aerosol or by lavage. The nucleic acid molecules of the invention may also be applied extracellularly
20 such as by direct injection into cells. The nucleic acid molecules encoding OX-2 are preferably prepared as a fusion with a nucleic acid molecule encoding an immunoglobulin (Ig) Fc region. As such, the OX-2 protein will be expressed *in vivo* as a soluble fusion protein.

The following non-limiting examples are illustrative of the
25 present invention:

EXAMPLES

EXAMPLE 1

Evidence of a role for CD200 in regulation of immune rejection of leukemic tumor cells in C56BL/6 mice

30 **MATERIALS and METHODS**

Mice: Male C3H/HeJ, BALB/c and C57BL/6 mice were purchased from the Jackson laboratories, Bar Harbour, Maine. Mice were housed 5/cage and allowed food and water ad libitum. All mice were used at 8-12 weeks of age.

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Monoclonal antibodies: The following monoclonal antibodies (mAbs) were obtained from Pharmingen (San Diego, CA, USA) unless stated otherwise: anti-IL-2 (S4B6, ATCC; biotinylated JES6-5H4); anti-IL-4 (11B11, ATCC; biotinylated BVD6-24G2); anti-IFN γ (R4-6A2, ATCC; biotinylated XMG1.2);
5 anti-IL-10 (JES5-2A5; biotinylated, SXC-1); anti-IL-6 (MP5-20F3; biotinylated MP5-32C11); anti-TNF α (G281-2626; biotinylated MP6-XT3); FITC anti-CD80, FITC anti-CD86 and FITC anti-CD40 were obtained from Cedarlane Labs, Hornby, Ontario. The hybridoma producing DEC205 (anti-mouse dendritic cells) was a kind gift from Dr. R. Steinman, and was directly labeled
10 with FITC. FITC anti-H2K^b, FITC anti-H2K^k, and anti-thy1.2 monoclonal antibodies (mAbs) were obtained from Cedarlane Labs, Hornby, Ontario. Unconjugated and PE-conjugated rat anti-mouse CD200 was obtained from BioSpark Inc., Mississauga, Ontario, Canada (28). CD200Fc was prepared in a Baculovirus expression system, using a cDNA encoding a murine IgG2aFc
15 region (a kind gift from Dr. T. Strom, Harvard, USA) which carried mutations to delete complement binding and FcR sites, as we described elsewhere (5). Rat monoclonal antibody to CD200^r was prepared from rats immunized with CHO cells transfected to express a cDNA encoding CD200^r (29). Anti-CD4 (GK1.5, rat IgG2b) and anti-CD8 (2.43, rat IgG2b) were both obtained from
20 ATCC, and used for in vivo depletion by iv infusion of 100 μ g Ig/mouse weekly. A control IgG2b antibody (R35.38), as well as streptavidin horse radish peroxidase and recombinant mouse GM-CSF, was purchased from Pharmingen (San Diego, CA).

Preparation of cells: Single cell spleen suspensions were prepared aseptically
25 and after centrifugation cells were resuspended in α -Minimal Essential Medium supplemented with 2-mercaptoethanol and 10% fetal calf serum (α F10). CD200^{r+} LPS splenic Mph, stained (>20%) with FITC-CD200Fc, were obtained by velocity sedimentation of cells cultured for 48 hrs with 1 μ g/ml LPS (13). Bone marrow cells were flushed from the femurs of donor mice,
30 washed and resuspended in α F10. Cells were depleted of mature T lymphocytes using anti-thy1.2 and rabbit complement.

C1498 (a spontaneous myeloid tumor) and EL4 (a radiation induced thymoma tumor) cells were obtained from The American Type

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Culture Collection (ATCC, Rockville, MD). Cells used for transplantation into mice were passaged weekly (5×10^6 cells/mouse) intraperitoneally in stock 8-week old C57BL/6 recipients. For experimental tumor challenge either 5×10^6 EL4 tumor cells, or 5×10^5 C1498 cells, were given intraperitoneally to groups of 6 mice (see results)-animals were sacrificed when they became moribund. EL4 cells stably transfected to express CD80 or CD86 were obtained from Dr.J.Allison, Cancer Research Labs, UC Berkeley, CA, while C1498 transfected with CD80/CD86 (cloned into pBK vectors) were produced in the author's laboratory. Tumor cells (parent and transfected) were stored at -80°C and thawed and cultured prior to use. Cells used for immunization, including the tumor cells transfected with CD80/CD86, were maintained in culture in aMEM medium supplemented with 10% FCS. Untransfected and transfected cells of each tumor line were used for immunization within 2 passages in culture. Over this time in culture transfected cells repeatedly showed stable expression (by FACS) of CD80/CD86 (>80% positive for each tumor assayed over a 6 month period with multiple vials thawed and cultured). Non-transfected tumor cells did not stain with these mAbs (<2%).

CD200^{r+} cells were obtained from lymphocyte-depleted murine spleen cells. Cells were treated with rabbit anti-mouse lymphocyte serum and complement (both obtained from Cedarlane Labs. Hornby, Ontario), cultured with LPS (10 $\mu\text{g/ml}$) for 24 hours, and separated into populations of different size by velocity sedimentation (13). Small CD200^{r+} cells stained >65% by FACS with anti-CD200^r antibody (29).

Bone marrow transplantation (BMT): C57BL/6 mice received 300mg/Kg cyclophosphamide iv 24 hrs before intravenous infusion of 20×10^6 T-depleted C3H or C57BL/6 bone marrow cells. Immediately prior to use for tumor transplantation (28 days following bone marrow engrafting), a sample of PBL (50 μl /mouse) was obtained from the tail vein of individual mice and analysed by FACS with FITC-anti-H2K^k or FITC-anti-H2K^b mAb. Cells from normal C57BL/6 or C57BL/6 reconstituted C57BL/6 mice were 100% H2K^b positive, as expected. In similar fashion, PBL from C3H mice were 100% H2K^k positive. H2K^k positive cells in the C3H-reconstituted C57BL/6 mice by FACS comprised $85\% \pm 8.5\%$ of the total cell population (mean over ~ 100 mice used

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in the studies described below). Mice in all groups were gaining weight and healthy.

Cytotoxicity and cytokine assays:

In allogeneic mixed leukocyte cultures (MLC) used to assess
 5 cytokine production or CTL, responder spleen cells were stimulated with
 equal numbers of mitomycin-C treated (45 min at 37°C) spleen stimulator
 cells in triplicate in α F10. Supernatants were pooled at 40hr from replicate
 wells and assayed in triplicate in ELISA assays for lymphokine production as
 follows, using capture and biotinylated detection mAbs as described above.
 10 Varying volumes of supernatant were bound in triplicate at 4°C to plates pre-
 coated with 100ng/ml mAb, washed x3, and biotinylated detection antibody
 added. After washing, plates were incubated with strepavidin-horse radish
 peroxidase (Cedarlane Labs), developed with appropriate substrate and
 OD₄₀₅ determined using an ELISA plate reader. Recombinant cytokines for
 15 standardization were obtained from Pharmingen (U.S.A.). All assays showed
 sensitivity in the range 40 to 4000 pg/ml. CTL assays were performed at 5
 days using cells harvested from the same cultures (as used for cytokine
 assays). Various effector:target ratios were used in 4hr ⁵¹Cr release tests with
 72 hr ConA activated spleen cell blasts of stimulator genotype.

20 Quantitation of CD200 mRNA by PCR:

RNA extraction from spleen tissue of tumor injected mice was
 performed using Trizol™ reagent. The OD280/260 of each sample was
 measured and reverse transcription performed using oligo (dT) primers (27-
 7858: Pharmacia, USA). cDNA was diluted to a total volume of 100μl with
 25 water and frozen at -70°C until use in PCR reactions with primers for mouse
 CD200 and GAPDH (3). Different amounts of standard cDNA from 24hr
 cultures of LPS stimulated peritoneal macrophages (known to express CD200
 and GAPDH) were amplified in six serial 1:10 dilutions for 30 cycles by PCR,
 in the presence of a tracer amount of ³²P. Samples were analysed in 12.5%
 30 polyacrylamide gels, the amplicons cut from the gel, and radioactivity
 measured in a β-counter. A standard curve was drawn for each set of primer
 pairs (amplicons). cDNAs from the various experimental groups were assayed
 in similar reactions using 0.1μl cDNA, and all groups were normalized to

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equivalent amounts of GAPDH. CD200 cDNA levels in the different experimental groups were then expressed relative to the cDNA standard (giving a detectable ^{32}P signal over five $\log_{10}$ dilutions). Thus a value of 5 (serial dilutions) indicates a test sample with approximately the same cDNA content as the standard, while a value of 0 indicates a test sample giving no detectable signal in an undiluted form ($<1/10^5$ the cDNA concentration of the standard).

RESULTS

Growth of EL4 or C1498 tumor cells in C57BL/6 mice, and in allogeneic (C3H)

10 BMT mice:

Groups of 6 C57BL/6 mice received iv infusion of 300mg/Kg cyclophosphamide (in 0.5 ml PBS). A control group received PBS only, as did a control group of 6 C3H mice. 24 hrs later cyclophosphamide treated C57BL/6 mice received iv injection of 20×10^6 T-depleted bone marrow cells pooled from C57BL/6 mice (syngeneic transplant), or C3H mice (allogeneic transplantation). All groups of animals received intraperitoneal injection (in 0.5 ml PBS) of 5×10^6 EL4 or 5×10^5 C1498 tumor cells (see Figure 1) 28 days later. Animals were monitored daily post tumor inoculation.

Data in Figure 1 (one of 2 such studies) show clearly that while C3H mice rejected both EL4 and C1498 (allogeneic) leukemia cell growth, 100% mortality was seen within 9-12 days in normal C57BL/6 mice, or in syngeneic bone marrow reconstituted mice. Interestingly, despite the absence of overt GVHD (as defined by weight loss and overall health), two-thirds of C3H reconstituted C57BL/6 mice rejected EL4 tumor cells, reflecting the existence of a graft versus leukemia effect (GVL) (panel a of Figure 1), and there was a marked delay of death for mice inoculated with C1498 leukemia cells (panel b of Figure). In separate studies similar findings were made using tumor inocula (for EL4/C1498 respectively) ranging from 2×10^6 - 10×10^6 , or 1.5×10^5 - 10×10^5 (RMG-unpublished).

30 Immunization of normal C57BL/6 mice for protection against EL4 tumor growth:

Blazar and co-workers reported immunization for protection from tumor growth in C57BL/6 mice using tumor cells transfected to over-express

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mouse CD80 (Blazar et al. 1997). Using CD80 and CD86 transfected EL4 cells obtained from this same group, or C1498 cells transfected with CD80/CD86 in our laboratory, we immunized groups of 6 C57BL/6 mice ip with Complete Freund's adjuvant (CFA) alone, or with CFA mixed with 5×10^6 mitomycin-C treated tumor cells, or CD80/CD86 transfected tumor. Animals received 2 injections at 14 day intervals. 10 days after the last immunization all mice received 5×10^6 EL4 tumor cells, or 5×10^5 C1498 cells, and mortality followed. Data are shown in Figure 2 (1 of 2 studies), for EL4 only.

In agreement with a number of other reports, mice pre-immunized with CD80-transfected EL4 survive significantly longer after challenge with viable EL4 tumor cells than non-immunized animals, or those immunized with non-transfected cells or CD86 transfected cells ($p < 0.05$)-see also Figure 5. Similar data were obtained using CD80-transfected C1498 cells (RMG-unpublished). In separate studies (not shown) mice immunized with tumor cells in the absence of Freund's Adjuvant failed to show any protection from tumor growth. However, equivalent protection (to that seen using Freund's Adjuvant) was also seen using concomitant immunization with poly(I:C) (100 μ g/mouse) as adjuvant (data not shown).

Role of CD4⁺ and/or CD8⁺ cells in modulation of tumor growth after BMT:

In order to investigate the effector cells responsible for leukemia growth-inhibition in mice transplanted with allogeneic bone marrow (see Figure 1), BMT recipients received weekly injections of 100 μ g/mouse anti-CD4 (GK1.5) or anti-CD8 (2.43) mAb, followed at 28 days by leukemia cell injection as described for Figure 1. Depletion of CD4 and CD8 cells in all mice with these treatments was >98% as defined by FACS analysis (not shown).

As shown in Figure 3 (data from one of 3 studies), and in agreement with data reported elsewhere (27), in this BMT model tumor growth inhibition for EL4 cells is predominantly a function of CD8 rather than CD4 cells, while for C1498 leukemia cells growth inhibition was equally, but not completely, inhibited by infusion of either anti-CD4 or anti-CD8 mAb (see pane b of Figure).

Evidence that tumor rejection in BMT mice is regulated by CD200:

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Previous studies in rodent transplant models have implicated expression of a novel molecule, CD200, in the regulation of an immune rejection response. Specifically, blocking functional expression of CD200 by a monoclonal antibody to murine CD200 prevented the increased graft survival which followed donor-specific pretransplant immunization, while a soluble form of CD200 linked to murine IgG Fc (CD200Fc) was a potent immunosuppressant (3,5). In order to investigate whether expression of CD200 was involved in regulation of tumor immunity, we studied first the effect of infusion of CD200Fc on suppression of resistance to growth of EL4 or C1498 tumor in BMT mice as described in Figure 1, and second the effect of CD200Fc infusion in mice immunized with CD80-transfected tumor cells as described in Figure 2. Note this CD200Fc lacks binding sites for mouse complement and FcR (see Materials and Methods, and (5)). In all cases control groups of mice received infusion of equivalent amounts of pooled normal mouse IgG. Data for these studies is shown in Figures 4 and 5 respectively (data from one of 2 studies in each case).

It is clear that suppression of growth of either EL4 or C1498 tumor cells in BMT mice is inhibited by infusion of CD200Fc, but not by pooled normal mouse IgG (Figure 4). CD200Fc also caused increased mortality in EL4 or C1498 injected normal C3H mice. Data in Figure 5 show that resistance to EL4 tumor growth in EL4-CD80 immunized mice (as documented in Figure 2) is also inhibited by infusion of CD200Fc. In separate studies (not shown) a similar inhibition of immunity induced by CD80 transfected C1498 was demonstrated using CD200Fc.

Effect of anti-CD200 mAb on resistance to tumor growth in mice immunized with CD80/CD86 transfected tumor cells:

As further evidence for a role for CD200 expression in tumor immunity in EL4-CD80/EL4-CD86, or C1498-CD80/C1498-CD86 immunized mice we examined the effect of infusion of an anti-CD200 mAb on EL4 or C1498 tumor growth in this model. Infusion of anti-CD200 into mice preimmunized with EL4-CD86, or C1498-CD86 uncovered evidence for resistance to tumor growth. Separate studies (not shown) revealed that anti-CD200 produced no significant perturbation of EL4 growth in the EL4 or EL4-

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CD80 immunized mice, or of C1498 growth in C1498 or C1498-CD86 immunized mice. We interpreted these data to suggest that immunization with EL4-CD86 or C1498-CD86 elicited an antagonism of tumor immunity resulting from increased expression of CD200. Thus blocking the functional increase of CD200 expression with anti-CD200 reversed the inhibitory effect. Note that in studies not shown we have reproduced these same effects of anti-CD200 (in mice immunized with CD86-transfected tumor cells) with F(ab')₂ anti-CD200 (RMG-unpublished).

To confirm that indeed immunization with CD86-transfected tumor cells was associated with increased expression of CD200, we repeated the study shown in Figure 6, and sacrificed 3 mice/group at 4 days following EL4 tumor injection. RNA was isolated from the spleen of all mice, and assayed by quantitative PCR for expression of CD200 (using GAPDH as "housekeeping" mRNA control). Data for this study are shown in Figure 7, and show convincingly that CD200 mRNA expression was >5-fold increased following preimmunization with EL4-CD86, a condition associated with increased tumor growth compared with EL4-immunized mice (Figures 2 and 6). In dual-staining FACS studies (not shown), with PE-anti-CD200 and FITC-DEC205, the predominant CD200⁺ population seen in control and immunized mice were DEC205⁺ (>80%)-see also (6). Similar results were obtained using C1498 tumor cells (data not shown).

Given this increase in CD200 expression following preimmunization with CD86-transfected cells, and the evidence that CD200 is associated with delivery of an immunosuppressive signal to antigen encountered at the same time, we also examined the response of spleen cells taken from these C57BL/6 mice to allostimulation (with mitomycin-C treated BALB/c spleen cells), in the presence/absence of anti-CD200 mAb. Data from one of 3 studies are shown in Table 1. Interestingly, mice preimmunized with EL4-CD86 cells show a decreased ability to generate CTL on alloimmunization with third-party antigen (BALB/c), and decreased type-1 cytokine production (IL-2, IFN γ), with some trend to increased type-2 cytokines (IL-4 and IL-10). These effects were reversed by inclusion of anti-CD200 in culture, consistent with the hypothesis that they result from

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increased delivery of an immunosuppressive signal via CD200 in spleen cells obtained from these animals (3).

Evidence for an interaction between CD200 and CD200^{r+} cells in inhibition of EL4 tumor growth:

5 Inhibition resulting from infusion of CD200Fc into mice follows an interaction with immunosuppressive CD200^{r+} cells (13). At least one identifiable functionally active population of suppressive CD200^{r+} cells was described as a small, F4/80⁺ cell in a pool of splenic cells following LPS stimulation (13)-F4/80 is a known cell surface marker for tissue macrophages.

10 In a further study we investigated whether signaling induced by CD200:CD200^r interaction (where CD200^{r+} cells were from lymphocyte-depleted, LPS stimulated, spleen cells) was behind the suppression of tumor immunity seen following CD200Fc injection. All groups of 6 recipient mice received tumor cells ip. In addition to infusion of CD200Fc as

15 immunosuppressant (in Figure 8), one group of C3H reconstituted animals received CD200^{r+} cells (Δ in Figure 8: >65% of these cells stained with an anti-CD200^r mAb), while a final group received a mixture of both CD200Fc and CD200^{r+} cells ($\blacktriangle$). It is clear from the Figure that it is this final group, in which interaction between CD200 and CD200^r is possible, which showed

20 maximum inhibition of tumor immunity compared with the C3H reconstituted control mice ($\bullet$).

Role of CD4⁺ and/or CD8⁺ cells in CD200 regulated modulation of tumor growth after BMT:

 Data in Figure 3 above, and elsewhere (27) show that tumor

25 growth inhibition for EL4 cells is predominantly a function of CD8 rather than CD4 cells, while for C1498 leukemia cells growth inhibition was equally, but not completely, inhibited by infusion of either anti-CD4 or anti-CD8 mAb. To investigate the role of CD200 in the protection mediated by different T cell subclasses, the following additional studies were performed. In the first,

30 C57BL/6 recipients of C3H BMT received (at 28 days post BMT) inoculations of EL4 or C1498 tumor cells along with anti-CD4, anti-CD8 or CD200Fc alone, or combinations of (anti-CD4+CD200Fc) or (anti-CD8+CD200Fc). Survival was followed as before (see Figure 9-one of 2 studies). In a second

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study (Figure 10-data from one of 2 such experiments) a similar treatment regimen of mAbs or CD200Fc alone or in combination was used to modify growth of EL4 tumor cells in mice preimmunized with EL4-CD80 cells as described earlier in Figure 2.

5 Data in panel a of Figure 9 confirm the effects previously documented in Figures 3 and 4, that CD200Fc and anti-CD8 each significantly impaired the growth inhibition in BMT recipients of EL4 cells, while anti-CD4 mAb was less effective. Combinations of CD200Fc and either anti-T cell mAb led to even more pronounced inhibition of tumor immunity in
10 the BMT recipients, to levels seen with non-allogeneic transplanted mice. Data with C1498 tumor cells (panel b) were somewhat analogous, though as in Figure 3, anti-CD4 alone produced equivalent suppression of growth inhibition to anti-CD8 with this tumor. As was the case for the EL4 tumor, combinations of CD200Fc and either anti-T cell mAb caused essentially
15 complete suppression of C1498 tumor growth inhibition. Both sets of data, from panels a and b, are consistent with the notion that CD200Fc blocks (residual) growth-inhibitory functional activity in both CD4 and CD8 cells, thus further inhibiting tumor immunity remaining after depletion of T cell subsets.

Data in Figure 10, using EL4 immunized BL/6 mice, also
20 showed combinations of CD200Fc and anti-CD4 treatment produced optimal suppression of tumor immunity to EL4 cells, consistent with an effect of CD200Fc on CD8⁺ cells. Anti-CD8 alone abolished tumor immunity in these studies, so any potential additional effects of CD200Fc on CD4⁺ cells could not be evaluated.

25 **DISCUSSION**

In the studies described above, the inventors have studied whether expression of the molecule CD200, previously reported to down-regulate rejection of tissue/organ allografts in rodents (3), was implicated in immunity to tumor cells in syngeneic hosts. Two model systems were used.
30 The one, in which tumor cells are injected into mice which had received an allogeneic bone marrow transplant following cyclophosphamide pre-conditioning, has been favoured as a model for studying potential innovative treatments of leukemia/lymphoma in man (27, 30-32). In the other EL4 or

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C1498 tumor cells were infused into BL/6 mice which had been preimmunized with tumor cells transfected to overexpress the costimulatory molecules CD80 or CD86. These studies were stimulated by the growing interest in such therapy for immunization of human tumor patients with autologous transfected
5 tumor cells (11, 23-26).

In both sets of models we found evidence for inhibition of tumor growth (Figures 1 and 2) which could be further modified by treatment designed to regulate expression of CD200. Infusion of CD200Fc suppressed tumor immunity (led to increased tumor growth, and faster mortality) in both
10 models (Figures 4 and 5), while anti-CD200 improved tumor immunity in mice immunized with CD86-transfected EL4 or C1498 tumor cells (Figure 6). The inventors interpreted this latter finding as suggesting that the failure to control tumor growth following immunization with EL4-CD86 or C1498-CD86 was associated with overexpression of endogenous CD200, a hypothesis which
15 was confirmed by quantitative PCR analysis of tissue taken from such mice (Figure 7). CD200 was predominantly expressed on DEC205⁺ cells in the spleen of these mice (see text), which was associated with a decreased ability of these spleen cell populations to respond to allostimulation in vitro (see Table 1). Non antigen-specific inhibition following CD200 expression formed
20 the basis of our previous reports that a soluble form of CD200 (CD200Fc) was a potent immunosuppressant (3). Consistent with the hypothesis that increased expression of CD200 in mice immunized with CD86-transfected tumor cells was responsible for the inhibition of alloreactivity seen in Table 1, suppression was abolished by addition of anti-CD200 mAb (see lower half of
25 Table 1). Earlier reports have already documented an immunosuppressive effect of CD200Fc on alloimmune responses (5), production of antibody in mice following immunization with sheep erythrocytes (5).

Maximum inhibition of tumor immunity was achieved by concomitant infusion of CD200Fc and CD200r⁺ cells (F4/80⁺ macrophages-
30 see Figure 8). The inventors next investigated the cell type responsible for tumor growth inhibition whose activity was regulated by CD200:CD200^r interactions. The data confirmed previous reports that EL4 growth inhibition was predominantly associated with CD8 immune cells, while immunity to

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C1498 was a function of both CD4⁺ and CD8⁺ cells (27). For both tumors in BMT models suppression of tumor growth inhibition was maximal following combined treatment with CD200Fc and either anti-T cell mAb, consistent with the idea that CD200 suppression acts on both CD4⁺ and CD8⁺ T cells.

5 A number of studies have examined immunity to EL4 or C1498 tumor cells in similar models to those described above (27, 33), concluding that CD8⁺ cells are important in (syngeneic) immunity to each tumor, and CD4⁺ T cells are also important in immunity to C1498 (27). Evidence to date suggests that NK cell mediated killing is not relevant to tumor growth inhibition
10 in BMT mice of the type used above (27). Other reports have addressed the issue of the relative efficiency of induction of tumor immunity in a number of models following transfection with CD80 or CD86, and also concluded that CD80 may be superior in induction of anti-tumor immunity (27, 34), while CD86 may lead to preferential induction of type-2 cytokines (8). This is of
15 interest given the cytokine production profile seen in EL4-CD86 immunized mice (Table 1), which is similar to the profile seen following CD200Fc treatment of allografted mice (3). EL4-CD86 immunized mice show increased expression of CD200 (Figure 7), with no evidence for increased resistance to tumor growth (Figure 2). Resistance is seen in these mice following treatment
20 with anti-CD200 (Figure 6A). Somewhat better protection from tumor growth is seen using viable tumor cells for immunization, rather than mitomycin-C treated cells as above (27). Whether this would improve the degree of protection from tumor growth in our model, and/or significantly alter the role of CD200:CD200^r interactions in its regulation, remains to be seen.

25 There are few studies exploring the manner in which suppression mediated by CD200:CD200^r interactions occurs. In a recent study in CD200 KO mice Hoek et al observed a profound increase in the presence of activated macrophages and/or macrophage-like cells (14), and we and others had previously found that CD200^r was expressed on
30 macrophages (13,35). The inventors also reported that CD200^r was present on a subpopulation of T cells, including the majority of activated $\gamma\delta$ TCR⁺ cells (13). $\gamma\delta$ TCR⁺ cells may mediate their suppressive function via cytokine production (36), while unpublished data (RMG-in preparation) suggests that

- 30 -

the CD200^{r+} macrophage cell population may exert its activity via mechanisms involving the indoleamine 2,3-dioxygenase (IDO) tryptophan catabolism pathway (37). We suggest that the mechanism by which CD200Fc leads to suppression of tumor growth inhibition in the models described is likely to be a function both of the tumor effector cell population involved (Figures 9, 10) as well as the CD200^r cell population implicated in suppression.

In a limited series of studies (not shown) we have used other BMT combinations (B10 congenic mice repopulated with B10.D2, B10.BR or B10.A bone marrow) to show a similar resistance to growth of EL4 or C1498 tumor cells, which is abolished by infusion of CD200Fc. Studies are in progress to examine whether DBA/2 or BALB/c mice can be immunized to resist growth of P815 syngeneic (H2^d) tumor cells by P815 cells transfected with CD80/CD86, and whether this too can be abolished by CD200Fc. Taken together, however, our data are consistent with the hypothesis that the immunomodulation following CD200:CD200^r interactions, described initially in a murine allograft model system, is important also in rodent models of tumor immunity. This has important clinical implications.

EXAMPLE 2

20 TRIM IN THE FSL SARCOMA LUNG METASTASIS MODEL: CUES FROM PREGNANCY

Allogeneic leukocyte-induced transfusion-related immunomodulation (TRIM) has been shown to enhance tumor growth (38, 39). OX-2 is expressed on a variety of cells in transfused blood (i.e. a subpopulation of dendritic cells and possibly B cells) (40, 41), thus the effect of anti-OX-2 on the TRIM enhancement of FSL10 lung nodules was examined.

MATERIALS and METHODS

Enhancement of lung nodules by TRIM

30 A dose response curve demonstrated a plateau in the TRIM enhancement of lung metastases with 50, 100 or 200 µl of BALB/c heparinized blood given 4 days after tail vein injection of the cultured tumor cell by tail vein (see Figure 11). A dose of 200 µl of BALB/c heparinized blood

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(about 15-20% of blood volume) was given 4 days after tail vein injection of the cultured tumor cells as a physiologically suitable model in which to screen for treatments that have a major abrogating effect on TRIM.

All animals were monitored for signs of illness daily, and 21 days after tumor inoculation, the mice were sacrificed, the lungs were removed and fixed in Bouin's solution, and the number of surface nodules was counted. To deal with variation in number of metastases between mice, 20-25 mice per group were used and medians were calculated (using log-transformed data, where 0 nodules was set at 0.1 for that animal). It was then possible to assess the significance of differences in log mean $\pm$ sem with respect to our *a priori* hypotheses using Student's t test, and to construct 95 % confidence intervals for the medians. Differences in the proportion of mice in different groups with no visible metastases was assessed by the χ^2 statistic, or by Fisher's Exact test where appropriate.

Figure 11A shows the median number of lung nodules in C57Bl/6J mice receiving the indicated dose of freshly-prepared allogeneic BALB/c strain blood by tail vein. The effect is seen if the blood is given 7 days prior to, or 4 days after a tail vein injection of 1×10^6 FSL sarcoma cells. FSL10 is a methylnanthrene-induced fibrosarcoma generated in C57Bl/6 mice and maintained by standard tissue culture *in vitro*. Such cells are weakly antigenic. Group size is 20-25 per group, and P values showing increased numbers to lung nodules are on the figure. Figure 11B shows proportion of mice with no tumor nodules. P values were determined by Student's t test for A, and by Chi-square or Fisher's Exact test for B.

25 The Role of Dendritic Cells

MAb to a myeloid DC/APC surface marker (5 μ g anti-CD11c) or lymphoid dendritic cells (DC) (5 μ g supernatant of DEC205 hybridoma, an amount shown to be sufficient using *in vitro* assays of DC function (42) was added to 200 μ l of BALB/c blood or to PBS. The TRIM enhancement of tumor growth was analyzed using the same method as above.

RESULTS

Enhancement of lung nodules by TRIM

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Figure 12A represents the effect of adding anti-OX-2 monoclonal antibody (3B6, 1 ug per million leukocytes) to the blood (or PBS control) before tumor cell transfer. A control is the same amount of 3B6 in PBS. The total dose was 3.3 ug per mouse. Figure 12A shows this amount of
5 anti-CD200 in PBS had no effect, whereas when added to blood, the stimulation of tumor nodule number was prevented, - indeed, it was reduced below control levels. Figure 12B shows % with no lung nodules. In this and subsequent studies, 2×10^6 FSL cells were used, and the blood was always given 4 days after this.

10 As illustrated in Figure 12, the enhancement of lung nodules in mice given 2×10^5 sarcoma cells by 200 μ l of BALB/c blood compared to phosphate buffered saline control (PBS) given 4 days after tumor injection, was completely blocked by adding 3.3 μ g of anti-OX-2 (3B6 monoclonal antibody (mAb)) to the blood before the transfusion. The mAb in PBS had no
15 effect. Interestingly, the proportion of mice with lung metastases was boosted by allogeneic blood compared to PBS but was reduced by blood to which anti-OX-2 had been added. The median number of nodules was greater in this study in part because we had doubled the tumor cell inoculum, but we do see experiment-to-experiment variation in the number of nodules in the
20 control group which has been important in executing large experiments, as will be discussed.

The Role of Dendritic Cells

Figure 13 is a repetition of the experiment in Figure 12A which confirms the effect of anti-OX-2, but with addition of antibodies to dendritic
25 cells. Anti-CD11c was used for myeloid dendritic cells, and DEC205 for lymphoid dendritic cells. The latter are usually CD8-positive. It can be readily appreciated that monoclonal antibody to lymphoid dendritic cells had no effect on the stimulation of lung metastases, whereas anti-CD11c blocked the effect. The reason the number of nodules is not below control is thought to be due to
30 the existence of OX-2-positive and OX-2-negative CD11c-type dendritic cells. The latter stimulate immunity, and this is seen when anti-OX-2 is used to block one of the subsets. Anti-CD11c leads to loss of both subsets.

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The result shows that anti-CD11c, but not DEC205, abrogated the TRIM effect (* indicates significant increase over control, ** indicates significant abrogation of TRIM, *** indicates significant decrease below control, $P < 0.05$). There was no effect of anti-OX-2, DEC205 or anti-CD11c in
5 PBS injected as a control (data not shown). Due to the large number of treatment groups in this experiment, it could not be done in a single day. Therefore, 5 mice in each group were treated in 4 experiments and the data was examined and pooled. The result therefore compensates for any effect of day-to-day variation in tumor cells, mice or blood used for transfusion.

10 While the present invention has been described with reference to what are presently considered to be the preferred examples, it is to be understood that the invention is not limited to the disclosed examples.

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TABLE 1

Preimmunization of mice with EL4-CD86 causes increased CD200 expression which leads to generalized suppression to newly encountered alloantigen

5

	Tumor used for Immunization ^a	% Lysis ^b	Cytokines in Supernatant ^c			
			IL-2	IL-4	IFN γ	IL-10
	NONE (control)	43 $\pm$ 5.5	980 $\pm$ 125	50 $\pm$ 10	455 $\pm$ 65	35 $\pm$ 10
	EL4	41 $\pm$ 6.2	890 $\pm$ 135	60 $\pm$ 15	515 $\pm$ 70	30 $\pm$ 10
10	EL4-CD80	46 $\pm$ 6.3	955 $\pm$ 140	45 $\pm$ 15	525 $\pm$ 55	30 $\pm$ 10
	EL4-CD86	16 $\pm$ 4.2*	420 $\pm$ 75*	125 $\pm$ 20*	240 $\pm$ 40*	120 $\pm$ 20*
	NONE (control) +	46 $\pm$ 5.8	950 $\pm$ 105	55 $\pm$ 15	490 $\pm$ 60	40 $\pm$ 10
	EL4 +	44 $\pm$ 4.9	940 $\pm$ 115	50 $\pm$ 20	530 $\pm$ 60	35 $\pm$ 10
15	EL4-CD80 +	44 $\pm$ 6.0	905 $\pm$ 120	60 $\pm$ 15	555 $\pm$ 75	35 $\pm$ 10
	EL4-CD86 +	39 $\pm$ 4.29	870 $\pm$ 125	75 $\pm$ 20	540 $\pm$ 65	40 $\pm$ 15

Footnotes:

- a. Spleen cells were pooled from 3 C57BL/6 mice/group, pretreated as described in the text, by immunization with 5×10^6 mitomycin-C treated EL4 tumor cells, or CD80/CD86-transfected tumor cells, in Complete Freund's Adjuvant 4 days earlier. 5×10^6 spleen cells were incubated in triplicate with equal numbers of mitomycin-C treated BALB/c spleen stimulator cells. + indicates anti-CD200 (anti-OX2) added to cultures (5 μ g/ml)
- b. % specific lysis in 4-hr ^{51} Cr release assays with 72-hr cultured BALB/c spleen Con A blast cells (effector:target ratio shown is 100:1).
- c. Cytokines in culture supernatants assayed in triplicate by ELISA at 40 hrs (see Materials and Methods). Data represent pg/ml except for IL-10 (ng/ml). *p<0.05 compared with all groups.

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cagaaaaaga aagctgtaag cccagaaaac atggtcacct tcagcgagaa ccatgggggtg 240
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accatcacct tctggaatat caccctggag gatgaagggt gttacatgtg tctcttcaat 360
acctttgggt ttgggaagat ctcaggaacg gcctgcctca ccgtctatgt acagcccata 420

- 45 -

```

gtatcccttc actacaaatt ctctgaagac cacctaaata tcacttgctc tgccactgcc      480
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actctgtctc acccaaattgg gaccacgtct gttaccagca tcctccatat caaagaccct      600
aagaatcagg tggggaagga ggtgatctgc caggtgctgc acctggggac tgtgaccgac      660
ttaagcaaaa ccgtcaacaa aggatattgg ttttcagttc cgctattgct aagcattggt      720
tccttggtaa ttctttctcat cctaactctca atcttactgt actggaaacg tcaccggaat      780
caggaccgag gtgaattgtc acagggagtt caaaaaatga cataa                        825

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<210> 6

<211> 274

<212> PRT

<213> Homo sapiens

<400> 6

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Val Ile Arg Met Pro Phe Ser His Leu Ser Thr Tyr Ser Leu Val Trp
1              5              10              15

Val Met Ala Ala Val Val Leu Cys Thr Ala Gln Val Gln Val Val Thr
                20              25              30

Gln Asp Glu Arg Glu Gln Leu Tyr Thr Thr Ala Ser Leu Lys Cys Ser
          35              40              45

Leu Gln Asn Ala Gln Glu Ala Leu Ile Val Thr Trp Gln Lys Lys Lys
      50              55              60

Ala Val Ser Pro Glu Asn Met Val Thr Phe Ser Glu Asn His Gly Val
65              70              75              80

Val Ile Gln Pro Ala Tyr Lys Asp Lys Ile Asn Ile Thr Gln Leu Gly
          85              90              95

Leu Gln Asn Ser Thr Ile Thr Phe Trp Asn Ile Thr Leu Glu Asp Glu
          100             105             110

Gly Cys Tyr Met Cys Leu Phe Asn Thr Phe Gly Phe Gly Lys Ile Ser
          115             120             125

Gly Thr Ala Cys Leu Thr Val Tyr Val Gln Pro Ile Val Ser Leu His
          130             135             140

```

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Tyr	Lys	Phe	Ser	Glu	Asp	His	Leu	Asn	Ile	Thr	Cys	Ser	Ala	Thr	Ala
145					150					155					160

Arg	Pro	Ala	Pro	Met	Val	Phe	Trp	Lys	Val	Pro	Arg	Ser	Gly	Ile	Glu
				165					170					175	

Asn	Ser	Thr	Val	Thr	Leu	Ser	His	Pro	Asn	Gly	Thr	Thr	Ser	Val	Thr
			180					185					190		

Ser	Ile	Leu	His	Ile	Lys	Asp	Pro	Lys	Asn	Gln	Val	Gly	Lys	Glu	Val
		195					200					205			

Ile	Cys	Gln	Val	Leu	His	Leu	Gly	Thr	Val	Thr	Asp	Phe	Lys	Gln	Thr
	210					215					220				

Val	Asn	Lys	Gly	Tyr	Trp	Phe	Ser	Val	Pro	Leu	Leu	Leu	Ser	Ile	Val
225					230					235					240

Ser	Leu	Val	Ile	Leu	Leu	Val	Leu	Ile	Ser	Ile	Leu	Leu	Tyr	Trp	Lys
				245					250					255	

Arg	His	Arg	Asn	Gln	Asp	Arg	Gly	Glu	Leu	Ser	Gln	Gly	Val	Gln	Lys
			260					265					270		

Met	Thr
-----	-----

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We Claim:

1. A use of an effective amount of an agent that inhibits a CD200
5 protein to prevent, inhibit or reduce leukemia cell growth, wherein the agent is
an antibody that specifically binds the CD200 protein or an antisense
oligonucleotide that is complementary to a nucleic acid sequence encoding
the CD200 protein.
- 10 2. The use according to claim 1 wherein the agent is an antibody.
3. The use according to claim 1 wherein the agent is an antisense
oligonucleotide that is complementary to a nucleic acid sequence encoding
the CD200 protein.

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Survival post EL4 or C1498 tumor inoculation in C57BL/6 bone marrow transplant recipients

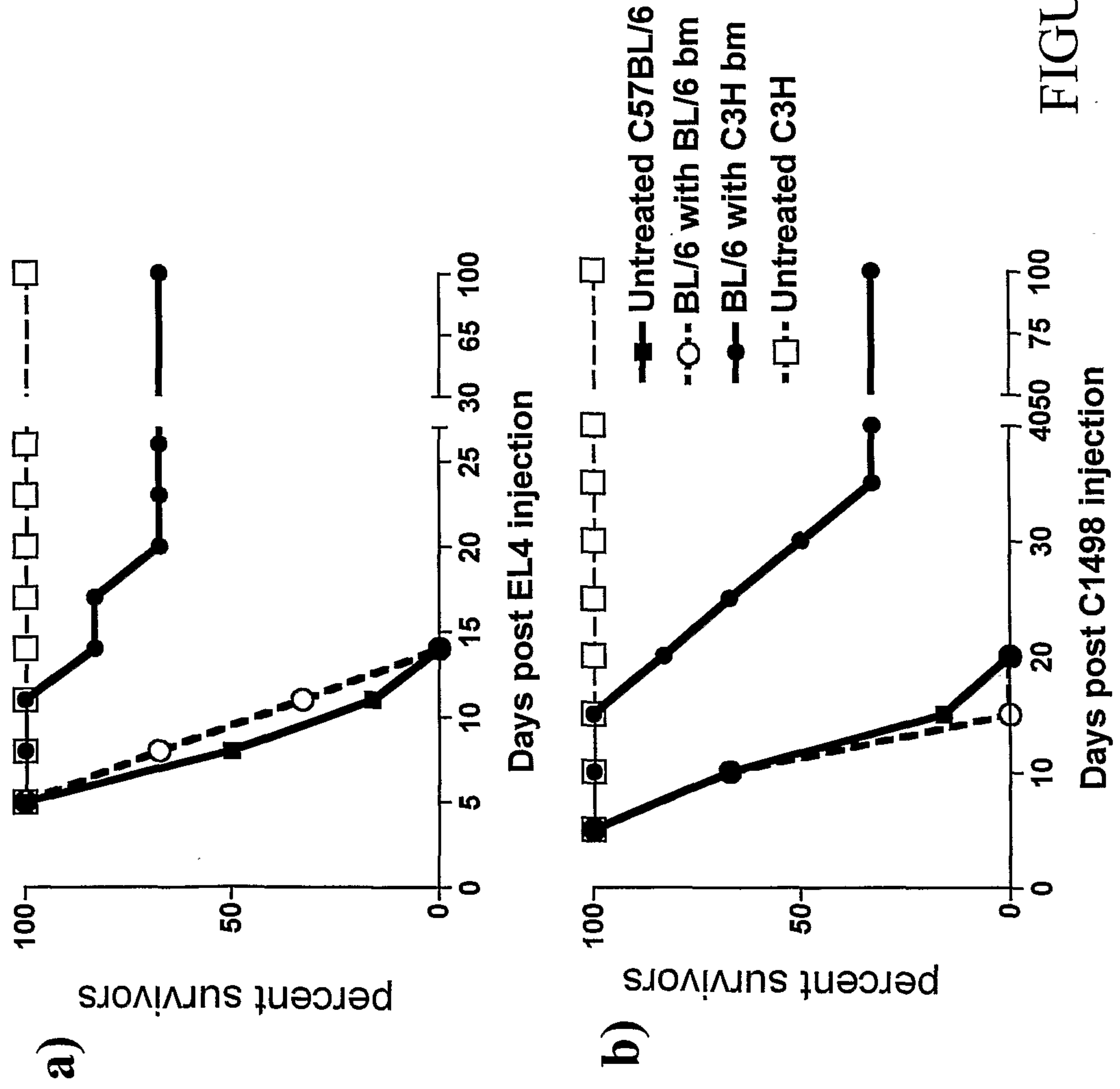


FIGURE 1

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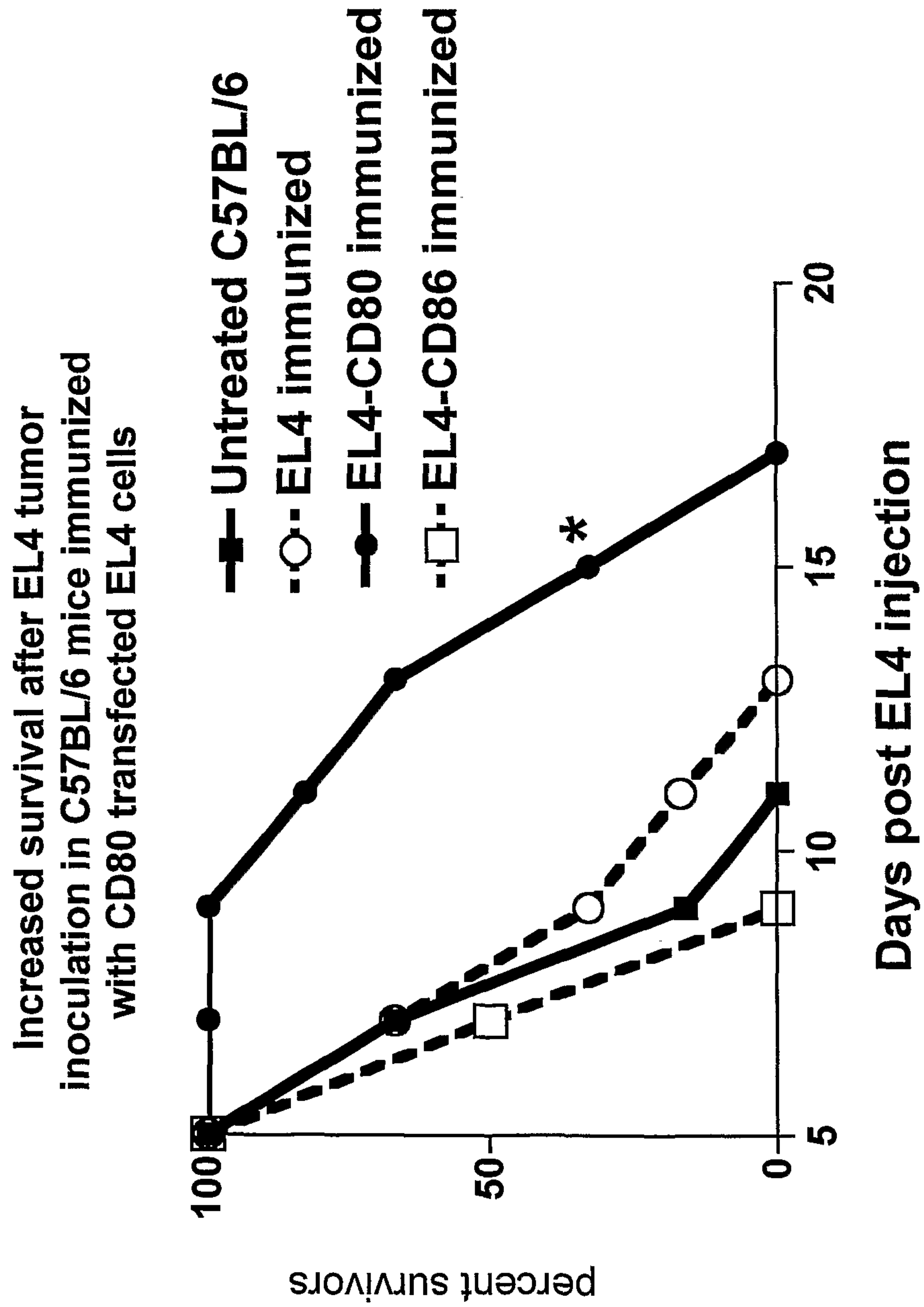


FIGURE 2

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Role of CD4⁺/CD8⁺ cells in tumor immunity in bone marrow transplant recipients

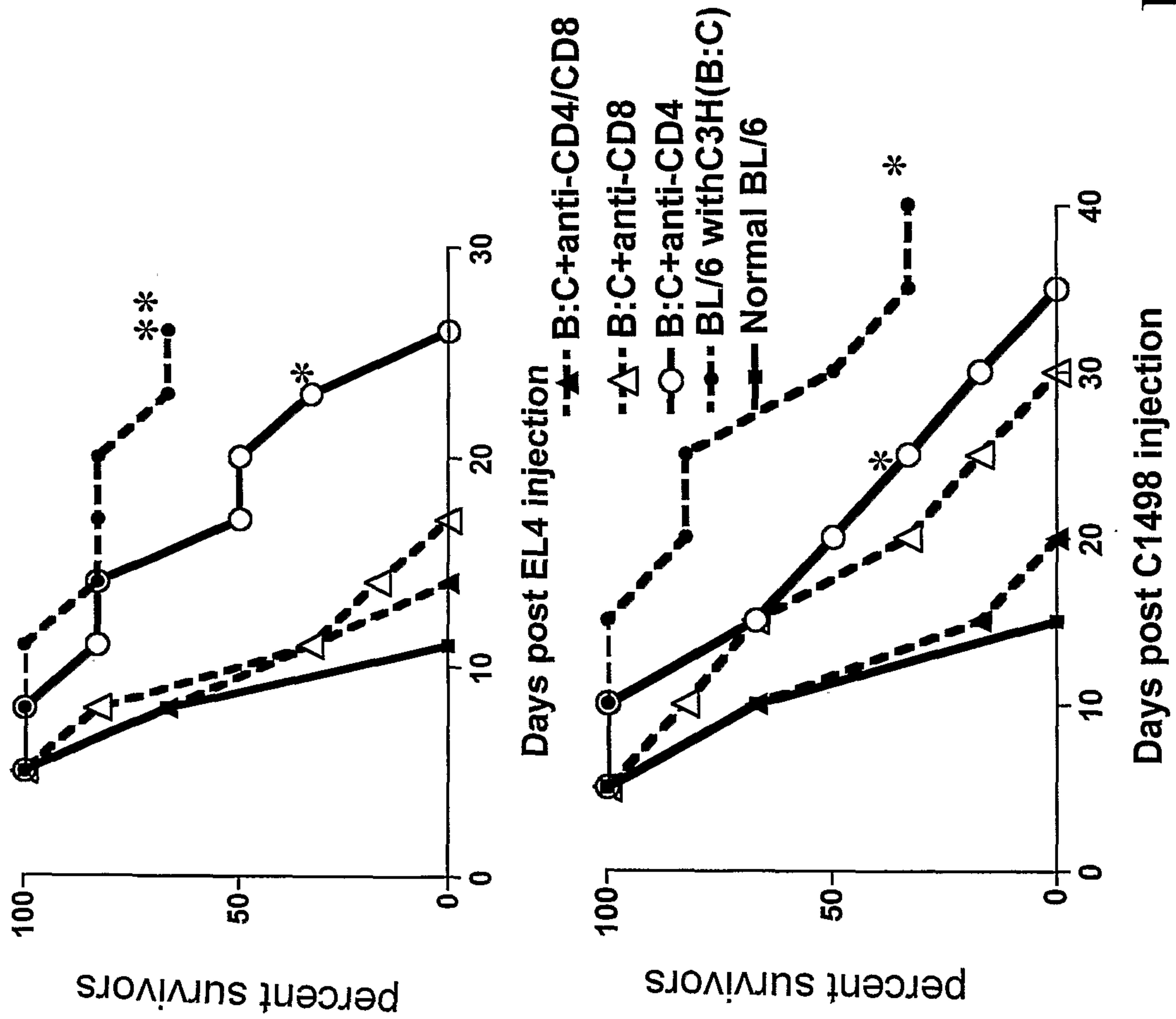


FIGURE 3

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CD200Fc reverses tumor protection in C3H recipients of C57BL/6 bone marrow

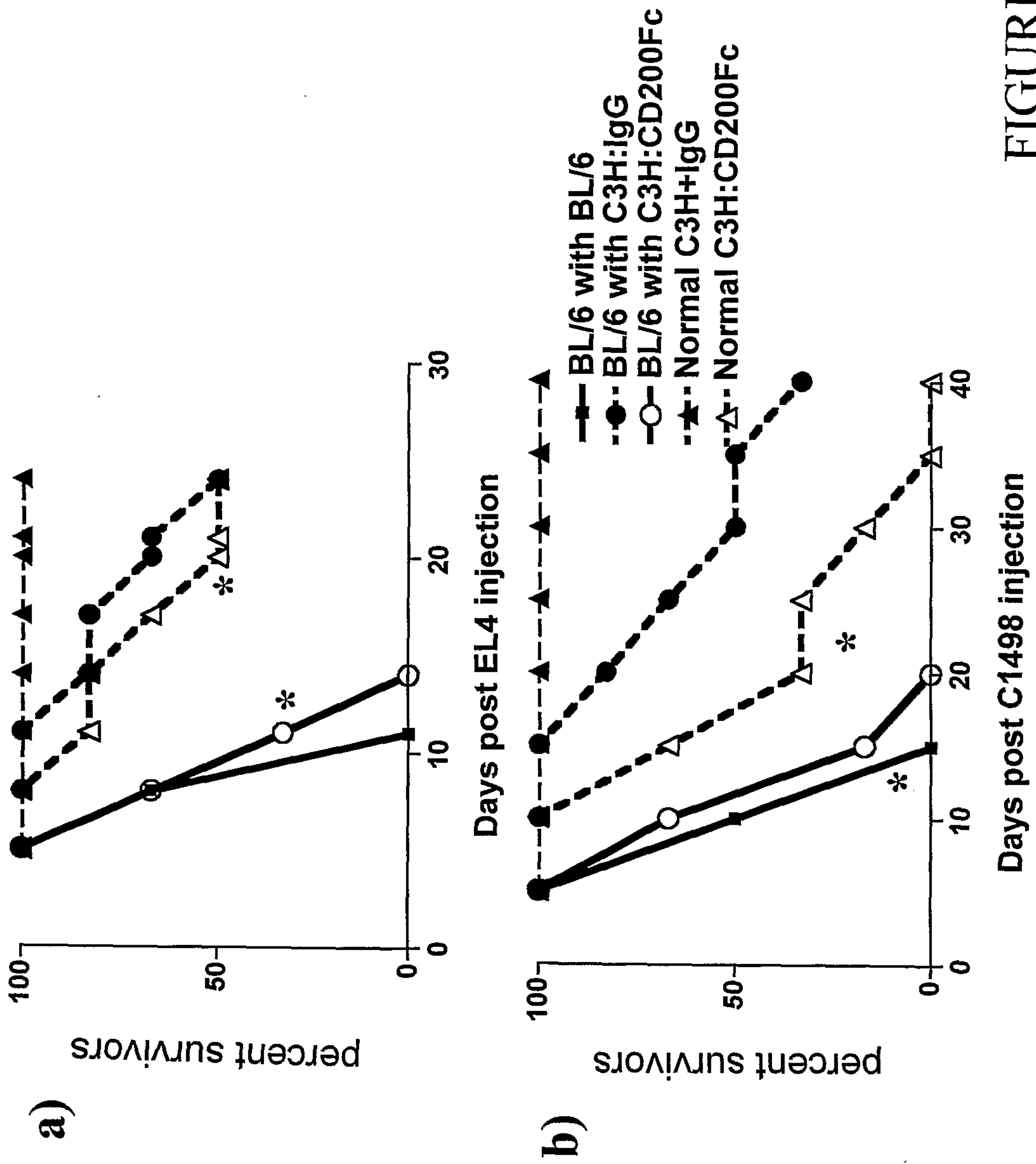


FIGURE 4

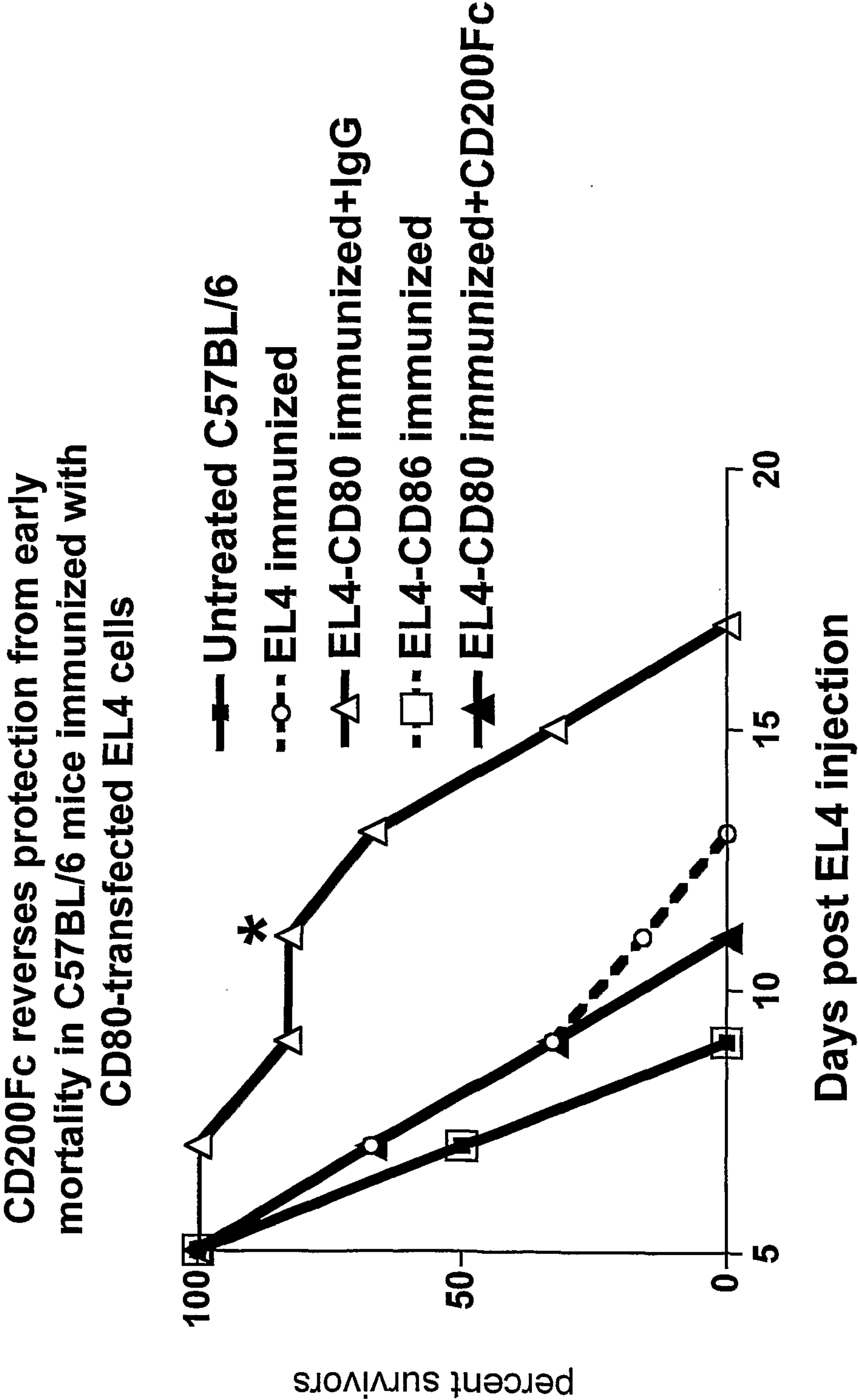


FIGURE 5

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Anti-CD200 reveals protection in mice preimmunized with CD86-tumor cells

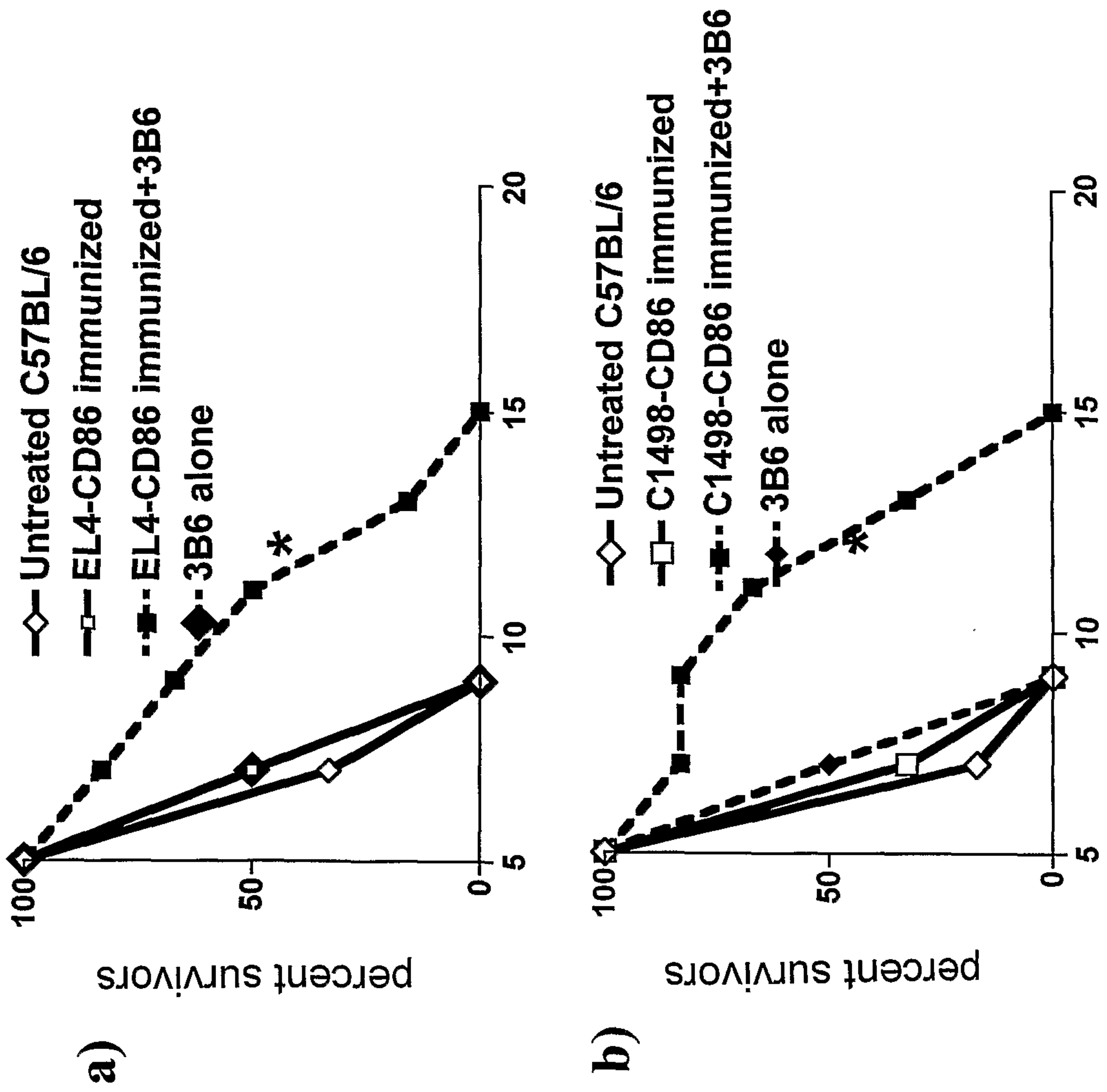
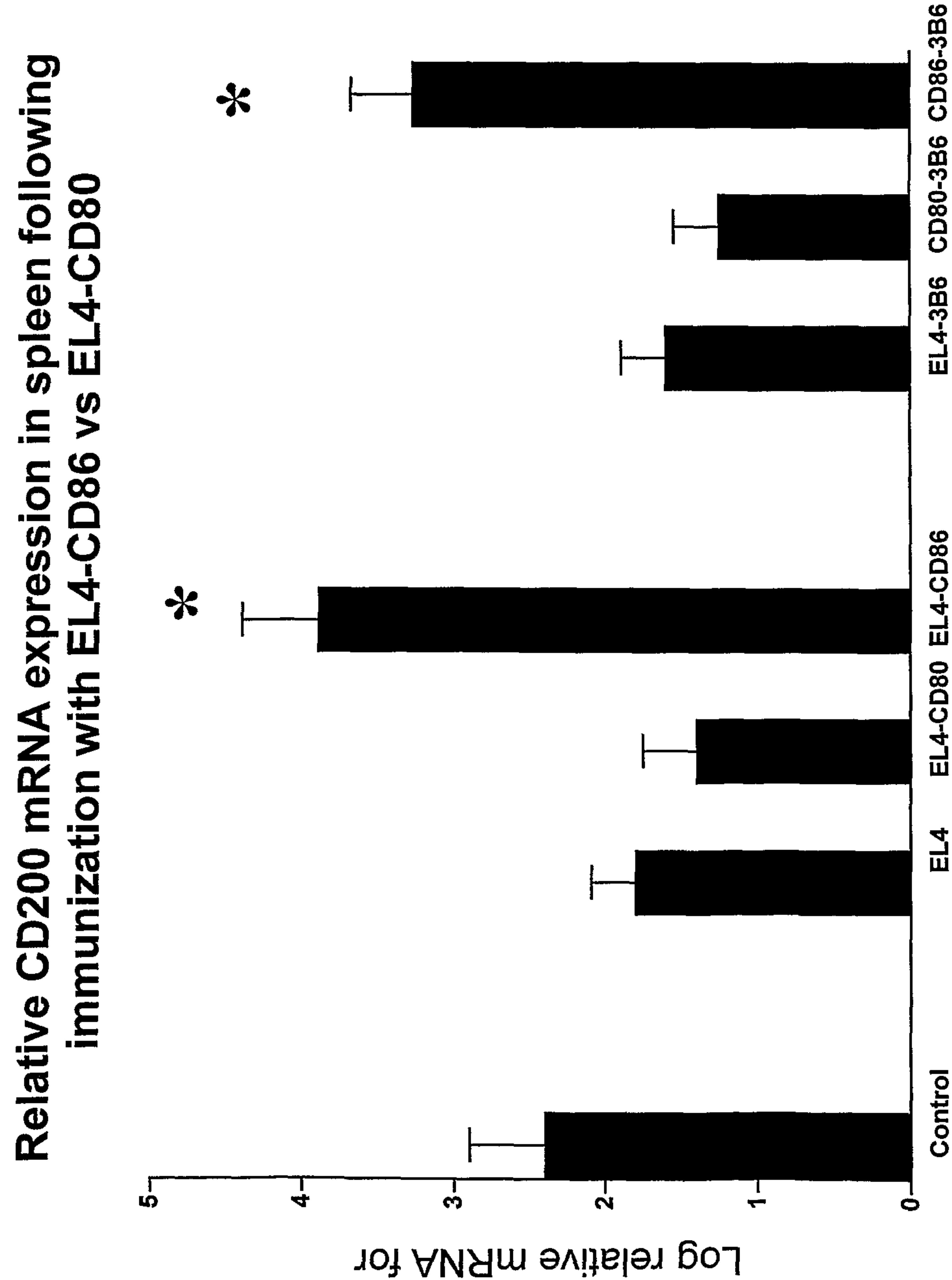


FIGURE 6

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Cells used for immunization of C57BL/6
FIGURE 7

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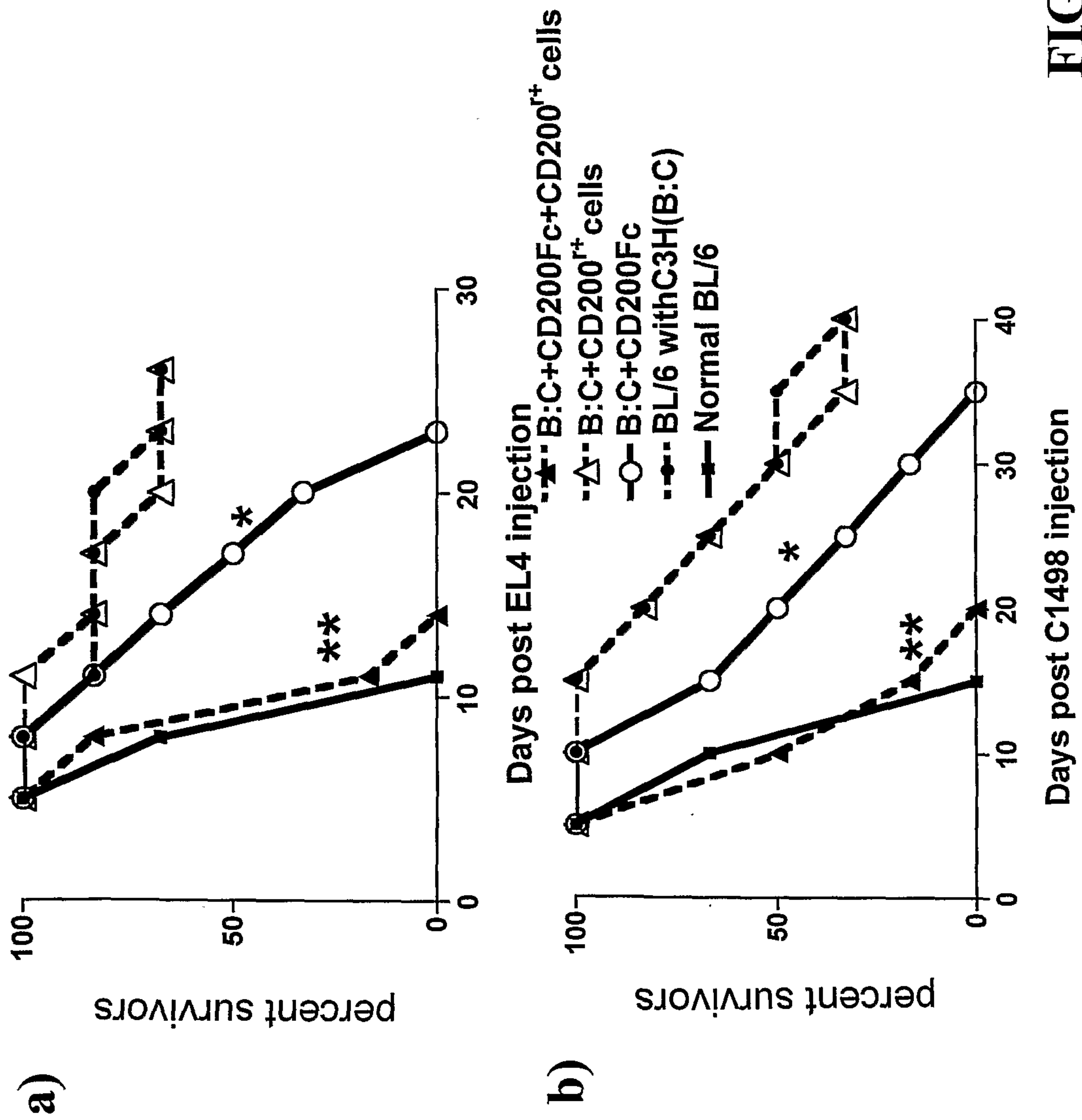
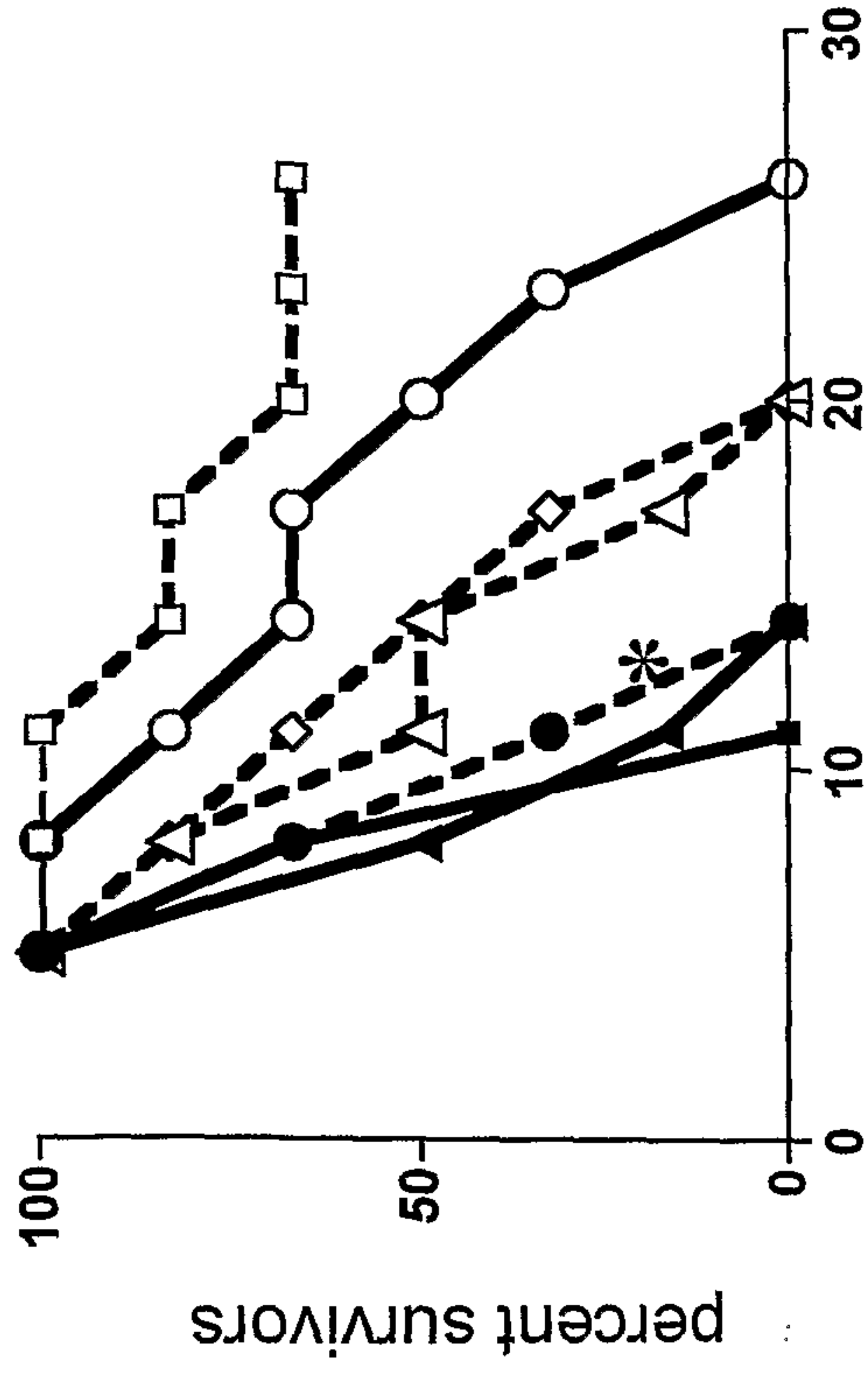
CD200:CD200^{r+} interactions block tumor immunity in BL/6 marrow recipients

FIGURE 8

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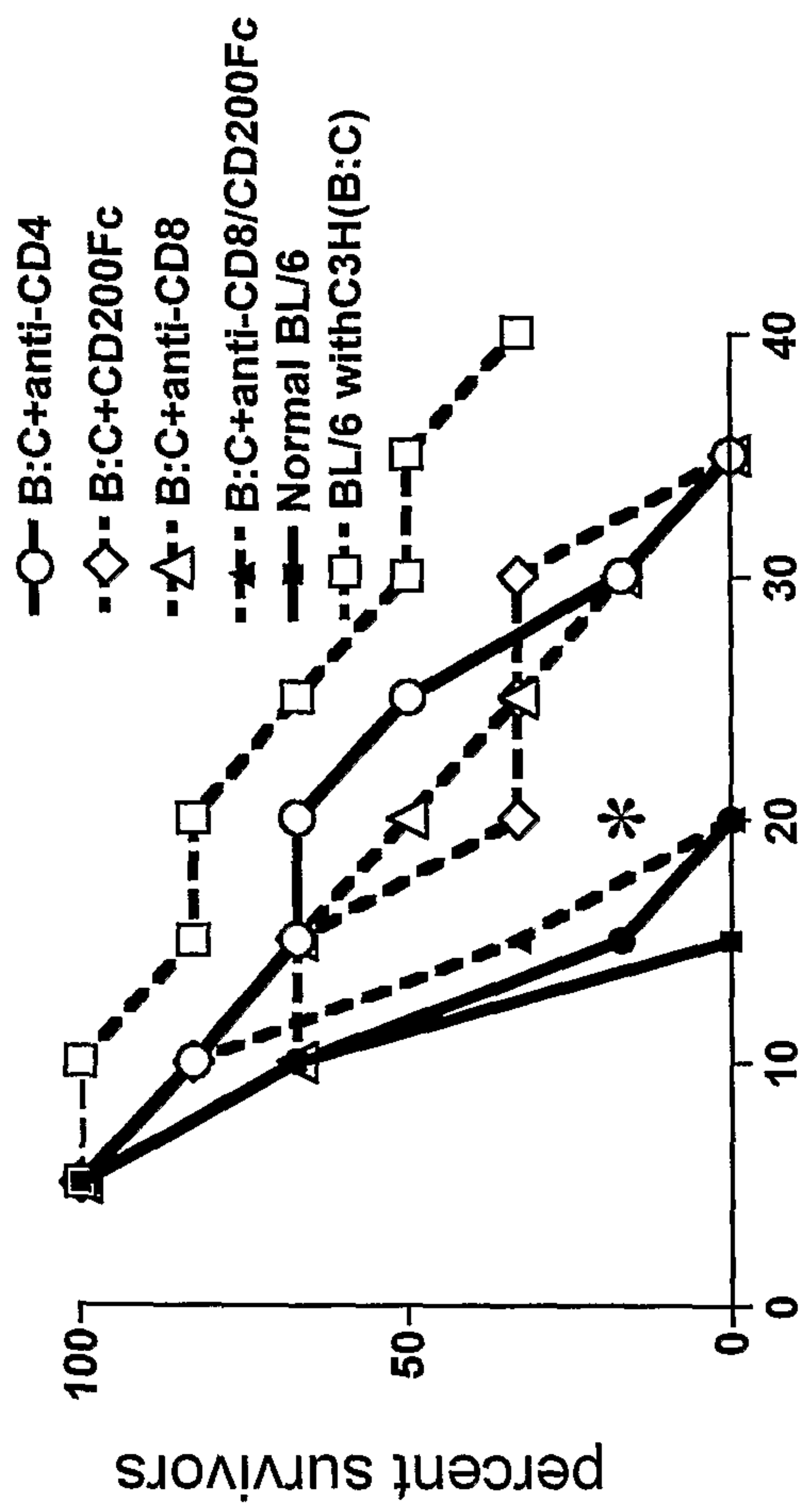
CD200Fc interacts with anti-CD4/CD8 to block tumor immunity

a)



Days post EL4 injection

b)



Days post C1498 injection

FIGURE 9

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CD200Fc reverses protection from early mortality in anti-CD4 treated C57BL/6 mice immunized with CD80-transfected EL4 cells

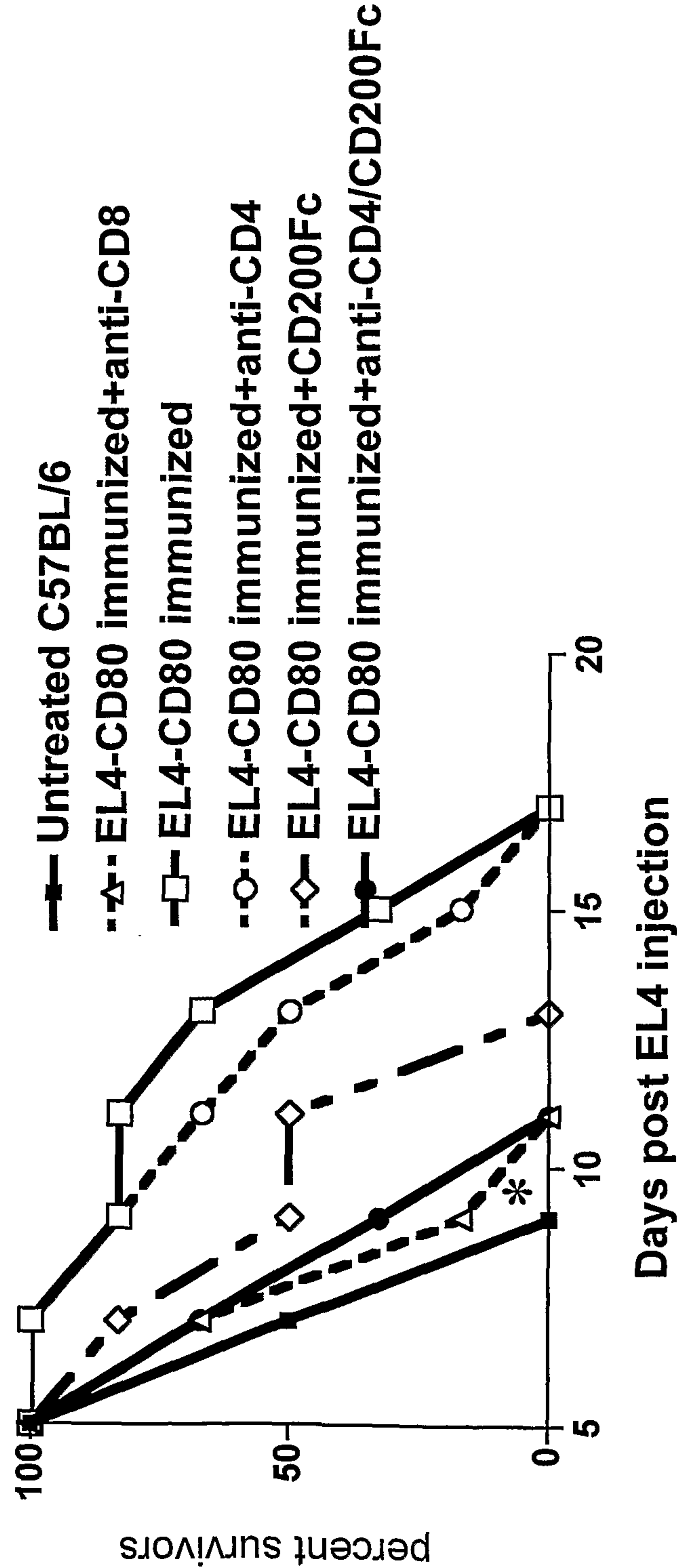
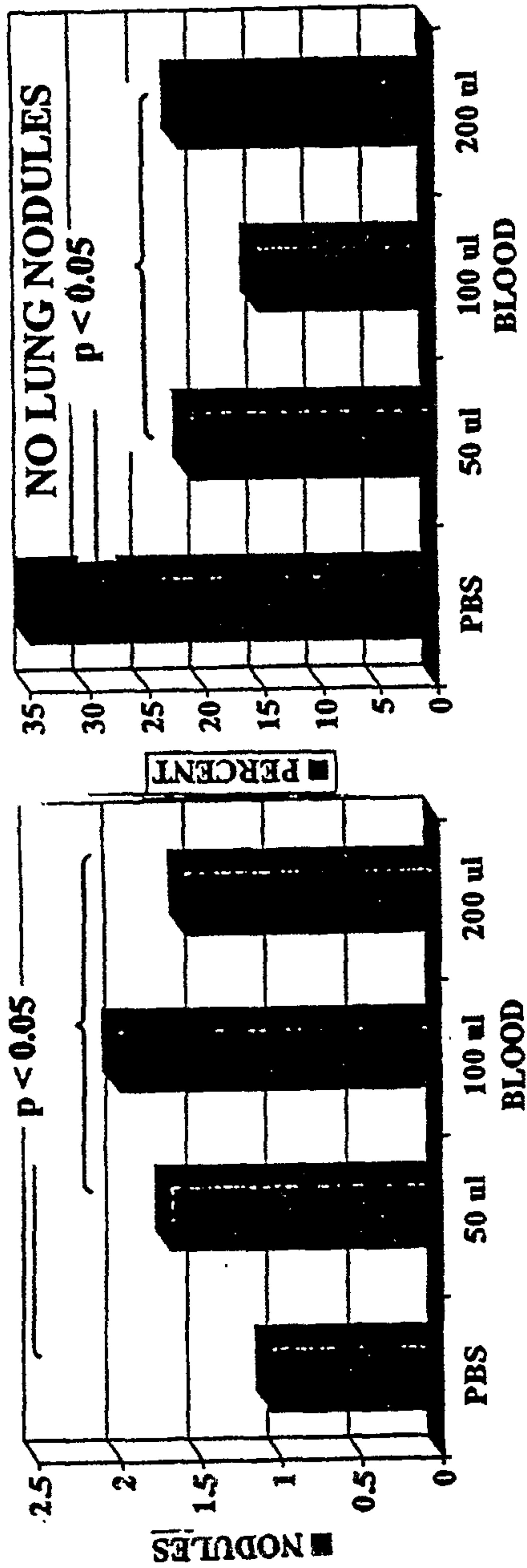


FIGURE 10

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FIGURE 11A and B

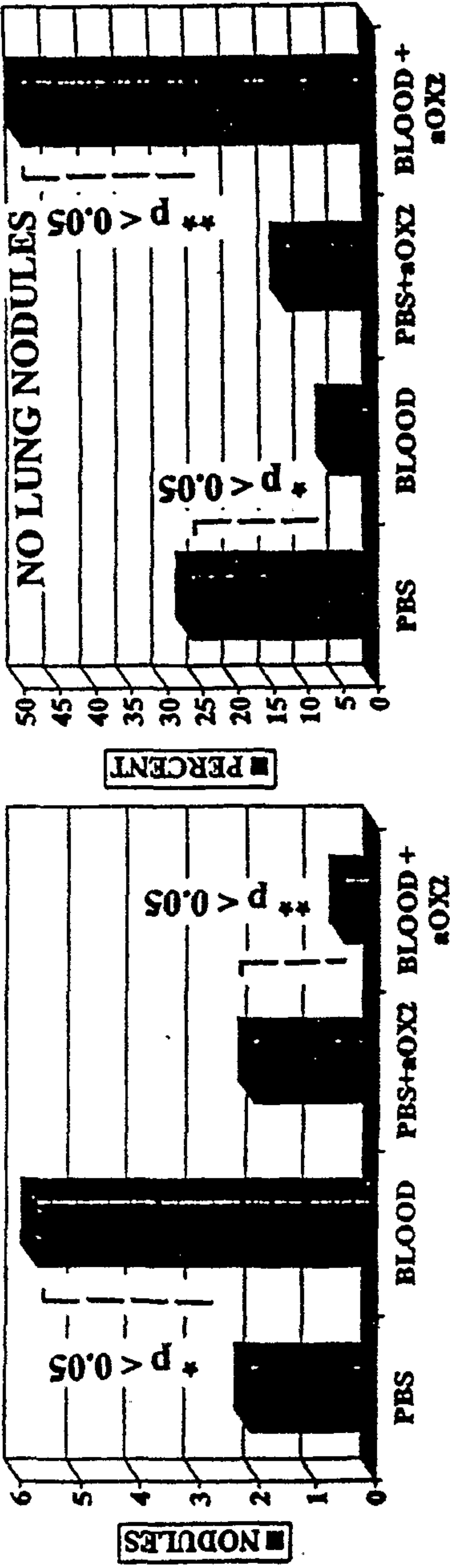


A

B

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FIGURE 12A and B

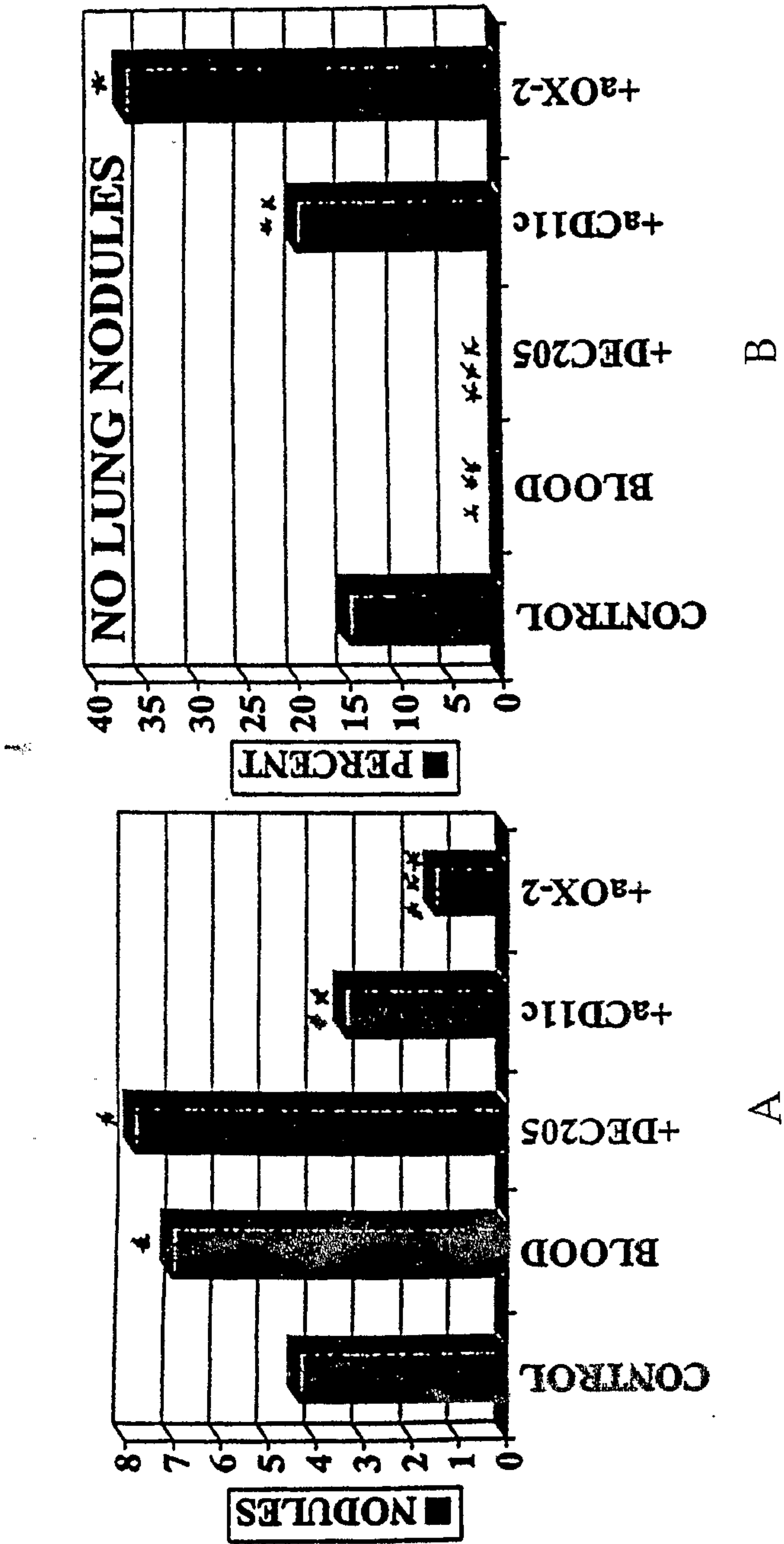


A

B

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FIGURE 13A and B



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FIGURE 14

	Leader -----	
RAT	ATGGGCAGTCCGGTATTCAGGAGACCTTTCTGCCATCTGTCCACCTACAGCCTGCTCTGGGCCATAG	67
MOU	-----T-----C-----A-T---G-----	67
HUM	--GA-----TG---C---CT-----T-----G-T---T---G-	55
	V-like domain -----	
RAT	CAGCAGTAGCGCTGAGCACAGCTCAAGTGGAAGTGGTGACCCAGGATGAAAGAAAGCTGCTGCACAC	134
MOU	-----GC-----	134
HUM	-----G-T---T-----A-----C-----A---T---	122
RAT	AACTGCATCCTTACGCTGTTCTCTAAAAACAACCCAGGAACCCCTTGATTGTGACATGGCAGAAAAAG	201
MOU	-----A-----T-----	201
HUM	-----T-----AAA-C---GC---ATG-----G---C-C-----	189
RAT	AAAGCCGTAGGCCCCAGAAAACATGGTCACTTACAGCAAAGCCCATGGGGTTGTCATTTCAGCCCACCT	268
MOU	-----GA-----C-----A-----A---C---TG---	268
HUM	-----T---A-----C-T---G-GAA-----G---G---C---TG---	256
RAT	ACAAAGACAGGATAAACATCACTGAGCTGGGACTCTTGAACACAAGCATCACCTTCTGGAACACAAC	335
MOU	-----TG---A-----G---T-----CA	335
HUM	-T---G---A-----T---CC-----C-A---T---C-----T-TC--	323
RAT	CCTGGATGATGAGGGTTGCTACATGTGTCTCTTCAACATGTTTGGATCTGGGAAGGTCTCTGGGACA	402
MOU	-A-T-GA---GA---C-----C---T---CA-----A---A---	402
HUM	-----G---A---G---T-----T-CC-----T-T-----A---A---G	390
	C-like domain -----	
RAT	GCTTGCCCTTACTCTCTATGTACAGCCCATAGTACACCTTCACTACAACCTATTTTGAAGACCACCTAA	469
MOU	-----C-----	469
HUM	--C-----C---CG-----TC-----A-TC-C-----	457
RAT	ACATCACGTGCTCTGCAACTGCCCGCCAGCCCCCTGCCATCTCCTGGAAGGGCACTGGGTCAGGAAT	536
MOU	-----T-----G-----T-----A-----T-----A-----	536
HUM	-T-----T-----C-----CATGG---T-----T-C-C-----	524
RAT	TGAGAATAGTACTGAGAGTCACTCCCATTCAAATGGGACTACATCTGTCACCAGCATCCTCCGGGTC	603
MOU	-----C-----T-----	603
HUM	---A-----A-T---C---TG---T---CC-----C---G-----T-----ATA--	591
RAT	AAAGACCCCAAACTCAGGTTGGAAAGGAAGTGATCTGCCAGGTTTATACTTGGGGAATGTGATTG	670
MOU	-----	670
HUM	-----T---G-A-----G---G-----GC-GC---C-----C---CC-	658
	Transmembrane region -----	
RAT	ACTACAAGCAGAGTCTGGACAAAGGATTTTGGTTTTTCAGTCCCAGTCTGCTGAGCATTGTTTCTCT	737
MOU	-----T-----T---A-----	737
HUM	---TT-----A-CCG-CA-----C-A-----T---G---AT---A-----C---	725
	Cytoplasmic region -----	
RAT	GGTAATTCTTCTGGTCTTGATCTCCATCTTATTATACTGGAAACGGCACCAGAAATCAGGAGCGGGGT	804
MOU	-----A-----C-----T-----	804
HUM	-----C---C-A---A---C-G-----T---G-----C---A---	792
RAT	GAGTCATCACAGGGGATGCAAAGAATGAAATAA	837
MOU	--A-----	837
HUM	----TG-----AG-T---A---C---	825

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FIGURE 15

Leader sequence-----

-30 -1

RAT M G S P V F R R P F C H L S T Y S L L W A I A A V A L S T A

MOU -----L-----I---G-----

HUM - I - M - - S - - - V - - V M - - - V - - C - -

|V-like domain (domain I) ----- *

RAT Q V E V V T Q D E R K L L H T T A S L R C S L K T T Q E P L

MOU -----A-----S-----

HUM ----Q-----E---Y-----K-----QNA----A--

31 **

RAT I V T W Q K K K A V G P E N M V T Y S K A H G V V I Q P T Y

MOU -----S-----T-----A--

HUM -----E N-----

61 ** **

RAT K D R I N I T E L G L L N T S I T F W N T T L D D G G C Y M

MOU -----V-----W--S-----H I G-----

HUM ----K-----Q-----Q---T-----I---E-----

91* ** |C-like domain (domain II)-----

RAT C L F N M F G S G K V S G T A C L T L Y V Q P I V H L H Y N

MOU -----T-----Q-----

HUM -----F G---I-----V-----S-----K

121 ** .

RAT Y F E H H L N I T C S A T A R P A P A I S W K G T G S G I E

MOU -----T-----T-----

HUM F S-----M V F-----V P R-----

151**

RAT N S T E S H S H S N G T T S V T S I L R V K D P K T Q V G K

MOU -----F-----

HUM -----V T L S--P-----H I-----N-----

181 . |Transmembrane region -----

RAT E V I C Q V L Y L G N V I D Y K Q S L D K G F W F S V P L L

MOU -----

HUM -----H---T--T--F---T V N---Y-----

211 |Cytoplasmic region -----

RAT L S I V S L V I L L V L I S I L L Y W K R H R N Q E R G E S

MOU -----I-----

HUM -----V-----D-----L

241

RAT S Q G M Q R M K

MOU -----

HUM -----V--K---T-

* invariant cysteine residues; ** invariant asparagine (N-linked oligosaccharides)