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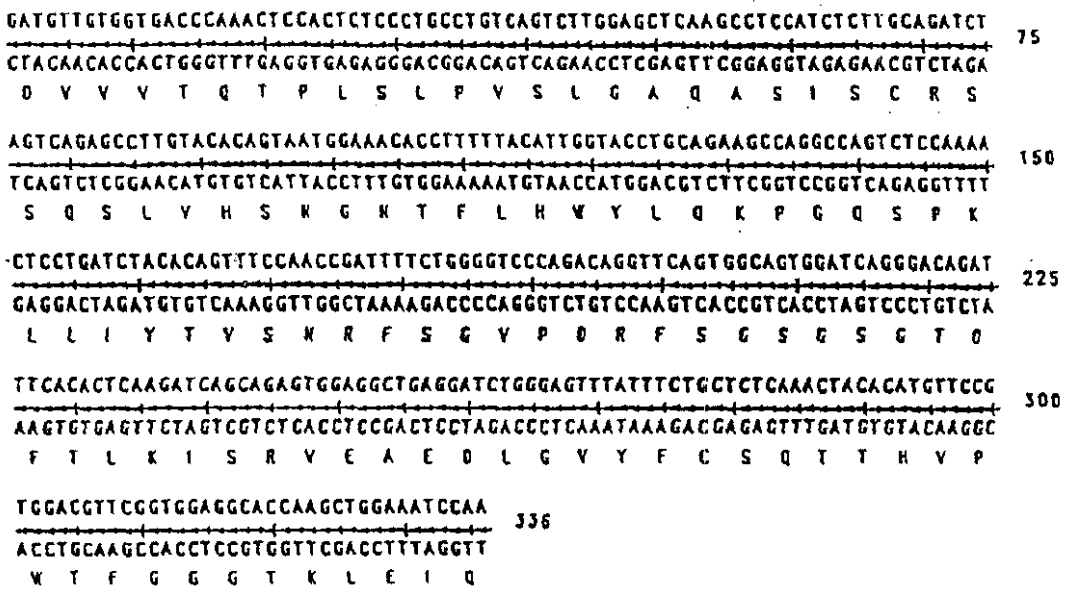
(54) Title: RECOMBINANT ANTI-CD40 ANTIBODY AND USES THEREOF

(57) Abstract: The present invention relates to methods and compositions for the prevention and treatment of cancer, inflammatory diseases and disorders or deficiencies of the immune system. The methods of the invention comprise administering a CD40 binding protein that potentiates the binding of CD40 to CD40 ligand.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CTACAACACCACTGGGTTTGAGGTGAGAGGGACGGACAGTCAGAACCTCGAGTTCGGAGGTAGAGAACGTCTAGA
D V V V T Q T P L S L P V S L G A Q A S I S C R S
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TCAGTCTCGGAACATGTGTCATTACCTTTGTGGAAAAATGTAACCATGGACGTCTTCGGTCCGGTCAGAGGTTTT
S Q S L V H S N G N T F L H W Y L Q K P G Q S P K
CTCCTGATCTACACAGTTTCCAACCGATTTTCTGGGGTCCCAGACAGGTTCAGTGGCAGTGGATCAGGGACAGAT
GAGGACTAGATGTGTCAAAGGTTGGCTAAAAGACCCCAGGGTCTGTCCAAGTCACCGTCACCTAGTCCCTGTCTA
L L I Y T V S N R F S G V P D R F S G S G S G T D
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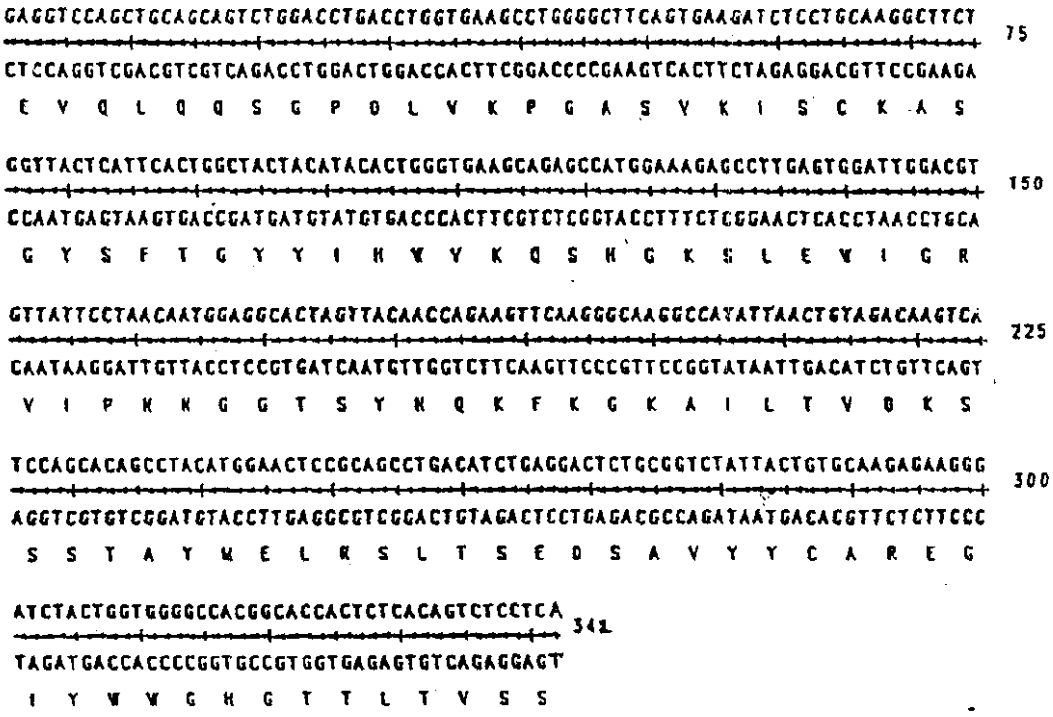
AAGTGTGAGTTCTAGTCGTCTCACCTCCGACTCCTAGACCCTCAAATAAAGACGAGAGTTTGATGTGTACAAGGC
F T L K I S R V E A E D L G V Y F C S Q T T H V P
TGGACGTTCGGTGGAGGCACCAAGCTGGAAATCCAA
ACCTGCAAGCCACCTCCGTGGTTCGACCTTTAGGTT
W T F G G G T K L E I Q
75
150
225
300
336

FIGURE 1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75
CTCCAGGTCGACGTCGTCAGACCTGGACTGGACCACTTCGGACCCCGAAGTCACTTCTAGAGGACGTTCCGAAGA
E V Q L Q Q S G P D L V K P G A S V K I S C K A S
GGTTACTCATTCACTGGCTACTACATACACTGGGTGAAGCAGAGCCATGGAAAGAGCCTTGAGTGGATTGGACGT
150
CCAATGAGTAAGTGACCGATGATGTATGTGACCCACTTCGTCTCGGTACCTTTCTCGGAACTCACCTAACCTGCA
G Y S F T G Y Y I H W V K Q S H G K S L E W I G R
GTTATTCCTAACAATGGAGGCACTAGTTACAACCAGAAGTTCAAGGGCAAGGCCATATTAACTGTAGACAAGTCA
225
CAATAAGGATTGTTACCTCCGTGATCAATGTTGGTCTTCAAGTTCCCGTTCCGGTATAATTGACATCTGTTCAGT
V I P K N G G T S Y N Q K F K G K A I L T V D K S
TCCAGCACAGCCTACATGGAACTCCGCAGCCTGACATCTGAGGACTCTGCGGTCTATTACTGTGCAAGAGAAGGG
300
AGGTCGTGTCGGATGTACCTTGAGGCGTCGGACTGTAGACTCCTGAGACGCCAGATAATGACACGTTCTCTTCCC
S S T A Y M E L R S L T S E D S A V Y Y C A R E G
ATCTACTGGTGGGGCCACGGCACCACTCTCACAGTCTCCTCA
341
TAGATGACCACCCCGGTGCCGTGGTGAGAGTGTCAGAGGAGT
I Y W W G H G T T L T V S S

FIGURE 2

S2C6 VL

DVVVTQTPLSLPVSLGAQASISCRSSQSLVHSNGNTFLHWYLQKPGQSPKL

CDR1

LIYTVSNRFSGVPDRFSGSGSGTDFTLKISRVEAEDLGVYFCSQTTHVPWT

CDR2 CDR3

FGGGTKLEIQ

S2C6 VH

EVQLQQSGPDLVKPGASVKISCKASGYSFTGYYIHWVKQSHGKSLEWIGRV

CDR1

IPNNGGTSYNQKFKGKAILTVDKSSSTAYMELRSLTSEDSAVYYCAREGIY

CDR2 CDR3

WWGHGTTLTVSS

FIGURES 3A-3B

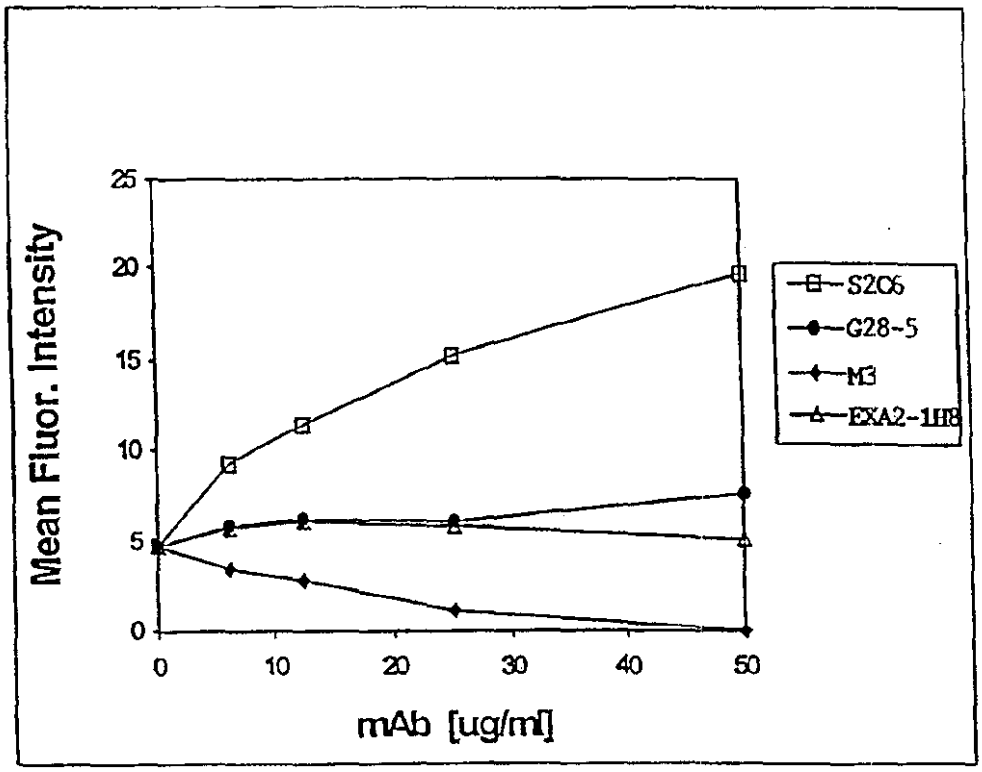

Mean Fluor. Intensity
25
20
15
10
5
0
0
10
20
30
40
50
mAb [ug/ml]
S2C6
G28-5
M3
EXA2-1H8

FIGURE 4

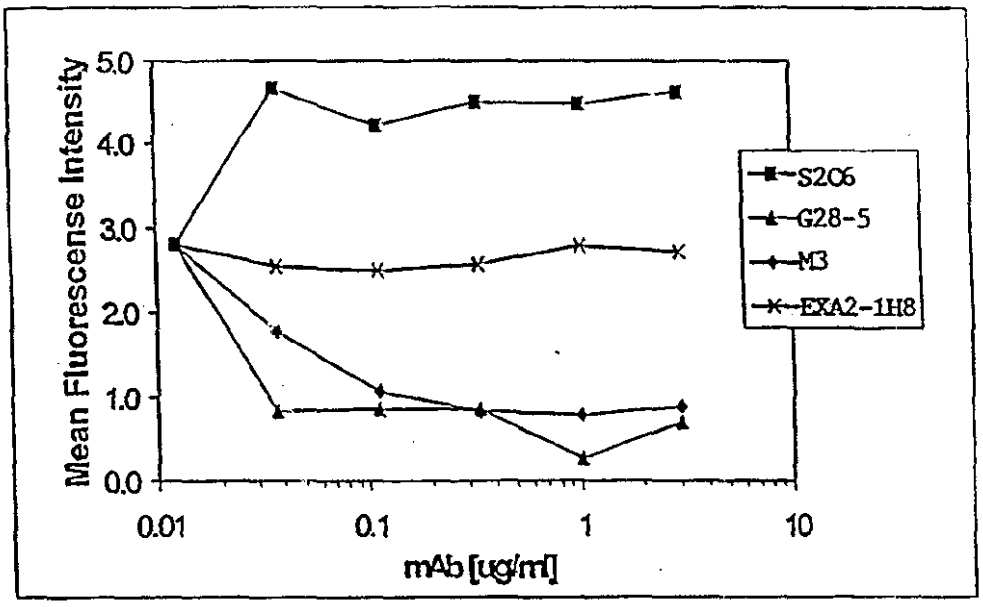

Mean Fluorescense Intensity
5.0
4.0
3.0
2.0
1.0
0.0
0.01
0.1
1
10
mAb [ug/ml]
S2C6
G28-5
M3
EXA2-1H8

FIGURE 5

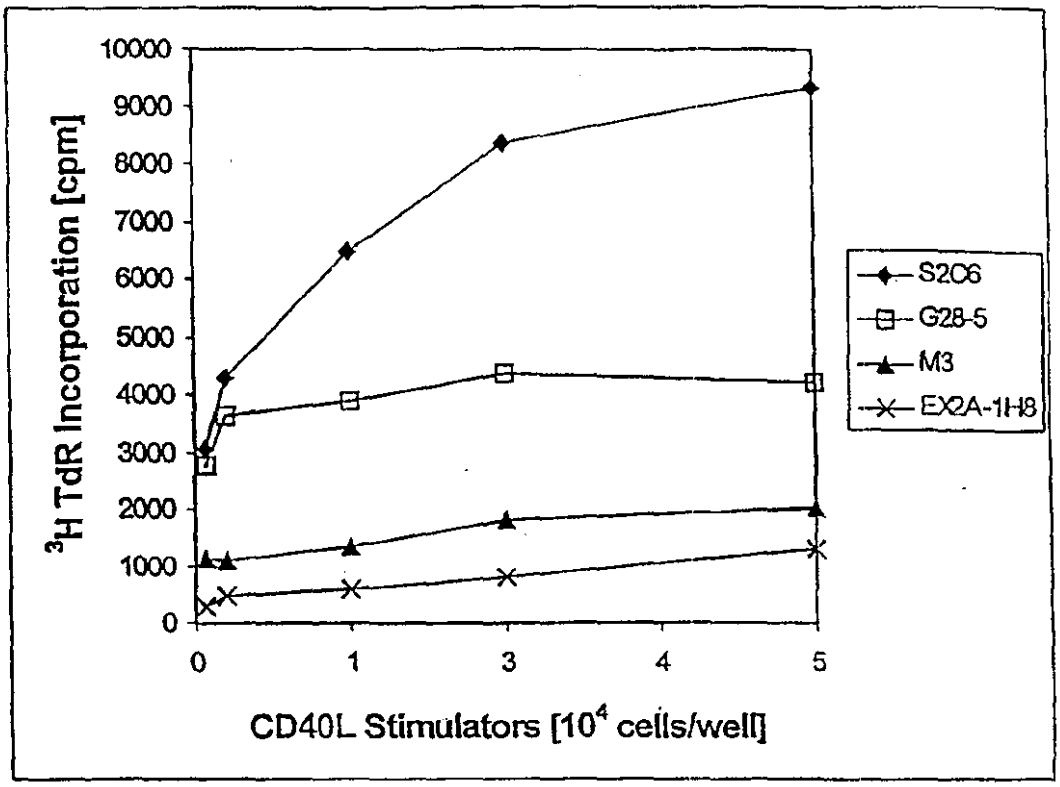

10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
$^3$H TdR Incorporation [cpm]
0
1
3
4
5
CD40L Stimulators [$10^4$ cells/well]
S2C6
G28-5
M3
EX2A-1H8

FIGURE 6

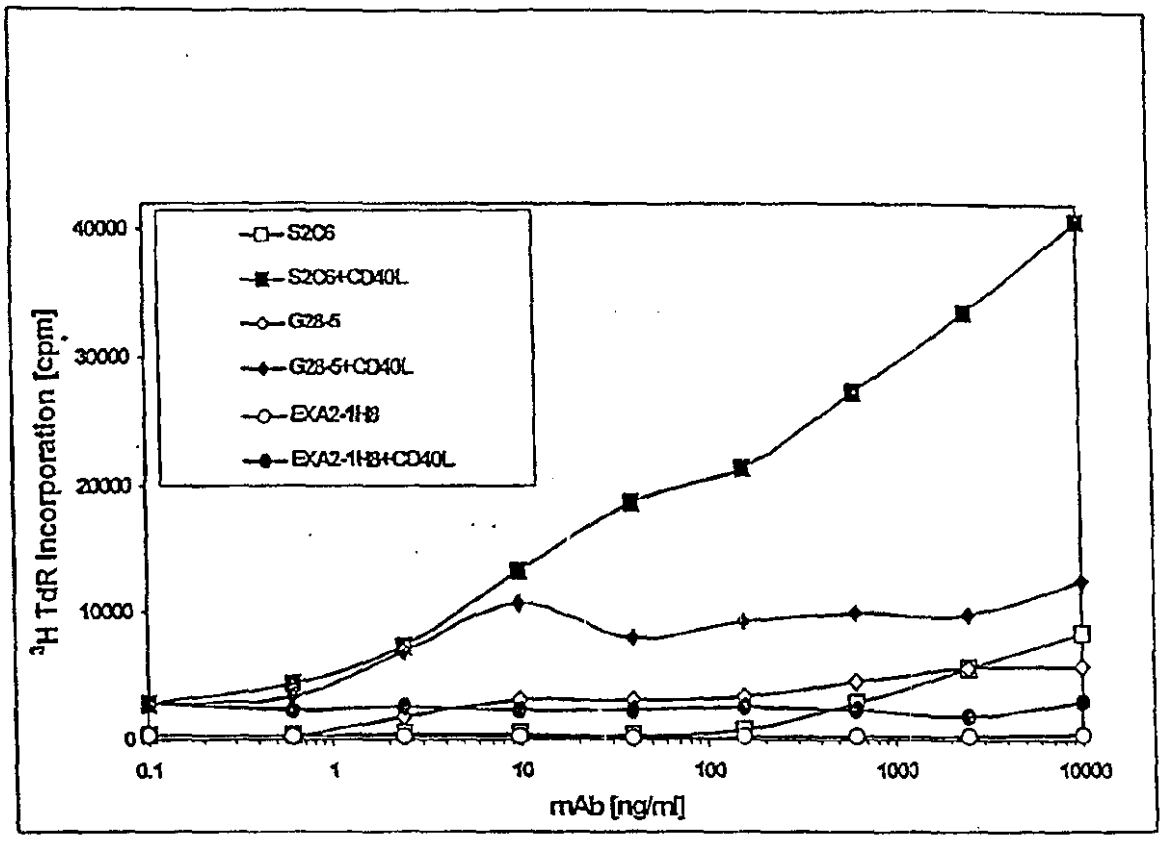
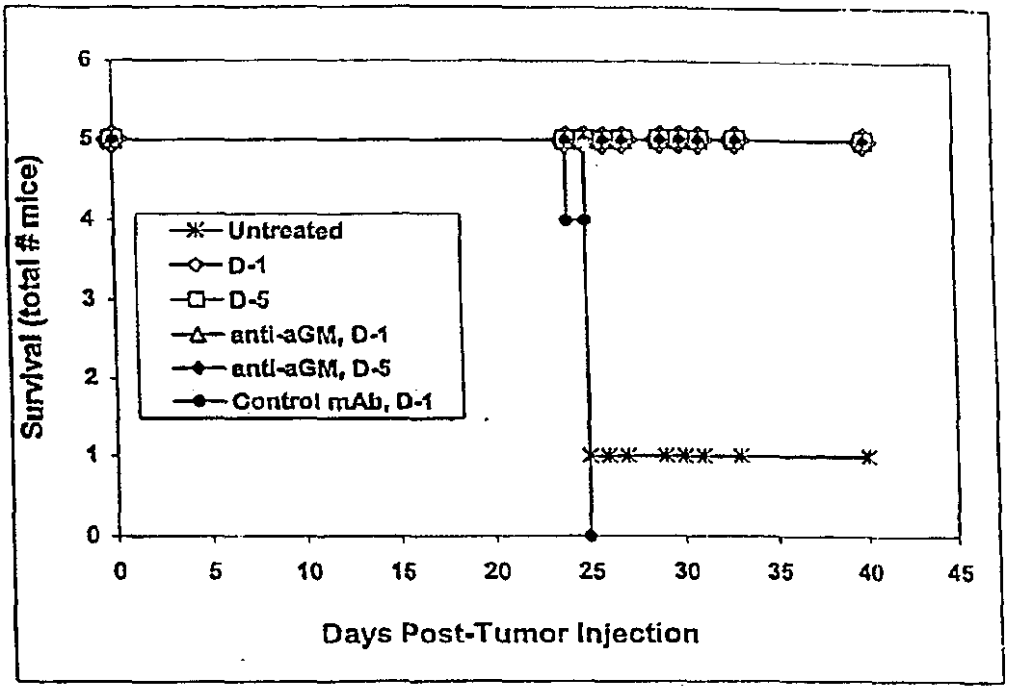
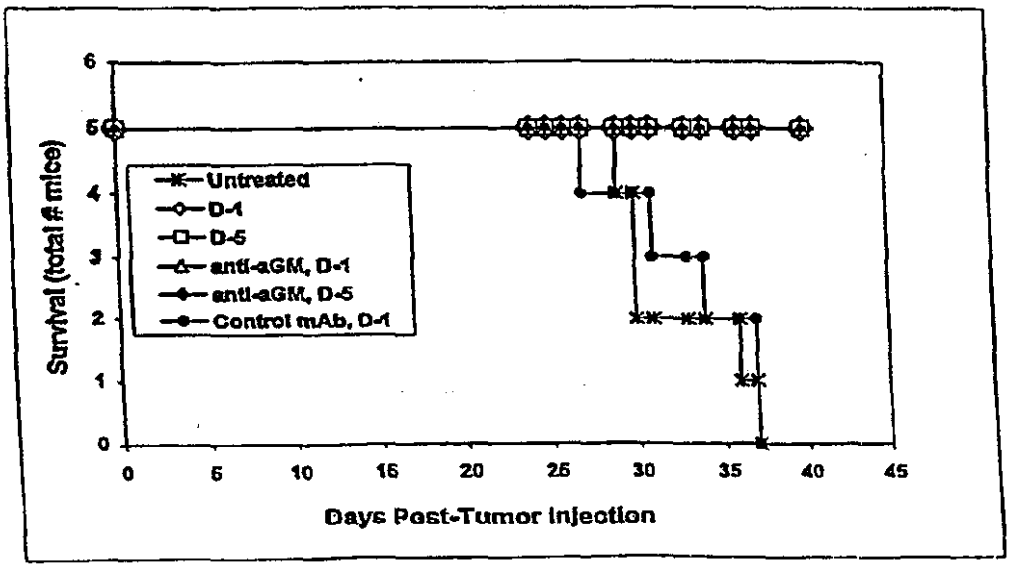

S2C6
S2C6+CD40L
G28-5
G28-5+CD40L
EXA2-1H8
EXA2-1H8+CD40L
40000
30000
20000
10000
0
0.1
1
10
100
1000
10000
$^3$H TdR Incorporation [cpm]
mAb [ng/ml]

FIGURE 7

A.




B.




FIGURES 8A-8B

C.

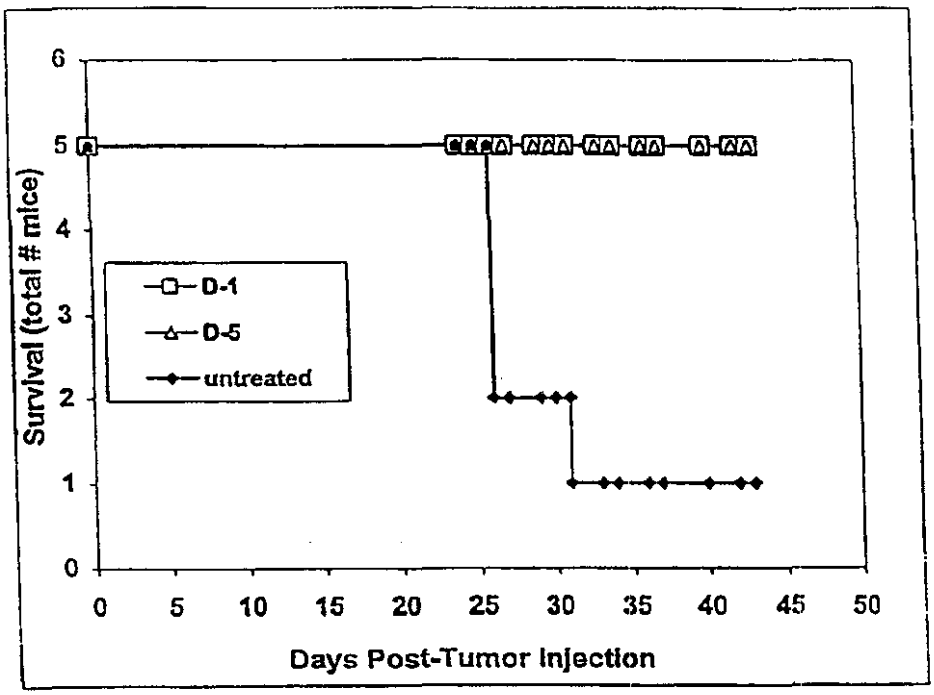

Survival (total # mice)
Days Post-Tumor Injection
D-1
D-5
untreated
0
1
2
3
4
5
6
0
5
10
15
20
25
30
35
40
45
50

FIGURE 8C

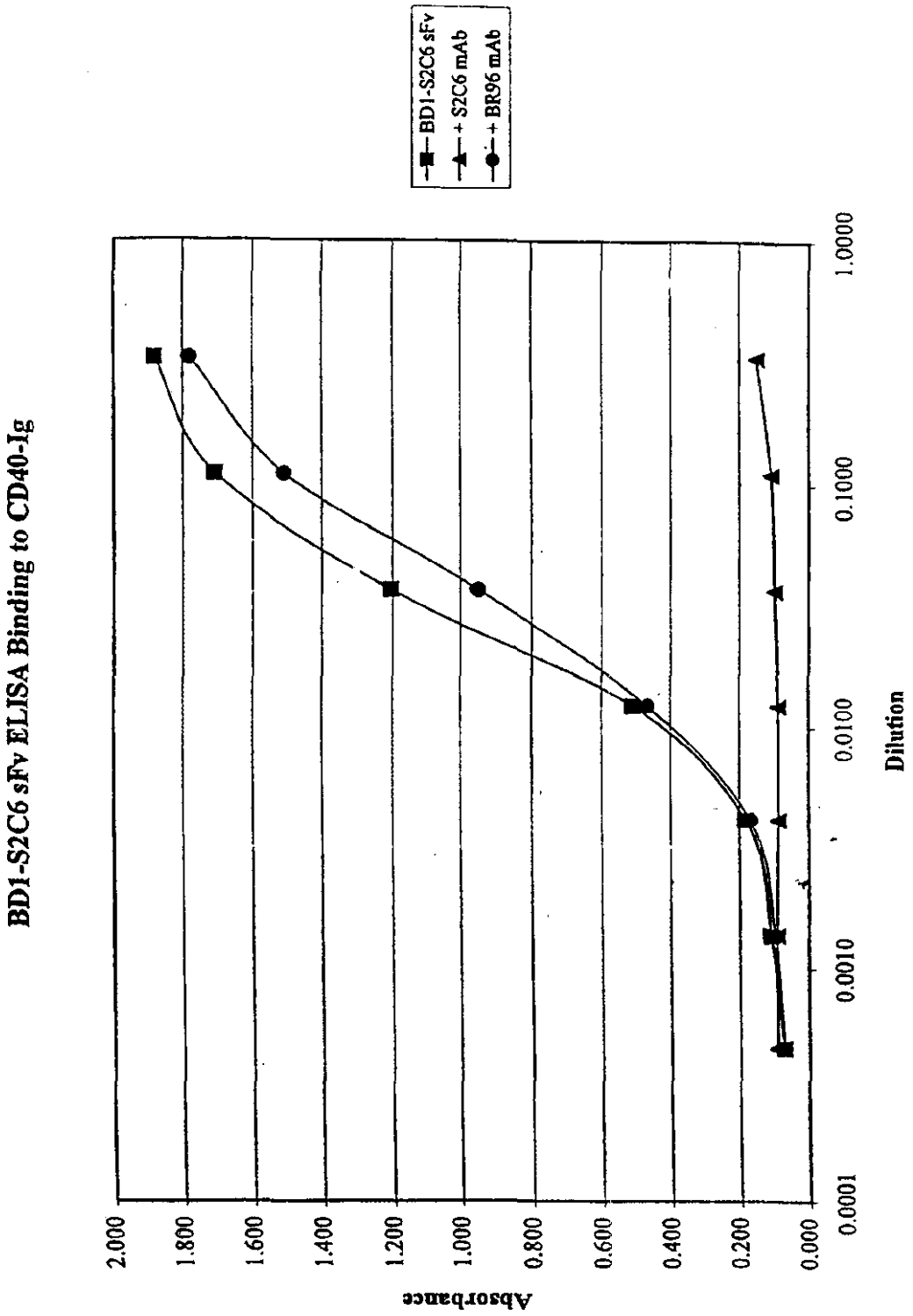

BD1-S2C6 sFv ELISA Binding to CD40-Ig
BD1-S2C6 sFv
+ S2C6 mAb
+ BR96 mAb
Absorbance
2.000
1.800
1.600
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
Dilution
1.0000
0.1000
0.0100
0.0010
0.0001

FIGURE 9

# RECOMBINANT ANTI-CD40 ANTIBODY AND USES THEREOF

## 1. FIELD OF THE INVENTION

The present invention relates to methods and compositions for the treatment of diseases and disorders, including cancer, inflammatory diseases or disorders and diseases or disorders of the immune system, comprising administering a CD40 binding protein which enhances binding of CD40 ligand to CD40. CD40 binding proteins include recombinant/variant forms of monoclonal antibody S2C6 and derivatives thereof.

## 2. BACKGROUND OF THE INVENTION

CD40 is a cell surface phosphorylated glycoprotein that is expressed on a variety of cell types, including B cells, B cell malignancies, follicular dendritic cells, basal epithelial cells, and carcinomas. CD40 binds CD40 ligand ("CD40L"). CD40L is expressed on activated T cells during inflammation and cancer (Younes et al., 1998, Br. J. Haematol. 100:135-141; for a review see Grewal and Flavell, 1998, Annu. Rev. Immunol. 16:111-135). The interaction of CD40 with CD40L results in B cell activation and proliferation of normal B cells; however CD40-mediated signaling in B cell-derived tumor lines can result in activation-induced cell death. The strength of the activation signal is key to activation-induced tumor cell death (Grafton et al., 1997, Cell. Immunol. 182:45-56). Therefore, compositions and methods for increasing receptor-ligand interaction and strength of activation signal between CD40 and CD40L would be of great value in treating disease.

### 2.1 CD40 AND CD40 LIGAND

CD40 is a member of the TNF receptor superfamily. This family includes TNFrII, CD40, CD30, LMP-1, LTBr, ATAR, OX-40 and 4-1BB receptors. CD40 is constitutively expressed on B-lymphocytes, macrophages and dendritic cells and is induced

by cytokine activation on fibroblasts, endothelial cells and epithelial cells (Van Kooten and Banchereau, 1997, Curr. Opin. Imunol., 9: 330-337). CD40 has also been shown to be highly expressed on many human carcinomas including lung, bladder, gastric, breast and ovarian cancers (Stamenkovic et al., 1989, EMBO J. 8:1403-1410).

The ligand for CD40 is a membrane protein that is expressed on activated T cells. Receptor binding of CD40L results in CD40 multimerization, the generation of activation signals (for antigen presenting cells such as dendritic cells, monocytes and B cells) and the generation of growth and differentiation signals (for cytokine-activated fibroblasts and epithelial cells). CD40 signals are transduced from the multimerized receptor via recruitment of a series of TNF receptor-associated factors ("TRAFs") (Kehry, 1996, J. Immunol. 156:2345-2348). Subsets of TRAFs interact differentially with TNF family members, including CD40, to provide stimuli to a wide variety of downstream pathways. TRAF1 and TRAF2 are implicated in the modulation of apoptosis (Speiser et al., 1997, J. Exp. Med. 185:1777-1783; Yeh et al., 1997, Immunity 7:715-725). TRAFs 2, 5, and 6 participate in proliferation and activation events, including NF-kB and c-Jun N-terminal kinase activation. In normal B cells, binding of CD40 recruits TRAF2 and TRAF3 to the receptor complex and induces down-regulation of other TRAFs (Kuhune et al., 1997, J. Exp. Med.186: 337-342). The effects of CD40 binding are also dependent on membrane density (De Paoli et al., 1997, Cytometry 30:33-38). Importantly, unlike the proliferative response seen with normal primary B cells, CD40 binding on neoplastic B cells can result in growth inhibition and activation-induced cell death (Funakoshi et al., 1994, Blood 83:2787-2794). Thus, CD40 activation in the context of different cell types, transformation, resident TRAFs and co-stimuli can induce responses ranging from activation and proliferation to growth inhibition and apoptosis.

## 2.2 ANTI-CD40 ANTIBODIES

With at least one exception, the anti-CD40 monoclonal antibodies ("mAbs") described to date are of three general classes: (1) those that block CD40/CD40L interaction by at least 90% and have anti-neoplastic properties(Armitage et al., U.S. Patent No. 5,674,492; Fanslow et al., 1995, Leukocyte Typing V, Schlossman et al., eds., 1:555-556); (2) those that antagonize signaling through CD40 (deBoer et al., U.S. Patent No. 5,677,165); and (3) those that deliver a stimulatory signal through CD40 but do not increase the interaction between CD40 and CD40L, *e.g.*, G28-5, (Ledbetter et al., U.S. Patent No. 5,182,368; PCT Publication WO 96/18413).

One mAb, CD40.4 (5C3)(PharMingen, San Diego, California), has been shown to increase the interaction between CD40 and CD40L by approximately 30-40% (Schlossman et al., eds., 1995, Leukocyte Typing V: White Cell Differentiation Antigens 1:547-556).

Armitage et al. (U.S. Patent No. 5,674,492) describes methods using CD40 binding proteins, including mAb HuCD40-M2, that are capable of binding CD40 and inhibiting the binding of CD40 to CD40L, for preventing or treating disease characterized by neoplastic cells expressing CD40.

DeBoer et al. (U.S. Patent No. 5,677,165) describes anti-CD40 mAbs that, being free of significant agonistic activity, bind to CD40 on the surface of B-cells, and block B-cell activation. An essential feature of U.S. Patent No. 5,677,165 is that upon binding of the anti-CD40 mAb to human CD40 on the surface of normal human B cells, the growth or differentiation of normal human B cells is inhibited.

Ledbetter et al. (U.S. Patent No. 5,182,368) describes a ligand, G28-5, that binds to the B cell surface antigen Bp50 (now designated CD40) and stimulates activated B cells to traverse the cell cycle such that B cell proliferation is augmented. However, G28-5 does not enhance activation of B cells in the presence of CD40L, and does not potentiate CD40/CD40L interaction.

S2C6 is an anti-CD40 mAb that was prepared against a human bladder carcinoma (Paulie et al., 1984, Cancer Immunol. Immunother. 17:165-179). S2C6 binds to the CD40 receptor expressed on a variety of cell types including B-lymphocytes, endothelial and epithelial cells. S2C6 has been shown to have specificity toward neoplastic urothelium and B cell-derived malignant lymphocytes. Reactivity with a prostatic carcinoma cell line, HS, and weak reactivity with a melanoma has also been shown (Paulie et al., 1984, Cancer Immunol. Immunother. 17:165-179). Studies have suggested the utility of S2C6 as a diagnostic marker for B cell malignancies (Paulie et al., 1984, Cancer Immunol. Immunother. 17:165-179; Paulie et al., 1985, Eur. J. Cancer. Clin. Oncol. 21:701-710). In addition to detecting B cell malignancies, S2C6 has been shown to deliver strong growth-promoting signals to B lymphocytes (Paulie et al., 1989, J. Immunol. 142:590-595).

S2C6 has agonistic activity on human peripheral B cells as demonstrated by its ability to stimulate primary B cell proliferation in a dose dependent manner (Paulie et al., 1989, J. Immunol. 142:590-595).

Although competition studies have shown that G28-5 and S2C6 bind the same or proximal epitopes, the antibodies have been determined to be functionally different based primarily on the stated magnitude of stimulation achieved by either mAb on previously stimulated tonsillar B cells (Clark and Ledbetter, 1986, Proc. Natl. Acad. Sci. USA 83:4494-4498; Ledbetter et al., U.S. Pat. No. 5,182,368). One hundred times more S2C6 compared to G28-5 was required to achieve tonsillar B cell activation under the specific conditions tested (Ledbetter et al., U.S. Patent No. 5,182,368).

There is a need in the art for therapeutics with increased efficacy to treat or prevent cancer, activate or augment the immune system or treat or prevent an immune deficiency or disorder, a need provided by the present invention.

Citation or identification of any reference herein shall not be construed as an admission that such reference is available as prior art to the present invention.

## 3. SUMMARY OF THE INVENTION

Applicants have made the unexpected discovery of a new class of anti-CD40 antibodies that, in addition to delivering a stimulatory signal, enhances the interaction between CD40 and CD40L, enhances CD40L-mediated stimulation and has *in vivo anti-neoplastic activity*. Production and use of these antibodies and related molecules are facilitated by the inventors' cloning and sequencing of the variable region of mAb S2C6, and identification of the CDR and framework regions therein.

The present invention relates to molecules comprising the variable domain of mAb S2C6 or one or more of the complementarity-determining regions (CDRs) thereof having novel sequences (SEQ ID NO: 3, 4, 8, 9 or 10), which molecules (a) immunospecifically bind CD40 and (b) comprise *one or more substitutions or insertions in primary amino acid* sequence relative to native monoclonal antibody S2C6 as secreted by the hybridoma deposited with the ATCC and assigned accession number PTA-110, or are not monoclonal antibody S2C6 as secreted by the hybridoma deposited with the ATCC and assigned accession number PTA-110 and do not result from cleavage of S2C6 with papain or pepsin. In a specific embodiment, the molecules are not native monoclonal antibody S2C6 and do not comprise the native heavy or light chain of said monoclonal antibody S2C6. In another specific *embodiment, the molecule is an antibody*. In another embodiment, the antibody is not isotype IgG1. In another specific embodiment, the molecule comprises a light chain variable domain, the amino acid sequence of SEQ ID NO:2, or a heavy chain variable domain, the amino acid sequence of SEQ ID NO:7.

The invention further relates to chimeric/fusion proteins comprising a fragment of mAb S2C6 fused to an amino

acid sequence of a second protein, as well as to molecules wherein a fragment of mAb S2C6 is covalently bound (e.g., by use of a crosslinking agent) to another chemical structure. In a specific embodiment, a molecule is provided that immunospecifically binds CD40, which molecule comprises the heavy and/or light chain variable domain of mAb S2C6 fused to a second protein comprising the amino acid sequence of bryodin 1 (BD1).

The invention further relates to proteins comprising an amino acid sequence that has at least 95% identity to SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, or SEQ ID NO:10, which proteins (a) immunospecifically bind CD40 and (b) comprise one or more substitutions or insertions in primary amino acid sequence relative to native monoclonal antibody S2C6 as secreted by the hybridoma deposited with the ATCC and assigned accession number PTA-110. In a specific embodiment, the proteins are not native monoclonal antibody S2C6 and do not comprise the native heavy or light chain of said monoclonal antibody S2C6.

The invention further relates to purified proteins, which proteins (a) compete for binding to CD40 with monoclonal antibody S2C6 as secreted by the hybridoma deposited with the ATCC and assigned accession number PTA-110, (b) increase the binding of CD40 ligand to CD40 by at least 45%, and (c) comprise one or more substitutions or insertions in primary amino acid sequence relative to native monoclonal antibody S2C6 as secreted by the hybridoma deposited with the ATCC and assigned accession number PTA-110, or are not monoclonal antibody S2C6 as secreted by the hybridoma deposited with the ATCC and assigned accession number PTA-110 and do not result from cleavage of S2C6 with papain or pepsin. In a specific embodiment, the proteins are not native monoclonal antibody S2C6 and do not comprise the native heavy or light chain of said monoclonal antibody S2C6.

The invention further relates to nucleic acids encoding such molecules and proteins or which hybridize to a DNA consisting of the nucleotide sequence encoding such proteins;

recombinant cells comprising such molecules and proteins; and methods of producing such proteins.

In an embodiment, the isolated nucleic acid comprises a nucleotide sequence encoding a protein comprising (a) a heavy chain variable domain of monoclonal antibody S2C6 as secreted by the hybridoma deposited with the ATCC and assigned accession number PTA-110, and (b) a human constant region.

In an embodiment, the isolated nucleic acid comprises a nucleotide sequence encoding a protein comprising (a) a light chain variable domain of monoclonal antibody S2C6 as secreted by the hybridoma deposited with the ATCC and assigned accession number PTA-110, and (b) a human constant region.

The invention further relates to recombinant cells containing a recombinant nucleic acid vector comprising a nucleotide sequence encoding a protein, which protein competes for binding to CD40 with monoclonal antibody S2C6 as secreted by the hybridoma deposited with the ATCC and assigned accession number PTA-110, and which protein increases the binding of CD40 ligand to CD40 by at least 45%. The invention also provides methods of producing such proteins comprising growing such cells such that the protein is expressed by the cell, and recovering the expressed protein.

The invention further relates to recombinant cells containing a recombinant nucleic acid vector comprising SEQ ID NO:1, SEQ ID NO:6, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, or SEQ ID NO:15 and methods of producing proteins comprising growing such cells, such that a protein encoded by the nucleotide sequence is expressed by the cell, and recovering the expressed protein.

Pharmaceutical compositions containing the molecules and antibodies of the invention, preferably in purified form, are also provided. In particular embodiments, the invention relates to pharmaceutical compositions comprising a molecule comprising SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, or SEQ ID NO:10, which molecule (i) immunospecifically binds CD40, (ii) increases

the binding of CD40 ligand to CD40, and (iii) comprises one or more substitutions or insertions in primary amino acid sequence relative to native monoclonal antibody S2C6 as secreted by the hybridoma deposited with the ATCC and assigned accession number PTA-110; and a pharmaceutically acceptable carrier. In a specific embodiment, the molecule is not native monoclonal antibody S2C6 and does not comprise the native heavy or light chain of said monoclonal antibody S2C6.

The invention further relates to pharmaceutical compositions comprising a purified protein, which protein (i) competes for binding to CD40 with mAb S2C6 as secreted by the hybridoma deposited with the ATCC and assigned accession number PTA-110, (ii) increases the binding of CD40 ligand to CD40 by at least 45%, and (iii) comprises one or more substitutions or insertions in primary amino acid sequence relative to native monoclonal antibody S2C6 as deposited with the ATCC and assigned accession number PTA-110, or is not monoclonal antibody S2C6 as secreted by the hybridoma deposited with the ATCC and assigned accession number PTA-110 and does not result from cleavage of S2C6 with papain or pepsin; and a pharmaceutically acceptable carrier. In a specific embodiment, the protein is not native monoclonal antibody S2C6 and does not comprise the native heavy or light chain of said monoclonal antibody S2C6.

In specific embodiments, the pharmaceutical compositions of the invention contain the molecules or antibodies of the invention in an amount effective for the treatment or prevention of cancer, or an amount effective for activating or augmenting an immune response, or an amount such that the immune response of the subject is activated or augmented.

In specific embodiments, the pharmaceutical compositions of the invention further comprise CD40 ligand. In a specific embodiment, the pharmaceutical composition comprises in an amount effective for the treatment or prevention of cancer or an immune disorder, or for activating or augmenting an immune response: (a) a molecule that immunospecifically binds CD40,

which molecule increases the binding of CD40 ligand to CD40; (b) CD40 ligand; and (c) a pharmaceutically acceptable carrier. In this embodiment, for example, the molecule can be native mAb S2C6 or native mAb 5C3 or an S2C6 derivative as described herein.

The invention further relates to methods for the treatment or prevention of cancer in a subject, for activating or augmenting an immune response in a subject, or for the treatment or prevention of an immune deficiency or disorder in a subject comprising administering to the subject a therapeutically effective amount of the molecules or antibodies of the invention, e.g., an amount of a molecule comprising SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, or SEQ ID NO:10, which molecule (i) immunospecifically binds CD40, (ii) increases the binding of CD40 ligand to CD40 by at least 45%, and comprises one or more substitutions or insertions in primary amino acid sequence relative to native monoclonal antibody S2C6 as secreted by the hybridoma deposited with the ATCC and assigned accession number PTA-110. In a specific embodiment, the molecule is not native monoclonal antibody S2C6 and does not comprise the native heavy or light chain of said monoclonal antibody S2C6.

The invention further relates to methods for the treatment or prevention of cancer in a subject, for activating or augmenting an immune response in a subject, or for the treatment or prevention of an immune deficiency or disorder in a subject comprising administering to the subject a purified protein, which protein (i) competes for binding to CD40 with monoclonal antibody S2C6 as secreted by the hybridoma deposited with the ATCC and assigned accession number PTA-110, (ii) increases the binding of CD40 ligand to CD40 by at least 45%, and (iii) comprises one or more substitutions or insertions in the primary amino acid sequence relative to native monoclonal antibody S2C6 as deposited with the ATCC and assigned accession number PTA-110, or is not monoclonal antibody S2C6 as secreted by the

hybridoma deposited with the ATCC and assigned accession number PTA-110 and does not result from cleavage of S2C6 with papain or pepsin. In a specific embodiment, the protein is not native monoclonal antibody S2C6 and does not comprise the native heavy or light chain of said monoclonal antibody S2C6.

In specific embodiments, the methods of the invention further comprise administering CD40 ligand to the subject.

The invention further relates to a method for the treatment or prevention of cancer or an immune disorder in a subject comprising administering to the subject, in an amount effective for said treatment or prevention: (a) a molecule that immunospecifically binds CD40, which molecule increases the binding of CD40 ligand to CD40; and (b) CD40 ligand, in which the molecule can be native mAb S2C6 or native mAb 5C3 or any of the S2C6 derivatives described herein.

In a preferred embodiment, the subject is a human.

The invention further relates to a transgenic non-human animal, plant, or an isolated cell containing one or more transgenes encoding a protein, which protein competes for binding to CD40 with monoclonal antibody S2C6 as secreted by the hybridoma deposited with the ATCC and assigned accession number PTA-110, and which protein increases the binding of CD40 ligand to CD40 by at least 45%.

## 4. BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Structure of the light chain variable region of S2C6. The nucleotide (SEQ ID NO:1) and amino acid (SEQ ID NO:2) sequences of the light chain variable region ("$V_L$") are shown.

Figure 2. Structure of the heavy chain variable region of S2C6. The nucleotide (SEQ ID NO:6) and amino acid (SEQ ID NO:7) sequences of the heavy chain variable region ("$V_H$") of S2C6 are shown.

Figures 3A-3B. Structure of variable regions of S2C6. (A) The amino acid sequence (SEQ ID NO:2) of S2C6 $V_L$ is shown. (B) The amino acid sequence (SEQ ID NO:7) of S2C6 $V_H$ is shown. Complementarity-determining regions ("CDR") are underlined.

The sequences of the four framework regions, adjacent to the CDRs, are shown. The amino acid sequences of $V_L$ CDRs 1-3 correspond to SEQ ID NOS:3-5, respectively. The amino acid sequences of $V_H$ CDRs 1-3 correspond to SEQ ID NOS:8-10, respectively.

Figure 4. S2C6 mAb augments CD40-Ig binding to CD40L-expressing Jurkat T cells. CD40-Ig (a soluble fusion protein of CD40 and human immunoglobulin) binding to surface CD40L was done in the presence of increasing concentrations of anti-CD40 monoclonal antibody ("mAb"). mAbs were pre-incubated for 1 hour with CD40-Ig followed by incubation for 1 hour with CD40L-expressing target cells. CD40-Ig binding to target cells was detected by flow cytometry using a fluorescein isothiocyanate ("FITC")-labeled anti-human Ig. The extent of CD40/CD40L binding was then determined from log mean fluorescent intensity ("MFI"). MFI minus background of each population is shown.

Figure 5. S2C6 mAb augments binding of soluble CD40L to B cell surface CD40. Ramos B cells, a human B cell lymphoma, were incubated in the presence of increasing concentrations of an anti-CD40 mAb: S2C6, G28-5, or M3 or an irrelevant control mAb, EXA2-1H8. The mAbs were pre-incubated for 1 hour with CD40-expressing target cells. Binding of the FITC-labeled CD40L to B cells was then detected directly by flow cytometry. The extent of CD40/CD40L binding was then determined from log mean fluorescent intensity. MFI minus background of each population is shown.

Figure 6. S2C6 enhances proliferative response of primary human peripheral B cells in the presence of CD40L$^+$ stimulator cells and an anti-CD40 mAb. Peripheral B cells ($1x10^5$/well) were combined with increasing numbers of non-proliferative CD40L$^+$ Jurkat T stimulator cells and 30 ng/ml of an anti-CD40 mAb: S2C6, G28-5, or M3 or the control mAb, EXA2-1H8. B cell proliferation was measured by $^3$H-TdR incorporation at 72 h following addition of stimulus.

Figure 7. Comparative proliferative response of primary human peripheral B cells to an anti-CD40 mAb in the presence

or absence of CD40L. Peripheral B cells were combined with non-proliferative CD40L$^+$ stimulator cells at a fixed ratio of 4:1 and increasing concentrations of an anti-CD40 mAb: S2C6 G28-5 or the control antibody, EXA2-1H8. B cell proliferation was measured by $^3$H-TdR incorporation at 72 h following addition of stimulus.

Figures 8A-8C. Anti-tumor activity of mAb S2C6 *in vivo*. Anti-tumor activity of S2C6 against (A) Ramos human B cell non-Hodgkin's lymphoma, (B) HS Sultan multiple myeloma, or (C) IM-9 multiple myeloma was assessed. SCID mice (5/group) were pretreated or not with anti-asialo-GM1 to inhibit natural killer ("NK") activity and treated with mAb on day 1 or day 5 following injection of $1x10^6$-$2x10^6$ tumor cells. Solid lines indicate the number of surviving mice over time.

Figure 9. BD1-S2C6 sFv specifically binds to immobilized CD40-Ig in ELISA. BD1-S2C6 sFv (single-chain anti-CD40 immunotoxin consisting of bryodin 1 (BD1) fused to the variable region of monoclonal antibody S2C6) was expressed in E. coli as inclusion bodies, denatured and refolded. The refolded protein was then isolated using Blue Sepharose followed by affinity chromatography over immobilized CD40-Ig. The purified protein was then tested for binding to immobilized CD40-Ig in ELISA. Microtiter plates were coated with CD40-Ig at 0.5 μg/ml followed by the addition of dilutions of purified BD1-S2C6 sFv in the presence of 25 μg/ml S2C6 mAb (▲), 25 μg/ml control antibody BR96 (●), or no excess antibody (■). Binding of BD1-S2C6 sFv to the immobilized receptor was detected by the addition of BD1-specific rabbit antiserum followed by the addition of horseradish peroxidase conjugated goat anti-rabbit Ig. The binding of BD1-S2C6 sFv to CD40-Ig was completely inhibited by the addition of excess S2C6 mAb but not by the addition of the control mAb.

## 5. DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to proteins encoded by and nucleotide sequences of S2C6 genes. The invention further

relates to fragments and other derivatives and analogs of such S2C6 proteins and nucleic acids. In various specific embodiments, the molecules (e.g., antibodies) of the invention comprise all or a portion of mAb S2C6 (the light chain and/or heavy chain, or light chain CDR 1 (SEQ ID NO:3) and/or 2 (SEQ ID NO:4), and/or heavy chain CDR 1 (SEQ ID NO:8), 2 (SEQ ID NO:9), and/or 3 (SEQ ID NO:10), or light chain CDR3 (SEQ ID NO:5) in combination with any of the other CDRs and/or one or more of the four heavy chain and four light chain framework regions, provided that such molecules are not native mAb S2C6 as deposited with the ATCC and assigned accession number PTA-110 or the heavy or light chain thereof. Such molecules may differ from S2C6 in sequence and/or in post-translational modification (glycosylation, amidation, peptide bonding or cross-linking to a non-S2C6 sequence, etc.). In various specific embodiments, a molecule of the invention immunospecifically binds CD40 (or when multimerized immunospecifically binds CD40), competes with native S2C6 for binding to CD40, and/or increases the binding of CD40 ligand to CD40 by at least 45%, 50%, 60% or 65%. Nucleic acids encoding such molecules, e.g., S2C6 fragments or derivatives, are also within the scope of the invention, as well as nucleic acids encoding native mAb S2C6. Production of the foregoing proteins, e.g., by recombinant methods, is provided.

The invention also relates to S2C6 proteins and derivatives including but not limited to fusion/chimeric proteins which are functionally active, i.e., which are capable of displaying one or more known functional activities associated with a full-length S2C6 mAb. Such functional activities include but are not limited to ability to bind CD40, delivery of a stimulatory signal to the CD40 signaling pathway (e.g., so as to cause B cell proliferation), potentiation of the interaction of CD40L with CD40; ability to inhibit tumor growth; and ability to induce an immune response.

Antibodies to CD40 comprising S2C6, its derivatives and analogs including but not limited to humanized antibodies; single chain antibodies; bispecific antibodies; and

antibodies conjugated to chemotherapeutic agents or biological response modifiers, are additionally provided.

The invention further relates to methods of treating or preventing cancer, inflammatory diseases and disorders of the immune system comprising administering a composition of the invention alone or in combination with CD40L.

The invention is illustrated by way of examples set forth in Sections 6-9 below which disclose, *inter alia*, the cloning and characterization of S2C6 genes; the potentiation of the CD40/CD40L interaction; inhibition of tumor growth; and binding of a single-chain anti-CD40 immunotoxin to CD40-Ig.

For clarity of disclosure, and not by way of limitation, the detailed description of the invention is divided into the subsections which follow.

## 5.1 ISOLATION OF S2C6 GENES

The invention relates to the nucleotide sequences of S2C6 nucleic acids. In specific embodiments, S2C6 nucleic acids comprise the cDNA sequences of SEQ ID NOS:1 and 6, or nucleic acids encoding an S2C6 protein (*e.g.*, a protein having the sequence of SEQ ID NOS:2 and 7). The invention provides purified nucleic acids consisting of at least 8 nucleotides (*i.e.*, a hybridizable portion) of an S2C6 gene sequence; in other embodiments, the nucleic acids consist of at least 25 (contiguous) nucleotides, 50 nucleotides, 100, or 200 nucleotides of an S2C6 sequence, or a full-length S2C6 variable region coding sequence. In the same or other embodiments, the nucleic acids are smaller than 50, 75, 100, or 200 or 5000 nucleotides in length. Nucleic acids can be single or double stranded. The invention also relates to nucleic acids hybridizable to or complementary to the foregoing sequences or their reverse complements, and in particular, such nucleic acids that encode proteins that bind to CD40, compete with S2C6 for binding to CD40, and/or increase the binding of CD40 ligand to CD40 by at least 45%, 50%, 60%, or 65%. In specific aspects, nucleic acids are provided which comprise a sequence complementary to at least

10, 25, 50, 100, or 200 nucleotides or the entire coding region of an S2C6 variable region gene.

Nucleic acids encoding derivatives and analogs of S2C6 proteins are additionally provided. As is readily apparent, as used herein, a "nucleic acid encoding a fragment or portion of an S2C6 protein" shall be construed as referring to a nucleic acid encoding only the recited fragment or portion of the S2C6 protein and not the other contiguous portions of the S2C6 protein as a continuous sequence.

## 5.2 CLONING PROCEDURES

Specific embodiments for the cloning of an S2C6 gene follow. In a specific embodiment, total RNA is isolated from a mAb S2C6-producing hybridoma and polymerase chain reaction is used to amplify desired variable region sequences, using primers based on the sequences disclosed herein. For an illustrative example, see Section 6, infra. By way of another example, mRNA is isolated from a mAb S2C6-producing hybridoma, cDNA is made and ligated into an expression vector (*e.g.*, a bacteriophage derivative) such that it is capable of being expressed by the host cell into which it is then introduced. Various screening assays can then be used to select for the expressed product. In one embodiment, selection is on the basis of hybridization to a labeled probe representing a portion of an S2C6 gene or its RNA or a fragment thereof (Benton and Davis, 1977, Science 196:180; Grunstein and Hogness, 1975, Proc. Natl. Acad. Sci. U.S.A. 72:3961). Those DNA fragments with substantial homology to the probe will hybridize. It is also possible to identify the appropriate fragment by restriction enzyme digestion(s) and comparison of fragment sizes with those expected according to a known restriction map if such is available. Further selection can be carried out on the basis of the properties of the gene.

Alternatively, the presence of the desired gene may be detected by assays based on the physical, chemical, or immunological properties of its expressed product. For example, cDNA clones, or DNA clones which hybrid-select the

proper mRNAs, can be selected and expressed to produce a protein that has, *e.g.*, similar or identical electrophoretic migration, isoelectric focusing behavior, proteolytic digestion maps, or functional activity, as known for an S2C6 protein. For example, ability to bind CD40 can be detected in an ELISA (enzyme-linked immunosorbent assay)-type procedure.

An S2C6 gene can also be identified by mRNA selection using nucleic acid hybridization followed by *in vitro* translation. In this procedure, fragments are used to isolate complementary mRNAs by hybridization. Functional assays (*e.g.*, binding to CD40, *etc.*) of the *in vitro* translation products of the isolated products of the isolated mRNAs identifies the mRNA and, therefore, the complementary DNA fragments that contain the desired sequences.

In another embodiment, the S2C6 cDNA can be chemically synthesized from the sequence disclosed herein. Other methods of isolating S2C6 genes known to the skilled artisan can be employed.

The identified and isolated S2C6 gene/cDNA can then be inserted into an appropriate cloning vector. A large number of vector-host systems known in the art may be used. Possible vectors include, but are not limited to, plasmids or modified viruses, but the vector system must be compatible with the host cell used. Such vectors include, but are not limited to, bacteriophages such as lambda derivatives, or plasmids such as PBR322 or pUC plasmid derivatives or the Bluescript vector (Stratagene). The insertion into a cloning vector can, for example, be accomplished by ligating the DNA fragment into a cloning vector which has complementary cohesive termini. However, if the complementary restriction sites used to fragment the DNA are not present in the cloning vector, the ends of the DNA molecules may be enzymatically modified. Alternatively, any site desired may be produced by ligating nucleotide sequences (linkers) onto the DNA termini; these ligated linkers may comprise specific chemically synthesized oligonucleotides encoding restriction

endonuclease recognition sequences. In an alternative method, the cleaved vector and an S2C6 gene may be modified by homopolymeric tailing, or by PCR with primers containing the appropriate sequences. Recombinant molecules can be introduced into host cells via transformation, transfection, infection, electroporation, etc., so that many copies of the gene sequence are generated.

In an alternative method, the desired gene may be identified and isolated after insertion into a suitable cloning vector in a "shot gun" approach. Enrichment for the desired gene, for example, by size fractionization, can be done before insertion into the cloning vector.

In specific embodiments, transformation of host cells with recombinant DNA molecules that incorporate an isolated S2C6 gene, cDNA, or synthesized DNA sequence enables generation of multiple copies of the gene. Thus, the gene may be obtained in large quantities by growing transformants, isolating the recombinant DNA molecules from the transformants and, when necessary, retrieving the inserted gene from the isolated recombinant DNA.

The S2C6 sequences provided by the instant invention include those nucleotide sequences encoding substantially the same amino acid sequences as found in native S2C6 variable regions, and those encoded amino acid sequences with functionally equivalent amino acids, as well as those encoding other S2C6 derivatives or analogs, as described below for S2C6 derivatives and analogs.

## 5.3 EXPRESSION OF S2C6 GENES

The nucleotide sequence coding for an S2C6 protein or a functionally active analog or fragment or other derivative thereof (*see* Section 5.6), can be inserted into an appropriate expression vector, *i.e.*, a vector which contains the necessary elements for the transcription and translation of the inserted protein-coding sequence. The necessary transcriptional and translational signals can also be supplied by the native S2C6 gene and/or its flanking regions.

A variety of host-vector systems may be utilized to express the protein-coding sequence. These include but are not limited to mammalian cell systems infected with virus (*e.g.*, vaccinia virus, adenovirus, *etc.*); insect cell systems infected with virus (*e.g.*, baculovirus); microorganisms such as yeast containing yeast vectors, or bacteria transformed with bacteriophage, DNA, plasmid DNA, or cosmid DNA; transgenic plants or transgenic non-human animals. The expression elements of vectors vary in their strengths and specificities. Depending on the host-vector system utilized, any one of a number of suitable transcription and translation elements may be used.

Any of the methods previously described for the insertion of DNA fragments into a vector may be used to construct expression vectors containing a chimeric gene consisting of appropriate transcriptional/translational control signals and the protein coding sequences. These methods may include *in vitro* recombinant DNA and synthetic techniques and *in vivo* recombinants (genetic recombination). Expression of a nucleic acid sequence encoding an S2C6 protein or peptide fragment may be regulated by a second nucleic acid sequence so that the S2C6 protein or peptide is expressed in a host transformed with the recombinant DNA molecule. For example, expression of an S2C6 protein may be controlled by any promoter/enhancer element known in the art. Promoters that are not native S2C6 gene promoters which may be used to control S2C6 gene expression include, but are not limited to, the SV40 early promoter region (Benoist and Chambon, 1981, Nature 290:304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto et al., 1980, Cell 22:787-797), the herpes thymidine kinase promoter (Wagner et al., 1981, Proc. Natl. Acad. Sci. U.S.A. 78:1441-1445), the regulatory sequences of the metallothionein gene (Brinster et al., 1982, Nature 296:39-42); prokaryotic expression vectors such as the β-lactamase promoter (Villa-Kamaroff et al., 1978, Proc. Natl. Acad. Sci. U.S.A. 75:3727-3731), or the *lac* promoter (DeBoer et al.,

1983, Proc. Natl. Acad. Sci. U.S.A. 80:21-25); *see also* "Useful proteins from recombinant bacteria" in Scientific American, 1980, 242:74-94; plant expression vectors comprising the nopaline synthetase promoter region (Herrera-Estrella et al., Nature 303:209-213) or the cauliflower mosaic virus 35S RNA promoter (Gardner et al., 1981, Nucl. Acids Res. 9:2871), and the promoter of the photosynthetic enzyme ribulose biphosphate carboxylase (Herrera-Estrella et al., 1984, Nature 310:115-120); promoter elements from yeast or other fungi such as the Gal 4 promoter, the ADC (alcohol dehydrogenase) promoter, PGK (phosphoglycerol kinase) promoter, alkaline phosphatase promoter, and the following animal transcriptional control regions, which exhibit tissue specificity and have been utilized in transgenic animals: elastase I gene control region which is active in pancreatic acinar cells (Swift et al., 1984, Cell 38:639-646; Ornitz et al., 1986, Cold Spring Harbor Symp. Quant. Biol. 50:399-409; MacDonald, 1987, Hepatology 7:425-515); a gene control region which is active in pancreatic beta cells (Hanahan, 1985, Nature 315:115-122), an immunoglobulin gene control region which is active in lymphoid cells (Grosschedl et al., 1984, Cell 38:647-658; Adames et al., 1985, Nature 318:533-538; Alexander et al., 1987, Mol. Cell. Biol. 7:1436-1444), mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder et al., 1986, Cell 45:485-495), albumin gene control region which is active in liver (Pinkert et al., 1987, Genes and Devel. 1:268-276), alpha-fetoprotein gene control region which is active in liver (Krumlauf et al., 1985, Mol. Cell. Biol. 5:1639-1648; Hammer et al., 1987, Science 235:53-58; alpha 1-antitrypsin gene control region which is active in the liver (Kelsey et al., 1987, Genes and Devel. 1:161-171), beta-globin gene control region which is active in myeloid cells (Mogram et al., 1985, Nature 315:338-340; Kollias et al., 1986, Cell 46:89-94; myelin basic protein gene control region which is active in oligodendrocyte cells in the brain (Readhead et al., 1987, Cell 48:703-712); myosin light chain-

2 gene control region which is active in skeletal muscle (Sani, 1985, Nature 314:283-286), and gonadotropic releasing hormone gene control region which is active in the hypothalamus (Mason et al., 1986, Science 234:1372-1378).

In a specific embodiment, a vector is used that comprises a promoter operably linked to an S2C6 gene nucleic acid, one or more origins of replication, and, optionally, one or more selectable markers (*e.g.*, an antibiotic resistance gene).

Expression vectors containing S2C6 gene inserts can be identified by three general approaches: (a) nucleic acid hybridization; (b) presence or absence of "marker" gene functions; and (c) expression of inserted sequences. In the first approach, the presence of an S2C6 gene inserted in an expression vector can be detected by nucleic acid hybridization using probes comprising sequences that are homologous to an inserted S2C6 gene. In the second approach, the recombinant vector/host system can be identified and selected based upon the presence or absence of certain "marker" gene functions (*e.g.*, thymidine kinase activity, resistance to antibiotics, transformation phenotype, occlusion body formation in baculovirus, *etc.*) caused by the insertion of an S2C6 gene in the vector. For example, if the S2C6 gene is inserted within the marker gene sequence of the vector, recombinants containing the S2C6 insert can be identified by the absence of the marker gene function. In the third approach, recombinant expression vectors can be identified by assaying the S2C6 product expressed by the recombinant. Such assays can be based, for example, on the physical or functional properties of the S2C6 protein in *in vitro* assay systems, *e.g.*, potentiation of CD40L binding with CD40; stimulation of proliferation of normal B cells; inhibition of tumor growth.

Once a particular recombinant DNA molecule is identified and isolated, several methods known in the art may be used to propagate it. Once a suitable host system and growth conditions are established, recombinant expression vectors

can be propagated and prepared in quantity. As previously explained, the expression vectors which can be used include, but are not limited to, the following vectors or their derivatives: human or animal viruses such as vaccinia virus or adenovirus; insect viruses such as baculovirus; yeast vectors; bacteriophage vectors (e.g., lambda phage), and plasmid and cosmid DNA vectors, to name but a few.

In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Expression from certain promoters can be elevated in the presence of certain inducers; thus, expression of the genetically engineered S2C6 protein may be controlled. Furthermore, different host cells have characteristic and specific mechanisms for the translational and post-translational processing and modification (e.g., glycosylation, phosphorylation of proteins. Appropriate cell lines or host systems can be chosen to ensure the desired modification and processing of the foreign protein expressed. For example, expression in a bacterial system can be used to produce a non-glycosylated core protein product. Expression in yeast will produce a glycosylated product. Expression in mammalian cells can be used to ensure "native" glycosylation of a heterologous protein. Furthermore, different vector/host expression systems may effect processing reactions to different extents.

In specific embodiments, the S2C6-related protein that is expressed is an antibody or fragment or derivative thereof. The recombinant antibody may contain a recombinant light chain variable domain, a recombinant heavy chain variable domain, or both. In a specific embodiment, both light and heavy chains or derivatives thereof are recombinantly expressed by a cell (see e.g., U.S. Patent No. 4,816,397 dated March 28, 1989 by Boss et al.) A variety of host-vector systems may be utilized to express the protein-coding sequence. These include but are not limited to mammalian cell systems infected with virus (e.g., vaccinia

virus, adenovirus, etc.); insect cell systems infected with virus (e.g., baculovirus); microorganisms such as yeast containing yeast vectors, or bacteria transformed with bacteriophage, DNA, plasmid DNA, or cosmid DNA; transgenic plants or transgenic non-human animals.

5.4 IDENTIFICATION AND PURIFICATION OF GENE PRODUCTS

In particular aspects, the invention provides amino acid sequences of S2C6 proteins and fragments and derivatives thereof which comprise a complementarity-determining region (CDR) or which are otherwise functionally active, as well as nucleic acid sequences encoding the foregoing. "Functionally active" S2C6 material as used herein refers to that material displaying one or more functional activities associated with a full-length (native) S2C6 protein, e.g., binding to CD40; stimulation of proliferation of normal B cells; inhibition of tumor growth; increase the binding of CD40 ligand to CD40 by at least 45%.

In specific embodiments, the invention provides fragments of an S2C6 protein consisting of at least 6 amino acids, 10 amino acids, 20 amino acids, 50 amino acids, 75 amino acids or of at least 100 amino acids and nucleic acids encoding the foregoing.

Once a recombinant which expresses the S2C6 gene sequence is identified, the gene product can be analyzed. This is achieved by assays based on the physical or functional properties of the product, including radioactive labeling of the product followed by analysis by gel electrophoresis, immunoassay; stimulation of proliferation of normal B cells; CD40 binding assays, promotion of the binding of CD40 ligand to CD40, inhibition of tumor growth, etc.

Once the S2C6 protein is identified, it may be isolated and purified by standard methods including chromatography (e.g., ion exchange, affinity, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for the purification of proteins. The functional properties may be evaluated using any suitable assay (see Section 5.7).

Alternatively, the S2C6 protein or derivative thereof can be synthesized by standard chemical methods known in the art based on the sequence disclosed herein (*e.g.*, *see* Hunkapiller et al., 1984, Nature 310:105-111).

In a specific embodiment of the present invention, such S2C6 proteins, whether produced by recombinant DNA techniques or by chemical synthetic methods or by purification of native proteins, include but are not limited to those containing, as a primary amino acid sequence, all or part of the amino acid sequence substantially as depicted in Figures 3A-3B (SEQ ID NOS:2 and 7), as well as fragments and other derivatives, and analogs thereof, including proteins homologous thereto.

## 5.5 STRUCTURE OF S2C6 GENES AND PROTEINS

The structure of S2C6 genes and proteins of the invention can be analyzed by various methods known in the art. Some examples of such methods are described below.

### 5.5.1 GENETIC ANALYSIS

The cloned DNA or cDNA corresponding to an S2C6 gene can be analyzed by methods including but not limited to Southern hybridization (Southern, 1975, J. Mol. Biol. 98:503-517), Northern hybridization (*see e.g.*, Freeman et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:4094-4098), restriction endonuclease mapping (Maniatis, 1982, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York), and DNA sequence analysis. Accordingly, this invention provides nucleic acid probes recognizing an S2C6 gene. For example, polymerase chain reaction (PCR; U.S. Patent Nos. 4,683,202, 4,683,195 and 4,889,818; Gyllenstein et al., 1988, Proc. Natl. Acad. Sci. U.S.A. 85:7652-7656; Ochman et al., 1988, Genetics 120:621-623; Loh et al., 1989, Science 243:217-220) followed by Southern hybridization with an S2C6 gene-specific probe can allow the detection of an S2C6 gene in DNA or cDNA from a cell (e.g., hybridoma). Methods of amplification other than PCR are commonly known and can also be employed. The stringency of the hybridization conditions for both Southern

and Northern hybridization can be manipulated to ensure detection of nucleic acids with the desired degree of relatedness to the specific S2C6 gene probe used. Modifications of these methods and other methods commonly known in the art can be used.

Restriction endonuclease mapping can be used to roughly determine the genetic structure of an S2C6 gene. Restriction maps derived by restriction endonuclease cleavage can be confirmed by DNA sequence analysis.

DNA sequence analysis can be performed by any techniques known in the art, including but not limited to the method of Maxam and Gilbert (1980, Meth. Enzymol. 65:499-560), the Sanger dideoxy method (Sanger et al., 1977, Proc. Natl. Acad. Sci. U.S.A. 74:5463), the use of T7 DNA polymerase (Tabor and Richardson, U.S. Patent No. 4,795,699), or use of an automated DNA sequenator (*e.g.*, Applied Biosystems, Foster City, California).

### 5.5.2 PROTEIN ANALYSIS

The amino acid sequence of an S2C6 protein can be derived by deduction from the DNA sequence, or alternatively, by direct sequencing of the protein, *e.g.*, with an automated amino acid sequencer.

An S2C6 protein sequence can be further characterized by a hydrophilicity analysis (Hopp and Woods, 1981, Proc. Natl. Acad. Sci. U.S.A. 78:3824). A hydrophilicity profile can be used to identify the hydrophobic and hydrophilic (potentially immunogenic) regions of the S2C6 protein and the corresponding regions of the gene sequence which encode such regions.

Secondary, structural analysis (Chou and Fasman, 1974, Biochemistry 13:222) can also be done, to identify regions of an S2C6 protein that assume specific secondary structures.

Manipulation, translation, and secondary structure prediction, open reading frame prediction and plotting, as well as determination of sequence homologies, can also be accomplished using computer software programs available in the art.

## 5.6 mAb S2C6 ANTIBODY DERIVATIVES

Described herein are methods for the production of S2C6 antibody derivatives capable of immunospecifically binding CD40.

Such antibodies include but are not limited to monoclonal, humanized, chimeric, single chain, bispecific, Fab fragments, $F(ab')_2$ fragments, fragments produced by a Fab expression library, anti-idiotypic (anti-Id) antibodies, and epitope-binding fragments of any of the above. In one embodiment, the S2C6 derivative comprises one or more deletions, additions and/or substitutions in primary amino acid sequence relative to the primary amino acid sequence of S2C6. In another embodiment, the S2C6 derivative does not result from cleavage of S2C6 with papain or pepsin. In yet another embodiment, the S2C6 derivative comprises one or more deletions, additions and/or substitutions in primary amino acid sequence relative to the primary amino acid sequence of S2C6 and does not result from cleavage of S2C6 with papain or pepsin. Guidance for selection of suitable deletions, additions and/or substitutions is provided in this section and in Section 5.7, *infra*.

For preparation of additional monoclonal antibodies to CD40, any technique that provides for the production of antibody molecules by continuous cell lines in culture may be used. These include but are not limited to the hybridoma technique of Kohler and Milstein, (1975, Nature 256, 495-497; and U.S. Patent No. 4,376,110), the human B-cell hybridoma technique (Kozbor *et al.*, 1983, Immunology Today 4, 72; Cole *et al.*, 1983, Proc. Natl. Acad. Sci. USA 80, 2026-2030), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole *et al.*, 1985, Monoclonal Antibodies And Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). Such antibodies or other anti-CD40 antibodies available in the art may, e.g., be used as the basis from which to clone and thus supply a complementary light chain if a S2C6 heavy chain is to be recombinantly expressed (the two chains may be recombinantly expressed in the same cell or combined in vitro

after separate expression and purification); alternatively, a light chain from an antibody of any specificity may be used. Nucleic acids (e.g., a plasmid) encoding a S2C6 heavy chain or encoding a molecule comprising a S2C6 heavy chain variable domain can be transfected into a cell expressing an antibody light chain or molecule comprising an antibody light chain, for expression of a multimeric protein; the antibody light chain can be recombinant or non-recombinant, and may or may not have anti-CD40 specificity. Alternatively, S2C6 heavy chains or molecules comprising the variable region thereof or a CDR thereof can optionally be expressed and used without the presence of a complementary light chain or light chain variable region. In various embodiments, the invention provides a S2C6 heavy chain with CD40 binding affinity, or a molecule consisting of or (alternatively) comprising one or more copies of heavy chain CDR 8, 9, and/or 10, or a protein (peptide or polypeptide) the sequence of which consists of, or comprises, one or more copies of CDR 8, 9 or 10. In a specific embodiment, such a protein can be N or C-terminal modified, e.g., by C-terminal amidation or N-terminal acetylation.

In addition, techniques developed for the production of "chimeric antibodies" (Morrison, et al., 1984, Proc. Natl. Acad. Sci., 81, 6851-6855; Neuberger, et al., 1984, Nature 312, 604-608; Takeda, et al., 1985, Nature 314, 452-454) by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can be used. A chimeric antibody is a molecule in which different portions are derived from different animal species, such as those having a variable region derived from a murine mAb and a human immunoglobulin constant region. (See, e.g., Cabilly et al., U.S. Patent No. 4,816,567; and Boss et al., U.S. Patent No. 5,816,397.) In a specific embodiment, the chimeric antibody comprises a variable domain of monoclonal antibody S2C6 secreted by the hybridoma as deposited with the ATCC and assigned accession number PTA-110, and a human

constant region. In specific embodiments the variable domain of the chimeric antibody comprises the S2C6 $V_L$ (SEQ ID NO:2) as depicted in Figure 3A and/or the S2C6 $V_H$ (SEQ ID NO:7) as depicted in Figure 3B.

In addition, techniques have been developed for the production of humanized antibodies. (See, *e.g.*, Queen, U.S. Patent No. 5,585,089 and Winter, U.S. Patent No. 5,225,539.) An immunoglobulin light or heavy chain variable region consists of a "framework" region interrupted by three hypervariable regions, referred to as complementarity-determining regions (CDRs). The extent of the framework region and CDRs have been precisely defined (see, "Sequences of Proteins of Immunological Interest", Kabat, E. et al., U.S. Department of Health and Human Services (1983). Briefly, humanized antibodies are antibody molecules from non-human species having one or more CDRs from the non-human species and framework regions from a human immunoglobulin molecule.

The invention encompasses an antibody or derivative thereof comprising a heavy or light chain variable domain, said variable domain comprising (a) a set of three complementarity-determining regions (CDRs), in which said set of CDRs are from monoclonal antibody S2C6, and (b) a set of four framework regions, in which said set of framework regions differs from the set of framework regions in monoclonal antibody S2C6, and in which said antibody or derivative thereof immunospecifically binds CD40. Preferably, the set of framework regions is from a human monoclonal antibody, e.g., a human monoclonal antibody that does not bind CD40.

In a specific embodiment, the invention encompasses an antibody or derivative thereof comprising a light chain variable domain, said variable domain comprising (a) a set of three complementarity-determining regions (CDRs), in which said set of CDRs comprises SEQ ID NO:3 or SEQ ID NO:4, and (b) a set of four framework regions, in which said set of framework regions differs from the set of framework regions

in the light chain of monoclonal antibody S2C6, and in which said antibody or derivative thereof immunospecifically binds CD40.

In a specific embodiment, the invention encompasses an antibody or derivative thereof comprising a heavy chain variable domain, said variable domain comprising (a) a set of three complementarity-determining regions (CDRs), in which said set of CDRs comprises SEQ ID NO:8, SEQ ID NO:9, or SEQ ID NO:10, and (b) a set of four framework regions, in which said set of framework regions differs from the set of framework regions in the heavy chain of monoclonal antibody S2C6, and in which said antibody or derivative thereof immunospecifically binds CD40.

Alternatively, techniques described for the production of single chain antibodies (U.S. Patent 4,946,778; Bird, 1988, Science 242, 423-426; Huston, et al., 1988, Proc. Natl. Acad. Sci. USA 85, 5879-5883; and Ward, et al., 1989, Nature 334, 544-546) can be adapted to produce single chain antibodies using S2C6 sequences. Single chain antibodies are formed by linking the heavy and light chain fragments of the Fv region via an amino acid bridge, resulting in a single chain polypeptide. In a specific embodiment, the single chain antibody comprises the amino acid sequences as depicted in Figure 3A and 3B (SEQ ID NOS:2 and 7, respectively).

In a specific embodiment, the antibody to a CD40 polypeptide, peptide or other derivative, or analog thereof comprising all or a portion of SEQ ID NO:1 or SEQ ID NO:6 is a bispecific antibody (see generally, *e.g.* Fanger and Drakeman, 1995, *Drug News and Perspectives* 8: 133-137). Such a bispecific antibody is genetically engineered to recognize both (1) an epitope and (2) one of a variety of "trigger" molecules, *e.g.* Fc receptors on myeloid cells, and CD3 and CD2 on T cells, that have been identified as being able to cause a cytotoxic T-cell to destroy a particular target. Such bispecific antibodies can be prepared either by chemical conjugation, hybridoma, or recombinant molecular biology techniques known to the skilled artisan. In a specific

embodiment, the bispecific antibody contains a molecule comprising the S2C6 heavy or light chain variable domain or a CDR sequence thereof, which molecule has the structure of an antibody heavy or light chain but which differs from the native S2C6 heavy or light chain (e.g., by having amino acid substitution(s) in the framework region or a human constant domain).

Antibody fragments that retain the ability to recognize CD40 may be generated by known techniques. For example, such fragments include but are not limited to: The $F(ab')_2$ fragments, which can be produced by pepsin digestion of the antibody molecule and the F(ab') fragments, which can be generated by reducing the disulfide bridges of the $F(ab')_2$ fragments. Alternatively, Fab expression libraries may be constructed (Huse, et al., 1989, Science 246, 1275-1281) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity.

## 5.7 S2C6 PROTEINS, DERIVATIVES AND ANALOGS

In addition to those antibody molecules/variants described in Section 5.6 above, the invention further relates to S2C6 proteins, derivatives (including but not limited to fragments), analogs, and molecules of S2C6 proteins. Nucleic acids encoding S2C6 protein derivatives and protein analogs are also provided. In one embodiment, the S2C6 proteins are encoded by the nucleic acids described in Section 5.1 above. In particular aspects, the proteins, derivatives, or analogs are encoded by the sequence of SEQ ID NO:1 or SEQ ID NO:6.

The production and use of derivatives and analogs related to an S2C6 protein are within the scope of the present invention. In a specific embodiment, the derivative or analog is functionally active, *i.e.*, capable of exhibiting one or more functional activities associated with a full-length, S2C6 protein. As one example, such derivatives or analogs which have the desired binding specificity can be used in immunoassays, or therapeutically for inhibition of tumor growth, etc. A specific embodiment relates to an S2C6

protein fragment that binds CD40 and potentiates binding of CD40L to CD40. Derivatives or analogs of an S2C6 protein can be tested for the desired activity by various immunoassays known in the art, including but not limited to competitive and non-competitive assay systems using techniques such as radioimmunoassays, enzyme linked immunosorbent assay (ELISA), "sandwich" immunoassays, Western blots, immunofluorescence assays, protein A assays, immunoelectrophoretic assays, etc.

In addition, assays known in the art can be used to detect or measure the ability to inhibit cell proliferation (*e.g.*, inhibition of tumor cell growth) or ability to stimulate cell proliferation (*e.g.*, proliferation of B cells) *in vivo* or *in vitro*.

In particular, S2C6 derivatives can be made by altering S2C6 sequences by substitutions, additions (*e.g.*, insertions) or deletions that provide for functionally equivalent molecules. Due to the degeneracy of nucleotide coding sequences, other DNA sequences which encode substantially the same amino acid sequence as an S2C6 gene may be used in the practice of the present invention. These include but are not limited to nucleotide sequences comprising all or portions of an S2C6 gene which is altered by the substitution of different codons that encode a functionally equivalent amino acid residue within the sequence, thus producing a silent change. Likewise, the S2C6 derivatives of the invention include, but are not limited to, those containing, as a primary amino acid sequence, all or part of the amino acid sequence of an S2C6 protein including altered sequences in which functionally equivalent amino acid residues are substituted for residues within the sequence resulting in a silent change. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity which acts as a functional equivalent, resulting in a silent alteration. Substitutions for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine,

leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. Such substitutions are generally understood to be conservative substitutions.

In a specific embodiment of the invention, proteins consisting of or comprising a fragment of an S2C6 protein consisting of at least 10 (continuous) amino acids of the S2C6 protein is provided. In other embodiments, the fragment consists of at least 20 or at least 50 amino acids of the S2C6 protein. In specific embodiments, such fragments are not larger than 50, 75, 100, or 200 amino acids. Derivatives or analogs of S2C6 proteins include but are not limited to those molecules comprising regions that are substantially homologous to an S2C6 protein or fragment thereof (e.g., in various embodiments, at least 60% or 70% or 80% or 90% or 95% identity over an amino acid sequence of identical size with no insertions or deletions or when compared to an aligned sequence in which the alignment is done by a computer homology program known in the art) or whose encoding nucleic acid is capable of hybridizing to a coding S2C6 gene sequence, under high stringency, moderate stringency, or low stringency conditions.

Specifically, by way of example computer programs for determining homology may include but are not limited to TBLASTN, BLASTP, FASTA, TEASTA, and CLUSTALW (Pearson and Lipman, 1988, Proc. Natl. Acad. Sci. USA 85(8):2444-8; Altschul et al., 1990, J. Mol. Biol. 215(3):403-10; Thompson, et al., 1994, Nucleic Acids Res. 22(22):4673-80; Higgins, et al., 1996, Methods Enzymol 266:383-402; Altschul, et al., 1990, J. Mol. Biol. 215(3):403-10). Default parameters for each of these computer programs are well known and can be utilized.

Specifically, Basic Local Alignment Search Tool (BLAST) (www.ncbi.nlm.nih.gov) (Altschul et al., 1990, J. of Molec. Biol., 215:403-410, "The BLAST Algorithm; Altschul et al., 1997, Nuc. Acids Res. 25:3389-3402) is a heuristic search algorithm tailored to searching for sequence similarity which ascribes significance using the statistical methods of Karlin and Altschul 1990, Proc. Natl Acad. Sci. USA, 87:2264-68; 1993, Proc. Nat'l Acad. Sci. USA 90:5873-77. Five specific BLAST programs perform the following tasks: 1) The BLASTP program compares an amino acid query sequence against a protein sequence database; 2) The BLASTN program compares a nucleotide query sequence against a nucleotide sequence database; 3) The BLASTX program compares the six-frame conceptual translation products of a nucleotide query sequence (both strands) against a protein sequence database; 4) The TBLASTN program compares a protein query sequence against a nucleotide sequence database translated in all six reading frames (both strands); 5) The TBLASTX program compares the six-frame translations of a nucleotide query sequence against the six-frame translations of a nucleotide sequence database.

Smith-Waterman (database: European Bioinformatics Institute wwwz.ebi.ac.uk/bic_sw/) (Smith-Waterman, 1981, J. of Molec. Biol., 147:195-197) is a mathematically rigorous algorithm for sequence alignments.

FASTA (see Pearson et al., 1988, Proc. Nat'l Acad. Sci. USA, 85:2444-2448) is a heuristic approximation to the Smith-Waterman algorithm. For a general discussion of the procedure and benefits of the BLAST, Smith-Waterman and FASTA algorithms see Nicholas et al., 1998, "A Tutorial on Searching Sequence Databases and Sequence Scoring Methods" (www.psc.edu) and references cited therein.

The S2C6 derivatives and analogs of the invention can be produced by various methods known in the art. The manipulations which result in their production can occur at the gene or protein level. For example, a cloned S2C6 gene sequence can be modified by any of numerous strategies known

in the art (Sambrook et al., 1989, *Molecular Cloning, A Laboratory Manual*, 2d ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York). The sequence can be cleaved at appropriate sites with restriction endonuclease(s), followed by further enzymatic modification if desired, isolated, and ligated *in vitro*. In the production of a modified gene encoding a derivative or analog of the S2C6 protein, care should be taken to ensure that the modified gene remains within the same translational reading frame as the native protein, uninterrupted by translational stop signals, in the gene region where the desired S2C6 protein activity is encoded.

Additionally, an S2C6 nucleic acid sequence can be mutated *in vitro or in vivo*, to create and/or destroy translation, initiation, and/or termination sequences, or to create variations in coding regions and/or to form new restriction endonuclease sites or destroy preexisting ones, to facilitate further *in vitro* modification. Any technique for mutagenesis known in the art can be used, including but not limited to, chemical mutagenesis, *in vitro* site-directed mutagenesis (Hutchinson et al., 1978, J. Biol. Chem. 253:6551), PCR with primers containing a mutation, etc.

Manipulations of an S2C6 protein sequence may also be made at the protein level. Included within the scope of the invention are S2C6 protein fragments or other derivatives or analogs which are differentially modified during or after translation, *e.g.*, by glycosylation, acetylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to an antibody molecule or other cellular ligand, etc. Any of numerous chemical modifications may be carried out by known techniques, including but not limited to specific chemical cleavage by cyanogen bromide, trypsin, chymotrypsin, papain, V8 protease, $NaBH_4$, acetylation, formylation, oxidation, reduction, metabolic synthesis in the presence of tunicamycin, etc.

In addition, analogs and derivatives of an S2C6 protein can be chemically synthesized. For example, a peptide corresponding to a portion of an S2C6 protein which comprises the desired domain, or which mediates the desired activity *in vitro*, can be synthesized by use of a peptide synthesizer. Furthermore, if desired, nonclassical amino acids or chemical amino acid analogs can be introduced as a substitution or addition into the S2C6 sequence. Non-classical amino acids include but are not limited to the D-isomers of the common amino acids, α-amino isobutyric acid, 4-aminobutyric acid, Abu, 2-amino butyric acid, γ-Abu, ε-Ahx, 6-amino hexanoic acid, Aib, 2-amino isobutyric acid, 3-amino propionic acid, ornithine, norleucine, norvaline, hydroxyproline, sarcosine, citrulline, cysteic acid, tbutyiglycine, t-butylalanine, phenyiglycine, cyclohexylalanine, β-alanine, fluoro-amino acids, designer amino acids such as β-methyl amino acids, Cα-methyl amino acids, Nα-methyl amino acids, and amino acid analogs in general. Furthermore, the amino acid can be D (dextrorotary) or L (levorotary).

In other specific embodiments, the S2C6 protein, fragment, analog, or derivative may be expressed as a fusion, or chimeric protein product (comprising the protein, fragment, analog, or derivative joined via a peptide bond to a heterologous protein sequence of a different protein). The heterologous protein sequence can comprise a biological response modifier, including but not limited to interferon-α, interferon γ, interleukin-2, interleukin-4, interleukin-6, and tumor necrosis factor, or a functionally active portion thereof. Alternatively, the heterologous protein sequence can comprise enzymes such as β-lactamase or carboxylesterases or toxins such as bryodin 1, Pseudomonas exotoxin A, or gelonin, or a functionally active portion thereof. Additionally, the S2C6 protein can be chemically linked to chemotherapeutic agents, including but not limited to alkylating agents (*e.g.* nitrogen mustards, nitrosoureas, triazenes); antimetabolites (*e.g.* folic acid analogs, pyrimidine analogs, purine analogs); natural products (*e.g.* antibiotics, enzymes, biological response modifiers);

miscellaneous agents (*e.g.* substituted urea, platinum coordination complexes); and hormones and antagonists (*e.g.* estrogens, androgens, antiandrogen, gonadotropin releasing hormone analog); or functionally active portion thereof (see, *e.g.*, Goodman and Gilman, The Pharmacological Basis of Therapeutics, Ninth Edition, McGraw-Hill, pp. 1225-1287, 1996). Such a chimeric product can be made by ligating the appropriate nucleic acid sequences encoding the desired amino acid sequences to each other by methods known in the art, in the proper coding frame, and expressing the chimeric product by methods commonly known in the art. Alternatively, such a chimeric product may be made by protein synthetic techniques, *e.g.*, by use of a peptide synthesizer. In different embodiments, the heterologous protein sequence can be covalently bound to the S2C6-related sequences by other than a peptide bond, e.g., by use of chemical crosslinking agents well known in the art.

In a specific embodiment, an S2C6 protein derivative is a chimeric or fusion protein comprising an S2C6 protein or fragment thereof (preferably consisting of at least a domain or motif of the S2C6 protein, or at least 10, 50 or 100 amino acids of the S2C6 protein) joined at its amino- or carboxy-terminus via a peptide bond to an amino acid sequence of a different protein. In a specific embodiment, the different protein is a toxin, enzyme or biological response modifier.

In specific embodiments, the amino acid sequence of the different protein is at least 6, 10, 20 or 30 continuous amino acids of the different protein or a portion of the different protein that is functionally active. In one embodiment, such a chimeric protein is produced by recombinant expression of a nucleic acid encoding the protein (comprising an S2C6-coding sequence joined in-frame to a coding sequence for a different protein). Such a chimeric product can be made by ligating the appropriate nucleic acid sequences encoding the desired amino acid sequences to each other by methods known in the art, in the proper coding frame, and expressing the chimeric product by methods commonly known in the art. Alternatively, such a chimeric

product may be made by protein synthetic techniques, *e.g.*, by use of a peptide synthesizer. Chimeric genes comprising portions of an S2C6 gene fused to any heterologous protein-encoding sequences may be constructed. A specific embodiment relates to a chimeric protein comprising a fragment of an S2C6 protein of at least 6 or 15 or 50 amino acids, or a fragment that displays one or more functional activities of the S2C6 protein (e.g., comprising copies of one or more CDRs).

In a specific embodiment, the S2C6 protein or derivative thereof is chemically linked to a chemotherapeutic drug including but not limited to doxorubicin, paclitaxel or docetaxel. Such a S2C6-drug conjugate can deliver the drug to cells expressing CD40. One or more drug molecules can be linked to the S2C6 protein or derivative. Linkages include but are not limited to hydrazone, peptide or carbohydrate linkages.

In another specific embodiment, the derivative is a molecule comprising a region of homology with an S2C6 protein. By way of example, in various embodiments, a first protein region can be considered "homologous" to a second protein region when the amino acid sequence of the first region is at least 30%, 40%, 50%, 60%, 70%, 75%, 80%, 90%, or 95% identical, when compared to any sequence in the second region of an equal number of amino acids as the number contained in the first region (without any insertions or deletions) or when compared to an aligned sequence of the second region that has been aligned by a computer homology program known in the art.

## 5.8 HYBRIDIZATION CONDITIONS

In a specific embodiment, a nucleic acid which is hybridizable to an S2C6 nucleic acid (*e.g.*, having a sequence as set forth in SEQ ID NOS:1 or 6), or to its reverse complement, or to a nucleic acid encoding an S2C6 derivative, or to its reverse complement under conditions of low stringency is provided. By way of example and not limitation, procedures using such conditions of low

stringency are as follows (*see also* Shilo and Weinberg, 1981, Proc. Natl. Acad. Sci. U.S.A. 78, 6789-6792). Filters containing DNA are pretreated for 6 h at 40°C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.1% PVP, 0.1% Ficoll, 1% BSA, and 500 μg/ml denatured salmon sperm DNA. Hybridizations are carried out in the same solution with the following modifications: 0.02% PVP, 0.02% Ficoll, 0.2% BSA, 100 μg/ml salmon sperm DNA, 10% (wt/vol) dextran sulfate, and 5-20 X $10^6$ cpm $^{32}$P-labeled probe is used. Filters are incubated in hybridization mixture for 18-20 h at 40°C, and then washed for 1.5 h at 55°C in a solution containing 2X SSC, 25 mM Tris-HCl (pH 7.4), 5 mM EDTA, and 0.1% SDS. The wash solution is replaced with fresh solution and incubated an additional 1.5 h at 60°C. Filters are blotted dry and exposed for autoradiography. If necessary, filters are washed for a third time at 65-68°C and re-exposed to film. Other conditions of low stringency which may be used are well known in the art (*e.g.*, as employed for cross-species hybridizations).

In a specific embodiment, a nucleic acid which is hybridizable to an S2C6 nucleic acid (*e.g.*, having a sequence as set forth in SEQ ID NOS:1 or 6), or to its reverse complement, or to a nucleic acid encoding an S2C6 derivative, or to its reverse complement under conditions of high stringency is provided. By way of example and not limitation, procedures using such conditions of high stringency are as follows. Prehybridization of filters containing DNA is carried out for 8 h to overnight at 65°C in buffer composed of 6X SSC, 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.02% BSA, and 500 μg/ml denatured salmon sperm DNA. Filters are hybridized for 48 h at 65°C in prehybridization mixture containing 100 μg/ml denatured salmon sperm DNA and 5-20 X $10^6$ cpm of $^{32}$P-labeled probe. Washing of filters is done at 37°C for 1 h in a solution containing 2X SSC, 0.01% PVP, 0.01% Ficoll, and 0.01% BSA. This is followed by a wash in 0.1X SSC at 50°C for 45 min before autoradiography. Other conditions of high stringency which may be used are well known in the art.

In a specific embodiment, a nucleic acid which is hybridizable to an S2C6 nucleic acid (*e.g.*, having a sequence as set forth in SEQ ID NOS:1 or 6), or to its reverse complement, or to a nucleic acid encoding an S2C6 derivative, or to its reverse complement under conditions of moderate stringency is provided. Selection of appropriate conditions for such stringencies is well known in the art (*see e.g.*, Sambrook et al., 1989, Molecular Cloning, A Laboratory Manual, 2d Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York; *see also*, Ausubel et al., eds., in the Current Protocols in Molecular Biology series of laboratory technique manuals, 1987-1997 Current Protocols,© 1994-1997 John Wiley and Sons, Inc.).

## 5.9 THERAPEUTIC USES

The invention provides for treatment or prevention of various diseases or disorders by administration of a therapeutic compound (termed herein "Therapeutic"). Such Therapeutics include but are not limited to: S2C6 antibodies and derivatives thereof (*e.g.*, as described hereinabove); and nucleic acids encoding such S2C6 antibodies and derivatives (*e.g.*, as described hereinabove). "Treatment" as used herein shall be deemed to include any clinically desirable or beneficial effect on the disease or disorder, including but not limited to alleviation of one or more symptoms, regression, slowing or cessation of progression, etc.

In specific embodiments of the invention, the Therapeutic is administered alone or in combination with CD40L for the treatment or prevention of malignancies (including but not limited to carcinoma and hematologic malignancies), inflammatory diseases, and disorders of the immune system. The Therapeutic and CD40L can, but need not be, contained within the same formulation, *i.e*, administration of the Therapeutic and CD40 can be performed separately but concurrently or during the same course of treatment. In a specific embodiment, the malignant cells express CD40. Alternatively, the cells of the malignancy need not express CD40, since endothelial cells of the

vasculature associated with a malignant tumor should express CD40 and thus the Therapeutic of the invention should provide treatment efficacy even for tumors that do not express CD40. In a preferred embodiment, the Therapeutic potentiates the binding of CD40L to CD40 by at least 45%, 50%, 60%, or 65%.

In specific embodiments, the Therapeutic is used to increase the immune response of an immunosuppressed individual, such as a person suffering from acquired immunodeficiency syndrome, from malignancy, or an infant or elderly person.

In other embodiments of the invention, the Therapeutic may be chemically modified so that cells that it binds to are killed. Such cells include but are not limited to multiple myeloma cells, lymphoma cells or carcinomas. Since all B-cells express CD40, this approach can result in suppression of the immune response. For example, a cytotoxic drug linked to S2C6 sequences (e.g., a fusion protein) may be used *in vivo* to cause immunosuppression in order to cross histocompatibility barriers in transplant patients; alternatively, these modified ligands may be used to control autoimmune diseases.

In other embodiments, the Therapeutic may be used to promote the proliferation and/or differentiation of CD40-bearing cells that are not B cells, for example, lung carcinoma cells, as a means of directly treating malignancy or as an adjunct to chemotherapy.

Malignancies which may be treated or prevented using a Therapeutic of the invention include but are not limited to those in Table 1:

TABLE 1
MALIGNANCIES AND RELATED DISORDERS

Leukemia
- acute leukemia
  - acute lymphocytic leukemia
  - acute myelocytic leukemia
    - myeloblastic
    - promyelocytic
    - myelomonocytic
    - monocytic

erythroleukemia
chronic leukemia
chronic myelocytic (granulocytic) leukemia
chronic lymphocytic leukemia
Polycythemia vera
Lymphoma
Hodgkin's disease
non-Hodgkin's disease
Multiple myeloma
Waldenström's macroglobulinemia
Heavy chain disease
Solid tumors
sarcomas and carcinomas
fibrosarcoma
myxosarcoma
liposarcoma
chondrosarcoma
osteogenic sarcoma
osteosarcoma
chordoma
angiosarcoma
endotheliosarcoma
lymphangiosarcoma
lymphangioendotheliosarcoma
synovioma
mesothelioma
Ewing's tumor
leiomyosarcoma
rhabdomyosarcoma
colon carcinoma
colorectal carcinoma
pancreatic cancer
breast cancer
ovarian cancer
prostate cancer
squamous cell carcinoma
basal cell carcinoma
adenocarcinoma
sweat gland carcinoma
sebaceous gland carcinoma
papillary carcinoma
papillary adenocarcinomas
cystadenocarcinoma
medullary carcinoma
bronchogenic carcinoma
renal cell carcinoma
hepatoma
bile duct carcinoma
choriocarcinoma
seminoma
embryonal carcinoma
Wilms' tumor
cervical cancer
uterine cancer
testicular tumor
lung carcinoma
small cell lung carcinoma
non small cell lung carcinoma
bladder carcinoma

epithelial carcinoma
glioma
astrocytoma
medulloblastoma
craniopharyngioma
ependymoma
pinealoma
hemangioblastoma
acoustic neuroma
oligodendroglioma
menangioma
melanoma
neuroblastoma
retinoblastoma
nasopharyngeal carcinoma
esophageal carcinoma

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Inflammatory diseases and deficiencies or disorders of the immune system which may be treated or prevented using a Therapeutic of the invention include but are not limited to those in Table 2:

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TABLE 2

INFLAMMATORY DISEASES AND
IMMUNE SYSTEM DISORDERS

systemic lupus erythematosus (SLE)
Scleroderma (*e.g.*, CRST syndrome)
inflammatory myositis
Sjögren's syndrome (SS)
mixed connective tissue disease (*e.g.*, MCTD, Sharp's syndrome)
rheumatoid arthritis
multiple sclerosis
inflammatory bowel disease (*e.g.*, ulcerative colitis, Crohn's disease)
acute respiratory distress syndrome
pulmonary inflammation
osteoporosis
delayed type hypersensitivity
asthma
primary biliary cirrhosis (PBC)
idiopathic thrombocytopenic purpura (ITP)

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5.9.1 EFFECTIVE DOSE

Toxicity and therapeutic efficacy of such Therapeutics can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the $LD_{50}$ (the dose lethal to 50% of the population) and the $ED_{50}$ (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio $LD_{50}/ED_{50}$. Therapeutics that exhibit large therapeutic indices are preferred. While Therapeutics that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. Exemplary doses include but are not limited to from 1 ng/kg to 100 mg/kg. The dosage of such Therapeutics lies preferably within a range of circulating concentrations that include the $ED_{50}$ with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For a Therapeutic, the therapeutically effective dose may preferably be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the $IC_{50}$ (*i.e.*, the concentration of the test compound that achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

### 5.9.2 FORMULATIONS

Pharmaceutical compositions for use in accordance with the present invention may be formulated in conventional manner using one or more physiologically acceptable carriers or excipients.

Thus, the Therapeutics and their physiologically acceptable salts and solvates may be formulated for administration by inhalation or insufflation (either through the mouth or the nose) or oral, buccal, parenteral or rectal administration.

For oral administration, the pharmaceutical compositions may take the form of, for example, tablets or capsules prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (*e.g.*, pregelatinised maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose); fillers (*e.g.*, lactose, microcrystalline cellulose or calcium hydrogen phosphate) lubricants (*e.g.*, magnesium stearate, talc or silica); disintegrants (*e.g.*, potato starch or sodium starch glycolate); or wetting agents (*e.g.*, sodium lauryl sulphate). The tablets may be coated by methods well known in the art. Liquid preparations for oral administration may take the form of, for example, solutions, syrups or suspensions, or they may be presented as a dry product for constitution with water or other suitable vehicles before use. Such liquid preparations may be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents (*e.g.*, sorbitol syrup, cellulose derivatives or hydrogenated edible fats); emulsifying agents (*e.g.*, lecithin or acacia); non-aqueous vehicles (*e.g.*, almond oil, oily esters, ethyl alcohol or fractionated vegetable oils); and preservatives (*e.g.*, methyl or propyl-p-hydroxybenzoates or sorbic acid). The preparations may also contain buffer salts, flavoring, coloring and sweetening agents as appropriate.

Preparations for oral administration may be suitably formulated to give controlled release of the active compound.

For buccal administration the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by inhalation, the Therapeutics for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from

pressurized packs or a nebulizer, with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, e.g., gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The Therapeutics may be formulated for parenteral administration by injection, e.g., by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multi-dose containers, with or without an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., sterile pyrogen-free water, before use.

The Therapeutics may also be formulated in rectal compositions such as suppositories or retention enemas, e.g., containing conventional suppository bases such as cocoa butter or other glycerides.

In addition to the formulations described previously, the Therapeutics may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the Therapeutics may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

The compositions may, if desired, be presented in a pack or dispenser device that may contain one or more unit dosage forms containing the active ingredient. The pack may for

example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration preferably for administration to a human.

In specific embodiments, the invention provides a pharmaceutical composition comprising a therapeutically effective amount of a Therapeutic in combination with CD40 ligand.

The invention is further described in the following examples which are in no way intended to limit the scope of the invention.

## 6. EXAMPLE: CLONING OF SC26 VARIABLE REGIONS

### 6.1 MATERIALS AND METHODS

The S2C6 light chain and heavy chain variable regions were cloned using methods essentially as described in Gilliland et al., 1996, Tissue Antigens 47:1-20. Total RNA was isolated from the S2C6 hybridoma. First strand complementary DNA (cDNA) was prepared for the mouse kappa light chain and heavy chain variable regions using reverse transcriptase and anti-sense primers that annealed approximately 100 base pairs downstream of the JC junction. A poly-G tail was added to the cDNA strands using terminal transferase and then double stranded DNA was synthesized using the polymerase chain reaction (PCR). The PCR primers, specific for the poly-G tail or a sequence approximately 50 bases inside the cDNA for the light chain or heavy chain, were designed to include unique restriction sites. After amplification, the PCR products were digested with *EcoRI* and *HindIII* cloned into pUC19 that had been digested with the same restriction enzymes. These reactions were ligated, transformed into *E. coli* DH5α, and the resulting clones were screened by restriction analysis. Clones that were positive by restriction digestion analysis were sequenced by DNA sequencing on a Li-Cor fluorescence sequencer. The

nucleotide (SEQ ID NO:1) and amino acid (SEQ ID NO:2) sequences of the light chain variable region ($V_L$) are shown in Figure 1. The nucleotide (SEQ ID NO:6) and amino acid (SEQ ID NO:7) sequences of the heavy chain variable region ($V_H$) are shown in Figure 2. Figures 3A-3B illustrate the amino acid sequence of S2C6 $V_L$ and S2C6 $V_H$ (Figure 3A and 3B, respectively). The CDRs are underlined. The amino acid sequences of $V_L$ CDRs 1-3 correspond to SEQ ID NOS:3-5, respectively. The amino acid sequences of $V_H$ CDRs 1-3 correspond to SEQ ID NOS:8-10, respectively.

The resulting DNA sequences were then compared to the light chain and heavy chain variable regions of other murine antibodies of the same isotype and the reading frame and corresponding amino acid sequences for the genes isolated from S2C6 were determined. To confirm the amino acid sequences, the light chain and heavy chain variable regions of S2C6 mAb were subjected to N-terminal amino acid analysis.

The amino acid sequences of S2C6 VL, S2C6 VH and the CDRs of both the VL and VH were submitted for BLASTP searches on April 21, 1999 using both the NR database (All non-redundant GenBank CDS translations+PDB+SwissProt+PIR+PRF) and the Kabat database (Kabat's database of sequences of immunological interest). The sequences found using the NR database can be retrieved using the Accession number at http://www.ncbi.nlm.nih.gov. The sequences found using the Kabat database can be retrieved using the Accession number at http://immuno.bme.nwu.edu/database_.html and SEQHUNT II. The results of these searches are shown below:

**BLASTP SEARCHES USING NR DATABASE**

**S2C6 VL** (SEQ ID NO:2): a BLASTP search of the NR database with S2C6 VL as the query yielded no hits with 100% identity and 6 hits with 94% (106/112) identity. These 6 are shown below:

pir||PT0359 IgG kappa chain V region (R4A.12) - mouse (fragment)

gi|196660 (M59949) immunoglobulin kappa-chain VJ region [Mus musculus]
gi|196954 (M12183) kappa-chain V-region [Mus musculus]>gi|2247 [Mus musculus]
pir||B34904 Ig kappa chain precursor V region (12-40 and 5-14) ...
emb|CAA80076| (Z22102) immunoglobulin variable region [Mus musculus]
dbj|BAA22172| (AB006833) anti-pseudouridine monoclonal antibody...

**VL CDR1** (SEQ ID NO:3): a BLASTP search with VL CDR1 as the query yielded no hits with 100% identity and numerous hits with 93% identity (15/16). The first 5 of these are shown below:
dbj|BAA03480| (D14627) immunoglobulin gamma-3 kappa chain [Mus musculus]
dbj|BAA22172| (AB006833) anti-pseudouridine monoclonal antibody...
gi|4101647 (AF005352) immunoglobulin V-region light chain [Mus musculus]
gi|3377681 (AF078800) single chain anti-HIV-1 Rev variable fragment...
gi|1870366 (U55625) anti-DNA immunoglobulin light chain IgM [Mus musculus]

**VL CDR2** (SEQ ID NO:4): a BLASTP search of the NR database with VL CDR2 as the query yielded no hits.

**VL CDR3** (SEQ ID NO:5): a BLASTP search of the NR database with VL CDR3 as the query yielded no hits.

**S2C6 VH** (SEQ ID NO:7): a BLASTP search of the NR database using S2C6 VH as a query yielded no hits with 100% identity and numerous hits with up to 88% identity the first 5 of which are shown below:

gi|3561044 (AF083186) anti-HIV-1 p24 antibody D2 heavy chain [Mus musculus]

pdb|1A6T|B Chain B, Fab Fragment Of Mab1-Ia Monoclonal Antibody

gi|2895955 (AF045895) IgG1 heavy chain mAB1-IA [Mus musculus]

emb|CAA80023| (Z22049) immunoglobulin variable region [Mus musculus]

gi|194510 (M91695) immunoglobulin gamma-1 chain [Mus musculus]

**VH CDR1** (SEQ ID NO:8): a BLASTP search of the NR database with VH CDR1 as the query yielded no hits.

**VH CDR2** (SEQ ID NO:9): a BLASTP search of the NR database with VH CDR2 as the query yielded no hits with 100% identity, 1 hit with 94% identity (16/17) and numerous hits with less than 94% identity. The 1 hit with 94% identity is shown:

gi|3561044 (AF083186) anti-HIV-1 p24 antibody D2 heavy chain [Mus musculus]

**VH CDR3** (SEQ ID NO:10): a BLASTP search of the NR database with VH CDR3 as the query yielded no hits.

## BLAST SEARCHES USING KABAT DATABASE

**S2C6 VL** (SEQ ID NO:2): a BLASTP search of the Kabat database using S2C6 VL as the query yielded no hits with 100% identity and numerous hits with 89-91% identity to the query. The first 5 are shown:

KADBID 005591, mouse IG KAPPA LIGHT CHAIN VARIABLE REGION (5-14...),

KADBID 005594, mouse IG KAPPA LIGHT CHAIN VARIABLE REGION (10VA...),

KADBID 005593, mouse IG KAPPA LIGHT CHAIN VARIABLE REGION (12-4...),

KADBID 005603, mouse IG KAPPA LIGHT CHAIN VARIABLE REGION (17s....),
KADBID 005588, mouse IG KAPPA LIGHT CHAIN VARIABLE REGION (TEPC...).

**VL CDR1** (SEQ ID NO:3): a BLASTP search of the Kabat database with VL CDR1 as the query yielded no hits with 100% identity and numerous hits with 93% identity (15/16). The first 5 are shown below:

KADBID 005720, mouse IG KAPPA LIGHT CHAIN VARIABLE REGION (BW24...),
KADBID 005614, mouse IG KAPPA LIGHT CHAIN VARIABLE REGION (PME7...),
KADBID 005624, mouse IG KAPPA LIGHT CHAIN VARIABLE REGION (C5-7...),
KADBID 005621, mouse IG KAPPA LIGHT CHAIN VARIABLE REGION (40-6...),
KADBID 005640, mouse IG KAPPA LIGHT CHAIN VARIABLE REGION (40-9...).

**VL CDR2** (SEQ ID NO:4): a BLASTP search of the Kabat database with VL CDR2 as the query yielded no hits.

**VL CDR3** (SEQ ID NO:5): a BLASTP search of the Kabat database with VL CDR3 as the query yielded 1 hit with 100% identity to the query:

KADBID 005681, mouse IG KAPPA LIGHT CHAIN VARIABLE REGION (NC10...).

**S2C6 VH** (SEQ ID NO:7): a BLASTP search of the Kabat database using S2C6 VH as the query yielded no hits with 100% identity and numerous hits with 79-85% identity to the query. The first 5 of the hits are shown below:

KADBID 001498, mouse IG HEAVY CHAIN VARIABLE REGION (HDEX24),
KADBID 001494, mouse IG HEAVY CHAIN VARIABLE REGION (HDEX5),

KADBID 001529, mouse IG HEAVY CHAIN VARIABLE REGION (163.72'CL),

KADBID 001500, mouse IG HEAVY CHAIN VARIABLE REGION (HDEX37),

KADBID 001597, mouse IG HEAVY CHAIN VARIABLE REGION (BB128'CL),

**VH CDR1** (SEQ ID NO:8): a BLASTP search of the Kabat database with VH CDR1 as the query yielded no hits

**VH CDR2** (SEQ ID NO:9): a BLASTP search of the Kabat database with VH CDR as the query yielded no hits with 100% identity and 10 hits with 87-88% identity to the query. The first 5 are shown:

KADBID 001535, mouse IG HEAVY CHAIN VARIABLE REGION (H10"CL),

KADBID 001534, mouse IG HEAVY CHAIN VARIABLE REGION (H81'CL),

KADBID 001533, mouse IG HEAVY CHAIN VARIABLE REGION (H50'CL),

KADBID 019741, mouse IG HEAVY CHAIN VARIABLE REGION (Clone F'CL),

KADBID 001529, mouse IG HEAVY CHAIN VARIABLE REGION (163.72'CL),

**VH CDR3** (SEQ ID NO:10): BLASTP search of the Kabat database with VH CDR3 as the query yielded no hits.

## 7. EXAMPLE:BIOLOGIC ACTIVITY OF S2C6

### 7.1 MATERIALS AND METHODS

#### 7.1.1 ANTI-CD40 ANTIBODY PREPARATION

The S2C6 hybridoma was cultured at 37°C in complete IMDM (Gibco BRL, Grand Island, NY) containing 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 mg/ml streptomycin. The culture was harvested by centrifugation and the supernatant was collected by filtration using a 0.2

micron filter. Subsequently the supernatant was loaded onto a GammaBind™ Sepharose column (Pierce), washed with phosphate buffered saline (PBS), and eluted with 0.1 M glycine pH 2.5. Immediately upon elution, the antibody was neutralized with 1 M Tris pH 8.0, dialyzed into PBS, and filter sterilized. MAb preparations were analyzed by size exclusion chromatography. Only samples of greater than 99% monomeric protein were used for the studies described herein.

### 7.1.2 HUMAN TUMOR XENOGRAFT MODELS

Ninety female C.B.-17 SCID mice were obtained (Taconic Labs, Germantown, NY) at age 6 to 8 weeks and quarantined for 2 weeks. Control groups of mice were injected intravenously (i.v.) with a human B cell tumor line: Ramos (non-Hodgkins lymphoma), HS Sultan (multiple myeloma) or IM-9 (multiple myeloma) cells ($1x10^6$-$2x10^6$ cells). The remaining mice were divided into two groups; half were treated with 200 $\mu$l of a 1:10 dilution of anti-asialo-GM1 (Wako Chemicals, Richmond, VA) i.v., one day prior to the injection of tumor cells, to remove host natural killer cells (Murphy et al., 1992, Eur. J. Immunol. 22:241). Mice in the two groups were injected i.v. with Ramos, HS Sultan or IM-9 cells ($1x10^6$-$2x10^6$cells). Mice in the test groups were then injected intraperitoneally (i.p.) with 1 mg/kg of S2C6 IgG prepared as described in Section 7.1.2 starting on day 1 or day 5 post tumor implant, according to the following schedule and were monitored for partial paralysis or other signs of disease.

**Xenograft Tumor Model Schedule**

| Group | Tumor cell line | Antibody (1mg/kg, i.p.) | Anti-asialo GM1 | Days treated with mAb |
|---|---|---|---|---|
| 1 Control | Ramos | - | - | - |
| 2 | Ramos | S2C6 | - | 1,5,9,13,17 |
| 3 | Ramos | S2C6 | - | 5,9,13,17,21 |
| 4 | Ramos | S2C6 | + | 1,5,9,13,17 |
| 5 | Ramos | S2C6 | + | 5,9,13,17,21 |
| 6 Control | HS Sultan | - | - | - |
| 7 | HS Sultan | S2C6 | - | 1,5,9,13,17 |

| Group | Tumor cell line | Antibody (1mg/kg, i.p.) | Anti-asialo GM1 | Days treated with mAb |
|---|---|---|---|---|
| 8 | HS Sultan | S2C6 | - | 5,9,13,17,21 |
| 9 | HS Sultan | S2C6 | + | 1,5,9,13,17 |
| 10 | HS Sultan | S2C6 | + | 5,9,13,17,21 |
| 11 Control | IM-9 | - | - | - |
| 12 | IM-9 | S2C6 | - | 1,5,9,13,17 |
| 13 | IM-9 | S2C6 | - | 5,9,13,17,21 |
| 14 | IM-9 | S2C6 | + | 1,5,9,13,17 |
| 15 | IM-9 | S2C6 | + | 5,9,13,17,21 |

### 7.1.3 PERIPHERAL BLOOD B CELL ISOLATION

Peripheral blood B cells were isolated by positive selection using immobilized antibodies against both CD19 and CD20. The final isolated cell population contained greater than 85% B cells as determined by flow cytometry. For storage, the cells were diluted to $4x10^7$ cells/ml in fetal bovine serum (FBS) containing 10% dimethyl sulfoxide and stored in a liquid nitrogen freezer.

### 7.1.4 B CELL PROLIFERATION ASSAY

Human peripheral blood B cells were thawed and incubated in 96-well tissue culture plates at $1x10^5$ per well in IMDM medium plus 10% FBS in the presence of 5 ng/ml recombinant human IL-4 (Biosource) and various dilutions of an anti-CD40 mAb: S2C6, G28-5 (Bristol-Myers Squibb) or M3 (Genzyme #80-3702-04). As a control, cells were incubated with IL-4 and an irrelevant control mAb, EXA2-1H8 (anti-*Pseudomonas* exotoxin). The plates were incubated at 37°C for 3 days and then pulsed for 16 h with 0.5 mCi $^3$H-thymidine/well. Cells were harvested onto 96-well glass fiber filters using a Filtermate 196 Harvester™ (Packard Instruments) and combined with scintillation fluid. The extent of $^3$H-thymidine incorporated into nascent DNA was measured by liquid scintillation counting using a Topcount LSC™ (Packard Instruments).

A Jurkat cell line selected to express constitutive high levels of CD40L ("Jurkat/CD40L"), was used as CD40L stimulator cells (Malik et al., 1996, J. Immunol. 156:3952-60). To eliminate proliferation of the stimulator cells,

they were treated with mitomycin C (50 mg/ml) in PBS for 20 min at 37°C followed by 3 washes in PBS prior to combining with B cells. B cells ($1x10^5$/well) were combined with Jurkat/CD40L cells and assayed as above. B cells and IL-4 were initially combined with stimulator cells ($2.5x10^4$/well) directly followed by addition of the anti-CD40 mAbs. Monoclonal antibodies were titrated with either a fixed concentration of stimulator cells or stimulator cells were titrated with a fixed concentration of mAb.

7.1.5 CD40/CD40L BINDING ASSAY

The Jurkat/CD40L cell line was used as a target cell line in these assays. Cells were adjusted to a density to $2x10^7$/ml at 50 µl per sample. Binding was performed in RPMI 1640 media (Gibco) + 10% FBS. To determine receptor saturation, Jurkat/CD40L cells were incubated with increasing concentrations of CD40-Ig (a soluble fusion protein of CD40 and human immunoglobulin) (Noelle et al., 1992, Proc. Natl. Acad. Sci. USA 89:6550-6554), washed and incubated with fluorescein isothiocyanate conjugated to anti-human immunoglobulin ("FITC-anti-human Ig"). The resultant binding was evaluated using a FacScan™ flow cytometer (Becton Dickinson). Recombinant soluble CD40-Ig (25 µg/ml) was pre-incubated for 1 h on ice with increasing concentrations of mAb S2C6. The anti-CD40 mAb G28-5; M3; and anti-*Pseudomonas* exotoxin, an isotype control, were used for comparison. The recombinant human soluble CD40 ligand (CD154-muCD8), produced as a fusion protein with murine CD8 and labeled with FITC, was obtained from Research Diagnostics, Inc. (Flanders, NJ). Dilutions of soluble CD40-Ig and anti-CD40 mAbs were made at a 4-fold final concentration, pre-incubated on ice for 1 h and then combined with Jurkat cells on ice for 1 h. Cells were washed and labeled with FITC-Goat anti-human $F(ab')_2$, (Jackson Labs, Fc-specific #109-096-098). The extent of CD40 binding was determined by flow cytometry.

## 7.2 RESULTS

### 7.2.1 *IN VITRO* STUDIES: mAb S2C6 PROMOTES CD40/CD40L INTERACTION

To evaluate the effect of anti-CD40 mAbs on the binding of soluble CD40 to CD40L expressed on the surface of activated T cells, increasing concentrations of various CD40 mAbs were pre-incubated with 25 μg/ml soluble CD40-Ig followed by incubation of the complexes with Jurkat/CD40L cells. CD40L expression on selected CD40L+ Jurkat T cells was initially verified by flow cytometry with FITC-labeled anti-CD40L (data not shown). CD40 binding to CD40L on these target cells was then determined by flow cytometry of the Jurkat/CD40L cells using FITC-goat anti-human Ig to detect the bound CD40-Ig. Titration with CD40-Ig showed receptor saturation at approximately 25 μg/ml CD40-Ig. Using saturating concentrations of soluble CD40, S2C6 complexed with CD40 at ratios ranging from 0.25 to 2:1 (mass:mass) resulted in a dose-dependent increase in CD40 binding to CD40L (approximately 50%, 100%, 146% and 220% at concentrations of approximately 6 μg/ml, 13 μg/ml, 25 μg/ml, and 50 μg/ml, respectively) (Figure 4). A similar titration with the inhibitory antibody M3 blocked CD40/CD40L binding in a dose dependent manner. mAb G28-5 showed no effect of CD40/CD40L binding at concentrations up to 25 μg/ml and was only slightly stimulatory at the highest concentration tested (50 μg/ml), relative to control EXA2-1H8 Ig.

These data clearly indicate mAb S2C6 promotes CD40/CD40L interaction. Further, S2C6 differs from G28-5 and M3 in its ability to increase CD40/CD40L interaction.

In a reciprocal assay, the effect of anti-CD40 mAbs on the binding of soluble CD40L to membrane-bound CD40 expressed on the surface of B cells was evaluated. Titration with soluble CD40L showed Ramos B cell surface CD40 saturation at approximately 10 μg/ml. Increasing concentrations of various anti-CD40 mAbs were pre-incubated with CD40-expressing B cells followed by incubation of the cells with FITC-labeled soluble CD40L. The labeled CD40L binding to CD40 on target B

cells was then determined by flow cytometry of the Ramos cells. Using saturating concentrations of soluble CD40L, mAb S2C6 complexed with CD40-expressing cells resulted in a maximal increase in CD40L binding of approximately 51% to 68% at concentrations ranging from 0.04 to 2 μg/ml (Figure 5).

In contrast to the above results with soluble CD40, in which mAb G28-5 had little effect on CD40/CD40L interaction, G28-5 showed inhibition of soluble ligand binding to CD40 at all concentrations tested. A similar titration with the inhibitory mAb M3 also blocked CD40L/CD40 binding in a dose dependent manner.

These data indicate that S2C6 differs surprisingly from G28-5 and M3 in its ability to increase CD40L/CD40 interaction. Moreover, under these conditions, both mAb G28-5 and mAb M3 inhibit the interaction of soluble CD40L with CD40 at concentrations as low as 40 ng/ml.

### 7.2.2 IN VITRO STUDIES: mAb S2C6 INCREASES B CELL RESPONSE TO CD40/CD40L

The growth response of primary peripheral B cells to CD40L-expressing cells was measured in the presence of an anti-CD40 mAb (S2C6, G28-5 or M3). First, B cells were combined with increasing numbers of non-proliferating, Jurkat/CD40L cells in the presence or absence of a fixed level (30 ng/ml) of the various mAbs. B cell activation in response to treatment was then measured by $^{3}$H-thymidine incorporation at 72 h post-stimulus. T cell titration in the presence of mAb M3 resulted in B cell proliferation similar to that seen with control Ig (Figure 6).

Although mAb G28-5 provided some B cell activation in the absence of ligand (Figure 7), CD40L$^{+}$ T cell titration in the presence of G28-5 only nominally increased B cell proliferation (1.3-fold) over the level seen with G28-5 alone. In contrast, B cell proliferation increased in the presence of S2C6 in a dose dependent manner with increasing numbers of T cell stimulator cells to 3-fold above mAb-only stimulation with a B cell to T cell stimulator ratio of 4:1.

These data demonstrate that unlike M3 and G28-5, S2C6 can surprisingly synergize with CD40L to promote B cell proliferation via CD40.

In a second assay of this type, B cells were either titrated with an anti-CD40 mAb or combined with non-proliferating CD40L$^+$ T stimulator cells at a fixed ratio of 4:1 (B:T) and titrated with an anti-CD40 mAb (Figure 7).

These results demonstrate that, under these conditions, activation of primary human peripheral blood B cells increased 2-fold at 10 μg/ml of mAb G28-5 and ligand, as compared to G28-5 alone. To a surprising degree, S2C6 was significantly more active and in the presence of ligand increased B cell proliferation in a dose dependent manner to 16.2-fold at 10 μg/ml (the highest level tested) as compared to S2C6 alone.

Taken together, these data indicate that S2C6 complexed to CD40 increases CD40L binding. Although S2C6 by itself will stimulate B cell proliferation in a manner similar to G28-5, S2C6 is distinguished from G28-5 by its ability to increase CD40L binding and the subsequent magnitude of the CD40L-mediated activation signal.

## 8. EXAMPLE: MONOCLONAL ANTIBODY S2C6 INHIBITS TUMOR GROWTH

To evaluate the anti-tumor activity of native mAb S2C6, female C.B.-17 SCID mice were divided into two groups (20 mice/group). Half of the mice of each group were treated with anti-asialo-GM1 to blunt host natural killer cell activity (Murphy et al., 1992, Eur. J. Immunol. 22:241). The following day, mice were injected i.v. with Ramos, HS Sultan or IM-9 cells ($1x10^6$ cells). Mice were then injected i.p. with 1 mg/kg of mAb S2C6 IgG, as described in Materials and Methods in Section 7 *supra* and monitored for partial paralysis or other signs of disease onset.

Monoclonal antibody S2C6 treatment of animals harboring Ramos human B cell lymphoma (Figure 8A), HS Sultan multiple myeloma (Figure 8B), or IM-9 multiple myeloma (Figure 8C), resulted in significant reduction in tumor mass and

subsequent tumor-related morbidity and mortality. In parallel studies, efficacy was sustained in the presence of anti-asialo-GM1, suggesting that the increased survival in the presence of mAb S2C6 was not due to nonspecific NK activity. The IM-9 cell line is an aggressive tumor model that, like multiple myeloma, secretes human Ig as a surrogate marker of disease.

Treatment of IM-9 diseased mice with mAb S2C6 significantly increased animal survival. These studies clearly demonstrate that S2C6 has potent anti-tumor activity against engrafted human tumors in mice.

## 9. EXAMPLE: A SINGLE-CHAIN ANTI-CD40 IMMUNOTOXIN FUSION PROTEIN BINDS CD40-Ig

BD1-S2C6 sFv (single-chain anti-CD40 immunotoxin, a fusion protein consisting of the amino acid sequence of bryodin 1 (BD1) (Francisco et al., 1997, J. Biol. Chem. 272(39):24165-24169) fused to the variable regions of monoclonal antibody S2C6) was expressed in E. coli as inclusion bodies, denatured and refolded.

Briefly, total RNA was isolated from S2C6 hybridoma cells using TRIZOL reagent (Life Technologies) following the manufacturer's recommendations. First strand cDNA synthesis of the light chain and heavy chain variable regions was performed essentially as described by Gilliland et al. (Tissue Antigens, 47:1-20(1996)) using primers which are complementary to sequences approximately 100 bases downstream of the J-C junctions. The first strands were then poly-G tailed and amplified by PCR using a poly-C anchor primer, which is complementary to the poly-G tail, and a primer nested approximately 50 bases inside the one used for first strand synthesis. The PCR primers were designed to generate unique restriction sites at the 5' and 3' ends of the PCR products. The two PCR products, containing the sequences coding for the light chain and heavy chain variable regions, were digested with *EcoRI* and *HINDIII* and ligated into pUC19 which had been digested with the same enzymes. The resulting

plasmids, pSG5 and pSG10, contain the DNA coding for S2C6 VL and S2C6 VH, respectively. The DNA of both plasmids was sequenced and verified to match the amino-terminal acid sequence of the parental monoclonal antibody.

The VH and VL fragments of S2C6 were "sewn" together (overlap extension PCR) as described by Gilliland et al. in the VH-VL orientation and ligated into a cloning vector. Subsequently the sFv fragment of BD1-G28-5 sFv (Francisco et al., 1997, *J. Biol. Chem.* 272:24165-24169) was removed from pSE151 by restriction digestion and S2C6 sFv was ligated in its place. The resulting plasmid, pSG40, contains the gene coding for BD1-S2C6 sFv under the control of the inducible T7 promoter.

For expression, pSG40 was transformed into competent *E. coli* strain BL21(DE3)pLysS cells and the cells were grown in T-broth at 37C°. When the culture reached $OD_{600}$ = 1.0 the cells were induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 h. Subsequently, the cells were harvested by centrifugation, lysed by sonication, and the BD1-S2C6 sFv fusion was isolated as insoluble inclusion bodies by centrifugation, which were denatured and refolded as follows: Inclusion bodies were solubilized in 7M guanidine at 5 mg/ml, refolded by rapid dilution (1:100) into PBS containing 0.3M L-arginine and 2 mM DTT, and dialyzed against 20 mM sodium phosphate buffer, pH 7.4, for subsequent purification.

The refolded protein was isolated using Blue Sepharose followed by affinity chromatography over immobilized CD40-Ig.

The purified protein was then tested for binding to immobilized CD40-Ig in ELISA. Microtiter plates were coated with CD40-Ig at 0.5 μg/ml followed by the addition of dilutions of purified BD1-S2C6 sFv in PBS (pH 7.4) with 1% bovine serum albumin and 0.05% Tween-20 in the presence of 25 μg/ml S2C6 mAb (▲), 25 μg/ml control antibody BR96 (●), or no excess antibody (■). Binding of BD1-S2C6 sFv to the immobilized receptor was detected by the addition of BD1-specific rabbit antiserum (Seattle Genetics, Inc., Bothell,

Washington) followed by the addition of horseradish peroxidase conjugated goat anti-rabbit Ig.

The binding of BD1-S2C6 sFv to CD40-Ig was completely inhibited by the addition of excess S2C6 mAb but not by the addition of the control mAb (Figure 9).

## 10. EXAMPLE: ADMINISTRATION OF RECOMBINANT S2C6 IN THE TREATMENT OF CD40-POSITIVE MALIGNANCIES

Patients with CD40-positive malignancies such as Non-Hodgkin's Lymphoma, Multiple Myeloma, and colon or other carcinomas are injected with recombinant humanized S2C6-anti-CD40 monoclonal antibody (with murine CDRs and human framework regions) or a recombinant chimeric antibody comprising the variable region of S2C6 and the constant region of a human antibody. The recombinant antibody is prepared *in vitro*. Treatment can commence at any time during the course of the disease, in the presence or absence of concomitant chemotherapy.

The treatment regimen includes weekly injections of the agent diluted in saline or other physiologically compatible solution.

The dosage used for recombinant S2C6 is in the range of 0.1 mg/m$^2$ (of body surface area of the patient) to 1000 mg/m$^2$ with the preferred dosage being 100-500 mg/m$^2$.

The route of injection is intravenous through either a peripheral IV access line or a central IV access line. The agent is administered as an infusion and not an IV push.

The effect of therapy with recombinant S2C6 is monitored by measuring: a) total lymphocyte and T and B lymphocyte counts in the peripheral blood; b) activity of T lymphocytes (helper T4 lymphocytes and cytolytic T8 lymphocytes) *in vitro*; and/or c) changes in the morphology of tumors using techniques such as a computed tomographic (CT) scan, magnetic resonance imaging (MRI) scan, x-radiographic imaging, bone scan imaging and tumor biopsy sampling including bone marrow aspiration (BMA).

Depending on the results obtained, the therapeutic regimen is developed to optimally treat CD40-positive malignancies with minimal impact on the competency of the immune system with the ultimate goal of achieving tumor regression and complete eradication of tumor cells.

## 11. DEPOSIT OF MICROORGANISM

Hybridoma S2C6, secreting native monoclonal antibody S2C6, was deposited on May 25, 1999, with the American Type Culture Collection (ATCC), 10801 University Boulevard, Manassass, Virginia 20110-2209, under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedures, and assigned accession number PTA-110.

## 12. SPECIFIC EMBODIMENTS, CITATION OF REFERENCES

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

Various references, including patent applications, patents, and scientific publications, are cited herein, the disclosures of which are incorporated herein by reference in their entireties.

## MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on pages 60, lines 7-14 of the description *

**A. IDENTIFICATION OF DEPOSIT ***

Further deposits are identified on an additional sheet *

Name of depositary institution *

American Type Culture Collection

Address of depositary institution (including postal code and country) *

10801 University Blvd.
Manassas, VA 20110-2209
US

Date of deposit * May 25, 1999 Accession Number * PTA-110

**B. ADDITIONAL INDICATIONS** * (leave blank if not applicable). This information is continued on a separate attached sheet

**C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE** * (if the indications are not all designated States)

**D. SEPARATE FURNISHING OF INDICATIONS** * (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later * (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☑ This sheet was received with the International application when filed (to be checked by the receiving Office)

Misty Walker (703)305-3682
(Authorized Officer) RO/US

☐ The date of receipt (from the applicant) by the International Bureau *

was

(Authorized Officer)

Form PCT/RO/134 (January 1981)

WHAT IS CLAIMED IS:

1. A molecule comprising SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:8, SEQ ID NO:9, or SEQ ID NO:10, which molecule (a) immunospecifically binds CD40, and (b) comprises one or more substitutions or insertions in primary amino acid sequence relative to native monoclonal antibody S2C6 as secreted by the hybridoma deposited with the ATCC and assigned accession number PTA-110.

2. A molecule comprising SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:8, SEQ ID NO:9, or SEQ ID NO:10, which molecule (a) immunospecifically binds CD40, and (b) is not monoclonal antibody S2C6 as secreted by the hybridoma deposited with the ATCC and assigned accession number PTA-110, and does not result from cleavage of S2C6 with papain or pepsin.

3. The molecule of claim 1 or 2 comprising the amino acid sequence of SEQ ID NO:2 or the amino acid sequence of SEQ ID NO:7 or the amino acid sequences of both SEQ ID NO:2 and NO:7.

4. The molecule of claim 1 or 2 which is an antibody.

5. The molecule of claim 1 or 2 which is a fusion protein comprising the amino acid sequence of a second molecule that is not an antibody.

6. The molecule of claim 5 that comprises an amino acid sequence of bryodin (BD1) fused to SEQ ID NO:7 fused to SEQ ID NO:2.

7. The molecule of claim 1 or 2 which is an antibody comprising a variable domain of monoclonal antibody S2C6 as secreted by the hybridoma deposited with the ATCC and

assigned accession number PTA-110, and a human constant region.

8. The molecule of any one of claims 1-4 which is purified.

9. A purified protein comprising an amino acid sequence that has at least 95% identity to SEQ ID NO:2 or SEQ ID NO:7, which protein (a) immunospecifically binds CD40; and (b) comprises one or more substitutions or insertions in primary amino acid sequence relative to native monoclonal antibody S2C6 as secreted by the hybridoma deposited with the ATCC and assigned accession number PTA-110.

10. A purified protein, which protein (a) competes for binding to CD40 with monoclonal antibody S2C6 as secreted by the hybridoma deposited with the ATCC and assigned accession number PTA-110, (b) increases the binding of CD40 ligand to CD40 by at least 45%, and (c) comprises one or more substitutions or insertions in primary amino acid sequence relative to native monoclonal antibody S2C6 as secreted by the hybridoma deposited with the ATCC and assigned accession number PTA-110.

11. A purified protein, which protein (a) competes for binding to CD40 with monoclonal antibody S2C6 as secreted by the hybridoma deposited with the ATCC and assigned accession number PTA-110, (b) increases the binding of CD40 ligand to CD40 by at least 45%, and (c) is not monoclonal antibody S2C6 as secreted by the hybridoma deposited with the ATCC and assigned accession number PTA-110, and does not result from cleavage of S2C6 with papain or pepsin.

12. An isolated nucleic acid comprising SEQ ID NO:1, SEQ ID NO:6, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, or SEQ ID NO:15.

13. An isolated nucleic acid comprising a nucleotide sequence encoding a protein comprising SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:8, SEQ ID NO:9, or SEQ ID NO:10.

14. The isolated nucleic acid of claim 13 comprising a nucleotide sequence encoding a protein comprising (a) a heavy chain variable domain of monoclonal antibody S2C6 as secreted by the hybridoma deposited with the ATCC and assigned accession number PTA-110, and (b) a human constant region.

15. An isolated nucleic acid comprising a nucleotide sequence encoding a protein comprising an amino acid sequence that has at least 95% identity to SEQ ID NO:2 or SEQ ID NO:7.

16. An isolated nucleic acid comprising a nucleotide sequence encoding a protein, which protein competes for binding to CD40 with monoclonal antibody S2C6 as secreted by the hybridoma deposited with the ATCC and assigned accession number PTA-110, and which protein increases the binding of CD40 ligand to CD40 by at least 45%.

17. An isolated nucleic acid comprising a nucleotide sequence encoding a fusion protein, said fusion protein comprising an amino acid sequence of bryodin 1 (BD1) fused to SEQ ID NO:7 fused to SEQ ID NO:2.

18. An isolated nucleic acid which hybridizes to the reverse complement of a DNA consisting of a coding DNA sequence encoding a protein consisting of an amino acid sequence selected from the group consisting of SEQ ID NO:2 and SEQ ID NO:7, under highly stringent conditions, which isolated nucleic acid encodes a protein that immunospecifically binds CD40.

19. A recombinant cell containing a recombinant nucleic acid vector comprising a nucleotide sequence encoding a protein, which protein competes for binding to CD40 with

monoclonal antibody S2C6 as secreted by the hybridoma deposited with the ATCC and assigned accession number PTA-110, and which protein increases the binding of CD40 ligand to CD40 by at least 45%.

20. A recombinant cell containing a recombinant nucleic acid vector comprising SEQ ID NO:1, SEQ ID NO:6, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, or SEQ ID NO:15.

21. A method of producing a protein comprising:

(a) growing a cell containing a recombinant nucleotide sequence encoding a protein, which protein competes for binding to CD40 with monoclonal antibody S2C6 as deposited with the ATCC and assigned accession number PTA-110, and which protein increases the binding of CD40 ligand to CD40 by at least 45%, such that the protein is expressed by the cell; and

(b) recovering the expressed protein.

22. A method of producing a protein comprising:

(a) growing a cell containing a recombinant nucleotide sequence encoding a protein comprising SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, or SEQ ID NO:10, such that a protein encoded by said nucleotide sequence is expressed by the cell; and

(b) recovering the expressed protein.

23. A pharmaceutical composition comprising:

(a) a molecule comprising SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, or SEQ ID NO:10, which molecule (i) immunospecifically binds CD40, (ii) increases the binding of CD40 ligand to CD40

by at least 45%, and (iii) comprises one or more substitutions or insertions in primary amino acid sequence relative to native monoclonal antibody S2C6 as secreted by the hybridoma deposited with the ATCC and assigned accession number PTA-110, in an amount effective for the treatment or prevention of cancer; and

(b) a pharmaceutically acceptable carrier.

24. A pharmaceutical composition comprising:

(a) a purified protein, which protein (i) competes for binding to CD40 with monoclonal antibody S2C6 as secreted by the hybridoma deposited with the ATCC and assigned accession number PTA-110, (ii) increases the binding of CD40 ligand to CD40 by at least 45%, and (iii) comprises one or more substitutions or insertions in primary amino acid sequence relative to native monoclonal antibody S2C6 as secreted by the hybridoma deposited with the ATCC and assigned accession number PTA-110, in an amount effective for the treatment or prevention of cancer; and

(b) a pharmaceutically acceptable carrier.

25. A pharmaceutical composition comprising:

(a) a purified protein, which protein (i) competes for binding to CD40 with monoclonal antibody S2C6 as secreted by the hybridoma deposited with the ATCC and assigned accession number PTA-110, (ii) increases the binding of CD40 ligand to CD40 by at least 45%, and (iii) is not monoclonal antibody S2C6 as secreted by the hybridoma deposited with the ATCC and assigned accession number PTA-110, and does not result from cleavage of S2C6 with papain

or pepsin, in an amount effective for the treatment or prevention of cancer; and

(b) a pharmaceutically acceptable carrier.

26. A pharmaceutical composition comprising:

(a) a molecule comprising SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, or SEQ ID NO:10, which molecule (i) immunospecifically binds CD40, (ii) increases the binding of CD40 ligand to CD40 by at least 45%, and (iii) comprises one or more substitutions or insertions in primary amino acid sequence relative to native monoclonal antibody S2C6 as secreted by the hybridoma deposited with the ATCC and assigned accession number PTA-110, in an amount effective for activating or augmenting an immune response; and

(b) a pharmaceutically acceptable carrier.

27. A pharmaceutical composition comprising:

(a) a purified protein, which protein (i) competes for binding to CD40 with monoclonal antibody S2C6 as secreted by the hybridoma deposited with the ATCC and assigned accession number PTA-110, (ii) increases the binding of CD40 ligand to CD40 by at least 45%, and (iii) comprises one or more substitutions or insertions in primary amino acid sequence relative to native monoclonal antibody S2C6 as secreted by the hybridoma deposited with the ATCC and assigned accession number PTA-110, in an amount effective for activating or augmenting an immune response; and

(b) a pharmaceutically acceptable carrier.

28. A pharmaceutical composition comprising:

(a) a purified protein, which protein (i) competes for binding to CD40 with monoclonal antibody S2C6 as secreted by the hybridoma deposited with the ATCC and assigned accession number PTA-110, (ii) increases the binding of CD40 ligand to CD40 by at least 45%, and (iii) is not monoclonal antibody S2C6 as secreted by the hybridoma deposited with the ATCC and assigned accession number PTA-110, and does not result from cleavage of S2C6 with papain or pepsin, in an amount effective for activating or augmenting an immune response; and

(b) a pharmaceutically acceptable carrier.

29. The pharmaceutical composition of any one of claims 23-28 further comprising CD40 ligand.

30. A method for the treatment or prevention of cancer in a subject comprising:

administering to the subject an amount of a molecule comprising SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, or SEQ ID NO:10, which molecule (i) immunospecifically binds CD40, (ii) increases the binding of CD40 ligand to CD40 by at least 45%, and (iii) comprises one or more substitutions or insertions in primary amino acid sequence relative to native monoclonal antibody S2C6 as secreted by the hybridoma deposited with the ATCC and assigned accession number PTA-110, which amount is effective for the treatment or prevention of cancer.

31. A method for the treatment or prevention of cancer in a subject comprising:

administering to the subject an amount of a purified protein, which protein (i) competes for

binding to CD40 with monoclonal antibody S2C6 as secreted by the hybridoma deposited with the ATCC and assigned accession number PTA-110, (ii) increases the binding of CD40 ligand to CD40 by at least 45%, and (iii) comprises one or more substitutions or insertions in primary amino acid sequence relative to native monoclonal antibody S2C6 as secreted by the hybridoma deposited with the ATCC and assigned accession number PTA-110, which amount is effective for the treatment or prevention of cancer.

32. A method for the treatment or prevention of cancer in a subject comprising:

administering to the subject an amount of a purified protein, which protein (i) competes for binding to CD40 with monoclonal antibody S2C6 as secreted by the hybridoma deposited with the ATCC and assigned accession number PTA-110, (ii) increases the binding of CD40 ligand to CD40 by at least 45%, and (iii) is not monoclonal antibody S2C6 as secreted by the hybridoma deposited with the ATCC and assigned accession number PTA-110, and does not result from cleavage of S2C6 with papain or pepsin, which amount is effective for the treatment or prevention of cancer.

33. A method for activating or augmenting the immune response of a subject comprising:

administering to the subject an amount of a molecule comprising SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, or SEQ ID NO:10, which molecule (i) immunospecifically binds CD40, (ii) increases the binding of CD40 ligand to CD40 by at least 45%, and (iii) comprises one or more substitutions or insertions in primary amino acid sequence relative to native monoclonal

antibody S2C6 as secreted by the hybridoma deposited with the ATCC and assigned accession number PTA-110, which amount is such that the immune response of the subject is activated or augmented.

34. A method for activating or augmenting the immune response of a subject comprising:

administering to the subject an amount of a purified protein, which protein (i) competes for binding to CD40 with monoclonal antibody S2C6 as secreted by the hybridoma deposited with the ATCC and assigned accession number PTA-110, (ii) increases the binding of CD40 ligand to CD40 by at least 45%, and (iii) comprises one or more substitutions or insertions in primary amino acid sequence relative to native monoclonal antibody S2C6 as secreted by the hybridoma deposited with the ATCC and assigned accession number PTA-110, which amount is such that the immune response of the subject is activated or augmented.

35. A method for activating or augmenting the immune response of a subject comprising:

administering to the subject an amount of a purified protein, which protein (i) competes for binding to CD40 with monoclonal antibody S2C6 as secreted by the hybridoma deposited with the ATCC and assigned accession number PTA-110, (ii) increases the binding of CD40 ligand to CD40 by at least 45%, and (iii) is not monoclonal antibody S2C6 as secreted by the hybridoma deposited with the ATCC and assigned accession number PTA-110, and does not result from cleavage of S2C6 with papain or pepsin, which amount is such that the immune response of the subject is activated or augmented.

36. A method for the treatment or prevention of an immune disorder in a subject comprising:

administering to the subject an amount of a molecule comprising SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, or SEQ ID NO:10, which molecule (i) immunospecifically binds CD40, (ii) increases the binding of CD40 ligand to CD40 by at least 45%, and (iii) comprises one or more substitutions or insertions in primary amino acid sequence relative to native monoclonal antibody S2C6 as secreted by the hybridoma deposited with the ATCC and assigned accession number PTA-110, which amount is effective for the treatment or prevention of an immune disorder.

37. A method for the treatment or prevention of an immune disorder in a subject comprising:

administering to the subject an amount of a purified protein, which protein (i) competes for binding to CD40 with monoclonal antibody S2C6 as secreted by the hybridoma deposited with the ATCC and assigned accession number PTA-110, (ii) increases the binding of CD40 ligand to CD40 by at least 45%, and (iii) comprises one or more substitutions or insertions in primary amino acid sequence relative to native monoclonal antibody S2C6 as secreted by the hybridoma deposited with the ATCC and assigned accession number PTA-110, which amount is effective for the treatment or prevention of an immune disorder.

38. A method for the treatment or prevention of an immune disorder in a subject comprising:

administering to the subject an amount of a purified protein, which protein (i) competes for binding to CD40 with monoclonal antibody S2C6 as secreted by the hybridoma deposited with the ATCC

and assigned accession number PTA-110, (ii) increases the binding of CD40 ligand to CD40 by at least 45%, and (iii) is not monoclonal antibody S2C6 as secreted by the hybridoma deposited with the ATCC and assigned accession number PTA-110, and does not result from cleavage of S2C6 with papain or pepsin, which amount is effective for the treatment or prevention of an immune disorder.

39. The method of any one of claims 30-38 further comprising administering CD40 ligand to the subject.

40. The method of any one of claims 30-38 in which the subject is a human.

41. The antibody of claim 4 which is not isotype IgG1.

42. A transgenic non-human animal, plant, or an isolated cell containing one or more transgenes encoding a protein, which protein competes for binding to CD40 with monoclonal antibody S2C6 as secreted by the hybridoma deposited with the ATCC and assigned accession number PTA-110, and which protein increases the binding of CD40 ligand to CD40 by at least 45%.

43. A pharmaceutical composition comprising in an amount effective for the treatment or prevention of cancer or an immune disorder, or for activating or augmenting an immune response: (a) a molecule that immunospecifically binds CD40, which molecule increases the binding of CD40 ligand to CD40; (b) CD40 ligand; and (c) a pharmaceutically acceptable carrier.

44. A method for the treatment or prevention of cancer or an immune disorder in a subject comprising administering to the subject, in an amount effective for said treatment or prevention: (a) a molecule that immunospecifically binds

CD40, which molecule increases the binding of CD40 ligand to CD40; and (b) CD40 ligand.

45. The method of claim 44, wherein the method is for the treatment of cancer.

46. The method of any one of claims 30-32 and 45, wherein the cancer is a solid tumor.

47. The method of any one of claims 36-38, wherein the immune disorder is rheumatoid arthritis.

SEQUENCE LISTING

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# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/15749

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(7) : C12N 15/63+, 15/00; G01N 33/53+; A61K 38/00+; C07K 5/00+, 14/00+, 16/00+; C07H 21/04.
US CL : 435/7.1, 69.1, 320.1; 514/2; 530/329, 329, 387.1, 387.3; 536/23.4, 23.5, 23.53.

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/7.1, 69.1, 320.1; 514/2; 530/329, 329, 387.1, 387.3; 536/23.4, 23.5, 23.53.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Office commercial protein and nucleic acid databases, WEST, Medline, SciSearch, embase.

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | US 5,677,165 A (DE BOER et al) 14 October 1997 (14.10.1997) , abstract, claims and columns 2-3, 5 and 9. | 1-7, 9-47 |
| Y | US 5,874,082 A (DE BOER et al) 23 February 1999 (23.02.1999), abstract and claims. | 1-7, 9-47 |
| X | US 6,056,959 A (DE BOER et al) 02 May 2000 (2.05.2000), claims and description. | 1-7, 9-18 |
| Y | BJORCK et al. CD40 Antibodies Defining Distinct Epitopes Display Qualitative Differences in Their Induction of B-cell Differentiation. Immunology. 1996, Vol. 87, pages 291-295, especially materials and methods and discussion. | 1-7, 9-47 |
| Y | BJORCK et al. Antibodies to Distinct Epitopes on the CD40 Molecule Co-operate in Stimulation and Can Be Used for the Detection of Soluble CD40. Immmunology. 1994, Vol. 83, pages 430-437, see entire document. | 1-7, 9-47 |
| X | CHEN et al. Nucleotide and Translated Amino Acid Sequences of cDNA Coding for the Variable Regions of the Light and Heavy Chains of Mouse Hybridoma Antibodies to Blood Group A and B Substances. The Journal of Biological Chemistry. 05 October 1987, Vol. 262, pages 13579-13583, especially Figure 1. | 1-7, 9-47 and SEQ ID NO:4. |
| X | TILLMAN et al. Both IgM and IgG Anti-DNA Antibodies are the Products of Clonally Selective B Cell Stimulation in (NZB x NZW)F1 Mice. J. Exp. Med., September 1992, Vol. 176, No. 3, pages 761-779, see entire document. | 1-7, 9-47 and SEQ ID NOs:4 and 12. |

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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权利要求书9页 说明书60页 附图页数10页

[54]发明名称 重组抗-CD40抗体及其应用

[57]摘要

本发明涉及预防和治疗癌症、炎症性疾病和免疫系统疾病或缺陷的方法和组合物。本发明的方法包括给予增强CD40与CD40配体结合的CD40结合蛋白。

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# 权 利 要 求 书

1. 一种包含 SEQ ID NO: 3、SEQ ID NO: 4、SEQ ID NO: 8、SEQ ID NO: 9 或 SEQ ID NO: 10 的分子，所述分子(a)免疫特异性地结合 CD40，和(b)与杂交瘤(保藏于 ATCC，指定的保藏号为 PTA-110)分泌的天然单克隆抗体 S2C6 相比，在一级氨基酸序列中包含一个或多个取代或插入。

2. 一种包含 SEQ ID NO: 3、SEQ ID NO: 4、SEQ ID NO: 8、SEQ ID NO: 9 或 SEQ ID NO: 10 的分子，所述分子(a)免疫特异性地结合 CD40，和(b)不是由杂交瘤(保藏于 ATCC，指定的保藏号为 PTA-110)分泌的单克隆抗体 S2C6，且不是由木瓜蛋白酶或胃蛋白酶切割 S2C6 产生。

3. 权利要求 1 或 2 的分子，所述分子含有 SEQ ID NO: 2 的氨基酸序列，或者含有 SEQ ID NO: 7 的氨基酸序列，或者既含有 SEQ ID NO: 2 的氨基酸序列，也含有 SEQ ID NO: 7 的氨基酸序列。

4. 权利要求 1 或 2 的分子，它是一种抗体。

5. 权利要求 1 或 2 的分子，它是一种融合蛋白，含有为非抗体的第二种分子的氨基酸序列。

6. 权利要求 5 的分子，它含有融合至 SEQ ID NO: 7 或融合至 SEQ ID NO: 2 的 bryodin (BD1)的氨基酸序列。

7. 权利要求 1 或 2 的分子，它是一种抗体，包含由杂交瘤(保藏于 ATCC，指定的保藏号为 PTA-110)分泌的单克隆抗体 S2C6 的可变区和人恒定区。

8. 权利要求 1-4 中任一项的分子，该分子是纯化的。

9. 一种纯化的蛋白，它含有的氨基酸序列与 SEQ ID NO: 2 或 SEQ ID NO: 7 具有至少 95%的同一性，所述蛋白(a)免疫特异性地结合 CD40；和(b)与杂交瘤(保藏于 ATCC，指定的保藏号为 PTA-110)分泌的天然单克隆抗体 S2C6 相比，在初级氨基酸序列中包含一个或多

个取代或插入。

10. 一种纯化的蛋白，所述蛋白(a)与杂交瘤(保藏于 ATCC，指定的保藏号为 PTA-110)分泌的单克隆抗体 S2C6 竞争结合 CD40，(b)使 CD40 配体与 CD40 的结合增加至少 45%，和(c)与杂交瘤(保藏于 ATCC，指定的保藏号为 PTA-110)分泌的天然单克隆抗体 S2C6 相比，在初级氨基酸序列中包含一个或多个取代或插入。

11. 一种纯化的蛋白，所述蛋白(a)与杂交瘤(保藏于 ATCC，指定的保藏号为 PTA-110)分泌的单克隆抗体 S2C6 竞争结合 CD40，(b)使 CD40 配体与 CD40 的结合增加至少 45%，和(c)不是由杂交瘤(保藏于 ATCC，指定的保藏号为 PTA-110)分泌的单克隆抗体 S2C6，且不是由木瓜蛋白酶或胃蛋白酶切割 S2C6 产生。

12. 一种分离的核酸，它含有 SEQ ID NO: 1、SEQ ID NO: 6、SEQ ID NO: 11、SEQ ID NO: 12、SEQ ID NO: 13、SEQ ID NO: 14 或 SEQ ID NO: 15。

13. 一种分离的核酸，它含有编码含 SEQ ID NO: 3、SEQ ID NO: 4、SEQ ID NO: 8、SEQ ID NO: 9 或 SEQ ID NO: 10 的蛋白的核苷酸序列。

14. 权利要求 13 的分离的核酸，它含有编码蛋白的核苷酸序列，所述蛋白包含(a)由杂交瘤(保藏于 ATCC，指定的保藏号为 PTA-110)分泌的单克隆抗体 S2C6 的重链可变区，和(b)人恒定区。

15. 一种分离的核酸，它含有编码蛋白的核苷酸序列，所述蛋白包含的氨基酸序列与 SEQ ID NO: 2 或 SEQ ID NO: 7 具有至少 95% 的同一性。

16. 一种包含编码蛋白的核苷酸序列的分离的核酸，所述蛋白与杂交瘤(保藏于 ATCC，指定的保藏号为 PTA-110)分泌的单克隆抗体 S2C6 竞争结合 CD40，并且所述蛋白增加 CD40 配体与 CD40 的结合至少 45%。

17. 一种包含编码融合蛋白的核苷酸序列的分离的核酸，所述融

合蛋白包含融合至 SEQ ID NO: 7 或 SEQ ID NO: 2 的 bryodin 1 (BD1) 的氨基酸序列。

18. 一种分离的核酸，它在高严格条件下与由编码 DNA 序列组成的 DNA 的反向互补物杂交，所述编码 DNA 序列对由选自 SEQ ID NO: 2 和 SEQ ID NO: 7 的氨基酸序列组成的蛋白进行编码，所述分离的核酸编码免疫特异性结合 CD40 的蛋白。

19. 一种重组细胞，它含有的重组核酸载体包含编码蛋白的核苷酸序列，所述蛋白与杂交瘤(保藏于 ATCC，指定的保藏号为 PTA-110)分泌的单克隆抗体 S2C6 竞争结合 CD40，并且所述蛋白增加 CD40 配体与 CD40 的结合至少 45%。

20. 一种重组细胞，它含有的重组核酸载体包含 SEQ ID NO: 1、SEQ ID NO: 6、SEQ ID NO: 11、SEQ ID NO: 12、SEQ ID NO: 13、SEQ ID NO: 14 或 SEQ ID NO: 15。

21. 一种生产蛋白的方法，该方法包括:

(a) 培养包含编码蛋白的重组核苷酸序列的细胞，所述蛋白与保藏于 ATCC，指定的保藏号为 PTA-110 的单克隆抗体 S2C6 竞争结合 CD40，并且所述蛋白增加 CD40 配体与 CD40 的结合至少 45%，以使所述蛋白由所述细胞表达；和

(b) 回收所表达的蛋白。

22. 一种生产蛋白的方法，该方法包括:

(a) 培养包含编码蛋白的重组核苷酸序列的细胞，所述蛋白包含 SEQ ID NO: 2、SEQ ID NO: 3、SEQ ID NO: 4、SEQ ID NO: 7、SEQ ID NO: 8、SEQ ID NO: 9 或 SEQ ID NO: 10，以使由所述核苷酸序列编码的蛋白由所述细胞表达；和

(b) 回收所表达的蛋白。

23. 一种药用组合物，它包括

(a) 包含 SEQ ID NO: 2、SEQ ID NO: 3、SEQ ID NO: 4、SEQ ID NO: 7、SEQ ID NO: 8、SEQ ID NO: 9 或 SEQ ID NO: 10 的一种分子，其量可有效治疗或预防癌症，所述分子(i)免疫特异性结合 CD40，(ii)使 CD40 配体与 CD40 的结合增加至少 45%，和(iii)与杂交瘤(保藏于 ATCC，指定的保藏号为 PTA-110)分泌的天然单克隆抗体 S2C6 相比，在初级氨基酸序列中包含一个或多个取代或插入；和

(b) 一种药学上可接受的载体。

24. 一种药用组合物，它包括

(a) 一种治疗或预防癌症有效量的纯化蛋白，所述蛋白(i)与杂交瘤(保藏于 ATCC，指定的保藏号为 PTA-110)分泌的单克隆抗体 S2C6 竞争结合 CD40，(ii)使 CD40 配体与 CD40 的结合增加至少 45%，和(iii)与杂交瘤(保藏于 ATCC，指定的保藏号为 PTA-110)分泌的天然单克隆抗体 S2C6 相比，在初级氨基酸序列中包含一个或多个取代或插入；和

(b) 一种药学上可接受的载体。

25. 一种药用组合物，它包括

(a) 一种治疗或预防癌症有效量的纯化蛋白，所述蛋白(i)与杂交瘤(保藏于 ATCC，指定的保藏号为 PTA-110)分泌的单克隆抗体 S2C6 竞争结合 CD40，(ii)使 CD40 配体与 CD40 的结合增加至少 45%，和(iii)不是由杂交瘤(保藏于 ATCC，指定的保藏号为 PTA-110)分泌的单克隆抗体 S2C6，且不是由木瓜蛋白酶或胃蛋白酶切割 S2C6 产生；和

(b) 一种药学上可接受的载体。

26. 一种药用组合物，它包括

(a) 包含 SEQ ID NO: 2、SEQ ID NO: 3、SEQ ID NO: 4、SEQ ID NO: 7、SEQ ID NO: 8、SEQ ID NO: 9 或 SEQ ID NO: 10 的一种分子，其量可有效激活或增强免疫应答，所述分

子(i)免疫特异性结合 CD40，(ii)使 CD40 配体与 CD40 的结合增加至少 45%，和(iii)与杂交瘤(保藏于 ATCC，指定的保藏号为 PTA-110)分泌的天然单克隆抗体 S2C6 相比，在初级氨基酸序列中包含一个或多个取代或插入；和

(b)一种药学上可接受的载体。

27. 一种药用组合物，它包括

(a)一种纯化蛋白，其量可有效激活或增强免疫应答，所述蛋白(i)与杂交瘤(保藏于 ATCC，指定的保藏号为 PTA-110)分泌的单克隆抗体 S2C6 竞争结合 CD40，(ii)使 CD40 配体与 CD40 的结合增加至少 45%，和(iii)与杂交瘤(保藏于 ATCC，指定的保藏号为 PTA-110)分泌的天然单克隆抗体 S2C6 相比，在初级氨基酸序列中包含一个或多个取代或插入；和

(b)一种药学上可接受的载体。

28. 一种药用组合物，它包括

(a)一种纯化蛋白，其量可有效激活或增强免疫应答，所述蛋白(i)与杂交瘤(保藏于 ATCC，指定的保藏号为 PTA-110)分泌的单克隆抗体 S2C6 竞争结合 CD40，(ii)使 CD40 配体与 CD40 的结合增加至少 45%，和(iii)不是由杂交瘤(保藏于 ATCC，指定的保藏号为 PTA-110)分泌的单克隆抗体 S2C6，且不是由木瓜蛋白酶或胃蛋白酶切割 S2C6 产生；和

(b)一种药学上可接受的载体。

29. 权利要求 23-28 中任一项的药用组合物，它还含有 CD40 配体。

30. 一种在患者中治疗或预防癌症的方法，该方法包括：

给予所述患者一定量的包含 SEQ ID NO：2、SEQ ID NO：3、SEQ ID NO：4、SEQ ID NO：7、SEQ ID NO：8、SEQ ID NO：

9 或 SEQ ID NO: 10 的一种分子，其量可有效治疗或预防癌症，所述分子(i)免疫特异性结合 CD40，(ii)使 CD40 配体与 CD40 的结合增加至少 45%，和(iii)与杂交瘤(保藏于 ATCC，指定的保藏号为 PTA-110)分泌的天然单克隆抗体 S2C6 相比，在初级氨基酸序列中包含一个或多个取代或插入。

31. 一种在患者中治疗或预防癌症的方法，该方法包括：

给予所述患者一定量的一种纯化蛋白，其量可有效治疗或预防癌症，所述蛋白(i)与杂交瘤(保藏于 ATCC，指定的保藏号为 PTA-110)分泌的单克隆抗体 S2C6 竞争结合 CD40，(ii)使 CD40 配体与 CD40 的结合增加至少 45%，和(iii)与杂交瘤(保藏于 ATCC，指定的保藏号为 PTA-110)分泌的天然单克隆抗体 S2C6 相比，在初级氨基酸序列中包含一个或多个取代或插入。

32. 一种在患者中治疗或预防癌症的方法，该方法包括：

给予所述患者一定量的一种纯化蛋白，其量可有效治疗或预防癌症，所述蛋白(i)与杂交瘤(保藏于 ATCC，指定的保藏号为 PTA-110)分泌的单克隆抗体 S2C6 竞争结合 CD40，(ii)使 CD40 配体与 CD40 的结合增加至少 45%，和(iii)不是由杂交瘤(保藏于 ATCC，指定的保藏号为 PTA-110)分泌的单克隆抗体 S2C6，且不是由木瓜蛋白酶或胃蛋白酶切割 S2C6 产生。

33. 一种激活或增强患者的免疫应答的方法，该方法包括：

给予所述患者一定量的包含 SEQ ID NO: 2、SEQ ID NO: 3、SEQ ID NO: 4、SEQ ID NO: 7、SEQ ID NO: 8、SEQ ID NO: 9 或 SEQ ID NO: 10 的一种分子，其量使得所述患者的免疫应答被激活或增强，所述分子(i)免疫特异性结合 CD40，(ii)使 CD40 配体与 CD40 的结合增加至少 45%，和(iii)与杂交瘤(保藏于 ATCC，指定的保藏号为 PTA-110)分泌的天然单克隆抗体 S2C6 相比，在初级氨基酸序列中包含一个或多个取代或

插入。

34. 一种在患者中激活或增强免疫应答的方法，该方法包括：给予所述患者一定量的一种纯化蛋白，其量使得所述患者的免疫应答被激活或增强，所述蛋白(i)与杂交瘤(保藏于ATCC，指定的保藏号为PTA-110)分泌的单克隆抗体S2C6竞争结合CD40，(ii)使CD40配体与CD40的结合增加至少45%，和(iii)与杂交瘤(保藏于ATCC，指定的保藏号为PTA-110)分泌的天然单克隆抗体S2C6相比，在初级氨基酸序列中包含一个或多个取代或插入。

35. 一种在患者中激活或增强免疫应答的方法，该方法包括：给予所述患者一定量的一种纯化蛋白，其量使得所述患者的免疫应答被激活或增强，所述蛋白(i)与杂交瘤(保藏于ATCC，指定的保藏号为PTA-110)分泌的单克隆抗体S2C6竞争结合CD40，(ii)使CD40配体与CD40的结合增加至少45%，和(iii)不是由杂交瘤(保藏于ATCC，指定的保藏号为PTA-110)分泌的单克隆抗体S2C6，且不是由木瓜蛋白酶或胃蛋白酶切割S2C6产生。

36. 一种在患者中治疗或预防免疫疾病的方法，该方法包括：给予所述患者一定量的包含SEQ ID NO：2、SEQ ID NO：3、SEQ ID NO：4、SEQ ID NO：7、SEQ ID NO：8、SEQ ID NO：9或SEQ ID NO：10的一种分子，其量可有效治疗或预防免疫疾病，所述分子(i)免疫特异性结合CD40，(ii)使CD40配体与CD40的结合增加至少45%，和(iii)与杂交瘤(保藏于ATCC，指定的保藏号为PTA-110)分泌的天然单克隆抗体S2C6相比，在初级氨基酸序列中包含一个或多个取代或插入。

37. 一种在患者中治疗或预防免疫疾病的方法，该方法包括：给予所述患者一定量的一种纯化蛋白，其量可有效治疗或预

防免疫疾病，所述蛋白(i)与杂交瘤(保藏于 ATCC，指定的保藏号为 PTA-110)分泌的单克隆抗体 S2C6 竞争结合 CD40，(ii)使 CD40 配体与 CD40 的结合增加至少 45%，和(iii)与杂交瘤(保藏于 ATCC，指定的保藏号为 PTA-110)分泌的天然单克隆抗体 S2C6 相比，在初级氨基酸序列中包含一个或多个取代或插入。

38. 一种在患者中治疗或预防免疫疾病的方法，该方法包括：给予所述患者一定量的一种纯化蛋白，其量可有效治疗或预防免疫疾病，所述蛋白(i)与杂交瘤(保藏于 ATCC，指定的保藏号为 PTA-110)分泌的单克隆抗体 S2C6 竞争结合 CD40，(ii)使 CD40 配体与 CD40 的结合增加至少 45%，和(iii)不是由杂交瘤(保藏于 ATCC，指定的保藏号为 PTA-110)分泌的单克隆抗体 S2C6，且不是由木瓜蛋白酶或胃蛋白酶切割 S2C6 产生。

39. 权利要求 30-38 中任一项的方法，该方法还包括给予所述患者 CD40 配体。

40. 权利要求 30-38 中任一项的方法，其中所述患者是人。

41. 权利要求 4 的抗体，它不是同种型 IgG1。

42. 一种人类以外的转基因动物、转基因植物或包含一种或多种编码蛋白的转基因的分离细胞，所述蛋白与杂交瘤(保藏于 ATCC，指定的保藏号为 PTA-110)分泌的单克隆抗体 S2C6 竞争结合 CD40，并且所述蛋白使 CD40 配体与 CD40 的结合增加至少 45%。

43. 一种药用组合物，它包含(a)免疫特异性结合 CD40 的一种分子，所述分子增加 CD40 配体与 CD40 的结合；(b)CD40 配体；和(c)一种药学上可接受的载体，它们的量可有效治疗或预防癌症或免疫疾病，或可有效激活或增强免疫应答。

44. 一种在患者中治疗或预防癌症或免疫疾病的方法，该方法包括给予所述患者(a)免疫特异性结合 CD40 的一种分子，所述分子增加 CD40 配体与 CD40 的结合；和(b)CD40 配体，它们的量对所述治疗或

预防有效。

45. 权利要求 44 的方法，其中所述方法是用于治疗癌症。

46. 权利要求 30-32 和 45 中任一项的方法，其中所述癌症为实体瘤。

47. 权利要求 36-38 中任一项的方法，其中所述免疫疾病为类风湿性关节炎。

# 说　明　书

## 重组抗-CD40 抗体及其应用

### 1. 发明领域

本发明涉及治疗包括癌症、炎性疾病和免疫系统疾病或紊乱在内的疾病的方法和组合物，包括给予增强 CD40 配体与 CD40 结合的 CD40 结合蛋白。CD40 结合蛋白包括重组/变异形式的单克隆抗体 S2C6 及其衍生物。

### 2. 发明背景

CD40 是一种在各种细胞类型上表达的细胞表面磷酸化糖蛋白，所述细胞类型包括 B 细胞、B 细胞恶性肿瘤、小结树突细胞、上皮基细胞和癌。CD40 结合 CD40 配体("CD40L")。在炎症和癌症过程中，CD40L 于活化 T 细胞上表达(Younes 等，1998，Br. J. Haematol. 100: 135-141；参见 Grewal 和 Flavell，1998，Annu. Rev. Immunol. 16: 111-135 的论述)。CD40 与 CD40L 的相互作用导致 B 细胞激活和正常 B 细胞的增殖；然而在 B 细胞衍生的肿瘤系中 CD40 介导的信号转导可以导致激活-诱导的细胞死亡。活化信号的强度是激活-诱导的肿瘤细胞死亡的关键(Grafton 等，1997，Cell. Immunol. 182: 45-56)。因此，用于增强 CD40 和 CD40L 之间的受体-配体相互作用以及活化信号强度的组合物和方法对于治疗疾病应当具有巨大的价值。

#### 2.1 CD40 和 CD40 配体

CD40 是 TNF 受体超家族的一员。该家族包括 TNFrII、CD40、CD30、LMP-1、LTBr、ATAR、OX-40 和 4-1BB 受体。CD40 组成型表达于 B-淋巴细胞、巨噬细胞和树突细胞上，并由在成纤维细胞、内皮细胞和上皮细胞上活化的细胞因子诱导(Van Kooten 和 Banchereau，1997，Curr. Opin. Immunol.，9: 330-337)。还已经表明，

CD40 高度表达于许多人类癌症，包括肺癌、膀胱癌、胃癌、乳癌和卵巢癌(Stamenkovic 等，1989，EMBO J. 8：1403-1410)。

CD40 配体是一种在活化 T 细胞上表达的膜蛋白。受体结合 CD40L 导致 CD40 多聚化，产生活化信号(对于抗原提呈(presenting)细胞如树突细胞、单核细胞和 B 细胞)和生长与分化信号(对于细胞因子活化的成纤维细胞和上皮细胞)。CD40 信号由多聚化受体经一系列 TNF 受体相关因子("TRAF")的募集而转导(Kehry，1996，J. Immunol. 156：2345-2348)。TRAF 的各种亚型与 TNF 家族成员(包括 CD40)的相互作用各有不同，以使向种类繁多的下游途径提供刺激物。TRAF1 和 TRAF2 参与编程性细胞死亡的调节(Speiser 等，1997，J. Exp. Med. 185：1777-1783；Yeh 等，1997，Immunity，7：715-725)。TRAF 2、5 和 6 参与增殖和活化过程，包括 NF-κB 和 c-Jun N 末端激酶活化。在正常 B 细胞中，CD40 的结合为受体复合物募集 TRAF2 和 TRAF3，并诱导其它 TRAF 的下调(Kuhune 等，1997，J. Exp. Med. 186：337-342)。CD40 结合的作用还取决于膜密度(De Paoli 等，1997，Cytometry 30：33-38)。重要的是，与在正常原始 B 细胞观察到的增殖反应不同，在肿瘤 B 细胞上的 CD40 结合可以导致生长抑制和活化-诱导的细胞死亡(Funakoshi 等，1994，Blood 83：2787-2794)。因此，在不同细胞类型中的 CD40 的活化、转化、残余 TRAF 和共刺激物可以诱导由活化和增殖至生长抑制和编程性细胞死亡的反应。

## 2.2 抗 CD40 抗体

除至少一个类型外，至今描述的抗 CD40 单克隆抗体("mAb")有三个基本类型：(1)以至少 90%封闭 CD40/CD40L 的相互作用并具有抗瘤特性的抗 CD40 单克隆抗体(Armitage 等，美国专利第 5,674,492 号；Fanslow 等，1995，Leukocyte Typing V，Schlossman 等编辑，1：555-556)；(2)通过 CD40 对抗信号转导的抗 CD40 单克隆抗体(deBoer 等，美国专利第 5,677,165 号)；和(3)通过 CD40 传递刺激信号但不增

加 CD40 和 CD40L 之间的相互作用的抗 CD40 单克隆抗体，例如 G28-5 (Ledbetter 等，美国专利第 5,182,368 号；PCT 说明书 WO 96/18413)。

已表明，一种单克隆抗体 CD40.4 (5C3)(PharMingen，San Diego，California)使 CD40 和 CD40L 之间的相互作用增加约 30-40% (Schlossman 等编辑，1995，Leukocyte Typing V：白细胞分化抗原 1：547-556)。

Armitage 等(美国专利第 5,674,492 号)介绍了使用 CD40 结合蛋白(包括单克隆抗体 HuCD40-M2)的方法，它们能够结合 CD40 并抑制 CD40 与 CD40L 的结合，以预防或治疗特征为肿瘤细胞表达 CD40 的疾病。

DeBoer 等(美国专利第 5,677,165 号)描述了抗 CD40 单克隆抗体，它没有明显的拮抗活性，结合 B 细胞表面上的 CD40 并阻断 B 细胞活化。美国专利第 5,677,165 号的基本特征在于当抗 CD40 单克隆抗体结合正常人 B 细胞表面上的人 CD40 时，正常人 B 细胞的生长或分化受到抑制。

Ledbetter 等(美国专利第 5,182,368 号)描述了一种配体 G28-5，它结合 B 细胞表面抗原 Bp50(现称为 CD40)并刺激活化的 B 细胞阻碍细胞周期，使得 B 细胞增殖扩大。然而，G28-5 在 CD40L 存在下没有增强 B 细胞的活化，也没有加强 CD40/CD40L 的相互作用。

S2C6 是一种制备用于抵抗人膀胱癌的抗 CD40 单克隆抗体(Paulie 等，1984，Cancer Immunol. Immunother. 17：165-179)。S2C6 结合在各种细胞类型上表达的 CD40 受体，包括 B-淋巴细胞、内皮细胞和上皮细胞。已经证明 S2C6 对尿路上皮癌和 B 细胞衍生的恶性淋巴细胞具有特异性。还已经证明 S2C6 与前列腺癌细胞系 HS 具有反应性，以及与黑素瘤具有弱反应性(Paulie 等，1984，Cancer Immunol. Immunother. 17：165-179)。已有研究证实 S2C6 作为 B 细胞恶性肿瘤诊断标记的用途(Paulie 等，1984，Cancer Immunol. Immunother. 17：165-179；Paulie 等，1985，Eur. J. Cancer. Clin. Oncol. 21：701-710)。

除了检测 B 细胞恶性肿瘤以外，还已经显示 S2C6 向 B 淋巴细胞传递强生长促进信号(Paulie 等，1989，J. Immunol. 142：590-595)。

S2C6 能以剂量依赖方式刺激原代 B 细胞增殖，由此证明 S2C6 对人外周 B 细胞具有激动活性(Paulie 等，1989，J. Immunol. 142：590-595)。

尽管竞争性研究已经显示 G28-5 和 S2C6 结合相同或最接近的表位，但经测定所述抗体的功能不同，这主要是依据所述的单克隆抗体之一所达到的对先前受刺激的扁桃体 B 细胞的刺激程度(Clark 和 Ledbetter，1986，Proc. Natl. Acad. Sci. USA 83：4494-4498；Ledbetter 等，美国专利第 5,182,368 号)。在具体的测试条件下，实现扁桃体 B 细胞活化需要的 S2C6 比 G28-5 多 100 倍(Ledbetter 等，美国专利第 5,182,368 号)。

本领域需要一种治疗方法，以增加治疗或预防癌症、激活或加强免疫系统或治疗或预防免疫缺陷或疾病的功效，而本发明提供这种需要。

本文引用或有关联的任一参考文献不应被视为允许该参考文献作为本发明的先有技术。

## 3. 发明概述

申请人获得的出乎意料的发现是一类新的抗 CD40 抗体，即除了传递刺激信号以外，还增强 CD40 和 CD40L 之间的相互作用、增强 CD40L 介导的刺激和具有体内抗癌活性。本发明人对 S2C6 单克隆抗体可变区的克隆和测序以及其中 CDR 区和构架区的鉴定，促进了这些抗体和其相关分子的产生和应用。

本发明涉及的分子包含 S2C6 单克隆抗体的可变区或一个或多个具有新序列(SEQ ID NO：3、4、8、9 或 10)的其互补性决定区(CDR)，所述分子(a)免疫特异性地结合 CD40 和(b)与杂交瘤(保藏于 ATCC，指定的保藏号为 PTA-110)分泌的天然单克隆抗体 S2C6 相比，在初

级氨基酸序列中包含一个或多个取代或插入，或不是由杂交瘤(保藏于 ATCC，指定的保藏号为 PTA-110)分泌的单克隆抗体 S2C6 且不是由木瓜蛋白酶或胃蛋白酶切割 S2C6 产生。在一个具体的实施方案中，所述分子不是天然单克隆抗体 S2C6，且不含有所述单克隆抗体 S2C6 的天然重链或轻链。在另一个具体的实施方案中，所述分子是一种抗体。在另一个实施方案中，所述抗体不是同种型 IgG1。在另一个具体的实施方案中，所述分子含有轻链可变区(SEQ ID NO: 2 的氨基酸序列)或重链可变区(SEQ ID NO: 7 的氨基酸序列)。

本发明还涉及包含融合至第二种蛋白的氨基酸序列的单克隆抗体 S2C6 的片段的嵌合/融合蛋白，以及涉及其中单克隆抗体 S2C6 的片段共价结合(例如通过使用交联剂)至另一种化学结构的分子。在一个具体的实施方案中，提供一种免疫特异性地结合 CD40 的分子，该分子含有单克隆抗体 S2C6 的重链和/或轻链可变区，它们融合至含有 bryodin 1(BD1)的氨基酸序列的第二种蛋白。

本发明还涉及其含有的氨基酸序列与 SEQ ID NO: 2、SEQ ID NO: 3、SEQ ID NO: 4、SEQ ID NO: 7、SEQ ID NO: 8、SEQ ID NO: 9 或 SEQ ID NO: 10 具有至少 95%的同一性的蛋白，所述蛋白(a)免疫特异性地结合 CD40 和(b)与杂交瘤(保藏于 ATCC，指定的保藏号为 PTA-110)分泌的天然单克隆抗体 S2C6 相比，在初级氨基酸序列中包含一个或多个取代或插入。在一个具体的实施方案中，所述蛋白不是天然单克隆抗体 S2C6，且不含有所述单克隆抗体 S2C6 的天然重链或轻链。

本发明还涉及纯化蛋白，所述蛋白(a)与杂交瘤(保藏于 ATCC，指定的保藏号为 PTA-110)分泌的单克隆抗体 S2C6 竞争结合 CD40，(b)使 CD40 配体与 CD40 的结合增加至少 45%，和(c)与杂交瘤(保藏于 ATCC，指定的保藏号为 PTA-110)分泌的天然单克隆抗体 S2C6 相比，在初级氨基酸序列中包含一个或多个取代或插入，或不是由杂交瘤(保藏于 ATCC，指定的保藏号为 PTA-110)分泌的单克隆抗体

S2C6，且不是由木瓜蛋白酶或胃蛋白酶切割 S2C6 产生。在一个具体的实施方案中，所述蛋白不是天然单克隆抗体 S2C6，且不含有所述单克隆抗体 S2C6 的天然重链或轻链。

本发明还涉及编码所述分子和蛋白的核酸或与包括编码核苷酸序列的所述蛋白的 DNA 杂交的核酸；包含所述分子和蛋白的重组细胞；以及生产所述蛋白的方法。

在一个实施方案中，所述分离的核酸包含编码蛋白的核苷酸序列，其中所述蛋白含有(a)由杂交瘤(保藏于 ATCC，指定的保藏号为 PTA-110)分泌的单克隆抗体 S2C6 的重链可变区，和(b)人恒定区。

在一个实施方案中，所述分离的核酸包含编码蛋白的核苷酸序列，其中所述蛋白含有(a)由杂交瘤(保藏于 ATCC，指定的保藏号为 PTA-110)分泌的单克隆抗体 S2C6 的轻链可变区，和(b)人恒定区。

本发明还涉及包含重组核酸载体的重组细胞，该载体含有编码蛋白的核苷酸序列，所述蛋白与杂交瘤(保藏于 ATCC，指定的保藏号为 PTA-110)分泌的单克隆抗体 S2C6 竞争结合 CD40，并使 CD40 配体与 CD40 的结合增加至少 45%。本发明还提供生产所述蛋白的方法，包括培养所述细胞，使得所述蛋白由所述细胞表达，并回收所表达的蛋白。

本发明还涉及含有重组核酸载体的重组细胞，所述载体包含 SEQ ID NO: 1、SEQ ID NO: 6、SEQ ID NO: 11、SEQ ID NO: 12、SEQ ID NO: 13、SEQ ID NO: 14 或 SEQ ID NO: 15，以及涉及生产蛋白的方法，包括培养所述细胞，使得由所述核苷酸序列编码的蛋白由所述细胞表达，并回收所表达的蛋白。

还提供优选为纯化形式的包含本发明分子和抗体的药用组合物。在具体的实施方案中，本发明涉及含有一种分子和一种药学上可接受的载体的药用组合物，所述分子含有 SEQ ID NO: 2、SEQ ID NO: 3、SEQ ID NO: 4、SEQ ID NO: 7、SEQ ID NO: 8、SEQ ID NO: 9 或 SEQ ID NO: 10，该分子(i)免疫特异性地结合 CD40，(ii)增加 CD40

配体与 CD40 的结合，和(iii)与杂交瘤(保藏于 ATCC，指定的保藏号为 PTA-110)分泌的天然单克隆抗体 S2C6 相比，在初级氨基酸序列中包含一个或多个取代或插入。在一个具体的实施方案中，所述分子不是天然单克隆抗体 S2C6，且不含有所述单克隆抗体 S2C6 的天然重链或轻链。

本发明还涉及包含一种纯化蛋白和一种药学上可接受的载体的药用组合物，所述蛋白(i)与杂交瘤(保藏于 ATCC，指定的保藏号为 PTA-110)分泌的单克隆抗体 S2C6 竞争结合 CD40，(ii)使 CD40 配体与 CD40 的结合增加至少 45%，和(iii)与杂交瘤(保藏于 ATCC，指定的保藏号为 PTA-110)分泌的天然单克隆抗体 S2C6 相比，在初级氨基酸序列中包含一个或多个取代或插入，或不是由杂交瘤(保藏于 ATCC，指定的保藏号为 PTA-110)分泌的单克隆抗体 S2C6，且不是由木瓜蛋白酶或胃蛋白酶切割 S2C6 产生。在一个具体的实施方案中，所述蛋白不是天然单克隆抗体 S2C6，且不含有所述单克隆抗体 S2C6 的天然重链或轻链。

在具体的实施方案中，本发明的药用组合物包含本发明的分子或抗体，其量可有效治疗或预防癌症，或可有效激活或增强免疫应答，或活化或增强患者的免疫应答。

在具体的实施方案中，本发明的药用组合物还包含 CD40 配体。在一个具体的实施方案中，所述药用组合物包含(a)一种分子，它免疫特异性地结合 CD40，增加 CD40 配体与 CD40 的结合；(b)CD40 配体；和(c)一种药学上可接受的载体，它们的量可有效治疗或预防癌症或免疫疾病，或可有效激活或增强免疫应答。在此实施方案中，例如，所述分子可以是天然单克隆抗体 S2C6 或天然单克隆抗体 5C3 或如本文所述的 S2C6 衍生物。

本发明还涉及在患者中治疗或预防癌症的方法，在患者中激活或增强免疫应答的方法或在患者中治疗或预防免疫缺陷或疾病的方法，包括给予所述患者治疗有效量的本发明的分子或抗体，例如一

定量的包含 SEQ ID NO: 2、SEQ ID NO: 3、SEQ ID NO: 4、SEQ ID NO: 7、SEQ ID NO: 8、SEQ ID NO: 9 或 SEQ ID NO: 10 的分子，所述分子(i)免疫特异性地结合 CD40，(ii)使 CD40 配体与 CD40 的结合增加至少 45%，并且与杂交瘤(保藏于 ATCC，指定的保藏号为 PTA-110)分泌的天然单克隆抗体 S2C6 相比，在初级氨基酸序列中包含一个或多个取代或插入。在一个具体的实施方案中，所述分子不是天然单克隆抗体 S2C6，且不含有所述单克隆抗体 S2C6 的天然重链或轻链。

本发明还涉及在患者中治疗或预防癌症的方法，在患者中激活或增强免疫应答的方法或在患者中治疗或预防免疫缺陷或疾病的方法，包括给予所述患者一种纯化的蛋白，所述蛋白(i)与杂交瘤(保藏于 ATCC，指定的保藏号为 PTA-110)分泌的单克隆抗体 S2C6 竞争结合 CD40，(ii)使 CD40 配体与 CD40 的结合增加至少 45%，和(iii)与杂交瘤(保藏于 ATCC，指定的保藏号为 PTA-110)分泌的天然单克隆抗体 S2C6 相比，在初级氨基酸序列中包含一个或多个取代或插入，或不是由杂交瘤(保藏于 ATCC，指定的保藏号为 PTA-110)分泌的单克隆抗体 S2C6，且不是由木瓜蛋白酶或胃蛋白酶切割 S2C6 产生。在一个具体的实施方案中，所述蛋白不是天然单克隆抗体 S2C6，且不含有所述单克隆抗体 S2C6 的天然重链或轻链。

在具体的实施方案中，本发明的方法还包括给予所述患者 CD40 配体。

本发明还涉及在患者中治疗或预防癌症或免疫疾病的方法，包括给予所述患者其量可有效用于所述治疗或预防的下述物质：(a)一种分子，它免疫特异性地结合 CD40，该分子增强 CD40 配体与 CD40 的结合；和(b) CD40 配体，其中所述分子可以是天然单克隆抗体 S2C6 或天然单克隆抗体 5C3 或如本文所述的任一种 S2C6 衍生物。

在一个优选的实施方案中，所述患者为人。

本发明还涉及人类以外的转基因动物、转基因植物或含有一种

或多种编码蛋白的转基因的分离细胞，所述蛋白与杂交瘤(保藏于ATCC，指定的保藏号为 PTA-110)分泌的单克隆抗体 S2C6 竞争结合 CD40，并且所述蛋白使 CD40 配体与 CD40 的结合增加至少 45%。

## 4. 附图简述

图 1. S2C6 的轻链可变区结构。显示了所述轻链可变区("$V_L$")的核苷酸序列(SEQ ID NO: 1)和氨基酸序列(SEQ ID NO: 2)。

图 2 S2C6 的重链可变区结构。显示了所述重链可变区("$V_H$")的核苷酸序列(SEQ ID NO: 6)和氨基酸序列(SEQ ID NO: 7)。

图 3A-3B. S2C6 的可变区结构。(A)显示了 S2C6 $V_L$ 的氨基酸序列(SEQ ID NO: 2)。(B)显示了 S2C6 $V_H$ 的氨基酸序列(SEQ ID NO: 7)。下划线标示互补性决定区("CDR")。显示了邻接 CDR 的四个构架区的序列。$V_L$ CDR 1-3 的氨基酸序列分别对应于 SEQ ID NOS: 3-5。$V_H$ CDR 1-3 的氨基酸序列分别对应于 SEQ ID NOS: 8-10。

图 4. S2C6 单克隆抗体增强 CD40-Ig 与表达 CD40L 的 Jurkat T 细胞的结合。在增加浓度的抗-CD40 单克隆抗体("mAb")存在下，CD40-Ig (CD40 和人免疫球蛋白的可溶性融合蛋白)结合表面 CD40L。单克隆抗体与 CD40-Ig 预温育 1 小时，接着与表达 CD40L 的靶细胞温育 1 小时。使用异硫氰酸荧光素("FITC")标记的抗人 Ig 利用流式细胞仪检测结合靶细胞的 CD40-Ig。然后根据对数平均荧光强度("MFI")测定 CD40/CD40L 结合的程度。显示了减去每组背景值的 MFI。

图 5. S2C6 单克隆抗体增强可溶性 CD40L 与 B 细胞表面 CD40 的结合。在增加浓度的抗-CD40 单克隆抗体：S2C6、G28-5 或 M3 或者无关对照单克隆抗体，EXA-1H8 存在下，温育 Ramos B 细胞、人 B 细胞淋巴瘤。将所述单克隆抗体与表达 CD40 的靶细胞预温育 1 小时。接着通过流式细胞仪直接检测 FITC 标记的 CD40L 与 B 细胞的结合。然后根据对数平均荧光强度测定 CD40/CD40L 结合的程度。

显示了减去每组背景值的 MFI。

图 6. 在 $CD40L^+$刺激细胞和抗-CD40 单克隆抗体存在下，S2C6 增强原代人外周 B 细胞的增殖反应。将外周 B 细胞($1 \times 10^5$/孔)与增加数目的非增殖性 $CD40L^+$ Jurkat T 刺激细胞和 30 ng/ml 的抗-CD40 单克隆抗体：S2C6、G28-5 或 M3 或对照单克隆抗体 EXA2-1H8 混合在一起。通过在加入刺激物后 72 小时掺入 $^3$H-TdR 检测 B 细胞增殖。

图 7. 在 CD40L 存在或不存在时，对比原代人外周 B 细胞对抗-CD40 单克隆抗体的增殖反应。将外周 B 细胞与 4:1 混合比率的非增殖性 $CD40L^+$刺激细胞和增加浓度的抗-CD40 单克隆抗体：S2C6、G28-5 或对照抗体，EXA2-1H8 混合在一起。通过在加入刺激物后 72 小时掺入 $^3$H-TdR 检测 B 细胞增殖。

图 8A-8C. 单克隆抗体 S2C6 的体内抗瘤活性。评价 S2C6 抗(A) Ramos 人 B 细胞非 Hodgkin 淋巴瘤，(B) HS Sultan 多发性骨髓瘤，或(C) IM-9 多发性骨髓瘤的抗瘤活性。用或不用抗-脱唾液酸-GM1 预处理 SCID 小鼠(5 只/组)，以抑制天然杀伤细胞("NK")活性，并在注射 $1 \times 10^6$-$2 \times 10^6$ 肿瘤细胞后的第 1 天或第 5 天用单克隆抗体处理所述小鼠。实线表示随时间的存活小鼠数目。

图 9. BD1-S2C6 sFv 在 ELISA 中特异性结合固定化 CD40-Ig。BD1-S2C6 sFv(由融合至单克隆抗体 S2C6 可变区的 bryodin 1(BD1)组成的单链抗-CD40 免疫毒素)在大肠杆菌中作为包含体表达，使其变性和重折叠。然后使用 Blue Sepharose 分离重折叠的蛋白，接着经固定化 CD40-Ig 亲和性层析。然后测试纯化蛋白在 ELISA 中结合固定化 CD40-Ig 的情况。用 0.5 μg/ml 的 CD40-Ig 包被微量滴定板，接着在 25 μg/ml S2C6 单克隆抗体(▲)、25 μg/ml 对照抗体 BR96 (●)或无过量抗体(■)存在下，加入纯化 BD1-S2C6 sFv 的稀释液。通过加入 BD1 特异性兔抗血清，接着加入缀合山羊抗兔 Ig 的辣根过氧化物酶，检测 BD1-S2C6 sFv 与固定化受体的结合。加入过量 S2C6 单克隆抗

体完全抑制 BD1-S2C6 sFv 与 CD40-Ig 的结合，但加入对照单克隆抗体不能完全抑制此种结合。

## 5. 发明详述

本发明涉及 S2C6 基因的核苷酸序列及其编码蛋白。本发明还涉及所述 S2C6 蛋白和核酸的片段以及其它衍生物和类似物。在各种具体的实施方案中，本发明的分子(例如抗体)包含全部或部分的 S2C6 单克隆抗体(轻链和/或重链，或轻链 CDR 1 (SEQ ID NO: 3)，和/或轻链 CDR 2 (SEQ ID NO: 4)，和/或重链 CDR 1 (SEQ ID NO: 8)、重链 CDR 2 (SEQ ID NO: 9)和/或重链 CDR 3 (SEQ ID NO: 10)，或轻链 CDR 3 (SEQ ID NO: 5)与任何其它 CDR 和/或四条重链构架区和四条轻链构架区中的一个或多个的组合)，前提是所述分子不是天然单克隆抗体 S2C6(保藏于 ATCC，获得的保藏号微 PTA-110)或其重链或轻链。所述分子与 S2C6 在序列和/或翻译后修饰(糖基化、酰胺化、肽键合或交联至非 S2C6 序列等)方面可以不同。在各种具体实施方案中，本发明的分子免疫特异性地结合 CD40 (或在多聚化时免疫特异性地结合 CD40)、与天然 S2C6 竞争结合 CD40，和/或使 CD40 配体与 CD40 的结合增加至少 45%、50%、60%或 65%。编码所述分子(例如 S2C6 片段或其衍生物)的核酸以及编码天然单克隆抗体 S2C6 的核酸也属于本发明的范围。提供例如通过重组方法来生产前述蛋白。

本发明还涉及 S2C6 蛋白及其衍生物，包括但不限于具有功能活性的融合/嵌合蛋白，即其能够显示一种或多种已知与全长 S2C6 单克隆抗体相关的功能活性。这样的功能活性包括但不限于结合 CD40 的能力、刺激信号至 CD40 信号转导途径的传递(例如便于引起 B 细胞增殖)、CD40L 与 CD40 相互作用的加强；抑制肿瘤生长的能力；以及诱导免疫应答的能力。

另外提供抗 CD40 的抗体，包括 S2C6、其衍生物和类似物，包

括但不限于人源化抗体、单链抗体、双特异性抗体和结合化疗剂或生物反应调节剂的抗体。

本发明还涉及治疗或预防癌症、炎性疾病和免疫系统疾病的方法，包括单独或与 CD40L 联合给予本发明组合物。

通过下文 6-9 部分阐明的实施例说明本发明，所述实施例特别公开了 S2C6 基因的克隆和鉴定特征；CD40/CD40L 的相互作用的增强；肿瘤生长的抑制；以及单链抗-CD40 免疫毒素与 CD40-Ig 的结合。

为了使公开内容更清晰明嘹而不是起限制作用，本发明的详述被分成以下的小单元。

## 5.1 分离 S2C6 基因

本发明涉及 S2C6 核酸的核苷酸序列。在具体的实施方案中，S2C6 核酸包括 SEQ ID NO: 1 和 6 的 cDNA 序列，或编码 S2C6 蛋白的核酸(例如具有 SEQ ID NO: 2 和 7 的序列的蛋白)。本发明提供的纯化核酸由 S2C6 基因序列的至少 8 个核苷酸(即可杂交部分)组成；在其它实施方案中，所述核酸由 S2C6 序列的至少 25 个(连续)核苷酸、50 个核苷酸、100 或 200 个核苷酸或全长 S2C6 可变区编码序列组成。在相同的或其它实施方案中，所述核酸的长度小于 50、75、100 或 200 或 5000 个核苷酸长度。核酸可以是单链或双链。本发明还涉及与前述序列或其反向互补物杂交或互补的核酸，具体而言，这种编码结合于 CD40 的蛋白的核酸与 S2C6 竞争结合 CD40，和/或使 CD40 配体与 CD40 的结合增加至少 45%、50%、60%或 65%。在具体方面，提供包含与至少 10、25、50、100 或 200 个核苷酸或 S2C6 可变区基因的完整编码区互补的序列的核酸。

另外提供编码 S2C6 蛋白的衍生物和类似物的核酸。显而易见，本文使用的“编码 S2C6 蛋白的片段或部分的核酸”应被解释为是指仅编码所提及的 S2C6 蛋白的片段或部分的核酸，而不是编码为连续

序列的 S2C6 蛋白的其它邻接部分的核酸。

5.2 克隆步骤

以下为克隆 S2C6 基因的具体实施方案。在一个具体的实施方案中，由产生单克隆抗体 S2C6 的杂交瘤分离总 RNA，并使用基于本文公开序列的引物，用聚合酶链式反应扩增所需的可变区序列。参见作为说明性实施例的下文 6 单元。至于另一个实施例，由产生单克隆抗体 S2C6 的杂交瘤分离 mRNA，制备 cDNA 并将其连接至表达载体(例如噬菌体衍生物)，使得其能够由宿主细胞表达，从而将其导入所述宿主细胞中。然后可以用各种筛选测定法选择表达产物。在一个实施方案中，选择是基于与代表一部分 S2C6 基因或其 RNA 或其片段的标记探针的杂交(Benton 和 Davis, 1977, Science 196: 180; Grunstein 和 Hogness, 1975, Proc. Natl. Acad. Sci. U.S.A. 72: 3961)。这些具有与探针大致同源性的 DNA 片段将与探针杂交。还可能通过限制酶消化来鉴定合适的片段，并按照已知的限制图谱(如果可以获得的话)比较其与预期片段的片段大小。可以根据所述基因的特性进行进一步的选择。

或者，通过基于所需基因表达产物的物理、化学或免疫学特性的测定，可以检测所需基因的存在。例如，可以选择并表达 cDNA 克隆或杂种选择合适 mRNA 的 DNA 克隆，以产生具有例如类似或等同于已知的 S2C6 蛋白的电泳迁移、等电聚焦特性、蛋白水解的消化图谱或功能活性的蛋白。例如，可在 ELISA(酶联免疫吸附测定)-型方法中检测结合 CD40 的能力。

还可以通过使用核酸杂交的 mRNA 选择，接着通过体外翻译，鉴定 S2C6 基因。在此方法中，使用片段通过杂交分离互补性 mRNA。分离 mRNA 的分离产物的体外翻译产物的功能测定(例如结合 CD40 等)鉴定所述 mRNA，因此所述互补性 DNA 片段包含所需的序列。

在另一个实施方案中，可以由本文公开的序列化学合成 S2C6

cDNA。可以使用技术人员已知的其它分离 S2C6 基因的方法。

然后可以将所鉴定和分离的 S2C6 基因/cDNA 插入到合适的克隆载体中。可以使用本领域已知的大量载体-宿主系统。可行的载体包括但不限于质粒或修饰病毒，但所述载体系统必须与使用的宿主细胞相适配。这样的载体包括但不限于噬菌体(如λ衍生物)或质粒，诸如 PBR322 或 pUC 质粒衍生物或 Bluescript 载体(Stratagene)。例如，可以通过将所述 DNA 片段连接至具有互补粘性末端的克隆载体，实现对克隆载体的插入。然而，如果用于所述 DNA 片段的互补限制位点在所述克隆载体中不存在，则可以经酶修饰所述 DNA 分子的末端。或者，通过将核苷酸序列(接头)连接至 DNA 末端上，可以产生任何需要的位点；这些连接接头可以含有编码限制性内切核酸酶识别序列的特异性化学合成的寡核苷酸。在一个替代的方法中，可以通过同聚物加尾或使用含合适序列的引物的 PCR，修饰切割的载体和 S2C6 基因。可以通过转化、转染、感染、电穿孔等将重组分子引入到宿主细胞中，以便产生许多拷贝的基因序列。

在一个替代的方法中，可在以“鸟枪”法将所需基因插入到合适的克隆载体后对其进行鉴定和分离。在插入所述克隆载体之前，可以通过例如大小分级进行所需的基因的富集。

在具体的实施方案中，用加入分离的 S2C6 基因、cDNA 或合成的 DNA 序列的重组 DNA 分子转化宿主细胞能够产生多拷贝的所述基因。因此，通过培养转化体、由转化体分离重组 DNA 分子并在需要时由分离的重组 DNA 中回收所插入的基因，可以获得大量的所述基因。

本发明提供的 S2C6 基因包括编码与天然 S2C6 可变区发现的基本相同的氨基酸序列的那些核苷酸序列、编码具有功能相同的氨基酸的氨基酸序列的那些核苷酸序列，以及编码其它 S2C6 衍生物或类似物(如下文对 S2C6 衍生物或类似物的描述)的那些核苷酸序列。

## 5.3 表达 S2C6 基因

编码 S2C6 蛋白或其功能活性类似物或片段或其它衍生物(参见 5.6 单元)的核苷酸序列，可以被插入到合适的表达载体中，即含有插入蛋白编码序列的转录和翻译必需的元件的载体。转录和翻译必需的信号还可以由天然 S2C6 基因和/或其侧翼区提供。可以使用各种宿主-载体系统表达所述蛋白编码序列。这些系统包括但不限于用病毒(例如痘苗病毒、腺病毒等)感染的哺乳动物细胞系统；用病毒(例如杆状病毒)感染的昆虫细胞系统；微生物，诸如含酵母载体的酵母或用噬菌体、DNA、质粒 DNA 或粘粒 DNA 转化的细菌；转基因植物或人类以外的转基因动物。载体的表达元件在其长度和特异性上有所变化。根据使用的宿主-载体系统，可以使用大量合适转录和翻译元件中的任一个。

可以使用先前所述的任一种将 DNA 片段插入到载体中的方法，构建含有嵌合基因(由合适的转录/翻译控制信号组成)和蛋白编码序列的表达载体。这些方法可包括体外重组体 DNA 和合成技术以及体内重组体(基因重组)。编码 S2C6 蛋白或肽片段的核酸序列的表达可以由第二种核酸序列调节，以便 S2C6 蛋白或肽在用重组体 DNA 分子转化的宿主中表达。例如，可以通过本领域已知的任何启动子/增强子元件控制 S2C6 蛋白的表达。可用于控制 S2C6 基因表达的非天然 S2C6 基因启动子包括但不限于，SV40 早期启动子区(Benoist 和 Chambon，1981，Nature 290：304-310)、包含在 Rous 肉瘤病毒的 3′长末端复制的启动子(Yamamoto 等，1980，Cell 22：787-797)、疱疹胸苷激酶启动子(Wagner 等，1981，Proc. Natl. Acad. Sci. U.S.A. 78：1441-1445)、金属硫蛋白基因的调节序列(Brinster 等，1982，Nature 296：39-42)；原核细胞表达载体如β-内酰胺酶启动子(Villa-Kamaroff 等，1978，Proc. Natl. Acad. Sci. U.S.A. 75：3727-3731)或 *lac* 启动子(DeBoer 等，1983，Proc. Natl. Acad. Sci. U.S.A. 80：21-25)；也参见“得自重组细菌的有用蛋白”，载于 Scientific American，1980，242：

74-94；含有胭脂碱合成酶启动子区的植物表达载体(Herrera-Estrella等，Nature 303：209-213)或花椰菜花叶病毒35S RNA启动子(Gardner等，1981，Nucl. Acids. Res. 9：2871)，以及光合成酶核酮糖二磷酸羧化酶启动子(Herrera-Estrella 等，1984，Nature 310：115-120)；得自酵母或其它真菌的启动子元件如 Gal 4 启动子、ADC(醇脱氢酶)启动子、PGK(磷酸甘油激酶)启动子、碱性磷酸酶启动子，和以下的显示出组织特异性并已应用于转基因动物的动物转录控制区：在胰腺腺泡细胞中有活性的弹性蛋白酶 I 基因控制区(Swift 等，1984，Cell 38：639-646；Ornitz 等，1986，Cold Spring Harbor Symp. Quant. Biol. 50：399-409；MacDonald，1987，Hepatology 7：425-515)、在胰腺β细胞中有活性的基因控制区(Hanahan，1985，Nature 315：115-122)、在淋巴细胞中有活性的免疫球蛋白基因控制区(Grosschedl 等，1984，Cell 38：647-658；Adames 等，1985，Nature 318：533-538；Alexander 等，1987，Mol. Cell. Biol. 7：1436-1444)、在睾丸、乳房、淋巴和肥大细胞中有活性的小鼠乳腺瘤病毒控制区(Leder 等，1986，Cell 45：485-495)、在肝脏中有活性的白蛋白基因控制区(Pinkert 等，1987，Genes and Devel. 1：268-276)、在肝脏中有活性的甲胎蛋白基因控制区(Krumlauf 等，1985，Mol. Cell. Biol. 5：1639-1648；Hammer 等，1987，Science 235：53-58)、在肝脏中有活性的α 1-抗胰蛋白酶基因控制区(Kelsey 等，1987，Genes and Devel. 1：161-171)、在骨髓细胞中有活性的β-球蛋白基因控制区(Mogram 等，1985，Nature 315：338-340；Kollias 等，1986，Cell 46：89-94)、在大脑的少突胶质细胞中有活性的髓鞘碱性蛋白基因控制区(Readhead 等，1987，Cell 48：703-712)、在骨骼肌中有活性的肌球蛋白轻链-2 基因控制区(Sani，1985，Nature 314：283-286)和在下丘脑中有活性的促性腺释放激素基因控制区(Mason 等，1986，Science 234：1372-1378)。

在一个具体的实施方案中，使用的载体包含有效连接至 S2C6 基因核酸的启动子、一个或多个复制起点以及任选含有一个或多个选

择性标记(例如抗生素抗性基因)。

可通过三种基本方法鉴定包含 S2C6 基因插入片段的表达载体: (a)核酸杂交; (b) "标记" 基因功能的存在或不存在; 和(c)插入序列的表达。在第一种方法中, 可通过使用含与插入的 S2C6 基因同源的序列的探针进行核酸杂交, 检测插入到表达载体中的 S2C6 基因的存在。在第二种方法中, 可根据所述载体中插入的 S2C6 基因产生的某些 "标记" 基因功能(例如胸苷激酶活性、抗生素抗性、转化表型、在杆状病毒中形成包含体等)的存在或不存在, 鉴定并选择重组载体/宿主系统。例如, 如果在所述载体的标记基因序列中插入 S2C6 基因, 则可通过标记基因功能的缺失鉴定含有 S2C6 插入片段的重组体。在第三种方法中, 可通过测定由所述重组体表达的 S2C6 产物, 鉴定重组表达载体。这样的测定可基于例如 S2C6 蛋白在体外测定系统中的物理和功能特性, 例如增强 CD40L 与 CD40 的结合、刺激正常 B 细胞增殖、抑制肿瘤生长。

一旦鉴定和分离出具体的重组 DNA 分子, 则可用几种本领域已知的方法进行增殖。一旦确定合适的宿主系统和培养条件, 则可以大量增殖和制备重组表达载体。如前所述, 可使用的表达载体包括但不限于以下的载体或其衍生物: 人或动物病毒如痘苗病毒或腺病毒、昆虫病毒如杆状病毒、酵母载体、噬菌体载体(例如λ噬菌体)以及质粒和粘粒 DNA 载体, 在此仅举几个例子。

此外, 可以选择调节插入序列的表达或以需要的特异性方式修饰和加工基因产物的宿主细胞株。在某些诱导物存在下可提升某些启动子的表达; 因此, 可以控制 S2C6 基因工程蛋白的表达。而且, 不同的宿主细胞具有用于翻译和翻译后加工和修饰的特征性和特异性机制(例如蛋白糖基化、磷酸化)。可以选择合适的细胞系或宿主系统, 以确保对表达的前述蛋白的所需修饰和加工。例如, 在细菌系统中的表达可用于产生非糖基化核心蛋白产物。在酵母中的表达可产生糖基化产物。在哺乳动物细胞中的表达可用于确保异源蛋白的

“天然”糖基化。而且，不同载体/宿主系统可不同程度地影响加工反应。

在具体的实施方案中，表达的 S2C6 相关蛋白为抗体或其片段或衍生物。重组抗体可含有重组轻链可变区、重组重链可变区或二者均包含在内。在一个具体的实施方案中，轻链和重链或其衍生物均由细胞重组表达(参见例如 Boss 等于 1989 年 3 月 28 日登记的美国专利第 4,816,397 号)。可使用各种宿主-载体系统表达蛋白编码序列。这些系统包括但不限于用病毒(例如痘苗病毒、腺病毒等)感染的哺乳动物细胞系统；用病毒(例如杆状病毒)感染的昆虫细胞系统；微生物，诸如含酵母载体的酵母或用噬菌体、DNA、质粒 DNA 或粘粒 DNA 转化的细菌；转基因植物或人类以外的转基因动物。

5.4 鉴定和纯化基因产物

在具体的方面，本发明提供 S2C6 蛋白及其片段和衍生物的氨基酸序列，以及编码它们的核酸序列，所述氨基酸序列含有互补决定区(CDR)或具有其它的功能活性。本文使用的“功能活性”的 S2C6 物质是指显示出一种或多种与全长(天然) S2C6 蛋白相关的功能活性的物质，所述功能活性例如为结合 CD40，刺激正常 B 细胞增殖，抑制肿瘤生长，使 CD40 配体与 CD40 的结合增加至少 45%。

在具体的实施方案中，本发明提供 S2C6 蛋白片段以及其编码核酸，其中所述片段由至少 6、10、20、50、75 或 100 个氨基酸组成。

一旦鉴定出表达 S2C6 基因序列的重组体，则可以分析基因产物。这可通过基于所述产物的物理或功能特性的测定来实现，所述测定包括放射性标记所述产物然后经凝胶电泳分析、免疫测定、正常 B 细胞增殖的刺激、CD40 结合测定、CD40 配体与 CD40 结合的促进、肿瘤生长的抑制等。

一旦鉴定出 S2C6 蛋白，则可通过标准方法对其进行分离和纯化，所述标准方法包括层析(例如离子交换层析、亲和性层析和分子

筛(sizing)柱层析)、离心、不同溶解性或任何其它用于蛋白纯化的标准技术。可用任一合适的测定评价功能特性(参见 5.7 单元)。

或者，可通过本领域已知的基于本文公开的序列的标准化学方法合成 S2C6 蛋白或其衍生物(例如参阅 Hunkapiller 等，1984，Nature 310：105-111)。

在本发明的一个具体实施方案中，这类 S2C6 蛋白，无论是通过重组 DNA 技术产生还是通过化学合成方法产生，或是通过天然蛋白纯化产生，都包括(但不限于)包含为初级氨基酸序列的基本如图 3A-3B (SEQ ID NO：2 和 7)所示的氨基酸序列的全部或部分的蛋白，以及其片段和其他衍生物和其类似物(包括其同源蛋白)。

## 5.5 S2C6 基因和蛋白的结构

可通过本领域已知的各种方法分析本发明的 S2C6 基因和蛋白的结构。以下描述这些方法的某些实例。

### 5.5.1 遗传分析

可分析对应于 S2C6 基因的克隆 DNA 或 cDNA 的方法包括但不限于 DNA 杂交(Southern hybridization)(Southern，1975，J. Mol. Biol. 98：503-517)、RNA 杂交(Northern hybridization)(参见例如 Freeman 等，1983，Proc. Natl. Acad. Sci. U.S.A. 80：4094-4098)、限制性内切核酸酶作图(Maniatis，1982，Molecular Cloning: A Laboratory Manual，Cold Spring Harbor Laboratory Press，Cold Spring Harbor: New York)以及 DNA 序列分析。因此，本发明提供识别 S2C6 基因的核酸探针。例如，在聚合酶链反应(PCR；美国专利第 4,683,202、4,683,195 和 4,889,818 号；Gyllenstein 等，1988，Proc. Natl. Acad. Sci. U.S.A. 85：7652-7656；Ochman 等，1988，Genetics 120：621-623；Loh 等，1989，Science 243：217-220)后用 S2C6 基因特异性探针进行 DNA 杂交，可检测来自细胞(例如杂交瘤)的 DNA 或 cDNA 中的 S2C6 基因。也可

以使用通常已知的非 PCR 扩增方法。可对 DNA 杂交和 RNA 杂交二者的杂交条件的严格性进行操作，以确保检测出与使用的特异性 S2C6 基因探针具有所需相关度的核酸。可使用这些方法和本领域通常已知的其它方法的改良方法。

可使用限制性内切核酸酶作图粗略估测 S2C6 基因的遗传结构。可通过 DNA 序列分析证实由限制性内切核酸酶切割产生的限制酶图谱。

可通过任何本领域已知的技术进行 DNA 序列分析，包括但不限于 Maxam 和 Gilbert 法(1980，Meth. Enzymol. 65：499-560)、Sanger 双脱氧法(Sanger 等，1977，Proc. Natl. Acad. Sci. U.S.A. 74：5463)、使用 T7 DNA 聚合酶(Tabor 和 Richardson，美国专利第 4,795,699 号)或使用自动化 DNA 测序仪(例如 Applied Biosystems，Foster City，California)。

### 5.5.2 蛋白分析

可通过 DNA 序列推导，或通过直接蛋白测序(例如使用自动化氨基酸测序仪)，得出 S2C6 蛋白的氨基酸序列。

可通过亲水性分析进一步特征鉴定 S2C6 蛋白序列(Hopp 和 Woods，1981，Proc. Natl. Acad. Sci. U.S.A. 78：3824)。亲水性分布图可用于鉴定 S2C6 蛋白的疏水区和亲水区(加强免疫原性)以及编码这些区的相应的基因序列区。

还可以进行二级结构分析(Chou 和 Fasman，1974，Biochemistry 13：222)，以鉴定表现为特异性二级结构的 S2C6 蛋白区。

还可以用本领域可得到的计算机软件程序完成操作、翻译和二级结构预测、可读构架预测和作图以及序列同源性测定。

## 5.6 单克隆抗体 S2C6 抗体衍生物

本文描述了用于产生能够免疫特异性结合 CD40 的 S2C6 抗体衍

生物的方法。

这样的抗体包括但不限于单克隆抗体、人源化抗体、嵌合抗体、单链抗体、双特异性抗体、Fab 片段、$F(ab')_2$ 片段、由 Fab 表达文库产生的片段、抗独特型(抗 Id)抗体以及以上任一抗体的表位结合片段。在一个实施方案中，S2C6 衍生物在与 S2C6 初级氨基酸序列相关的初级氨基酸序列中包含一个或多个缺失、添加和/或取代。在另一个实施方案中，S2C6 衍生物不是用木瓜蛋白酶或胃蛋白酶切割 S2C6 产生的。在再另一个实施方案中，S2C6 衍生物在与 S2C6 初级氨基酸序列相关的初级氨基酸序列中包含一个或多个缺失、添加和/或取代，且不是用木瓜蛋白酶或胃蛋白酶切割 S2C6 产生的。在本单元和下文的 5.7 单元提供选择合适的缺失、添加和/或取代的引导。

为制备另外的抗 CD40 的单克隆抗体，可使用在培养基中由传代细胞系生产抗体分子的任何技术。这些技术包括但不限于 Kohler 和 Milstein 的杂交瘤技术(1975，Nature 256，495-497；以及美国专利第 4,376,110 号)、人 B 细胞杂交瘤技术(Kozbor 等，1983，Immunology Today 4，72；Cole 等，1983，Proc. Natl. Acad. Sci. USA 80，2026-2030)以及生产人单克隆抗体的 EBV-杂交瘤技术(Cole 等，1985，单克隆抗体和癌症治疗，Alan R. Liss，Inc.，第 77-96 页)。这样的抗体或本领域可获得的其它抗 CD40 抗体例如可用作克隆的基础，并因此在 S2C6 重链重组表达时提供互补轻链(两条链可在同一细胞中重组表达，或在分开表达和纯化后体外组合)；或者，可使用来自任何特异性抗体的轻链。可将编码 S2C6 重链或编码含 S2C6 重链可变区的分子的核酸(例如质粒)转染入表达抗体轻链或含抗体轻链的分子的细胞中，用于表达多聚体蛋白；所述抗体轻链可以是重组体或非重组体，并可以具有或不具有抗-CD40 特异性。或者，可任选在互补轻链或轻链可变区不存在的情况下，表达和使用 S2C6 重链或含其可变区或其 CDR 的分子。在各种实施方案中，本发明提供具有 CD40 结合亲和性的 S2C6 重链或一种分子(其含有一个或多个拷贝的重链 CDR 8、

9 和/或 10 或由其组成)或一种蛋白(肽或多肽)，所述蛋白的序列含有一个或多个拷贝的 CDR 8、9 和/或 10 或由其组成。在一个具体的实施方案中，这种蛋白可以通过例如 C 末端酰胺化或 N 末端乙酰化对 N 或 C 末端进行修饰。

另外，可使用通过将合适抗原特异性的小鼠抗体分子基因与合适生物活性的人抗体分子基因剪接在一起开发的用于产生“嵌合抗体”的技术(Morrison 等，1984，Proc. Natl. Acad. Sci.，81，6851-6855；Neuberger 等，1984，Nature 312，604-608；Takeda 等，1985，Nature 314，452-454)。嵌合抗体是一种其中不同的部分得自不同的动物物种的分子，例如具有鼠类单克隆抗体的可变区和人免疫球蛋白恒定区的分子。(参阅例如美国专利第 4,816,567 号；以及 Boss 等，美国专利第 5,816,397 号)。在一个具体的实施方案中，所述嵌合抗体包含由杂交瘤(保藏于 ATCC，指定的保藏号为 PTA-110)分泌的单克隆抗体 S2C6 的可变区和人恒定区。在具体的实施方案中，所述嵌合抗体的可变区含有示于图 3A 的 S2C6 $V_L$ (SEQ ID NO: 2)和/或示于图 3B 的 S2C6 $V_H$ (SEQ ID NO: 7)。

另外，已开发了产生人源化抗体的技术。(参阅例如 Queen，美国专利第 5,585,089 号和 Winter，美国专利第 5,225,539 号)。免疫球蛋白轻链或重链可变区由被三个高变区间隔的“构架”区组成，称为互补性决定区(CDR)。已精确定义了构架区和 CDR 的范围(参阅“目的免疫球蛋白的序列”，Kabat, E.等，美国公共医疗卫生与人类健康部(1983))。简而言之，人源化抗体是得自具有一个或多个人类以外物种的 CDR 和人免疫球蛋白分子的构架区的人类以外物种的抗体分子。

本发明包括含重链或轻链可变区的抗体或其衍生物，所述可变区包含(a)一组三个互补性决定区(CDR)，其中所述 CDR 组来自单克隆抗体 S2C6，和(b)一组四个构架区，其中所述构架区组与单克隆抗体 S2C6 中的构架区组不同，并且其中所述抗体或其衍生物免疫特异

性地结合 CD40。所述构架区组最好是得自人单克隆抗体，例如不结合 CD40 的人单克隆抗体。

在一个具体的实施方案中，本发明包括含轻链可变区的抗体或其衍生物，所述可变区包含(a)一组三个互补性决定区(CDR)，其中所述 CDR 组包含 SEQ ID NO: 3 或 SEQ ID NO: 4，和(b)一组四个构架区，其中所述构架区组与单克隆抗体 S2C6 中的轻链构架区组不同，并且其中所述抗体或其衍生物免疫特异性地结合 CD40。

在一个具体的实施方案中，本发明包括含重链可变区的抗体或其衍生物，所述可变区包含(a)一组三个互补性决定区(CDR)，其中所述 CDR 组包含 SEQ ID NO: 8、SEQ ID NO: 9 或 SEQ ID NO: 10，和(b)一组四个构架区，其中所述构架区组与单克隆抗体 S2C6 的重链中的构架区组不同，并且其中所述抗体或其衍生物免疫特异性结合 CD40。

或者，可采用所描述的用于产生单链抗体的技术(美国专利第 4,946,778 号; Bird, 1988, Science 242, 423-426; Huston 等, 1988 Proc. Natl. Acad. Sci. USA 85, 5879-5883; 和 Ward 等, 1989, Nature 334, 544-546)，使用 S2C6 序列产生单链抗体。通过经氨基酸桥连接 Fv 区的重链和轻链片段形成单链抗体，产生单链多肽。在一个具体的实施方案中，所述单链抗体包含示于图 3A 和图 3B 的氨基酸序列(分别为 SEQ ID NOS: 2 和 7)。

在一个具体的实施方案中，含有 SEQ ID NO: 1 或 SEQ ID NO: 6 的全部或部分的抗 CD40 抗体、多肽、肽或其它衍生物或其类似物是一种双特异性抗体(一般参见例如 Fanger 和 Drakeman, 1995, *Drug News and Perspectives* 8: 133-137)。对这样的双特异性抗体进行基因工程改造，以便识别(1)表位和(2)众多“触发”分子中的一种，例如骨髓细胞上的 Fc 受体和 T 细胞上的 CD3 和 CD2，它们已被鉴定为能够使细胞毒性 T 细胞破坏特定靶。可通过化学接合、杂交瘤或技术人员已知的重组分子生物学技术制备这种双功能抗体。在一个具

体的实施方案中，所述双功能抗体包含的分子含有 S2C6 重链或轻链可变区或其 CDR 序列，所述分子具有抗体重链或轻链的结构，但不同于天然 S2C6 重链或轻链(例如在构架区或人恒定区中具有氨基酸取代)。

可通过已知技术产生保留识别 CD40 能力的抗体片段。例如，这样的片段包括但不限于：可通过胃蛋白酶消化抗体分子产生的 $F(ab')_2$ 片段以及可通过还原 $F(ab')_2$ 片段的二硫键产生的 F(ab')片段。或者，可构建 Fab 表达文库(Huse 等，1989，Science 246：1275-1281)，以允许快速且容易地鉴定具有所需的特异性的单克隆 Fab 片段。

5.7 S2C6 蛋白、衍生物和类似物

除了在以上 5.6 单元描述的那些抗体分子/变异体以外，本发明还涉及 S2C6 蛋白、衍生物(包括但不限于片段)、类似物和 S2C6 蛋白分子。还提供编码 S2C6 蛋白衍生物和 S2C6 蛋白类似物的核酸。在一个实施方案中，S2C6 蛋白由上文 5.1 单元描述的核酸所编码。在具体的方面，所述蛋白、衍生物或类似物由 SEQ ID NO：1 或 SEQ ID NO：6 的序列所编码。

与 S2C6 蛋白相关的衍生物和类似物的生产和应用属于本发明的范围。在一个具体的实施方案中，所述衍生物和类似物具有功能活性，即能够表现出一种或多种与全长 S2C6 蛋白相关的功能活性。作为一个实施例，可在免疫测定或治疗性抑制肿瘤生长等中应用这样的具有所需结合特异性的衍生物或类似物。一个具体的实施方案涉及结合 CD40 并增强 CD40L 与 CD40 结合的 S2C6 蛋白片段。可通过本领域已知的各种免疫测定检测 S2C6 蛋白的衍生物或类似物的所需活性，所述免疫测定包括但不限于使用以下技术的竞争性测定系统和非竞争性测定系统：诸如放射免疫测定、酶联免疫吸附测定(ELISA)、“夹心”免疫测定、蛋白质印迹、免疫荧光测定、A 蛋白测定、免疫电泳测定等。

另外，可使用本领域已知的测定检测或测定体内或体外抑制细胞增殖(例如抑制肿瘤生长)的能力或刺激细胞增殖(例如 B 细胞增殖)的能力。

具体而言，可通过提供功能等同分子的取代、添加(例如插入)或缺失改变 S2C6 序列，制备 S2C6 衍生物。由于核苷酸编码序列的简并性，所以编码与 S2C6 基因大致相同的氨基酸序列的其它 DNA 序列可用于本发明的实践。这些 DNA 序列包括但不限于包含 S2C6 基因的全部或部分的核苷酸序列，它们是通过编码序列中功能等同氨基酸残基的不同密码子的取代，由此产生沉默变化而得到改变。同样地，本发明的 S2C6 衍生物包括但不限于包含为初级氨基酸序列的 S2C6 蛋白的全部或部分氨基酸序列的那些物质，包括其中功能等同氨基酸残基取代所述序列的残基导致沉默变化的改变序列。例如，所述序列中的一个或多个氨基酸残基可被另一个起功能等价作用的相似极性氨基酸取代，产生沉默改变。所述序列中的氨基酸取代可选自所述氨基酸所属类型的其它成员。例如，非极性(疏水性)氨基酸包括丙氨酸、亮氨酸、异亮氨酸、缬氨酸、脯氨酸、苯丙氨酸、色氨酸和甲硫氨酸。极性中性氨基酸包括甘氨酸、丝氨酸、苏氨酸、半胱氨酸、酪氨酸、天冬酰胺和谷氨酰胺。带正电(碱性)氨基酸包括精氨酸、赖氨酸和组氨酸。带负电(酸性)氨基酸包括天冬氨酸和谷氨酸。这类取代通常被理解为保守取代。

在本发明的一个具体实施方案中，提供由 S2C6 蛋白片段组成或包含 S2C6 蛋白片段的蛋白，其中所述 S2C6 蛋白片段由 S2C6 蛋白的至少 10 个(连续的)氨基酸组成。在其它实施方案中，所述片段由 S2C6 蛋白的至少 20 个或至少 50 个氨基酸组成。在具体的实施方案中，这种片段不长于 50、75、100 或 200 个氨基酸。S2C6 蛋白的衍生物或类似物包括但不限于那些含有与 S2C6 蛋白或其片段大致同源(例如在各种实施方案中，在没有插入或缺失的相同大小的氨基酸序列上的同一性至少为 60%或 70%或 80%或 90%或 95%，或与通过本

领域已知的计算机同源性程序进行序列对比的对比序列相比较，同一性至少为 60%或 70%或 80%或 90%或 95%)的区的分子，或者其编码核酸能够在高、中或低严格条件下与编码 S2C6 基因序列杂交的分子。

具体而言，用于测定同源性的计算机程序实例包括但不限于 TBLASTN、BLASTP、FASTA、TEASTA 和 CLUSTALW (Pearson 和 Lipman，1988，Proc. Natl. Acad. Sci. USA 85 (8)：2444-8；Altschul 等，1990，J. Mol. Biol. 215 (3)：403-10；Thompson 等，1994，Nucleic. Acids. Res. 22 (22)：4673-80；Higgins 等，1996，Methods Enzymol 266：383-402；Altschul 等，1990，J. Mol. Biol. 215 (3)：403-10)。这些计算机程序中的每一种程序的默认参数均为熟知的，并可加以利用。

具体而言，基本局部序列对比检索工具 (BLAST) (www.ncbi.nlm.nih.gov) (Altschul 等，1990，J. of Molec. Biol. 215：403-410，“BLAST 算法；Altschul 等，1997，Nuc. Acids. Res. 25：3389-3402)是一种专门用于检索序列相似性的启发性检索算法，该算法可归结于使用 Karlin 和 Altschul 等，1990，Proc. Natl. Acad. Sci. USA 87：2264-68；1993，Proc. Nat'l Acad. Sci. USA，90：5873-77 的统计学方法的显著性。使用五种特定的 BLAST 程序执行以下的任务：1) BLASTP 程序比较氨基酸查询(query)序列与蛋白质序列数据库；2) BLASTN 程序比较核苷酸查询序列与核苷酸序列数据库；3) BLASTX 程序比较核苷酸查询序列(双链)的 6-读框(frame)概念翻译产物与蛋白质序列数据库；4) TBLASTN 程序比较蛋白查询序列与在全部 6 个读框中翻译的核苷酸序列数据库(双链)；5) TBLASTX 程序比较核苷酸查询序列的 6-读框翻译产物与核苷酸序列数据库的 6-读框翻译产物。

Smith-Waterman (数据库：欧洲生物信息研究所 wwwz.ebi.ac.uk/bic_sw/) (Smith-Waterman，1981，J. of Molec. Biol.，147：195-197)是一种用于序列对比的数学上严密的算法。

FASTA (参见 Pearson 等，1988，Proc. Nat'l Acad. Sci. USA，85：

2444-2448)是一种近似于 Smith-Waterman 算法的启发性算法。关于 BLAST、Smith-Waterman 和 FASTA 算法的的步骤和优势的一般性论述，参见 Nicholas 等，1998，“序列数据库检索指南和序列记分方法”(www.psc.edu)及其中引用的参考文献。

本发明的 S2C6 衍生物和类似物可通过本领域已知的各种方法产生。可在基因或蛋白水平上进行导致其产生的操作。例如，可通过本领域已知的众多策略中的任一种，修饰克隆的 S2C6 基因序列(Sambrook 等，1989，*Molecular Cloning: A Laboratory Manual*，第二版，Cold Spring Harbor Laboratory Press，Cold Spring Harbor, New York)。可用限制性内切核酸酶在合适的位点切割所述序列，接着如果需要的话进一步进行酶修饰、分离并体外连接。在产生编码 S2C6 蛋白衍生物或类似物的修饰基因时，应小心确保所述修饰基因保留在与天然蛋白相同的翻译读框中，而不致于在编码所需的 S2C6 蛋白活性的基因区中被翻译终止信号间断。

另外，S2C6 核酸序列可在体外或体内突变，以产生和/或破坏翻译序列、起始序列和/或终止序列，或在编码区产生变异和/或形成新的限制性内切核酸酶位点或破坏预先存在的限制性内切核酸酶位点，以易于进一步的体外修饰。可以使用任一种本领域已知的诱变技术，包括但不限于化学诱变、体外定点诱变(Hutchinson 等，1978，J. Biol. Chem. 253: 6551)、使用含有诱变的引物的 PCR 等。

还可以在蛋白水平上操作 S2C6 蛋白序列。包括在本发明范围内的 S2C6 蛋白片段或其它衍生物或类似物在翻译过程中或在翻译后被分别修饰，例如通过糖基化、乙酰化、磷酸化、酰胺化、利用已知的保护基/封闭基团衍生化、蛋白酶剪切、连接至抗体分子或其它细胞配体等。可通过已知技术进行众多化学修饰中的任一种，包括但不限于利用溴化氰、胰蛋白酶、胰凝乳蛋白酶、木瓜蛋白酶、V8 蛋白酶、$NaBH_4$ 进行特异性化学切割、乙酰化、甲酰化、氧化、还原、在衣霉素存在下的代谢合成等。

另外，可化学合成 S2C6 蛋白的类似物和衍生物。例如，可使用肽合成仪合成对应于一部分、含有所需的结构域或在体外介导所需的活性的 S2C6 蛋白的肽。而且，如果需要的话，可将非典型氨基酸或化学氨基酸类似物以取代或添加引入到 S2C6 序列中。非典型氨基酸包括但不限于一般氨基酸的 D-异构体、α-氨基异丁酸、4-氨基丁酸、Abu、2-氨基丁酸、γ-Abu、ε-Ahx、6-氨基己酸、Aib、2-氨基异丁酸、3-氨基丙酸、鸟氨酸、正亮氨酸、正缬氨酸、羟脯氨酸、肌氨酸、瓜氨酸、半胱磺酸、叔丁基甘氨酸、叔丁基丙氨酸、苯甘氨酸、环己基丙氨酸、β-丙氨酸、氟代氨基酸、designer 氨基酸(诸如β-甲基氨基酸、Cα-甲基氨基酸、Nα-甲基氨基酸)和一般的氨基酸类似物。而且，所述氨基酸可以是 D(右旋)氨基酸或 L(左旋)氨基酸。

在其它的具体实施方案中，S2C6 蛋白、片段、类似物或衍生物可作为融合或嵌合蛋白产物(包含通过肽键连接不同蛋白的异源蛋白序列的所述蛋白、片段、类似物或衍生物)表达。所述异源蛋白序列可含有生物反应调节剂，包括但不限于α干扰素、γ干扰素、白介素-2、白介素-4、白介素-6 和肿瘤坏死因子或其功能活性部分。或者，所述异源蛋白序列可包含酶(如β-内酰胺酶或羧酸酯酶)或毒素(如 bryodin 1、假单胞菌外毒素 A 或 gelonin)或其功能活性部分。或者，S2C6 蛋白可与化疗剂化学连接，所述化疗剂包括但不限于烷化剂(例如氮芥、亚硝基脲、三氮烯)；抗代谢物(例如叶酸类似物、嘧啶类似物、嘌呤类似剂)；天然产物(例如抗生素、酶、生物反应调节物)；其它物质(例如取代的脲、铂配位复合物)；以及激素和拮抗剂(例如雌激素、雄激素、抗雄激素、促性腺素释放激素类似物)；或其功能活性部分(参见例如 Goodman 和 Gilman，治疗的药理基础，第九板，McGraw-Hill，第 1225-1287 页，1996)。可通过本领域已知的方法，将编码所需的氨基酸序列的合适核酸序列在正确的编码框内互相连接，并通过本领域通常已知的方法表达所述嵌合产物，从而制备这样的嵌合产物。或者，可通过蛋白合成技术(例如使用肽合成仪)制备这样的嵌合产

物。在不同的实施方案中，异源蛋白序列可通过非肽键(例如使用本领域众所周知的化学交联剂)与 S2C6 相关序列共价结合。

在一个具体的实施方案中，S2C6 蛋白衍生物是一种含有 S2C6 蛋白或其片段(优选由 S2C6 蛋白的至少一个结构域或基序组成，或由 S2C6 蛋白的至少 10、50 或 100 个氨基酸组成)的嵌合或融合蛋白，其中所述 S2C6 蛋白或其片段在其氨基末端或羧基末端通过肽键连接至不同蛋白的氨基酸序列。在一个具体的实施方案中，所述不同蛋白是毒素、酶或生物反应调节剂。

在具体的实施方案中，所述不同蛋白的氨基酸序列为所述不同蛋白的至少 6、10、20 或 30 个连续的氨基酸或所述不同蛋白的功能活性部分，在一个实施方案中，通过重组表达编码所述蛋白(包含在读框内连接至不同蛋白的编码序列的 S2C6 编码序列)的核酸产生这样的嵌合蛋白。可通过本领域已知的方法，将编码所需的氨基酸序列的合适核酸序列在正确的编码框内互相连接，并通过本领域通常已知的方法表达所述嵌合产物，从而制备这样的嵌合产物。或者，可通过蛋白合成技术(例如使用肽合成仪)制备这样的嵌合产物。可构建含有融合至任何异源蛋白编码序列的 S2C6 基因部分的嵌合基因。一个具体的实施方案涉及的嵌合蛋白含有的 S2C6 蛋白片段至少为 6 或 15 或 50 个氨基酸，或具有 S2C6 蛋白的一种或多种功能活性(例如包含一个或多个 CDR 的拷贝)。

在一个具体的实施方案中，S2C6 蛋白或其衍生物化学连接至化疗药物(包括但不限于阿霉素、紫杉醇或 docetaxel)。这种 S2C6-药物结合体可将所述药物传递至表达 CD40 的细胞。S2C6 蛋白或衍生物可连接一种或多种药物分子。连接包括但不限于脲、肽或糖连接。

在另一个具体的实施方案中，所述衍生物是一种含有与 S2C6 蛋白同源的区的分子。作为实施例，在各种实施方案中，当第一个蛋白区的氨基酸序列(没有任何插入或缺失)与第二个蛋白区(其含有的氨基酸数目与所述第一个区中含有的氨基酸数目相等)的任何序列相

比至少 30%、40%、50%、60%、70%、75%、80%、90%或 95%相同，或当第一个蛋白区的氨基酸序列与第二个区的已通过本领域已知的计算机同源性程序进行比较的对比序列相比至少 30%、40%、50%、60%、70%、75%、80%、90%或 95%相同时，则可认为所述第一个蛋白区与所述第二个蛋白区同源。

5.8 杂交条件

在一个具体的实施方案中，提供在低严格条件下与 S2C6 核酸(例如具有 SEQ ID NOS: 1 或 6 所提供的序列)或其反向互补物或者编码 S2C6 衍生物的核酸或其反向互补物杂交的核酸。作为非限制性实例，以下为使用这类低严格条件的方法(也参见 Shilo 合 Weinberg，1981，Proc. Natl. Acad. Sci. U.S.A. 78，6789-6792)。于 40℃在含 35%甲酰胺、5X SSC、50 mM Tris-HCl (pH 7.5)、5 mM EDTA、0.1% PVP、0.1% Ficoll、1% BSA 和 500 μg/ml 变性鲑精 DNA 的溶液中预处理含 DNA 的滤器 6 小时。在具有以下改变的相同溶液中进行杂交：0.02% PVP、0.02% Ficoll、0.2% BSA、100 μg/ml 鲑精 DNA、10%(重量/体积)硫酸葡聚糖，并使用 5-20 × $10^6$ cpm $^{32}$P 标记的探针。滤器于 40℃在杂交混合物中温育 18-20 小时，然后于 55℃在含 2X SSC、25 mM Tris-HCl (pH 7.4)、5 mM EDTA 和 0.1% SDS 的溶液中漂洗 1.5 小时。用新鲜溶液替换漂洗溶液，并于 60℃再温育 1.5 小时。吸干滤器并进行放射自显影曝光。如果必要，于 65-68℃第三次清洗滤器并对胶片重新曝光。可以使用的其它低严格条件是本领域众所周知的(例如采用种间杂交)。

在一个具体的实施方案中，提供在高严格条件下与 S2C6 核酸(例如具有 SEQ ID NOS: 1 或 6 所提供的序列)或其反向互补物或者编码 S2C6 衍生物的核酸或其反向互补物杂交的核酸。作为非限制性实例，以下为使用这类高严格条件的方法。于 65℃在由 6X SSC、50 mM Tris-HCl (pH 7.5)、1 mM EDTA、0.02% PVP、0.02% Ficoll、0.02% BSA

和 500 μg/ml 变性鲑精 DNA 组成的缓冲液中预杂交含 DNA 的滤器 8 小时至过夜。于 65℃使滤器在含 100 μg/ml 变性鲑精 DNA 和 5-20 × $10^6$ cpm $^{32}P$ 标记的探针的预杂交混合物中杂交 48 小时。于 37℃在含 2X SSC、0.01% PVP、0.01% Ficoll 和 0.01% BSA 的溶液中漂洗滤器 1 小时。接着于 50℃在 0.1X SSC 中漂洗 45 分钟，然后放射自显影。可以使用的其它高严格条件是本领域众所周知的。

在一个具体的实施方案中，提供在中等严格条件下与 S2C6 核酸(例如具有 SEQ ID NOS: 1 或 6 所提供的序列)或其反向互补物或者编码 S2C6 衍生物的核酸或其反向互补物杂交的核酸。对合适的中等严格条件的选择是本领域众所周知的(参见例如 Sambrook 等，1989, Molecular Cloning: A Laboratory Manual，第二版，Cold Spring Harbor Laboratory Press，Cold Spring Harbor: New York; 还参见 Ausubel 等编辑，载于：实验室技术手册分子生物学系列现时方案，1987-1997 Current Protocols,© 1994-1997 John Wiley and Sons，Inc.)。

5.9 治疗应用

本发明通过给予治疗性化合物(本文称为“治疗剂”)提供对各种疾病或紊乱的治疗和预防。这样的治疗剂包括但不限于：S2C6 抗体及其衍生物(例如上文所述)；以及编码这种 S2C6 抗体及其衍生物的核酸(例如上文所述)。本文使用的“治疗”实际上应包括任何对所述疾病或紊乱有临床需要或有益的作用，包括但不限于缓解一种或多种症状、抑制、减缓或终止恶化等。

在本发明的具体实施方案中，所述治疗剂或单独给予或与 CD40L 一起给予，以治疗或预防恶性肿瘤(包括但不限于癌和血癌)、炎性疾病以及免疫系统疾病。所述治疗剂和 CD40L 可以(但不是必须)包含在同一制剂中，即所述治疗剂和 CD40 可分别给予，但也可同时给予或在同一治疗过程中给予。在一个具体的实施方案中，所述恶性细胞表达 CD40。或者，所述恶性细胞不必表达 CD40，因为与恶性肿

瘤相连的脉管系统的内皮细胞应表达 CD40，因此本发明的治疗剂甚至对不表达 CD40 的肿瘤也能提供治疗功效。在一个优选的实施方案中，所述治疗剂使 CD40L 与 CD40 的结合增强至少 45%、50%、60 或 65%。

在具体的实施方案中，所述治疗剂用于增强免疫受抑制的个体(如罹患获得性免疫缺陷综合征、恶性肿瘤的人或婴儿或老年人)的免疫应答。

在本发明的其它实施方案中，所述治疗剂可进行化学修饰，以便杀死其结合的细胞。这样的细胞包括但不限于多发性骨髓瘤细胞、淋巴瘤细胞或癌细胞。因为所有的 B 细胞都表达 CD40，所以该方法可对免疫应答产生抑制。例如，为了越过移植患者的组织相容性屏障，可使用连接 S2C6 序列的细胞毒性药物(例如融合蛋白)，以在体内引起免疫抑制；或者可使用这些修饰的配体控制自身免疫性疾病。

在其它的实施方案中，所述治疗剂可用于促进非 B 细胞的携带 CD40 的细胞(例如肺癌细胞)的增殖和/或分化，作为直接治疗恶性肿瘤的工具或作为化疗辅助剂。

可使用本发明的治疗剂治疗或预防的恶性肿瘤包括但不限于表 1 中的那些恶性肿瘤：

表 1

恶性肿瘤和相关疾病

白血病

急性白血病

急性淋巴细胞白血病

急性骨髓细胞白血病

急性成髓细胞白血病

急性前髓细胞白血病

急性骨髓单核细胞白血病

急性单核细胞白血病

急性红白血病

慢性白血病

慢性骨髓细胞(粒细胞)白血病

慢性淋巴细胞白血病

脾大性红细胞增多

淋巴瘤

何杰金氏病

非何杰金氏病

多发性骨髓瘤

Waldenström 氏巨球蛋白血症

重链疾病

实体瘤

肉瘤和癌症

纤维肉瘤

粘液肉瘤

脂肪肉瘤

软骨肉瘤

骨原性肉瘤

骨肉瘤

脊索瘤

血管肉瘤

内皮肉瘤

淋巴管肉瘤

淋巴管内皮肉瘤

滑膜瘤

间皮瘤

Ewing 氏瘤

平滑肌肉瘤

横纹肌肉瘤

结肠癌

结肠直肠癌

胰腺癌

乳癌

卵巢癌

前列腺癌

鳞状细胞癌

基细胞癌

腺癌

汗腺癌

皮脂腺癌

乳头状癌

乳头状腺癌

囊腺癌

髓样癌

支气管原性癌

肾细胞癌

肝细胞瘤

胆管癌

绒膜癌

精原细胞瘤

胚胎癌

Wilms 氏肿瘤

颈癌

子宫癌

睾丸癌

肺癌

小细胞肺癌

非小细胞肺癌

膀胱癌

上皮细胞癌

神经胶质瘤

星细胞瘤

成神经管细胞瘤

颅咽管瘤

室管膜细胞瘤

松果体瘤

成血管细胞瘤

听神经瘤

寡枝神经胶质细胞瘤

脑膜瘤(menangioma)

黑素瘤

成神经细胞瘤

眼癌

鼻咽癌

食道癌

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可使用本发明的治疗剂治疗或预防的炎性疾病和免疫系统缺陷或疾病包括但不限于在表 2 中的那些病症:

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表 2

炎性疾病和免疫系统疾病

系统性红斑狼疮(SLE)

硬皮病(例如 CRST 综合征)

炎症性肌炎

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| |
|---|
| Sjögren 氏综合征(SS) |
| 混合性结缔组织疾病(例如 MCTD、Sharp 综合征) |
| 类风湿性关节炎 |
| 多发性硬化 |
| 炎性肠道疾病(例如溃疡性结肠炎、节段性结肠炎) |
| 急性呼吸窘迫综合征 |
| 肺炎 |
| 骨质疏松症 |
| 迟发型过敏症 |
| 哮喘 |
| 夏科氏肝硬变(PBC) |
| 自发性血小板减少性紫癜(ITP) |

5.9.1 有效剂量

可通过标准药学方法测定所述治疗剂在细胞培养物或实验性动物中的毒性和治疗功效，例如测定 $LD_{50}$ (使 50%的群体致死的剂量)和 $ED_{50}$ (在 50%的群体中治疗有效的剂量)。毒性和疗效之间的剂量比率为治疗指数，它可以表示为 $LD_{50}/ED_{50}$ 比率。优选具有大治疗指数的治疗剂。尽管可以使用具有毒性副作用的治疗剂，但应小心设计将所述化合物靶向受累组织部位的传递系统，以使对未感染细胞的潜在伤害最小，并因此减少副作用。

在配制人类使用的剂量范围时，可使用由细胞培养测定和动物研究所获得的数据。典型剂量包括但不限于 1 ng/kg 至 100 mg/kg。所述治疗剂的剂量优选位于包括几乎无毒或无毒的 $ED_{50}$ 的循环浓度范围内。所述剂量可根据使用的剂型和利用的给药途径而在此范围内有所变化。对于一种治疗剂，最好先由细胞培养测定评价治疗有效剂量。可配制一种剂量，以在动物模型中达到循环血浆浓度范围，包括在细胞培养中测定的 $IC_{50}$ (即达到对综合征最大抑制一半时的测

试化合物浓度)。可使用这种信息，以更精确地测定在人类中有用的剂量。例如可通过高效液相色谱检测在血浆中的水平。

### 5.9.2 配制

可以常规方式，使用一种或多种生理学上可接受的载体或赋形剂，配制按照本发明使用的药用组合物。

因此，可配制所述治疗剂及其生理学上可接受的盐和溶剂化物，以通过吸入或吹入(或通过嘴或通过鼻)给药或口服、口腔含化、胃肠外或直肠给药。

对于口服给药，所述药用组合物可采用例如通过常规方法利用药学上可接受的赋形剂制备的片剂或胶囊剂剂型，其中所述赋形剂有例如粘合剂(例如预凝胶化的玉米淀粉、聚乙烯吡咯烷酮或羟丙基甲基纤维素)；填充剂(例如乳糖、微晶纤维素或磷酸氢钙)；润滑剂(例如硬脂酸镁、滑石粉或二氧化硅)；崩解剂(例如马铃薯淀粉或羟基乙酸淀粉钠)；或润湿剂(例如十二烷基硫酸钠)。片剂可通过本领域广为人知的方法进行包衣。口服的液体制剂可采用的剂型例如为溶液剂、糖浆剂或悬浮剂，或者它们可作为在使用前用水或其它合适溶媒稀释的干燥产品存在。这样的液体制剂可通过常规方法，用药学上可接受的添加剂制备，其中所述添加剂例如为悬浮剂(例如山梨醇糖浆、纤维素衍生物或氢化可食用脂肪)；乳化剂(例如卵磷脂或阿拉伯胶)；非水溶性载体(例如杏仁油、油酯、乙醇或分馏植物油)；以及防腐剂(例如对羟基苯甲酸甲酯或羟基苯甲酸丙酯或山梨酸)。所述制剂在合适时还可以含有缓冲盐、调味剂、着色剂和甜味剂。

可适当地配制口服给药制剂，以获得控释的活性化合物。

对于口腔含化给药，所述组合物可采用以常规方式配制的片剂或锭剂剂型。

对于吸入给药，按照本发明使用的所述治疗剂通常以由加压填充器或喷雾器使用合适的抛射剂提供的气溶胶喷雾剂形式传递，所

述抛射剂例如为二氯二氟甲烷、三氯氟甲烷、二氯四氟乙烷、二氧化碳或其它合适的气体。在加压气溶胶的情况下，可通过提供一种计量的量传递的阀确定剂量单位。用于吸入剂或吹入剂的胶囊和药筒(例如明胶)，可配制成含有所述化合物的粉末混合物和一种合适的粉末基，诸如乳糖或淀粉。

所述治疗剂可配制为通过注射胃肠外给予，例如通过大剂量注射或连续输注。注射制剂可以是有或没有添加的防腐剂的单位剂型(例如在安瓿或多剂量容器中)。所述组合物可采用诸如在油性或水性溶媒中的悬浮液、溶液或乳液的形式，并可以含有配制剂如悬浮剂、稳定剂和/或分散剂。或者，所述活性成分可以为在使用前用合适溶媒(例如灭菌无热源水)配制的粉末形式。

所述治疗剂还可以被配制为直肠组合物，诸如栓剂或保留灌肠剂，例如含常规栓剂基质(如可可酯或其它甘油酯)的组合物。

除了先前描述的制剂以外，所述治疗剂还可以被配制为包埋长效(depot)制剂。这种长效制剂可通过植入(例如皮下或肌内)或肌内注射给药。因此，例如所述治疗剂可用合适的聚合物或疏水性物质(例如在可接受的油中的乳液)或离子交换树脂配制，或配制为微溶衍生物，例如微溶盐。

如果需要的话，所述组合物可存在于包装或分散装置中，所述装置可含有一种或多种含所述活性成分的单位剂型。所述包装例如可包括金属或塑料薄片，诸如泡罩包装。所述包装或分散装置可附带有优选给予人的给药说明。

在具体的实施方案中，本发明提供包含治疗有效量的治疗剂与CD40配体的药用组合物。

在以下的实施例中进一步描述本发明，但这些实施例决不是限制本发明的范围。

## 6. 实施例：克隆 S2C6 可变区

## 6.1 材料与方法

使用基本如 Gilliland 等，1996，Tissue Antigens 47: 1-20 所述的方法，克隆 S2C6 轻链和重链可变区。由 S2C6 杂交瘤分离总 RNA。使用逆转录酶和退火 JC 接点(junction)下游大约 100 个碱基对的反义引物，制备小鼠κ轻链和重链可变区的互补 DNA(cDNA)第一链。使用末端转移酶将聚鸟苷酸尾(poly-G tail)加入到所述 cDNA 链中，然后使用聚合酶链反应(PCR)合成双链 DNA。设计对所述聚鸟苷酸尾或轻链或重链 cDNA 内部约 50 个碱基的序列特异性的 PCR 引物，使之能包括独特限制酶切位点。扩增后，将用 *EcoRI* 和 *HindIII* 消化的 PCR 产物克隆入已用相同限制酶消化的 pUC19 中。连接这些反应物，转化到大肠杆菌 DH5α中，并通过限制酶切分析筛选获得的克隆。通过在 Li-Cor 荧光测序仪上进行 DNA 测序，对限制酶消化分析为阳性的克隆测序。在图 1 中显示了轻链可变区($V_L$)的核苷酸序列(SEQ ID NO: 1)和氨基酸序列(SEQ ID NO: 2)。在图 2 中显示了重链可变区($V_H$)的核苷酸序列(SEQ ID NO: 6)和氨基酸序列(SEQ ID NO: 7)。图 3A-3B 图解显示了 S2C6 $V_L$ 和 S2C6 $V_H$ 的氨基酸序列(分别为图 3A 和 3B)。CDR 标出下划线。$V_L$ CDR 1-3 的氨基酸序列分别对应于 SEQ ID NOS: 3-5。$V_H$ CDR 1-3 的氨基酸序列分别对应于 SEQ ID NOS: 8-10。

然后比较获得的 DNA 序列与相同同种型的其它鼠抗体的轻链和重链可变区，并测定分离自 S2C6 的基因的读框和对应的氨基酸序列。为证实所述氨基酸序列，对 S2C6 单克隆抗体的轻链和重链可变区进行 N-末端氨基酸分析。

1999 年 4 月 21 日提交 S2C6 VL、S2C6 VH 以及 VL 和 VH 二者 CDR 的氨基酸序列，使用 NR 数据库(全部非丰余 GenBank CDS 翻译物 + PDB + SwissProt + PIR + PRF)和 Kabat 数据库(Kabat 的有关免疫球蛋白序列数据库)进行 BLASTP 检索。在 http://www.ncbi.nlm.nih.gov 使用登录号可获取使用 NR 数据库发现的

序列。在 http://immuno.bme.nwu.edu/database_.html 和 SEQHUNT II 使用登录号可获取使用 Kabat 数据库发现的序列。这些检索结果如下所示:

**使用 NR 数据库进行 BLASTP 检索**

**S2C6 VL** (SEQ ID NO: 2): 用 S2C6 VL 作为查询序列对 NR 数据库进行 BLASTP 检索,在 100%同一性时未得到命中序列,而在 94% (106/112)同一性时获得 6 个命中序列。这 6 个序列示于以下:

pir||PT0359 IgG κ链 V 区(R4A.12) – 小鼠(片段)

gi|196660 (M59949)免疫球蛋白κ链 VJ 区[鼠类肌肉]

gi|196954 (M12183) κ链 V 区[鼠类肌肉] > gi|2247[鼠类肌肉]

pir||B34904 Ig κ链前体 V 区(12-40 和 5-14)...

emb|CAA80076|(Z22102)免疫球蛋白可变区[鼠类肌肉]

dbj|BAA22172|(AB006833)抗假尿苷单克隆抗体...

**VL CDR1** (SEQ ID NO: 3): 用 VL CDR1 作为查询序列进行 BLASTP 检索,在 100%同一性时未得到命中序列,而在 93% (15/16) 同一性时获得无数命中序列。其中前 5 个序列示于以下:

dbj|BAA03480|(D14627)免疫球蛋白γ-3 κ链[鼠类肌肉]

dbj|BAA22172|(AB006833)抗假尿苷单克隆抗体...

gi|4101647 (AF005352)免疫球蛋白 V 区轻链[鼠类肌肉]

gi|3377681 (AF078800)单链抗 HIV-1 Rev 可变区片段

gi|1870366 (U55625)抗 DNA 免疫球蛋白轻链 IgM [鼠类肌肉]

**VL CDR2** (SEQ ID NO: 4): 用 VL CDR2 作为查询序列对 NR 数据库进行 BLASTP 检索,未得到命中序列。

**VL CDR3** (SEQ ID NO: 5): 用 VL CDR3 作为查询序列对 NR

数据库进行 BLASTP 检索，未得到命中序列。

**S2C6 VH** (SEQ ID NO: 7): 用 S2C6 VH 作为查询序列对 NR 数据库进行 BLASTP 检索，在 100%同一性时未得到命中序列，而在最多可至 88%同一性时获得无数命中序列，其中的前 5 个序列示于以下:

gi|3561044 (AF083186)抗 HIV-1 p24 抗体 D2 重链[鼠类肌肉]

pdb|1A6T|B 链 B，Mab1-Ia 单克隆抗体的 Fab 片段

gi|2895955 (AF045895) IgG1 重链 mAB1-IA[鼠类肌肉]

emb|CAA80023|(Z22049)免疫球蛋白可变区[鼠类肌肉]

gi|194510 (M91695)免疫球蛋白γ-1 链 [鼠类肌肉]

**VH CDR1** (SEQ ID NO: 8): 用 VH CDR1 作为查询序列对 NR 数据库进行 BLASTP 检索，未得到命中序列。

**VH CDR2** (SEQ ID NO: 9): 用 VH CDR2 作为查询序列对 NR 数据库进行 BLASTP 检索，在 100%同一性时未得到命中序列，在 94% (16/17)同一性时获得 1 个命中序列，而在低于 94%的同一性时得到无数个命中序列。94%同一性时获得的 1 个命中序列如下所示:

gi|3561044 (AF083186)抗 HIV-1 p24 抗体 D2 重链[鼠类肌肉]

**VH CDR3** (SEQ ID NO: 10): 用 VH CDR3 作为查询序列对 NR 数据库进行 BLASTP 检索，未得到命中序列。

**使用 KABAT 数据库进行 BLASTP 检索**

**S2C6 VL** (SEQ ID NO: 2): 用 S2C6 VL 作为查询序列对 Kabat 数据库进行 BLASTP 检索，在 100%同一率时未得到命中序列，而在 89-91%同一率时获得无数个命中序列。前 5 个序列示于以下:

KADBID 005591，小鼠 IG κ轻链可变区(5-14...),

KADBID 005594，小鼠 IG κ轻链可变区(10VA...)，

KADBID 005593，小鼠 IG κ轻链可变区(12-4...)，

KADBID 005603，小鼠 IG κ轻链可变区(17s...)，

KADBID 005588，小鼠 IG κ轻链可变区(TEPC...)。

**VL CDR1** (SEQ ID NO: 3)：用 VL CDR1 作为查询序列对 Kabat 数据库进行 BLASTP 检索，在 100%同一率时未得到命中序列，而在 93% (15/16)同一率时获得无数命中序列。前 5 个序列示于以下：

KADBID 005720，小鼠 IG κ轻链可变区(BW24...)，

KADBID 005614，小鼠 IG κ轻链可变区(PME7...)，

KADBID 005624，小鼠 IG κ轻链可变区(C5-7...)，

KADBID 005621，小鼠 IG κ轻链可变区(40-6...)，

KADBID 005640，小鼠 IG κ轻链可变区(40-9...)。

**VL CDR2** (SEQ ID NO: 4)：用 VL CDR2 作为查询序列对 Kabat 数据库进行 BLASTP 检索，未得到命中序列。

**VL CDR3** (SEQ ID NO: 5)：用 VL CDR3 作为查询序列对 Kabat 数据库进行 BLASTP 检索，在与所述查询序列的同一性为 100%时得到 1 个命中序列：

KADBID 005681，小鼠 IG κ轻链可变区(NC10...)。

**S2C6 VH** (SEQ ID NO: 7)：用 S2C6 VH 作为查询序列对 Kabat 数据库进行 BLASTP 检索，在与所述查询序列的同一性为 100%时未得到命中序列，而在与所述查询序列的同一性为 79-85%时获得无数命中序列。前 5 个命中序列示于以下：

KADBID 001498，小鼠 IG 重链可变区(HDEX24)，

KADBID 001494，小鼠 IG 重链可变区(HDEX5)，

KADBID 001529，小鼠 IG 重链可变区(163. 72′CL)，
KADBID 001500，小鼠 IG 重链可变区(HDEX 37)，
KADBID 001597，小鼠 IG 重链可变区(BB128′CL)。

**VH CDR1** (SEQ ID NO: 8)：用 VH CDR1 作为查询序列对 Kabat 数据库进行 BLASTP 检索，未得到命中序列。

**VH CDR2** (SEQ ID NO: 9)：用 VH CDR 作为查询序列对 Kabat 数据库进行 BLASTP 检索，在与所述查询序列的同一率为 100%时未得到命中序列，而在与所述查询序列的同一率为 87-88%时获得 10 个命中序列。前 5 个命中序列如下所示：
KADBID 001535，小鼠 IG 重链可变区(H10″CL)，
KADBID 001534，小鼠 IG 重链可变区(H81′CL)，
KADBID 001533，小鼠 IG 重链可变区(H50′CL)，
KADBID 019741，小鼠 IG 重链可变区(克隆 F′CL)，
KADBID 001529，小鼠 IG 重链可变区(163. 72′CL)。

**VH CDR3** (SEQ ID NO: 10)：用 VH CDR3 作为查询序列对 Kabat 数据库进行 BLASTP 检索，未得到命中序列。

## 7. 实施例：S2C6 的生物活性

### 7.1 材料与方法

#### 7.1.1 抗-CD40 抗体的制备

于 37℃在含 10%胎牛血清(FBS)、100 单位/ml 青霉素和 100 mg/ml 链霉素的完全 IMDM(Gibco-BRL，Grand Island，NY)中培养 S2C6 杂交瘤。通过离心收获培养物，并通过使用 0.2 μm 滤膜过滤收集上清液。随后将所述上清液上 GammaBind™ Sepharose 柱(Pierce)，用磷酸缓冲盐水(PBS)洗涤，并用 0.1 M 甘氨酸 pH 2.5 洗脱。在洗脱后立

即用 1 M Tris pH 8.0 中和抗体，透析到 PBS 中，并除菌过滤。通过大小排阻层析分析单克隆抗体制品。在本文所述的研究中只使用单体蛋白超过 99%的样品。

### 7.1.2 人肿瘤异种移植模型

得到 90 只 6-8 周龄的雌性 C.B.-17 SCID 小鼠(Taconic Labs, Germantown, NY)，并隔离检疫 2 周。对照组小鼠静脉内(i.v.)注射人 B 细胞肿瘤系：Ramos(非何杰金氏淋巴瘤)细胞、HS Sultan(多发性骨髓瘤)细胞或 IM-9(多发性骨髓瘤)细胞($1 \times 10^6$-$2 \times 10^6$ 细胞)。余下的小鼠被分为两组；一半在注射肿瘤细胞前 1 天静脉注射 200 μl 1:10 稀释的抗-脱唾液酸-GM1 (Wako Chemicals, Richmond, VA)，以去除宿主天然杀伤细胞(Murphy 等，1992，Eur. J. Immunol. 22: 241)。两组小鼠静脉注射 Ramos 细胞、HS Sultan 细胞或 IM-9 细胞($1 \times 10^6$-$2 \times 10^6$ 细胞)。然后由肿瘤移植后第 1 天或第 5 天开始，按照以下的时间表对测试组小鼠腹膜内(i.p.)注射 1 mg/kg 的如 7.1.2 单元中所述制备的 S2C6 IgG，并监测测试组小鼠的部分瘫痪或其它疾病体征。

**异种移植模型时间表**

| 组别 | 肿瘤细胞系 | 抗体 (1 mg/kg, i.p.) | 抗-脱唾液酸-GM1 | 用 mAb 处理天数 |
|---|---|---|---|---|
| 1 对照 | Ramos | - | - | - |
| 2 | Ramos | S2C6 | - | 1, 5, 9, 13, 17 |
| 3 | Ramos | S2C6 | - | 5, 9, 13, 17, 21 |
| 4 | Ramos | S2C6 | + | 1, 5, 9, 13, 17 |
| 5 | Ramos | S2C6 | + | 5, 9, 13, 17, 21 |
| 6 对照 | HS Sultan | - | - | - |
| 7 | HS Sultan | S2C6 | - | 1, 5, 9, 13, 17 |
| 8 | HS Sultan | S2C6 | - | 5, 9, 13, 17, 21 |
| 9 | HS Sultan | S2C6 | + | 1, 5, 9, 13, 17 |
| 10 | HS Sultan | S2C6 | + | 5, 9, 13, 17, 21 |

| | | | | |
|---|---|---|---|---|
| 11 对照 | IM-9 | - | - | - |
| 12 | IM-9 | S2C6 | - | 1, 5, 9, 13, 17 |
| 13 | IM-9 | S2C6 | - | 5, 9, 13, 17, 21 |
| 14 | IM-9 | S2C6 | + | 1, 5, 9, 13, 17 |
| 15 | IM-9 | S2C6 | + | 5, 9, 13, 17, 21 |

### 7.1.3 外周血 B 细胞分离

使用抗 CD19 和 CD20 二者的固定化抗体，通过正选择分离外周血 B 细胞。经流式细胞仪测定，最终分离的细胞群含有的 B 细胞超过 85%。为了储存，将所述细胞在含 10%二甲基亚砜的胎牛血清(FBS)中稀释至 $4 \times 10^7$ 细胞/ml，并储存在液氮冷冻装置中。

### 7.1.4 B 细胞增殖测定

解冻人外周血 B 细胞，并在 5 ng/ml 重组人 IL-4 (Biosource)和各种稀释度的抗-CD40 单克隆抗体：S2C6、G28-5(Bristol-Myers Squibb)或 M3 (Genzyme #80-3702-04)存在的情况下，在 IMDM 培养基 + 10% FBS 中以 $1 \times 10^5$ 细胞/孔在 96 孔组织培养板中温育。作为对照，细胞与 IL-4 和不相关对照单克隆抗体 EXA2-1H8 (抗假单胞菌外毒素)温育。该板于 37℃温育 3 天，然后以 0.5 mCi $^3$H-胸苷/孔脉冲 16 小时。使用 Filtermate 196 Harvester™ (Packard Instruments)将细胞收获在 96 孔玻璃纤维滤器上，并与闪烁液混合。使用 Topcount LSC™ (Packard Instruments)通过液闪记数检测 $^3$H-胸苷掺入到新生 DNA 中的程度。

将选择用来组成型表达高水平 CD40L 的 Jurkat 细胞系("Jurkat/CD40L")用作 CD40L 刺激细胞(Malik 等，1996，J. Immunol. 156: 3952-60)。为消除刺激细胞增殖，将它们用丝裂霉素(50 mg/ml)的 PBS 溶液于 37℃处理 20 分钟，接着在 PBS 中洗涤 3 次，然后与 B 细胞混合。将 B 细胞($1 \times 10^5$ 细胞/孔)与 Jurkat/CD40L 细胞混合，如上所述进行测定。首先将 B 细胞和 IL-4 与刺激细胞($2.5 \times 10^4$ 细胞/

孔)混合，接着立即加入抗 CD40 单克隆抗体。用固定浓度的刺激细胞滴定单克隆抗体，或者用固定浓度的单克隆抗体滴定刺激细胞。

### 7.1.5 CD40/CD40L 结合测定

在这些测定中使用 Jurkat/CD40L 细胞系作为靶细胞系。将细胞浓度调节为 $2 \times 10^7$/ml，每个样品 50 μl。在 RPMI 1640 培养基(Gibco) + 10% FBS 中进行结合。为测定受体饱和度，将 Jurkat/CD40L 细胞与增加浓度的 CD40-Ig (CD40 和人免疫球蛋白的可溶性融合蛋白)温育(Noelle 等，1992，Proc. Natl. Acad. Sci. USA 89：6550-6554)，洗涤并与缀合抗人免疫球蛋白的异硫氰酸荧光素("FITC-抗-人 Ig")温育。使用 FacScan™流式细胞仪(Becton Dickinson)评价产生的结合。将重组可溶性 CD40-Ig (25 μg/ml)在冰上与增加浓度的 S2C6 单克隆抗体预温育 1 小时。抗-CD40 单克隆抗体 G28-5、M3 和抗假单胞菌外毒素(一种同种型对照品)被用于比较。可溶性重组人 CD40 配体(CD154-muCD8)作为与鼠 CD8 的融合蛋白产生并用 FITC 标记，该配体得自 Research Diagnostics，Inc.(Flanders，NJ)。以 4 倍终浓度稀释可溶性 CD40-Ig 和抗-CD40 单克隆抗体，在冰上预温育 1 小时，然后在冰上与 Jurkat 细胞混合 1 小时。洗涤细胞，并用 FITC-山羊抗人 $F(ab')_2$ (Jackson Labs，Fc 特异性#109-096-098)标记。通过流式细胞仪测定 CD40 结合的程度。

## 7.2 结果

### 7.2.1 体外研究：S2C6 单克隆抗体促进 CD40/CD40L 的相互作用

为评价抗 CD40 单克隆抗体对可溶性 CD40 与在激活 T 细胞表面表达的 CD40L 的结合的影响，将增加浓度的各种 CD40 单克隆抗体与 25 μg/ml 可溶性 CD40-Ig 预温育，接着与 Jurkat/CD40L 细胞的复合体(complex)温育。首先通过流式细胞仪用 FITC 标记的抗 CD40L

核实在选择的 $CD40L^+$ Jurkat T 细胞上的 CD40L 表达(未列出数据)。然后使用 FITC-山羊抗人 Ig 对 Jurkat/CD40L 细胞进行流式细胞计量，测定结合在这些靶细胞上的 CD40L 的 CD40，以检测结合的 CD40-Ig。用 CD40-Ig 滴定显示受体饱和度约为 25 μg/ml CD40-Ig。使用饱和浓度的可溶性 CD40，S2C6 与 CD40 以 0.25-2：1(质量：质量)的比率混合，导致 CD40 与 CD40L 的结合剂量依赖性增加(在约 6 μg/ml、13 μg/ml、25 μg/ml 和 50 μg/ml 的浓度分别为约 50%、100%、146%和 220%)(图 4)。采用抑制性抗体 M3 的相似滴定以剂量依赖方式阻断 CD40/CD40L 结合。相对于对照 EXA2-1H8 Ig，G28-5 单克隆抗体在高至 25 μg/ml 的浓度显示对 CD40/CD40L 结合没有影响，在测试的最高浓度(50 μg/ml)仅具有轻微刺激性。

这些数据清楚表明，S2C6 单克隆抗体促进 CD40/CD40L 相互作用。此外，S2C6 在增加 CD40/CD40L 相互作用的能力方面与 G28-5 和 M3 不同。

在交互测定中，评价抗 CD40 单克隆抗体对可溶性 CD40L 与在 B 细胞表面表达的膜结合 CD40 的结合的影响。用可溶性 CD40L 滴定显示，Ramos B 细胞表面 CD40 饱和度约为 10 μg/ml。将增加浓度的各种抗 CD40 单克隆抗体与表达 CD40 的 B 细胞预温育，接着将所述细胞与 FITC 标记的可溶性 CD40L 温育。然后通过对 Ramos 细胞进行流式细胞计量，测定与靶 B 细胞上的 CD40 结合的标记 CD40L。使用饱和浓度的可溶性 CD40L，将浓度范围为 0.04-2 μg/ml 的 S2C6 单克隆抗体与表达 CD40 的细胞混合，导致 CD40L 结合最大增加约 51%-68%(图 5)。

在以上可溶性CD40的结果中，单克隆抗体G28-5对CD40/CD40L的相互作用几乎没有影响，与其相反，G28-5 在所有的测试浓度均显示抑制可溶性配体与 CD40 的结合。采用抑制性单克隆抗体 M3 的相似滴定也以剂量依赖方式阻断 CD40L/CD40 结合。

这些数据表明，S2C6 在增加 CD40L/CD40 相互作用的能力方面

令人惊奇地与 G28-5 和 M3 不同。而且，在这些条件下，单克隆抗体 G28-5 和单克隆抗体 M3 在低至 40 ng/ml 的浓度抑制可溶性 CD40L 与 CD40 的相互作用。

7.2.2 体外研究：单克隆抗体 S2C6 增加 B 细胞对 CD40/CD40L 的反应

在存在抗 CD40 单克隆抗体(S2C6、G28-5 或 M3)的情况下，检测原代外周 B 细胞对表达 CD40L 的细胞的生长应答。首先，在存在或不存在固定水平(30 ng/ml)的各种单克隆抗体的情况下，将 B 细胞与增加数目的非增殖性 Jurkat/CD40L 细胞混合。然后通过在刺激后 72 小时加入 $^{3}$H-胸苷，检测 B 细胞对处理的活化反应。存在单克隆抗体 M3 时滴定 T 细胞导致 B 细胞增殖，与对照 Ig 观察到的相似(图 6)。

尽管在没有配体时单克隆抗体 G28-5 使某些 B 细胞活化(图 7)，但在存在 G28-5 时 CD40L$^{+}$ T 细胞滴定仅略微地增加 B 细胞增殖，超过单独用 G28-5 观察到的水平 1.3 倍。相反，在 S2C6 存在下 B 细胞增殖随着 T 细胞刺激细胞数目的增加以剂量依赖方式增加，当 B 细胞对 T 细胞刺激物的比率为 4: 1 时，B 细胞增殖增加至仅用单克隆抗体刺激时的 3 倍以上。

这些数据证明，与 M3 和 G28-5 不同，S2C6 令人惊奇地可与 CD40L 协同作用，通过 CD40 促进 B 细胞增殖。

在该类型的第二个测定中，或者用抗 CD40 单克隆抗体滴定 B 细胞，或者将 B 细胞与非增殖性 CD40L$^{+}$ T 刺激细胞以固定比率 4: 1(B: T)混合，并用抗 CD40 单克隆抗体滴定 B 细胞(图 7)。

这些结果证明，在这些条件下，和单独的 G28-5 相比，10 μg/ml 的单克隆抗体 G28-5 和配体使原始人外周血 B 细胞的活化增加 2 倍。S2C6 明显更具有活性，与单独的 S2C6 相比，在配体存在下 10 μg/ml(最高测试水平)的 S2C6 以剂量依赖方式使 B 细胞增殖增加至 16.2 倍，

达到令人吃惊的程度。

总而言之，这些数据表明，S2C6 与 CD40 混合增加 CD40L 结合。尽管 S2C6 自身以类似于 G28-5 的方式刺激 B 细胞增殖，但 S2C6 在其增加 CD40L 结合的能力以及随后的 CD40L 介导的活化信号强度方面与 G28-5 不同。

8. 实施例：单克隆抗体 S2C6 抑制肿瘤生长

为评价天然单克隆抗体 S2C6 的抗肿瘤活性，将雌性 C.B.-17 SCID 小鼠分为两组(20 只小鼠/组)。每组的一半小鼠用抗-脱唾液酸-GM1 处理，以钝化宿主天然杀伤细胞活性(Murphy 等，1992，Eur. J. Immunol. 22: 241)。第二天，用 Ramos、HS Sultan 或 IM-9 细胞($1 \times 10^6$ 细胞)静脉内注射小鼠。然后用 1 mg/kg 的如前文第 7 单元的材料和方法中所述的单克隆抗体 S2C6 IgG 腹膜内注射小鼠，并监测部分瘫痪或其它疾病发作体征。

用单克隆抗体 S2C6 治疗患有 Ramos 人 B 细胞淋巴瘤(图 8A)、HS Sultan 多发性骨髓瘤(图 8B)或 IM-9 多发性骨髓瘤(图 8C)的动物，明显减小了肿瘤块以及所后的与肿瘤相关的发病率和死亡率。在平行研究中，存在抗-脱唾液酸-GM1 时疗效得到维持，提示在单克隆抗体 S2C6 存在时存活率增加不是缘于非特异性 NK 细胞的活性。IM-9 细胞系是一种类似于多发性骨髓瘤的侵袭性肿瘤模型，该细胞系分泌作为疾病替代标志的人 Ig。

用单克隆抗体 S2C6 治疗患 IM-9 疾病的小鼠明显增加动物的存活。这些研究清楚显示，S2C6 对小鼠中移入的人肿瘤具有有效的抗肿瘤活性。

9. 实施例：单链抗-CD40 免疫毒素融合蛋白结合 CD40-Ig

在大肠杆菌中以包含体表达 BD1-S2C6 sFv(单链抗-CD40 免疫毒素，一种由 bryodin 1(BD1)的氨基酸序列(Francisco 等，1997，J. Biol.

Chem. 272(39): 24165-24169)融合至单克隆抗体 S2C6 可变区组成的融合蛋白)，使其变性和重折叠。

简而言之，使用 TRIZOL 试剂(Life Technologies)，按照生产商的建议由 S2C6 杂交瘤细胞分离总 RNA。基本上按照 Gilliland 等所述(Tissue Antigens, 47: 1-20(1996))，使用与 J-C 接点下游约 100 个碱基的序列互补的引物，进行轻链可变区和重链可变区的 cDNA 第一链的合成。然后给所述第一链加聚鸟苷酸尾，并通过使用与聚鸟苷酸尾互补的 poly-C 锚式引物和将约 50 个碱基嵌套入用于第一链合成的引物中的引物的 PCR 进行扩增。设计 PCR 引物以在 PCR 产物的 5′和 3′末端产生独特限制位点。用 *EcoRI* 和 *HindIII* 消化含有轻链可变区编码序列和重链可变区编码序列的两个 PCR 产物，并将其连接入已用相同酶消化的 pUC19 中。获得的质粒 pSG5 和 pSG10 包含分别编码 S2C6 VL 和 S2C6 VH 的 DNA。对两种质粒的 DNA 测序，并验证其与亲代单克隆抗体的氨基酸末端序列匹配。

按照 Gilliland 等所述以 VH-VL 取向将 S2C6 的 VH 片段和 VL 片段“缝合”在一起(重叠延伸 PCR)，并连接到克隆载体中。随后通过限制酶消化将 BD1-G28-5 sFv 的 sFv 片段(Francisco 等, 1997, *J. Biol. Chem.* 272: 24165-24169)从 pSE151 中去除，并在其原位连接 S2C6 sFv。获得的质粒 pSG40 含有在诱导型 T7 启动子控制下的 BD1-S2C6 sFv 编码基因。

为了表达 pSG40，将其转化入感受态大肠杆菌菌株 BL21(DE3)pLysS 细胞中，并在 T-肉汤培养基中于 37℃培养所述细胞。当培养物达到 $OD_{600}$=1.0 时，用 1 mM 异丙基-β-D-硫代半乳糖苷(IPTG)诱导细胞 3 小时。随后，通过离心收获细胞，经超声波裂解，并通过离心分离为不溶性包含体的 BD1-S2C6 sFv 融合蛋白，如下对其进行变性和重折叠：在 7M 胍中以 5 mg/ml 溶解包含体，通过快速稀释(1: 100)到含 0.3 M L-精氨酸和 2 mM DTT 的 PBS 中重折叠，并对 20 mM 磷酸钠缓冲液(pH 7.4)透析，以随后纯化。

使用 Blue Sepharose 随后在固定化 CD40-Ig 上亲和层析，分离重折叠的蛋白。

然后在 ELISA 中测试纯化蛋白对固定化 CD40-Ig 的结合。用 0.5 μg/ml 的 CD40-Ig 包被微量滴定板，接着在 25 μg/ml S2C6 单克隆抗体(▲)、25 μg/ml 对照抗体 BR96 (●)或无过量抗体(■)存在下，加入纯化 BD1-S2C6 sFv 在含 1%牛血清白蛋白和 0.05% Tween-20 的 PBS (pH 7.4)中的稀释液。通过加入 BD1 特异性兔抗血清(Seattle Genetics, Inc., Bothell, Washington)，接着加入缀合山羊抗兔 Ig 的辣根过氧化物酶，检测 BD1-S2C6 sFv 与固定化受体的结合。

加入过量 S2C6 单克隆抗体完全抑制 BD1-S2C6 sFv 与 CD40-Ig 的结合，但加入对照单克隆抗体不能完全抑制 BD1-S2C6 sFv 与 CD40-Ig 的结合(图 9)。

10. 实施例: 在治疗 CD40 阳性恶性肿瘤时给予重组 S2C6

给患 CD40 阳性恶性肿瘤(例如非何杰金氏淋巴瘤、多发性骨髓瘤和结肠癌或其它癌症)的患者注射重组人源化 S2C6-抗-CD40 单克隆抗体(具有鼠 CDR 和人构架区)或重组嵌合抗体(包含 S2C6 可变区和人抗体恒定区)。体外制备重组抗体。治疗可在病程中的任意时间开始，可伴随或不伴随化疗。

治疗方案包括每周注射一次在盐水或其它生理上相容的溶液中稀释的所述药物。

重组 S2C6 使用的剂量范围为 0.1 mg/m$^2$ (患者的体表面积)至 1000 mg/m$^2$，优选剂量为 100-500 mg/m$^2$。

注射途径为通过或外周静脉连线(access line)或中枢静脉连接线静脉内注射。所述药物作为输注液而不是静脉推注给药。

通过检测 a)在外周血中的总淋巴细胞数目以及 T 淋巴细胞和 B 淋巴细胞的数目； b) T 淋巴细胞(辅助 T4 淋巴细胞和溶细胞 T8 淋巴细胞)的体外活性; 和/或 c)使用诸如计算层析 X 射线照相术(CT)扫描、

磁共振成像(MRI)扫描、X 射线成像、骨扫描成像和肿瘤活检采样包括骨髓抽提(BMA)的技术检测肿瘤形态学的变化，监测重组 S2C6 的治疗功效。

根据以上获得的结果，开发最适宜治疗 CD40 阳性恶性肿瘤的治疗方案，即对免疫系统能力的影响最小，并达到肿瘤消退和完全根除肿瘤细胞的最终目标。

## 11. 微生物保藏

根据国际承认的用于专利程序的微生物保藏的布达佩斯条约的规定，1999 年 5 月 25 日将分泌天然单克隆抗体 S2C6 的杂交瘤 S2C6 保藏于美国典型培养物保藏中心(ATCC)，10801 University Boulevard，Manassass，Virginia 20110-2209，指定的的保藏号为 PTA-110。

## 12. 具体的实施方案、参考文献的引用

本文所述的具体实施方案不限制本发明的范围。实际上，除了本文描述的本发明以外，根据前述和附图对本发明进行各种修改对本领域技术人员而言是显而易见的。这样的修改应包括在所附权利要求书的范围内。

本文引用的各种参考文献，包括专利申请、专利和科学出版物，其公开的内容都通过引用整体结合到本文中。

# 说明书的核苷酸和氨基酸序列

<110>SEATTLE GENETICS, INC.

<120> 重组抗-CD40 抗体及其用途

<130> 9632-009-228

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<170> PatentIn Ver. 2.0

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gct caa gcc tcc atc tct tgc aga tct agt cag agc ctt gta cac agt   96
Ala Gln Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Leu Val His Ser
             20                  25                  30

aat gga aac acc ttt tta cat tgg tac ctg cag aag cca ggc cag tct   144
Asn Gly Asn Thr Phe Leu His Trp Tyr Leu Gln Lys Pro Gly Gln Ser
```

35 40 45

cca aaa ctc ctg atc tac aca gtt tcc aac cga ttt tct ggg gtc cca 192
Pro Lys Leu Leu Ile Tyr Thr Val Ser Asn Arg Phe Ser Gly Val Pro
50 55 60

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Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile
65 70 75 80

agc aga gtg gag gct gag gat ctg gga gtt tat ttc tgc tct caa act 288
Ser Arg Val Glu Ala Glu Asp Leu Gly Val Tyr Phe Cys Ser Gln Thr
85 90 95

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20 25 30

Asn Gly Asn Thr Phe Leu His Trp Tyr Leu Gln Lys Pro Gly Gln Ser
35 40 45

Pro Lys Leu Leu Ile Tyr Thr Val Ser Asn Arg Phe Ser Gly Val Pro

50 55 60

Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile
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Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Ser Phe Thr Gly Tyr
20 25 30

tac ata cac tgg gtg aag cag agc cat gga aag agc ctt gag tgg att 144
Tyr Ile His Trp Val Lys Gln Ser His Gly Lys Ser Leu Glu Trp Ile

35 40 45

gga cgt gtt att cct aac aat gga ggc act agt tac aac cag aag ttc 192
Gly Arg Val Ile Pro Asn Asn Gly Gly Thr Ser Tyr Asn Gln Lys Phe
50 55 60

aag ggc aag gcc ata tta act gta gac aag tca tcc agc aca gcc tac 240
Lys Gly Lys Ala Ile Leu Thr Val Asp Lys Ser Ser Ser Thr Ala Tyr
65 70 75 80

atg gaa ctc cgc agc ctg aca tct gag gac tct gcg gtc tat tac tgt 288
Met Glu Leu Arg Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Tyr Cys
85 90 95

gca aga gaa ggg atc tac tgg tgg ggc cac ggc acc act ctc aca gtc 336
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35 40 45

Gly Arg Val Ile Pro Asn Asn Gly Gly Thr Ser Tyr Asn Gln Lys Phe
50 55 60

Lys Gly Lys Ala Ile Leu Thr Val Asp Lys Ser Ser Ser Thr Ala Tyr
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85 90 95

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1 5

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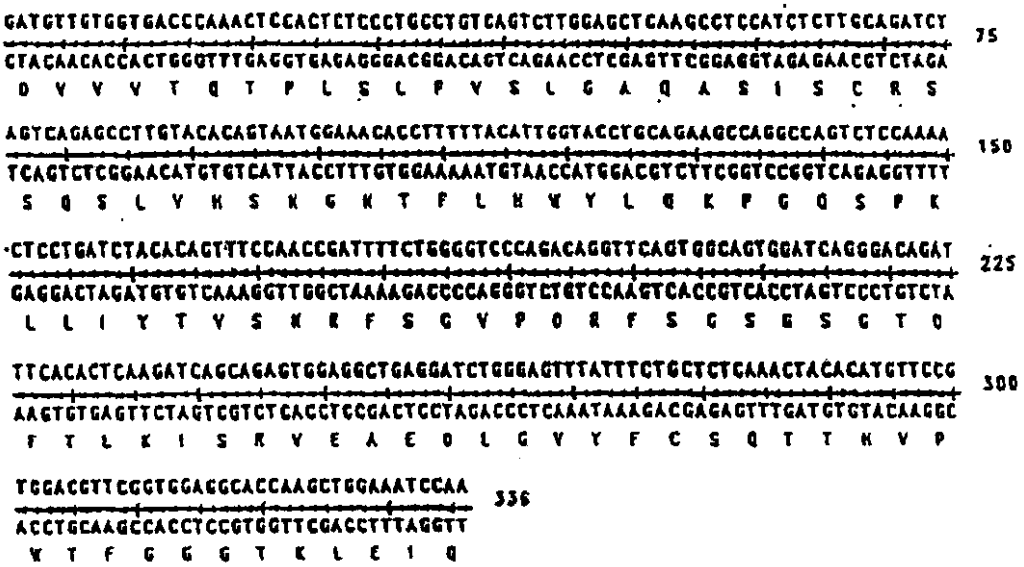
# 说 明 书 附 图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S Q S L V H S N G N T F L H W Y L Q K P G Q S P K
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L L I Y T V S N R F S G V P D R F S G S G S G T D
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F T L K I S R V E A E D L G V Y F C S Q T T H V P
TGGACGTTCGGTGGAGGCACCAAGCTGGAAATCCAA
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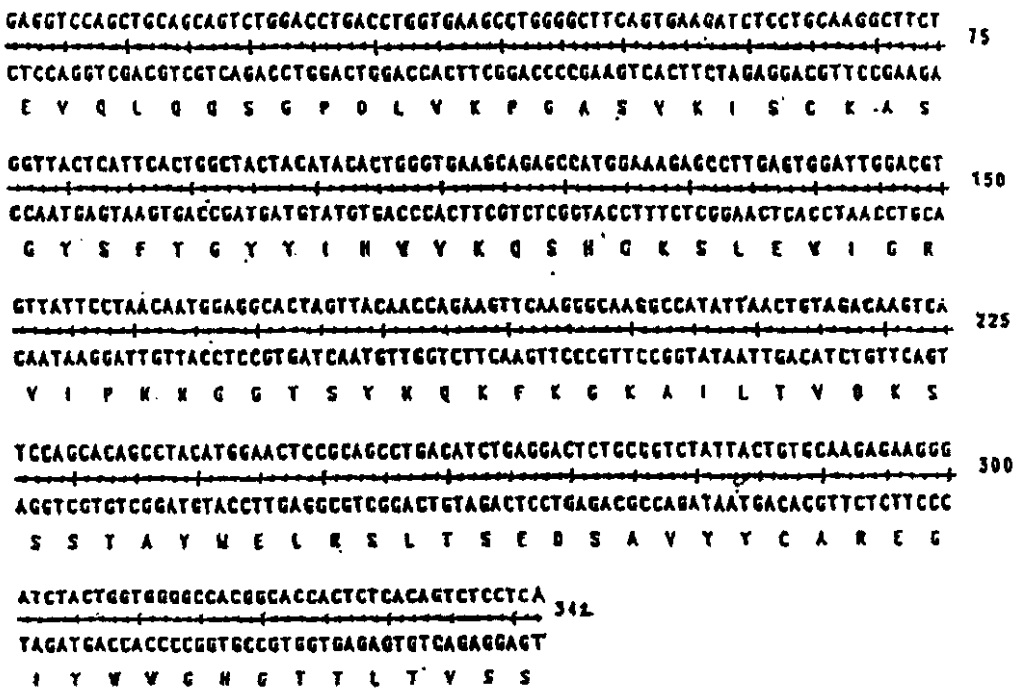
150
225
300
336

图 1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150
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G Y S F T G Y Y I H W V K Q S H G K S L E W I G R
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225
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V I P K N G G T S Y N Q K F K G K A I L T V D K S
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300
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342
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I Y W W G H G T T L T V S S

图 2

S2C6 VL

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CDR1

LIYTVSNRFSGVPDRFSGSGSGTDFTLKISRVEAEDLGVYFCSQTTHVPWT

CDR2 CDR3

FGGGTKLEIQ

S2C6 VH

EVQLQQSGPDLVKPGASVKISCKASGYSFTGYYIHWVKQSHGKSLEWIGRV

CDR1

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CDR2 CDR3

WWGHGTTLTVSS

# 图 3A-3B

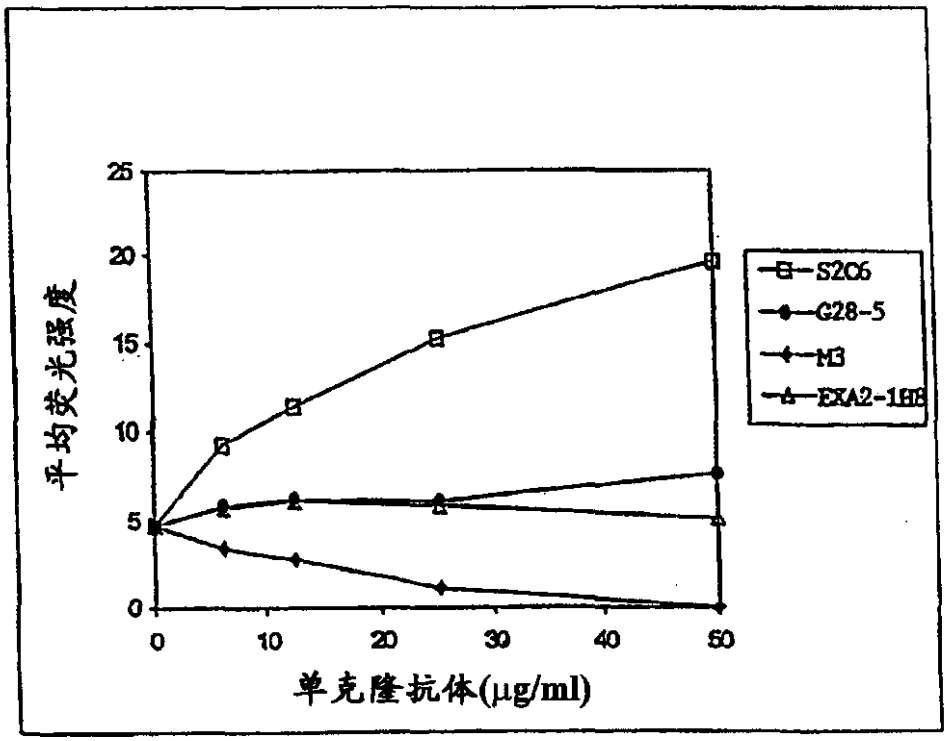
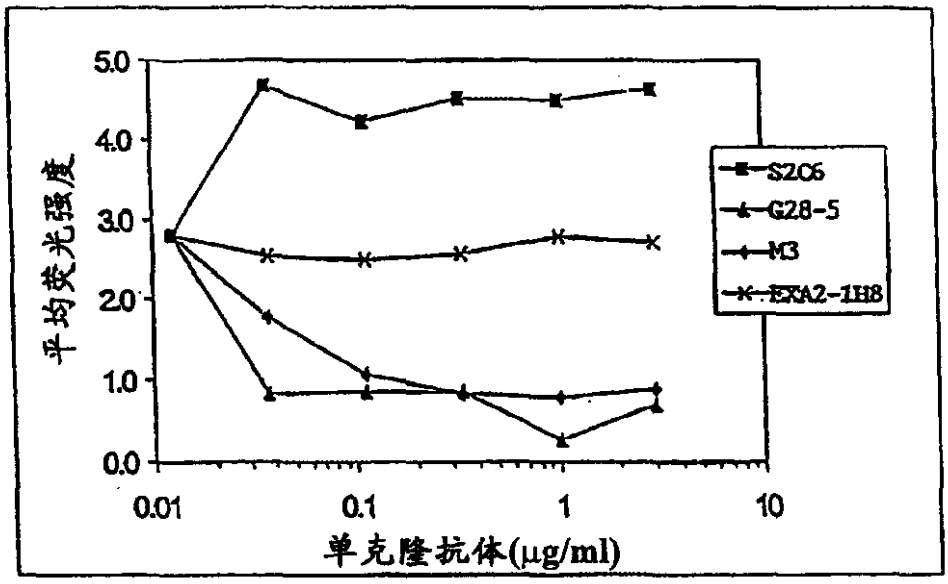

平均荧光强度
25
20
15
10
5
0
0
10
20
30
40
50
单克隆抗体(μg/ml)
S2C6
G28-5
M3
EXA2-1H8

图 4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4.0
3.0
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单克隆抗体(μg/ml)
S2C6
G28-5
M3
EXA2-1H8

图 5

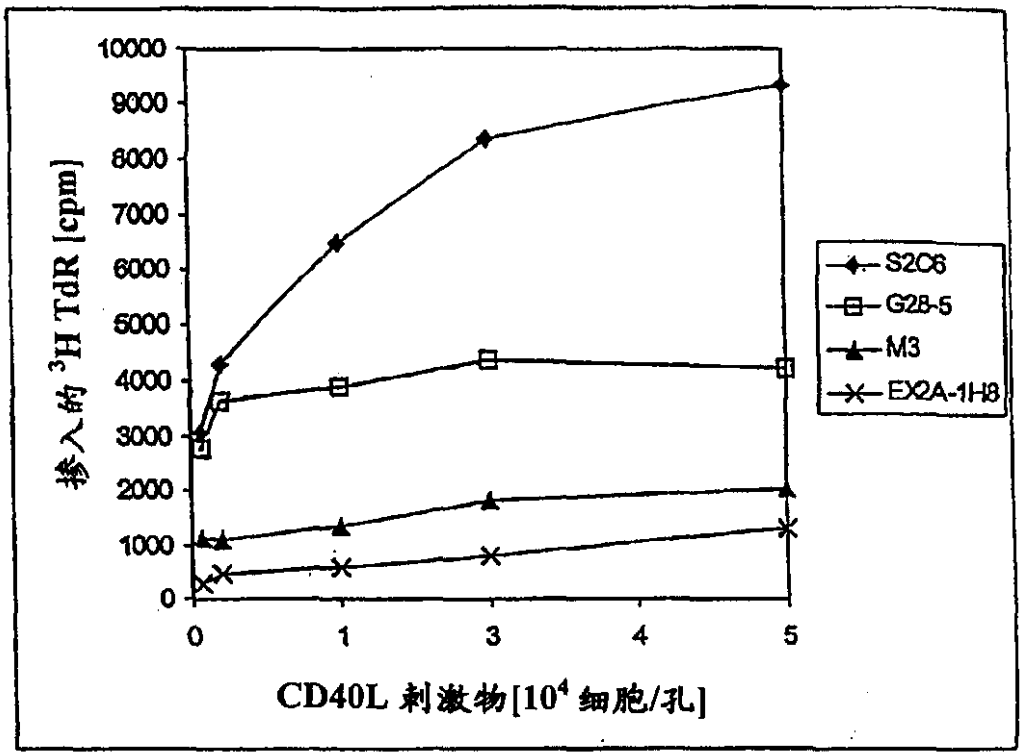

10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
掺入的 $^3$H TdR [cpm]
0
1
3
4
5
CD40L 刺激物[$10^4$ 细胞/孔]
S2C6
G28-5
M3
EX2A-1H8

图 6

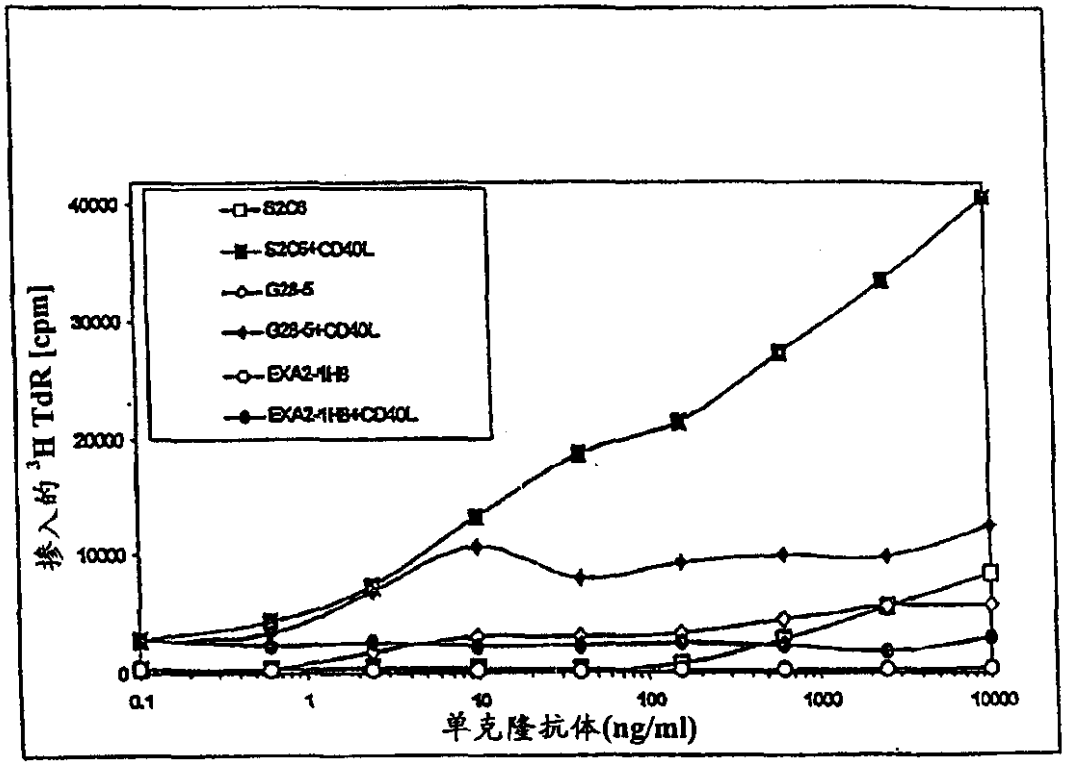
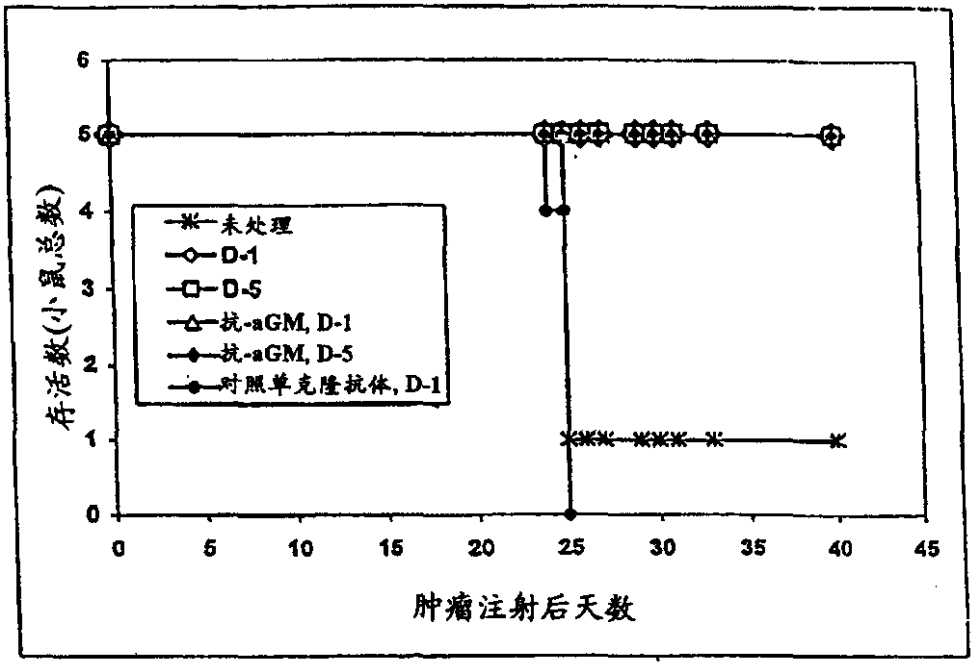

S2C6
S2C6+CD40L
G28-5
G28-5+CD40L
EXA2-1HB
EXA2-1HB+CD40L
掺入的 $^3$H TdR [cpm]
40000
30000
20000
10000
0
0.1
1
10
100
1000
10000
单克隆抗体(ng/ml)

图 7

A.


存活数(小鼠总数)
肿瘤注射后天数
未处理
D-1
D-5
抗-aGM, D-1
抗-aGM, D-5
对照单克隆抗体, D-1

B.

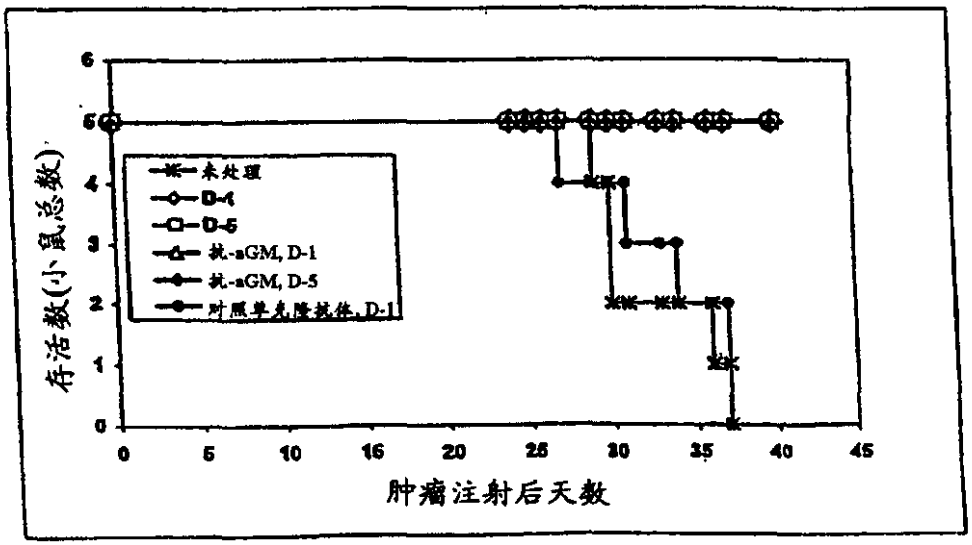

存活数(小鼠总数)
肿瘤注射后天数
未处理
D-1
D-5
抗-aGM, D-1
抗-aGM, D-5
对照单克隆抗体, D-1

图 8A-8B

C.

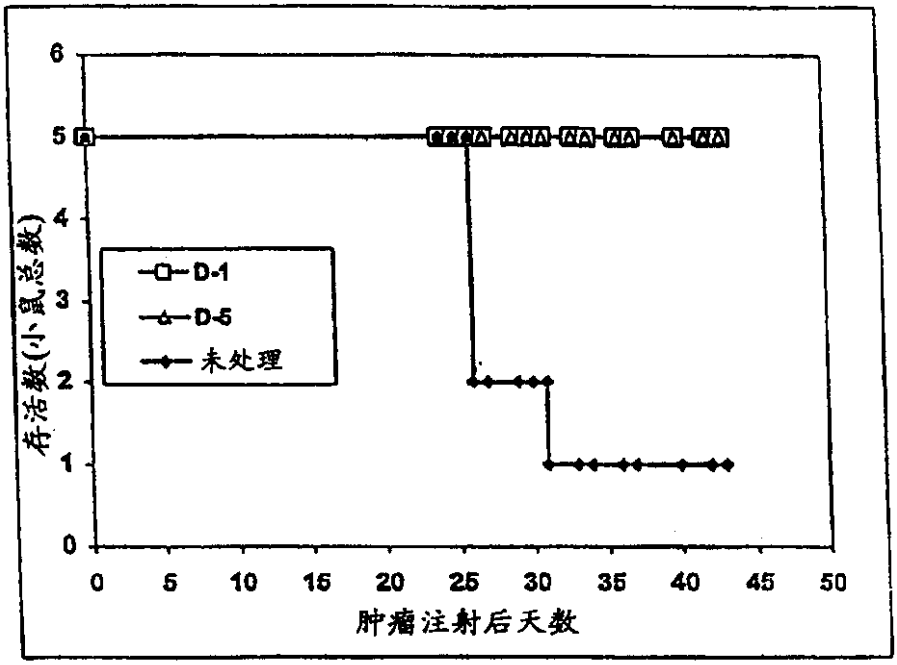
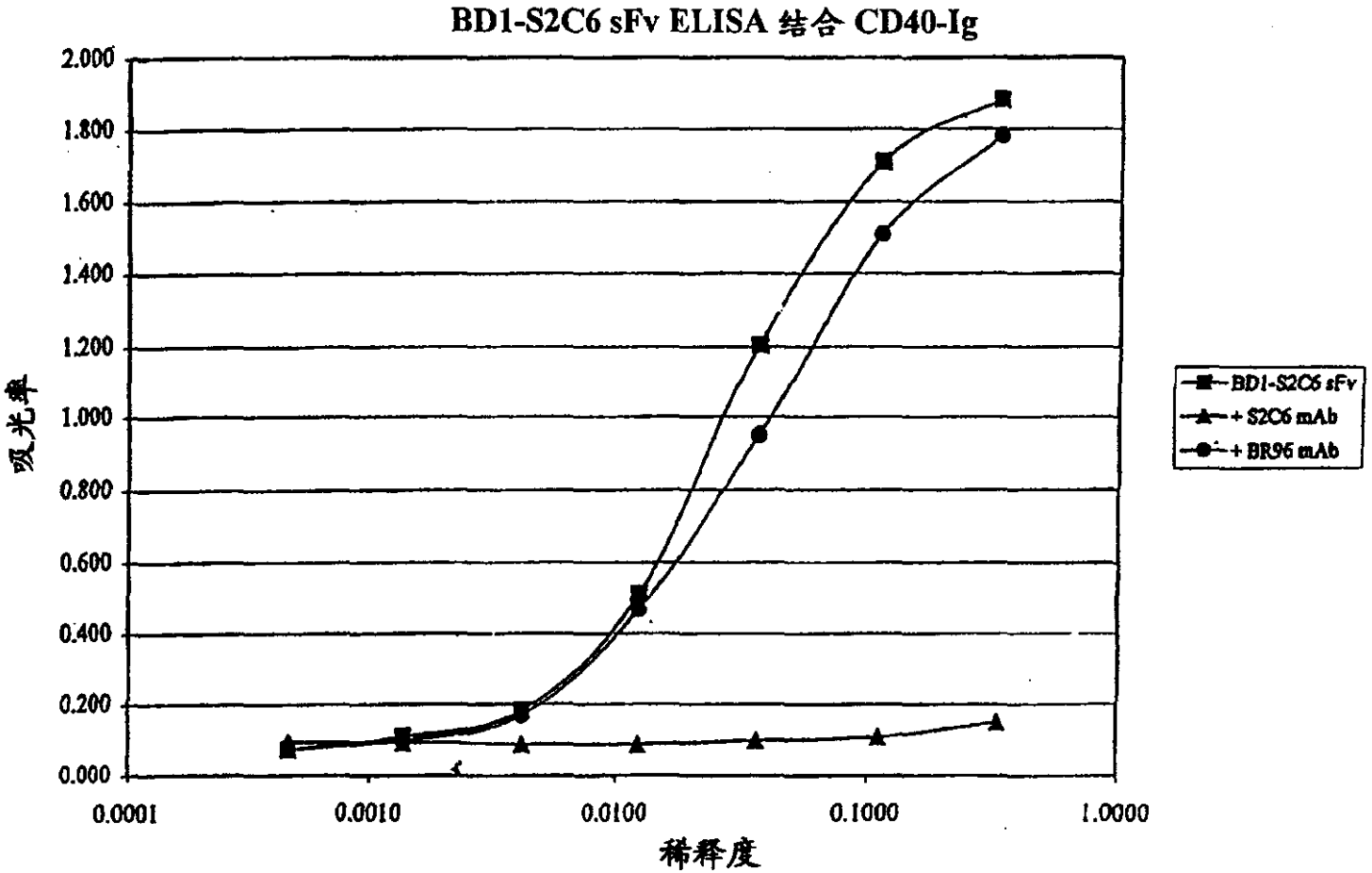



图 8C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2.000
1.800
1.600
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
吸光率
0.0001
0.0010
0.0100
0.1000
1.0000
稀释度
BD1-S2C6 sFv
+ S2C6 mAb
+ BR96 mAb

图 9