Title: CD40 VARIANTS AND USES THEREOF

Abstract: Disclosed are newly discovered CD40 variant molecules, their polypeptide sequences, and the polynucleotides encoding the polypeptide sequences. Also provided are procedures for producing such polypeptides by recombinant techniques employing, for example, vectors and host cells. Also disclosed are methods for utilizing such polypeptides and modulators thereof for the treatment of diseases, including cancer, immune diseases, infectious diseases, and ischemic diseases.
CD40 VARIANTS AND USES THEREOF

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the priority benefit of the provisional patent application U.S. Serial No. 60/557,092 filed March 26, 2004, which is incorporated herein by reference in its entirety.

TECHNICAL FIELD

[0002] The present invention relates to newly identified CD40 splice variant polypeptides, muteins, polynucleotides encoding the polypeptides, sequences thereof, vectors, host cells, compositions and kits containing such, and their methods of use in diagnostic, prophylactic and therapeutic applications.

BACKGROUND OF THE INVENTION

[0003] CD40 is a 48-kDa, 277 amino acid residue, phosphorylated transmembrane glycoprotein belonging to the tumor necrosis factor (TNF) receptor superfamily, as described in Inwald, D.P. (2003, Circ. Res. Vol. 92:1041-8). It is expressed on the surface of a number of cell types including B cells, dendritic cells, normal epithelium, some carcinomas, hematopoietic progenitor cells, and bone cells, such as osteoblasts and osteocytes, as described in U.S. 6,482,411.

[0004] A soluble form of CD40 (sCD40) has been reported to co-exist with the membrane-anchored form (mCD40), presumably as a natural antagonist of mCD40/CD154 interaction. Contin C. et al (2003, J. Biol. Chem. Vol. 278:32801-9) reported that recombinant metalloproteinase disintegrin tumor necrosis factor-alpha converting enzyme (TACE) cleaved purified CD40 ectodomain/Fc chimeric protein in vitro, giving rise to a sCD40 form similar to that shed from B cell cultures. Contin et al. supra further found that spontaneous production of sCD40 by membrane-anchored form of CD40 (mCD40)-transfected human embryonic kidney cells (constitutively expressing TACE) was enhanced by the over-expression of TACE and abrogated by co-transfection with a dominant-negative TACE mutant.
The ligand for CD40, referred to as CD40 ligand or CD40L or CD154, is said to be expressed on a number of cell types as well, including activated T lymphocytes, human dendritic cells, human vascular endothelial cells, smooth muscle cells, and macrophages. CD40L when trimerized, induces oligomerization of its receptor upon binding.

The CD40L has been reported to be also present on platelets and to be released therefrom upon activation, as described in Heeschen, C. et al. [(2003) Soluble CD40 ligand in acute coronary syndromes, N. Engl. J. Med. 348(12): 1104-11]. It is believed that the platelets are the most abundant blood source of CD40L, as reported in Welt, F.G., [(2004) GP IIb/IIIa inhibition with eptifibatide lowers levels of soluble CD40L and RANTES after Percutaneous coronary intervention, Catheter Cardiovasc. Interv. 61(2): 185].

Interaction between CD40, for example, on B cells, and CD40L, for example, on T cells, activates the B cells. CD40-activated B cells results in secretion of a number of cytokines, according to WO 01/05967, including IL-1, IL-5, IL-8, IL-10, IL-12, TNF-α, and matrix metalloproteinase (MMP). Consistent with this finding, CD40L or CD40 deficient mice or mice treated with anti-CD40L antibodies were said to show absence of germinal center formation and impaired development of B cell memory to T dependent antigens.

More recently, it was reported that platelets expressed CD40 as well. Danese, S. et al. (2004, J. Immunol. Vol. 172:2011-5) co-cultured platelets with resting or activated autologous T cells and assessed their activation. They found that CD40L-positive T cells induced platelet activation through a contact-mediated CD40-dependent pathway resulting in RANTES release, which bound to endothelial cells and mediated T cell recruitment.

See also, U.S. Pat. Nos. 5,633,145; 6,194,151; 6,197,584; 6,235,872; 6,376,459; 6,455,040; 6,469,144; 6,472,510; 6,500,938; 6,555,111; 6,602,993; 6,607,879; 6,682,739; and 6,720,182 and PCT patent publications WO 00/37102; WO 01/05967; WO 02/068579; WO 03/004646; WO 03/011324; WO 03/038129; WO 03/049759; and WO 03/070768.

Antibodies directed to CD40L failed in clinical trials because of adverse side effects, presumably because thrombotic events cause the death of patients. It would be desirable to develop compositions and methods that can block CD40/CD40L interaction.
[0011] All references and patent publications cited herein are hereby incorporated by reference in their entirety.

**SUMMARY OF THE INVENTION**

[0012] The present invention provides newly identified isolated CD40 splice variant polypeptides, muteins of CD40 splice variants, and fusion molecules comprising such polypeptides, isolated polynucleotides encoding the polypeptides, the sequences thereof, expression vectors containing the isolated polynucleotides, host cells containing such polynucleotides, polypeptides and vectors, as well as compositions containing such, and methods of making and using such for diagnosis, prevention and treatment of diseases, syndromes, or conditions, including, for example, cancer, inflammation, metabolic diseases such as diabetes, infection and immune cell related disorders.

[0013] Thus, provided herein are methods and compositions for treatment, prevention and diagnosis of diseases, syndromes or conditions (hereafter, collectively, “diseases”) associated with the polypeptides and polynucleotides of the invention, as well as active fragments, antibodies or other modulators thereof.

[0014] In some examples a method is provided for producing the disclosed polypeptides and polynucleotides, for example, by cell free expression of the encoding polynucleotides or by culturing host cells transfected with a recombinant expression vector that contains the polynucleotides described herein under conditions appropriate for replication of the polynucleotides or expression of polypeptides, then recovering the polynucleotides or expressed polypeptides from the culture.

[0015] In other examples modulators of the polypeptides of the invention are provided herein, including but not limited to antibody and small molecule agonists or antagonists thereto, for treatment, prevention and diagnosis of diseases associated with over-activation or under-activation of the CD40/CD40L system.

[0016] In yet other examples further uses for the polypeptides of the invention are identified, as well as the isolated polynucleotides encoding the polypeptides and modulators thereto.

[0017] Thus, in some examples provided herein are isolated polypeptides or compositions containing the isolated polypeptides of the invention, wherein the compositions additionally contain a vehicle, including one or more pharmaceutically
acceptable carriers or excipients, where the compositions are useful for diagnosis, treatment, or prophylaxis of diseases in animals.

[0018] In other examples, provided herein are modulators, wherein the modulators include antibodies specific for the CD40 splice variants polypeptides of the present invention or antibodies specific for active fragments thereof.

[0019] In some examples, provided herein are antibodies that specifically bind to or interfere with the activity of polypeptides of the invention. The antibody can also be a neutralizing antibody that inhibits signaling through the CD40/CD40L system.

[0020] In some examples, provided herein are methods for treating, preventing, or ameliorating the symptoms of an infection in a subject by use of the isolated polypeptides of the invention, as well as by use of the isolated polynucleotides encoding the polypeptides. In some examples, a polypeptide of the invention is present in monomer form and in other examples, is present as a multimer, such as dimer or trimer.

[0021] In other examples, provided herein are methods for modulating an immune response in a subject by use of an agonist or an antagonist of the polypeptides of the invention.

[0022] In other examples, provided herein are methods of treatment of diseases, and/or ameliorating the symptoms of diseases such as inflammatory diseases; autoimmune diseases; ischemia-related disorders, such as stroke, myocardial infarction, and fulminant liver failure; metabolic diseases, such as diabetes; cancer; and infectious diseases.

[0023] The invention identifies nucleotide and polypeptide targets for diagnosis and therapeutic intervention of the disease states described herein, and provides methods for diagnosis and treatment of these diseases by intervening with these targets. The invention provides the nucleic acid and amino acid sequences of these targets in Appendices A-C. In some examples, the CD40 splice variant polypeptides, muteins thereof, and fusion molecules that comprise such CD40 splice variants and muteins are more potent than the wild-type CD40 and in some examples, have fewer adverse side effects.

[0024] Accordingly, provided herein are isolated polypeptides that are capable of binding a CD40 ligand, wherein said polypeptide is selected from the group consisting of:
a) CD40 splice variants selected from the group consisting of CD40sv1, CD40sv2, and CD40sv3; b) muteins of the CD40 splice variants of a); c) CD40 splice variant fusion molecules comprising the CD40 splice variants of a); d) mutein fusion molecules comprising the muteins of b); e) polypeptides comprising the sequence SPGQWALEKA;
f) polypeptides comprising the sequence VRPKTWLCNQRQAQTRLMLSVVSPGQWALEKA; and g) polypeptides comprising the extracellular domain of CD40sv2. In some examples, the polypeptide competes with wild-type CD40 for binding with a CD40 ligand. In some examples, a CD40 splice variant or mutein is in monomer form and in other examples, is in multimer form. In other examples, a fusion molecule is in multimer form, such as for example, dimer or trimer form.

[0025] In some examples the polypeptide comprises a wild-type human CD40 domain selected from the group consisting of domain 1; domain 2; domain 3; domains 1 and 2; domains 2 and 3; domains 1 and 3; and domains 1, 2 and 3 of wild-type CD40. In other examples, the polypeptide is soluble. In yet other examples, the polypeptide comprises part or all of a transmembrane domain. In some examples, a polypeptide is between about 100 and about 160 acids in length, between about 120 and about 150 amino acids in length, between about 130 and 145 amino acids in length and between about 140 and about 145 amino acids in length. In other examples, a polypeptide comprises the sequence for CD40sv1 as shown in SEQ ID NO:10, the sequence for CD40sv2 as shown in SEQ ID NO:15, or the sequence for CD40sv3 as shown in SEQ ID NO:14.

[0026] In further examples, the polypeptide is a mutein of the CD40 splice variants. In yet additional examples, the mutein is soluble. In some examples, a mutein is in monomer form and in other examples, is a multimer, such as dimer or trimer.

In some examples, the mutein is selected from the group consisting of SEQ ID NO:95, SEQ ID NO:96, SEQ ID NO:97, SEQ ID NO:98, SEQ ID NO:99, SEQ ID NO:100, SEQ ID NO:101, SEQ ID NO:102, SEQ ID NO:103, SEQ ID NO:104, SEQ ID NO:105, SEQ ID NO:106, SEQ ID NO:107, SEQ ID NO:108, SEQ ID NO:109, SEQ ID NO:110, SEQ ID NO:111, SEQ ID NO:112, SEQ ID NO:113, SEQ ID NO:114, SEQ ID NO:115, SEQ ID NO:116, SEQ ID NO:117, SEQ ID NO:118, and SEQ ID NO:119. In further examples, the mutein comprises the amino acid sequence
SPGQWALEKA. In yet further examples, the mutein comprises the amino acid sequence VRPKTWLCNRQAQTRMLSVVSPGQWALEKA. In additional examples, the mutein comprises or consists essentially of the extracellular domain of CD40sv2. In some examples, the mutein comprises a modification of the amino acid residue Cys at position 125 and/or 143 as shown in Figure 1. In some examples, the modification is a deletion of the amino acid residue Cys at position 125 and/or 143 as shown in Figure 1 and in other examples, is a substitution of the amino acid residue Cys at position 125 and/or 143 as shown in Figure 1 for any other amino acid. In some examples, the mutein comprises the amino acid sequence SEQ ID NO:118 or SEQ ID NO:119. In some examples, a polypeptide comprises a heterologous signal sequence.

In other examples, the polypeptide is a CD40 splice variant fusion molecule. In additional examples, the CD40 splice variant fusion molecule comprises a fusion partner directly linked to a CD40 splice variant. In some examples, a CD40 splice variant fusion molecule is a multimer, such as dimer or trimer. In other examples, the CD40 splice variant fusion molecule is soluble. In some examples, the fusion partner is an oligomerization domain selected from the group consisting of a Fc domain, a leucine zipper domain, a trimerization domain from tetranectin, and a trimerization domain from a mannose binding protein, such as for example mannose binding protein 1 (MBP1). In some examples, the CD40 splice variant fusion molecule has the sequence as shown in SEQ ID NO:121, SEQ ID NO:123, SEQ ID NO:125, SEQ ID NO:127, SEQ ID NO:128, SEQ ID NO:129, SEQ ID NO:132 or SEQ ID NO:133, SEQ ID NO:136, SEQ ID NO:138, SEQ ID NO:140, SEQ ID NO:142, SEQ ID NO:144, or SEQ ID NO:146.

In some examples, the polypeptide is a mutein fusion molecule. In some examples, the mutein fusion molecule is soluble. In some examples, a mutein fusion molecule is a multimer, such as dimer or trimer.

In other examples, the mutein fusion molecule comprises a fusion partner directly linked to the mutein. In further examples, the fusion partner is an oligomerization domain selected from the group consisting of a Fc domain, a leucine zipper domain, a trimerization domain from tetranectin, and a trimerization domain from mannose binding protein1 (MBP1).
[0030] In further examples, a polypeptide comprises the sequence SPGQWALEKA, VRPKTWLCRNRAQTRLMSVVSPPGQWALEKA, or the extracellular domain of CD40 sv2.

[0031] Provided herein are fusion molecules comprising a polypeptide disclosed herein linked to a fusion partner. In some examples, the fusion partner is selected from the group consisting of Fc domain, a leucine zipper domain, a trimerization domain from tetrancin, and a trimerization domain from a mannone binding protein such as for example, mannone binding protein 1 (MBP1).

[0032] Provided herein are isolated polynucleotides encoding a) CD40 splice variants selected from the group consisting of CD40sv1, CD40 sv2, and CD40sv3; b) muteins of the CD40 splice variants of a); c) CD40 splice variant fusion molecules comprising the CD40 splice variants of a); d) mutein fusion molecules comprising the muteins of b); e) polypeptides comprising the sequence SPGQWALEKA; f) polypeptides comprising the sequence VRPKTWLCRNRAQTRLMSVVSPPGQWALEKA; and g) polypeptides comprising the extracellular domain of CD40sv2. In some examples, polynucleotides comprise or consist essentially of SEQ ID NO:1, SEQ ID NO:19, SEQ ID NO:6, SEQ ID NO:24, SEQ ID NO:5, SEQ ID NO:23, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49, SEQ ID NO:50, SEQ ID NO:51, SEQ ID NO:52, SEQ ID NO:53, SEQ ID NO:54, SEQ ID NO:55, SEQ ID NO:56, SEQ ID NO:57, SEQ ID NO:58, SEQ ID NO:59, SEQ ID NO:60, SEQ ID NO:61, SEQ ID NO:62, SEQ ID NO:64, SEQ ID NO:66, SEQ ID NO:68, SEQ ID NO:70, SEQ ID NO:71, SEQ ID NO:72, SEQ ID NO:75, SEQ ID NO:76, SEQ ID NO:79, SEQ ID NO:81, SEQ ID NO:83, SEQ ID NO:85, SEQ ID NO:87, or SEQ ID NO:89, or a complement thereof.

[0033] The present invention also provide compositions comprising polynucleotides and polypeptides provided herein, as well as host cells comprising polynucleotides and polypeptides provided herein. In some examples, the present invention provides compositions comprising a polypeptide selected from the group consisting of a) CD40 splice variants selected from the group consisting of CD40sv1, CD40 sv2, and CD40sv3; b) muteins of the CD40 splice variants of a); c) CD40 splice variant fusion molecules comprising the CD40 splice variants of a); d) mutein fusion
molecules comprising the muteins of b); e) polypeptides comprising the sequence SPGQWALEKA;
f) polypeptides comprising the sequence VRPKTWLCNRQAQTRMLSVVSPGQWALEKA; and g) polypeptides comprising the extracellular domain of CD40sv2 bound to a CD40 ligand. In some examples, the CD40 splice variant or mutein polypeptide is a monomer and in other examples is a multimer, such as a dimer or trimer. In other examples, a fusion molecule is a multimer. In some examples, the compositions further comprise a buffer or a pharmaceutically acceptable buffer. The present invention also provides vectors comprising the polynucleotide disclosed herein. In some examples, the vector is an insect vector and other examples is a mammalian vector. The present invention also provides complexes comprising a polypeptide described herein bound to a CD40 ligand. In some examples, the CD40 ligand is CD154.

[0034] The present invention also provide antibodies that specifically bind polypeptides selected from the group consisting of a) CD40 splice variants selected from the group consisting of CD40sv1, CD40 sv2, and CD40sv3; b) muteins of the CD40 splice variants of a); c) CD40 splice variant fusion molecules comprising the CD40 splice variants of a); d) mutein fusion molecules comprising the muteins of b); e) polypeptides comprising the sequence SPGQWALEKA;
f) polypeptides comprising the sequence VRPKTWLCNRQAQTRMLSVVSPGQWALEKA; and g) polypeptides comprising the extracellular domain of CD40sv2 and do not bind wild type human CD40 (SEQ ID NO:13) or SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:150, SEQ ID NO:152, SEQ ID NO:154, SEQ ID NO:155 and SEQ ID NO:156. In some examples, an antibody is a monoclonal antibody and in other examples, is a polyclonal antibody.

[0035] The present invention also provides methods of inhibiting binding of a CD40 ligand with a wild-type human CD40 (SEQ ID NO:13) comprising contacting the CD40 ligand with a polypeptide provided herein under conditions suitable for binding of said CD40 ligand to said polypeptide. In some examples, the polypeptide is selected from the group consisting of a) CD40 splice variants selected from the group consisting of CD40sv1, CD40 sv2, and CD40sv3; b) muteins of the CD40 splice variants of a); c) CD40 splice variant fusion molecules comprising the CD40
splice variants of a); d) mutein fusion molecules comprising the muteins of b); e) polypeptides comprising the sequence SPGQWALEKA;

f) polypeptides comprising the sequence VRPKTWLCNRQAQTRLMLSVVSPGQWALEKA; and g) polypeptides comprising the extracellular domain of CD40sv2. In some examples, the contacting is performed in the presence of said wild-type CD40. In other examples, the CD40 ligand is present on a cell. The present invention also provide methods for blocking wild-type CD40 activity, comprising contacting the wild-type human CD40 (SEQ ID NO: 13) with a polypeptide provided herein in the presence of CD40 ligand and under conditions suitable to inhibit binding of the CD40 ligand with said wild-type CD40, thereby blocking wild-type CD40 activity. In some examples, the wild-type CD40 is present on a cell. In other examples, the polypeptide is selected from the group consisting of a) CD40 splice variants selected from the group consisting of CD40sv1, CD40 sv2, and CD40sv3; b) muteins of the CD40 splice variants of a); c) CD40 splice variant fusion molecules comprising the CD40 splice variants of a); d) mutein fusion molecules comprising the muteins of b); e) polypeptides comprising the sequence SPGQWALEKA;

f) polypeptides comprising the sequence VRPKTWLCNRQAQTRLMLSVVSPGQWALEKA; and g) polypeptides comprising the extracellular domain of CD40sv2. In some examples, a CD40 splice variant or mutein is in monomer form, and in other examples, is a multimer, such as a dimer or trimer. In other examples, a fusion molecule is a multimer, such as for example a dimer or trimer or oligomer.

[0036] The present invention also provides methods of treatment and/or amelioration of the symptoms of a disease or condition in a subject that are associated with undesirable interaction of wild-type CD40 with CD40 ligand comprising administering a polypeptide disclosed herein to said subject. In some examples, the disease includes autoimmune diseases, such as for example, transplant rejection; cardiovascular diseases, such as for example, atherosclerosis; and oncological disease, such as for example, B cell malignancy. The present invention also provides methods of treatment and/or amelioration of the symptoms of an immune cell related disease in a subject comprising administering a polypeptide disclosed herein to said subject, wherein immune cell proliferation in said subject is inhibited. In some examples, a polypeptide is selected from the group consisting of a) CD40 splice variants selected
from the group consisting of CD40sv1, CD40 sv2, and CD40sv3; b) muteins of the CD40 splice variants of a); c) CD40 splice variant fusion molecules comprising the CD40 splice variants of a); d) mutein fusion molecules comprising the muteins of b); e) polypeptides comprising the sequence SPGQWALEKA;

f) polypeptides comprising the sequence VRPKTWLCNRQAQTRLMLSVVSPGQWALEKA; and g) polypeptides comprising the extracellular domain of CD40sv2. In some examples, the polypeptide is a monomer and in other examples, is a multimer, such as a dimer or trimer. Provided herein are use of a CD40 splice variant, mutein thereof or fusion molecule comprising a CD40 splice variant or mutein, or antibody as disclosed herein in the manufacture of a medicament for ameliorating the symptoms of a disease in a subject associated with an undesirable interaction between wild-type human CD40 and CD40 ligand. Also provided herein is the use of a CD40 splice variant, mutein thereof or fusion molecule comprising a CD40 splice variant or mutein, or antibody as disclosed herein in the manufacture of a medicament for inhibiting immune cell proliferation in a subject associated with an undesirable interaction between wild-type human CD40 and CD40 ligand.

[0037] Also provided herein are kits comprising a polynucleotide or polypeptide or antibody as disclosed herein. The present invention also provides methods of producing polypeptides disclosed herein.

DESCRIPTION OF THE FIGURES

[0038] Figure 1 shows the polypeptide alignment of the three newly identified CD40 splice variants (sv) of the invention. CLN00236172_5pv1.a, herein CD40sv1; CLN00162416, herein CD40sv2; and CLN00265506, herein CD40sv3.

[0039] Figure 2 shows the polypeptide alignment of the three newly identified CD40 splice variants of the invention and six known CD40 molecules. Human wild-type Cd40 (277 amino acids in length) is NP_001241_NM_001250. Domains are shown for wild-type CD40.

[0040] Figure 3 shows the polypeptide sequence alignment of two CD40 splice variants, CD40sv1 and CD40sv2 with wild-type CD40. Cysteines in domain 3 are shown at amino acid position 125 and 143 for CD40sv1 and wild-type CD40.

[0041] Figures 4A-4B show the structure of the CD40 splice variant 1 molecule, CD40sv1, and the structure of a wild-type CD40 molecule. Amino acid
residues reported to be required for CD40-CD40L interaction (that is binding) are shown at amino acid residues 84(D); 114(E); and 117(E).

Figures 5A-5B(i-v). Fig. 5A shows the structure of the pTT5 backbone vector used to generate pTT5-Gateway vectors pTT5-A, pTT5-B, pTT5-D, pTT5-E and pTT5-H. Figure 5Bi-v show the multiple cloning site flanking sequences for the following pTT5 vectors, respectively: pTT5-A; pTT5-B; pTT5-D; pTT5-E; and pTT5-H.

Figures 6A-6B show protein expression of CD40 extracellular domain (ECD) and CD40sv1 constructs in 293-T cells. Cells were transfected with constructs no. 1 through 9 of Table 7 and cultured for 4 days. Cells (Fig. 6A) and supernatants (Fig. 6B) were harvested and analyzed for expression by SDS-PAGE and Western blotting methods. Samples from cells and supernatants were matched by cell number to estimate secretion efficiency. Molecular weight markers (kD) are shown on left.

Figures 7A-7B show expression of CD40sv1 proteins in 293-6E cells. Cells were transfected with construct no. 2 (CD40sv1 construct) and no. 3 (CD40sv1-Fc) from Table 7 and cultured for 6 days. Cells (Fig. 7A) and supernatants (Fig. 7B) were harvested and analyzed for expression by SDS-PAGE and Western blotting methods. Samples for cells and supernatants were matched by cell number to estimate secretion efficiency. Molecular weight markers (kD) are shown on left.

Figure 8 shows expression of FPSP-CD40sv1-Fc construct in 293-6E cells. Cells were transfected with construct no. 4 from Table 7 and cultured for 6 days. Cells (lanes 1-4) and supernatants (lanes 5-15) were harvested and analyzed for expression by SDS-PAGE and Western blotting methods. Lane 1- cell lysate after 4 days culture; lane 2- cell lysate after 5 days culture; lane 3- cell lysate after 6 days culture; lane 4- cell lysate of vector control after 5 days culture; lanes 5 and 6- supernatant after 4 days culture; lanes 7 and 8- supernatant after 5 days culture; lanes 9 and 10- supernatant after 6 days culture; and lane 11- supernatant of vector control after 5 days culture. 15 ul and 3.8 ul supernatant were loaded per sample, respectively. A commercially available CD40-Fc protein (R&D Systems, Minneapolis, MN) was used as a standard for quantification. Lane 12- 1.3 ng protein/lane; lane 13- 2.5 ng protein/lane; lane 14- 5 ng protein/lane; and lane 15- 10 ng protein/lane. Molecular weight markers (kD) are shown on left.

Figures 9A-9B show protein expression of CD40ECD-Fc and CD40sv1-Fc in HighFive™ insect cells. Cells were transfected with constructs no.
10, no. 11 and no. 12 (vector control) from Table 7 and cultured for 4 days as described. Cells (Fig. 9A) and supernatant (Fig. 9B) were harvested as described and analyzed for expression by SDS-PAGE and Western blotting methods using a monoclonal mouse anti-human CD40 antibody (BD Biosciences, San Jose, CA). Samples for cells and supernatants are matched by cell number to estimate secretion efficiency. Molecular weight markers (kD) are shown on left.

Figure 10 shows transient and stable expression of CD40sv1-Fc and CD40ECD-Fc in HighFive™ insect cells. Cells were transfected as described and protein expression analyzed by western blotting methods using a monoclonal mouse anti-human CD40 antibody (BD Biosciences, San Jose, CA). Lane 1-supernatant of adherent HighFive™ cells transiently expressing CD40ECD-Fc for 4 days; lane 2-supernatant of suspension HighFive™ cells stably expressing CD40ECD-Fc for 2 days; lane 3-supernatant of suspension HighFive™ cells stably expressing CD40sv1-Fc for 2 days; lane 4-untransfected control; lane 5-1.3 ng CD40-Fc; lane 6: 2.5 ng CD40-Fc; lane 7: 5 ng CD40-Fc; and lane 8: 10 ng CD40-Fc. 10ul supernatant per sample was loaded. Molecular weight markers (kD) are shown on left.

Figure 11 shows purification of CD40sv1-Fc fusion protein. Supernatant (Sup) from HighFive™ insect cells containing CD40sv1-Fc were concentrated (Conc. Sup.), precipitates were removed by centrifugation (Start) and loaded onto a Protein A-Sepharose column. The protein was eluted with a pH gradient (Eluted Fractions). Further, the column flow through (FT), wash fraction (Wash) and the permeate from the concentration (Perm.) are shown. Molecular weight markers (kD) are shown on left.

Figure 12 shows the inhibition of human B cell activity by a CD40sv1-Fc fusion protein. The human B cells were stimulated with CHO cells transfected with human CD40L. RLU-Relative light units. Protein concentration is in ng/ml.

Figure 13 shows the lack of inhibition of human B cell activity by a CD40sv1-Fc fusion protein when the B cells were stimulated with CHO cells without CD40L. RLU-Relative light units. Protein concentration is in ng/ml.

Figures 14A-14H show CD40sv1 and muteins. The domains of CD40wt; CD40sv1; and 6 muteins are described herein. Fig. 14A represents wild-type CD40; Fig. 14B represents CD40sv1; and Fig. 14C-14H represent muteins as described herein. For each mutein depicted schematically, Appendix B provides various amino acid sequences. Loops depict disulfide bonds between Cysteines.
Light gray shading in domain 1, 2, 3 and 4 represents wild-type human CD40 (SEQ ID NO:13) sequences; amino acid differences between wild-type CD40 and a CD40sv1 (as shown herein in Figure 2); or muteins is represented by dark gray shading and the boxed-in region at the end of the new domain represents the amino acid sequence SPGWALEKA. Mutein5 (L142I) and mutein6 (N144E/R145P) have the amino acid substitutions as shown.

Figures 15A-15D show CD40sv2, mutein7 and mutein 8. Domains of CD40wt, CD40sv2 and muteins are described herein. For each mutein depicted schematically, Appendix B provides various amino acid sequences. The legend is shown at the bottom. Light gray shading in domain 1, 2, 3 and 4 represents wild-type human CD40 (SEQ ID NO:13) sequences; the dotted regions depict gap/deletion; and curved lines above domains depict disulfide bonds between Cysteines.

Tables and Appendices are found in Examples. Appendix A shows the nucleotide sequence of the open reading frame (i.e., N1 sequences) of the polypeptides disclosed herein.

Appendix B shows amino acid sequences of the polypeptides disclosed herein (i.e., P1 sequences).

Appendix C shows the polynucleotide sequences of the nucleic acid inserts encoding the polypeptides disclosed herein and may include 5' and/or 3' untranslated sequences (i.e., N0 sequences).

Appendix D show SEQ ID NOs 149 to 156 from PCT publication WO 03/070768.

Table 1 (SEQ ID NO: Table): column 1 shows a designation ID number (FP ID); column 2 shows the nucleotide sequence ID number for the open reading frame ("ORF") for the nucleic acid sequence (N1); column 3 shows the amino acid sequence ID number for the polypeptide sequence (P1); column 4 shows the nucleotide sequence ID number for the entire nucleic acid sequence (N0); column 5 shows the polypeptide ID number of the source clone or sequence.

Table 2 shows the public annotation of polypeptide sequences (from Appendices A-B). Column 1 shows a designation ID number of the polypeptide (FP ID); column 2 shows the source ID number of the polypeptide (clone ID); column 3 shows the predicted length of the polypeptide; column 4 shows the public ID number of the hits found in the public database NR (all non-redundant GenBank +EMPL+DDBJ+PDB sequences) (human); column 5 shows the annotation of the ID number set forth in column 4 (human); column 6 shows the percent identity between
the polypeptide and the sequence set forth in column 4 (human); column 7 shows the
length of the match between the polypeptide and the sequence shown in column 4.

[0059] Table 3 shows pfam (Protein family), signal peptide and
transmembrane data, and other information about the polypeptides of the invention.
Column 1 shows a designation ID number of the polypeptide (FP ID); column 2
shows the source ID number of the polypeptide (clone ID); column 3 shows the
cluster ID number of the polypeptide; column 4 shows the classification of the
polypeptide; column 5 shows the predicted protein length (in number of amino acid
residues); column 6 shows an internal parameter "the treevote"; column 7 shows the
mature protein coordinates (numbered in terms of amino acid residues, starting from
number 1 at the N-terminus); column 8 shows the alternate mature protein coordinates
(numbered in terms of amino acid residues, starting from number 1 at the N-
terminus); column 9 shows the signal peptide coordinates (numbered in terms of
amino acid residues, starting from number 1 at the N-terminus); column 10 shows the
number of transmembrane domains; column 11 shows the coordinates of
transmembrane domains (numbered in terms of amino acid residues, starting from
number 1 at the N-terminus); column 12 shows the coordinates of non-transmembrane
domains (numbered in terms of amino acid residues, starting from number 1 at the N-
terminus); column 13 shows the names of pfam domains within the polypeptide.

[0060] Table 4 shows the coordinates of the predicted pfam domains in the
CD40 polypeptides. Column 1 shows a designation ID number of the polypeptide (FP
Patent ID); column 2 shows the source ID number of the polypeptide; column 3
shows the name of the pfam domain; column 4 shows the start and stop coordinates of
the pfam domain in the polypeptide (numbered in terms of amino acid residues,
starting from number 1 at the N-terminus).

[0061] Table 5 shows SEQ ID NOs: 28-32 that identify particular CD40
regions.

[0062] Table 6A-6B shows expression constructs for example, of CD40wt,
CD40ECD, CD40sv1 and CD40sv2. Column 1 shows an ID number for the
polypeptide (FP ID); column 2 shows the nucleotide sequence ID number for the open
reading frame for the nucleic acid sequence (N1); column 3 shows the amino acid
sequence ID number for the polypeptide sequence (P1); column 4 shows the
nucleotide sequence ID number for the entire nucleic acid sequence (N0); column 5
shows the polypeptide ID number of the source clone or sequence (Clone ID); and column 6 shows polypeptide or construct designation.

Table 7 shows CD40ECD and CD40sv1 constructs tested for protein expression in 293-T, 293-6E and HighFive™ insect cells. Column 1 shows the polypeptide ID number of the source clone or sequence; column 2 shows the polypeptide construct; and column 3 shows an annotation for the construct.

**DETAILED DESCRIPTION OF THE INVENTION**

The present inventors have discovered three naturally occurring CD40 splice variants while making cDNA libraries based on mRNA isolated from different tissues, including inflamed tonsils. One such naturally occurring variant of the present invention, HG1015485 (CLN00236172_5pv1.a or “CD40sv1”), designated herein as a “soluble CD40” or “sCD40” was found to be a 166-amino acid residue protein that contains a signal peptide, a TNFR_c6 protein family (“pfam”) domain, but is missing the transmembrane and cytoplasmic domains. Additionally, this sCD40 varies from the wild type CD40, such as NP_001241 NM_001250, at the C-terminus of the sCD40 molecule. In particular, CD40sv1 comprises the sequence SPGWALEKA; and VRPKTWLCNRQATRLMLSVVSPGWALEKA.

A second CD40 splice variant of the present invention, HG1015500 (CLN00265506) designated herein as “CD40sv3” is 233 amino acid residues in length. A third CD40 splice variant of the present invention, HG1015501 (CLN00162416) designated herein as “CD40sv2”, is 226 amino acid residues in length. These 2 variants differ from the first splice variant above, CD40sv1, in that these contain the transmembrane domain of the wild type CD40, but are truncated in other respects. Compared with the wild-type CD40, splice variant CD40sv3 is truncated at its N-terminus and missing a portion of the TNFR_pfam domain. Also compared with the wild-type CD40, CD40sv2, is truncated in part of the extracellular domain. The sequences are shown in Figures 1-3.

Also provided herein are muteins of CD40 splice variants, that is, muteins of CD40sv1, CD40sv2, and CD40sv3. Also provided herein are fusion molecules that comprise the CD40 splice variants and muteins.

*General Techniques*

The present invention may be more clearly understood in light of the following definitions.

By “nucleotide sequence” of a nucleic acid molecule or polynucleotide is intended, for a DNA molecule or polynucleotide, a sequence of deoxyribonucleotides, and for an RNA molecule or polynucleotide, the corresponding sequence of ribonucleotides (A, G, C and U), where each thymidine deoxyribonucleotide (T) in the specified deoxyribonucleotide sequence is replaced by the ribonucleotide uridine (U). A “nucleic acid” molecule can include both double- and single-stranded sequences and refers to, but is not limited to, cDNA from viral, procaryotic or eucaryotic mRNA, genomic DNA sequences from viral (e.g. DNA viruses and retroviruses) or procaryotic DNA, and also includes synthetic nucleic acid sequences such as DNA sequences. The term also captures sequences that include any of the known base analogs of DNA and RNA. A nucleotide sequence encompasses aptamers, RNAi, ribozymes, and anti-sense molecules.

By “isolated” nucleic acid molecule(s) is intended a nucleic acid molecule, DNA or RNA, which has been removed from its native environment. In some examples this means a nucleic acid molecule is removed from at least one component within its native environment. For example, recombinant DNA molecules
contained in a vector are considered isolated for the purposes of the present invention. Further examples of isolated DNA molecules include recombinant DNA molecules maintained in heterologous host cells or purified (partially or substantially) DNA molecules in solution. Isolated RNA molecules include in vivo or in vitro RNA transcripts of the DNA molecules of the present invention. Isolated nucleic acid molecules according to the present invention further include such molecules produced synthetically.

[0071] “CD40,” as used herein, refers to the molecules of the invention, the wild-type 277 amino acid residue molecule (SEQ ID NO:13) and all variants described herein (See Appendices A-C).

[0072] “sCD40” or “soluble CD40” refers to a specific variant molecule of the invention that does not contain a transmembrane domain, i.e., the transmembrane domain has been spliced out. In some examples a transmembrane domain is removed by any means including via genetic engineering techniques. In some examples, a sCD40 is an extracellular domain (ECD) that has been removed from the full-length molecule.

[0073] “CD40 splice variant” or “CD40sv” as used herein refers to the splice variants referred to as CD40sv1, CD40sv2, and CD40sv3. In some examples, a CD40 splice variant comprises a transmembrane domain and in other examples, does not comprise a transmembrane domain and is soluble.

[0074] “CD40 TM splice variants” refers to specific variant molecules of the invention that contain a transmembrane domain but truncated in other portions of the molecule when compared to the wild type 277 amino acid CD40 molecule. In some examples, a CD40 TM splice variant contains a transmembrane domain and is modified in other portions of the molecule when compared to the wild type 277 amino acid CD40 molecule.

[0075] A “mutein” refers to a wild-type CD40 polypeptide or a CD40 splice variant polypeptide that comprises an amino acid modification, such as for example, an amino acid insertion, deletion, substitution or point mutation, within the CD40 region, with the proviso that the CD40 mutein is not identical to wild-type CD40 (NP_001241 NM_001250: SEQ ID NO:13) and not identical to SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:150, SEQ ID NO:152, SEQ ID NO:154, SEQ ID NO:155 and SEQ ID NO:156. As used herein, a “mutein” does not include muteins known in the art as of the filing date of the patent.
application and is not identical to muteins known in the art as of the filing date of the patent application. In some examples, a CD40 mutein comprises a transmembrane domain and in other examples, is soluble.

[0076] “CD40 ligand” or “CD40L” refers to a ligand that binds to CD40, such as that expressed on the surface of T cells. CD40 ligand is also referred to as CD154.

[0077] A “modulator” of a polypeptide or polynucleotide or an “agent” as used herein encompasses agonists or antagonists that interfere with the binding or activity of such polypeptide or polynucleotide. Within this context, a modulator either increases or decreases the binding or activity of such polypeptides or polynucleotides. Such modulators or agents include, for example, polypeptide variants, whether agonist or antagonist; antibodies, whether agonist or antagonist; soluble receptors, usually antagonists; small molecule drugs, whether agonist or antagonist; RNAi, usually an antagonist; antisense molecules, usually an antagonist; and ribozymes, usually an antagonist. In some embodiments, an agent is a subject polypeptide, that is, a CD40 splice variant or mutein thereof, where the subject polypeptide itself is administered to an individual. In some examples, a subject polypeptide is a sCD40. In some embodiments, for example, an agent is an antibody specific for a subject “target” polypeptide, e.g. for example, an antibody specific for CD40sv1. In some embodiments, an agent is a chemical compound such as a small molecule that may be useful as an orally available drug. In some instances, such modulation includes direct and indirect modulation of binding or activity and includes the recruitment of other molecules that directly affect the modulation. For example, an antibody that modulates the activity of a subject polypeptide that is a receptor on a cell surface may bind to the receptor and fix complement, activating the complement cascade and resulting in lysis of the cell. An agent which modulates a biological activity of a subject polypeptide or polynucleotide increases or decreases the activity or binding at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 50%, at least about 80%, or at least about 2-fold, at least about 5-fold, or at least about 10-fold or more when compared to a suitable control.

[0078] “Disease,” “disorder,” “syndrome” or “condition,” or any combination of these terms as used herein, refers to any disease, disorder, syndrome or conditions that affects the health and well being of an animal and includes, but is not limited to, metabolic disorders such as: diabetes, including type II diabetes mellitus, type I diabetes mellitus, impaired glucose tolerance, or other metabolic syndrome;
cardiovascular disease, such as atherosclerosis, coronary artery disease, acute coronary syndrome, myocardial infarction, chronic heart failure and stroke; restenosis; inflammation, such as inflammatory bowel disease, Crohn’s disease, ulcerative colitis, inflammation due to coronary stenting or associated with familial hypercholesterolemia, or inflammation of the central nervous system; Alzheimer’s disease; Parkinson’s disease; Lou Gehrig’s disease; immune cell related diseases, such as an autoimmune disease, including systemic lupus erythematosis, immune or idiopathic thrombocytopenic purpura, rheumatoid arthritis, multiple sclerosis, and psoriasis; allograft rejection; cancer, such as hematological malignancy, carcinomas, such as gastric carcinoma, ovarian carcinoma, colon carcinoma, breast carcinoma, lung carcinoma, sarcomas, such as osteosarcoma, Ewing sarcoma; thrombosis; ischemic diseases, such as acute cerebral ischemia; mixed connective tissue disease; Kawasaki disease; myasthenia gravis and bleeding.

[0079] "Therapeutic agents” include agents that are conventionally used for treatment of a disease or that are considered standard of care and include, for example, statins, chemotherapeutic agents, radiation therapy, etc.

[0080] "Neoangiogenesis” or “neovascularization” as used herein, refers to growth of new blood vessels, for example, as in tumors or retinopathy.

[0081] "Bone loss,” as used herein, refers to absorption or loss of bone tissues which may be due to infection or an over-active immune system.

[0082] "Fusion molecule,” as used herein, refers to a non-naturally occurring molecule that comprises a CD40, e.g. a CD40 splice variant, or mutein and a fusion partner. A fusion partner may be a polypeptide or other moiety, such as for example, PEG. In some examples the fusion molecule is made by directly or indirectly linking one or part of one molecule to another or another part of another molecule to produce a single combination. A fusion molecule, such as a fusion polypeptide may be made by linkage of a polynucleotide encoding one molecule with the polynucleotide encoding a second molecule, and introducing the resulting polynucleotide molecule in a vector for transfecting host cells that are capable of expressing the fusion molecule. A fusion molecule may or may not contain a linker between the molecules. A fusion molecule as used herein refers to a molecule that is synthesized by any means. In some examples, a fusion molecule comprises a CD40, e.g., a CD40 splice variant or mutein, and a fusion partner, such as a heterologous domain.
“Adjuvant effect to an antigen,” as used herein, refers to the ability to enhance immunogenicity of an antigen so as to generate a more robust immune response, such as an adjuvant for vaccination purposes.

“Modulating a level of active subject polypeptide” includes increasing or decreasing activity of a subject polypeptide; increasing or decreasing a level of active polypeptide protein; and increasing or decreasing a level of mRNA encoding active subject polypeptide.

“Treatment,” as used herein, covers any treatment of a condition or disease in a mammal, including a human, and includes preventing the condition or disease from occurring or recurring in a subject who may be predisposed to the condition or disease but has not yet been diagnosed as having it, inhibiting the condition or disease, i.e., arresting its development, or relieving the condition or disease, i.e., causing regression of the condition or disease, or stabilizing the condition or disease, i.e., maintain health status, or restoring or repairing a lost, missing or defective function, or stimulating an inefficient process.

In the context of cancer, the term “treating” includes any or all of: preventing or inhibiting growth of tumor cells or cancer cells, preventing or inhibiting replication of tumor cells or cancer cells, lessening of overall tumor burden, inducing apoptosis and ameliorating one or more symptoms associated with the disease.

In the context of an autoimmune disease, the term “treating” includes any or all of: preventing or inhibiting replication of cells associated with an immune cell related disease or condition, such as an autoimmune disease state including, but not limited to, cells capable of producing an autoimmune antibody, lessening the autoimmune-antibody burden and ameliorating one or more symptoms of an autoimmune disease.

In the context of an infectious disease, the term “treating” includes any or all of preventing or inhibiting the growth, multiplication or replication of the pathogen that causes the infectious disease and ameliorating one or more symptoms of an infectious disease.

In the context of an inflammatory disease, the term “treating” includes any or all of preventing or inhibiting the growth, multiplication or replication of cells or production of the agents, such as inflammatory cytokines, that cause(s) the inflammatory disease and ameliorating one or more symptoms of an inflammatory disease.
In the context of an ischemic disease, the term “treating” includes any or all of preventing or inhibiting the growth, multiplication or replication of the cells, replication of the pathogen or production of agents that causes or stimulates the ischemic disease, such as the formation of plaques or clots, and ameliorating one or more symptoms of an ischemic disease.

“Operably linked” refers to an arrangement of elements wherein the components so described are configured so as to perform their desired function. Thus, a given promoter operably linked to a coding sequence is capable of effecting the expression of the coding sequence when the proper transcription factors, etc., are present. The promoter need not be contiguous with the coding sequence, so long as it functions to direct the expression thereof. Thus, for example, intervening untranslated yet transcribed sequences can be present between the promoter sequence and the coding sequence, as can translated introns, and the promoter sequence can still be considered "operably linked" to the coding sequence.

“Recombinant” as used herein to describe a nucleic acid molecule means a polynucleotide of genomic, cDNA, viral, semi-synthetic, or synthetic origin which, by virtue of its origin or manipulation is not associated with all or a portion of the polynucleotide with which it is associated in nature. The term "recombinant" as used with respect to a protein or polypeptide means a polypeptide produced by expression of a recombinant polynucleotide.

A “control element” refers to a polynucleotide sequence which aids in the expression of a coding sequence to which it is linked. The term includes promoters, transcription termination sequences, upstream regulatory domains, polyadenylation signals, and when appropriate, leader sequences and enhancers, which collectively provide for the transcription and translation of a coding sequence in a host cell.

A “promoter” as used herein is a DNA regulatory region capable of binding RNA polymerase in a mammalian cell and initiating transcription of a downstream (3' direction) coding sequence operably linked thereto. For purposes of the present invention, a promoter sequence includes the minimum number of bases or elements necessary to initiate transcription of a gene of interest at levels detectable above background. Within the promoter sequence is a transcription initiation site, as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase. Eucaryotic promoters will often, but not always, contain “TATA”
boxes and "CAT" boxes. Promoters further include those that are naturally contiguous to a nucleic acid molecule and those that are not naturally contiguous to a nucleic acid molecule. Additionally, a promoter includes inducible promoters, conditionally active promoters, such as a cre-lox promoter, constitutive promoters and a tissue specific promoter(s).

[0095] By "selectable marker" is meant a gene which confers a phenotype on a cell expressing the marker, such that the cell can be identified under appropriate conditions. Generally, a selectable marker allows selection of transformed cells based on their ability to thrive in the presence or absence of a chemical or other agent that inhibits an essential cell function. Suitable markers, therefore, include genes coding for proteins which confer drug resistance or sensitivity thereto, impart color to, or change the antigenic characteristics of those cells transfected with a molecule encoding the selectable marker, when the cells are grown in an appropriate selective medium. For example, selectable markers include: cytotoxic markers and drug resistance markers, whereby cells are selected by their ability to grow on media containing one or more of the cytotoxins or drugs; auxotrophic markers by which cells are selected by their ability to grow on defined media with or without particular nutrients or supplements, such as thymidine and hypoxanthine; metabolic markers by which cells are selected for, e.g., their ability to grow on defined media containing the appropriate sugar as the sole carbon source, or markers which confer the ability of cells to form colored colonies on chromogenic substrates or cause cells to fluoresce.

[0096] "Transformation," or "transfection" as used herein, refers to the insertion of an exogenous polynucleotide into a host cell, irrespective of the method used for insertion: for example, transformation by direct uptake, transfection, infection, and the like. For particular methods of transfection, see further below. The exogenous polynucleotide may be maintained as a nonintegrated vector, for example, an episome, or alternatively, may be integrated into the host genome.

[0097] The terms "polypeptide" and "protein" refer to a polymer of amino acid residues and are not limited to a minimum length of the product. Thus, peptides, oligopeptides, dimers, multimers, and the like, are included within the definition. Both full-length proteins and fragments thereof are encompassed by the definition. The terms also include post-expression modifications of the polypeptide, for example, glycosylation, acetylation, phosphorylation and the like. Furthermore, for purposes of the present invention, a modified "polypeptide" refers to a protein which includes
modifications, such as deletions, additions and substitutions (generally conservative in nature), to the native sequence, as long as the protein maintains the desired activity. These modifications may be deliberate, as through site-directed mutagenesis, or may be accidental, such as through mutations of hosts which produce the proteins or errors due to PCR amplification.

[0098] A “gene,” for the purposes of the present disclosure, includes a DNA region encoding a gene product, as well as all DNA regions which regulate the production of the gene product, whether or not such regulatory sequences are adjacent to coding and/or transcribed sequences. Accordingly, a gene includes, but is not necessarily limited to, promoter sequences, terminators, translational regulatory sequences such as ribosome binding sites and internal ribosome entry sites, enhancers, silencers, insulators, boundary elements, replication origins, matrix attachment sites and locus control regions.

[0099] “Gene expression” refers to the conversion of the information, contained in a gene, into a gene product. A gene product can be the direct transcriptional product of a gene (e.g., mRNA, tRNA, rRNA, antisense RNA, ribozyme, structural RNA or any other type of RNA) or a protein produced by translation of an mRNA. Gene products also include RNAs which are modified, by processes such as capping, polyadenylation, methylation, and editing, and proteins modified by, for example, methylation, acetylation, phosphorylation, ubiquitination, ADP-ribosylation, myristilation, and glycosylation.

[0100] A “coding sequence” or a sequence which “encodes” a selected polypeptide, is a nucleic acid molecule which is transcribed (in the case of DNA) and translated (in the case of mRNA) into a polypeptide in vivo when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5′ (amino) terminus and a translation stop codon at the 3′ (carboxy) terminus. A coding sequence can include, but is not limited to, cDNA from viral, procaryotic or eucaryotic mRNA, genomic DNA sequences from viral (e.g. DNA viruses and retroviruses) or procaryotic DNA, and especially synthetic DNA sequences. A transcription termination sequence may be located 3′ to the coding sequence.

[0101] As used herein, the term “antibody” encompasses polyclonal and monoclonal antibody preparations, as well as preparations including hybrid antibodies, altered antibodies, chimeric antibodies and, humanized antibodies, as well
as: hybrid (chimeric) antibody molecules (see, for example, Winter et al., *Nature* 349:293-299 (1991); and U.S. Patent No. 4,816,567); antibody fragments, such as, F(ab')₂ and F(ab) fragments; Fv molecules (noncovalent heterodimers, see, for example, Inbar et al., *Proc Natl Acad Sci USA* 69:2659-2662 (1972)); and Ehrlich et al. (1980) *Biochem* 19:4091-4096; single-chain Fv molecules (sFv) (see, e.g., Huston et al., *Proc Natl Acad Sci USA* 85:5879-5883 (1980)); dimeric and trimeric antibody fragment constructs; minibodies (see, e.g., Pack et al., *Biochem* 31:1579-1584 (1992); Cumber et al., *J. Immunology* 149B:120-126 (1992)); humanized antibody molecules (see, e.g., Riechmann et al., *Nature* 332:323-327 (1988); Verhoeyan et al., *Science* 239:1534-1536 (1988)); and, any functional fragments obtained from such molecules, wherein such fragments retain specific binding.

[0102] As used herein, the term “monoclonal antibody” refers to an antibody composition having a homogeneous antibody population. The term is not limited regarding the species or source of the antibody, nor is it intended to be limited by the manner in which it is made. The term encompasses whole immunoglobulins.

[0103] Methods of making polyclonal and monoclonal antibodies are known in the art. Polyclonal antibodies are generated by immunizing a suitable animal, such as a mouse, rat, rabbit, sheep or goat, with an antigen of interest, for examples a stem cell transformed with a gene encoding an antigen. In order to enhance immunogenicity, the antigen can be linked to a carrier prior to immunization. Suitable carriers are typically large, slowly metabolized macromolecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, lipid aggregates (such as oil droplets or liposomes), and inactive virus particles. Such carriers are well known to those of ordinary skill in the art. Furthermore, the antigen may be conjugated to a bacterial toxoid, such as toxoid from diphtheria, tetanus, cholera, etc., in order to enhance the immunogenicity thereof.

[0104] In addition, techniques developed for the production of “chimeric antibodies” (Morrison et al., PNAS, 81:851-855 (1984); Neuberger et al., *Nature*, 312:604-608 (1984); Takeda et al., *Nature*, 314:452-454 (1985)) by splicing 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
monoclonal antibody and a human immunoglobulin constant region, for example, humanized antibodies, and insertion/deletions relating to CDR and framework regions.

[0105] The term “subject,” as used herein, refers to a subject, such as a living animal, including a human and a non-human animal. The subject is an organism possessing immune cells capable of responding to antigenic stimulation and stimulatory and inhibitory signaling transduction through cell surface receptor binding. In preferred embodiments, the subject is a mammal, including humans and non-human mammals such as dogs, cats, pigs, cows, sheep, goats, horses, rats, and mice. In the most preferred embodiment, the subject is a human. The term “subject” does not preclude individuals that are entirely normal with respect to a disease, or normal in all respects.

[0106] By “isolated” is meant, when referring to a polynucleotide or polypeptide of the invention, that the indicated molecule is substantially separated, e.g., from the whole organism in which the molecule is found or from the cell culture in which the antibody is produced, or is present in the substantial absence of other biological macromolecules of the same type.

[0107] The term “specifically binds,” refers to high avidity and/or high affinity binding of an antibody to a specific polypeptide, or more accurately, to an epitope of an antigen. Antibody specifically binding to such epitope on the receptor can be stronger than binding of the same antibody to any other epitopes, particularly other epitopes that can be present in molecules in association with, or in the same sample as the receptor of interest. For example, when an antibody binds more strongly to one epitope than to another, adjusting the binding conditions can result in antibody binding almost exclusively to the specific epitope and not to any other epitopes on the same polypeptide, and not to any other polypeptide, which does not comprise the epitope.

[0108] The term “agonist,” in the context of a polypeptide, for example a CD40 splice variant or mutein thereof, refers to a molecule that mimics, enhances, stimulates or activates the function of a molecule with which the agonist interacts. Agonists include, but are not limited to, analogues and fragments thereof.

[0109] The term “agonist,” in the context of an antibody, refers to a molecule that mimics, enhances, stimulates or activates the function of a molecule with which
the agonist interacts. Agonists include, but are not limited to, analogues and 
fragments thereof.

[0110] The term “antagonist,” in the context of a polypeptide, for example a 
CD40 splice variant or mutein thereof, refers to a molecule that competes, inhibits or 
interferes with the activity of a molecule with which the antagonist interacts. For 
example, an antagonist antibody can bind to the receptor, but does not induce an 
active response. Antagonists include, but are not limited to, analogues and fragments 
thereof.

[0111] The term “antagonist,” in the context of an antibody, refers to a 
molecule that competes, inhibits or interferes with the activity of a molecule with 
which the antagonist interacts. For example, an antagonist antibody can bind to the 
receptor, but does not induce an active response. Antagonists include, but are not 
limited to, analogues and fragments thereof.

[0112] By “fragment” is intended, for example a polypeptide consisting of 
only a part of the intact full-length polypeptide sequence and structure. The fragment 
can include a C-terminal deletion, an N-terminal deletion, and/or an internal deletion 
of the native polypeptide. A fragment of a protein will generally include at least 
about 5-10 contiguous amino acid residues of the full-length molecule, in some 
examples, at least about 15-25 contiguous amino acid residues of the full-length 
molecule, and in other examples at least about 20-50 or more contiguous amino acid 
residues of the full-length molecule, or any integer between 5 amino acids and the 
full-length sequence.

[0113] By “fragment” is intended, for example a nucleic acid molecule 
comprising only a part of the intact full-length polynucleotide sequence encoding a 
polypeptide. In some examples, a fragment of a polynucleotide will generally include 
at least about 15-30 contiguous nucleic acids of the full-length polynucleotide, in 
some examples, at least about 45-75 contiguous nucleic acids of the full-length 
polynucleotide, and in other examples at least about 60-150 or more contiguous 
nucleic acids of the full-length polynucleotide, or any integer between 15 nucleic 
acids and the full-length sequence.

[0114] As used herein, the phrase “pharmaceutically acceptable carrier” is 
intended to include substances that can be co-administered with the compositions of 
the invention that allows the composition or active molecule therein to perform its 
intended function. Examples of such carriers include solutions, solvents, buffers,
Nucleic Acid and Polypeptides

[0115] The present invention provides isolated nucleic acid molecules comprising a polynucleotide encoding newly identified CD40 splice variant polypeptides having the amino acid sequences as shown in Appendices A-C (CD40sv1 (SEQ ID NO:10), CD40sv2 (SEQ ID NO:14); and CD40sv3 (SEQ ID NO:15)). With respect to Figure 1, the mature polypeptide, that is the polypeptide without a signal sequence, for CD40sv1 and CD40sv2 begins at about the (P) at amino acid residue 20. The isolated CD40 splice variants of the invention were identified in bioinformatics analyses of all of the CD40 clones that were identified. Specifically, the CD40 variant polypeptides are structurally related to members of the tumor necrosis factor receptor_c6 ("TNFR_c6") family.

[0116] Accordingly, provided herein are nucleic acid molecules comprising a polynucleotide having a sequence chosen from among the sequences as shown in: (A) SEQ ID NO:1, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:19, SEQ ID NO:23 and SEQ ID NO:24; (B) polynucleotide sequences encoding polypeptides having the sequences as shown in SEQ ID NO:10, SEQ ID NO:14, SEQ ID NO:15, and SEQ ID NO:28-34; (C) biologically active fragments thereof; and (D) a complement thereof.

In some examples provided herein a polynucleotide comprising a sequence chosen from among the sequences as shown in: (A) SEQ ID NO:1, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:19, SEQ ID NO:23 and SEQ ID NO:24; (B) polynucleotide sequences encoding polypeptides having the sequence as shown in SEQ ID NO:10, SEQ ID NO:14, SEQ ID NO:15, and SEQ ID NO:28-34; (C) biologically active fragments thereof; and (D) a complement thereof is a nucleic acid molecule chosen from among: a cDNA molecule, genomic DNA molecule, a cRNA molecule, a siRNA molecule, a RNAi molecule, a mRNA molecule, an antisense molecule, and a ribozyme.

[0117] Fragments of the described nucleic acid molecules encoding CD40 splice variants may be used as hybridization probes for cDNA libraries to isolate the full length gene and to isolate other genes which have a high sequence similarity to the gene or similar biological activity. Probes of this type preferably have at least 30
bases and may contain, for example, 50 or more bases. The probe may also be used to identify a cDNA clone corresponding to a full length transcript and a genomic clone or clones that contain the complete CD40 splice variant including regulatory and promoter regions, exons, and introns. In some examples, a screen comprises isolating the coding regions of a particular CD40 splice variant by using the known nucleic acid sequence to synthesize an oligonucleotide probe. Labeled oligonucleotides having a sequence complementary to that of the gene of the present invention are used to screen a library of human cDNA, genomic DNA or mRNA to determine which members of the library the probe hybridizes to. In some examples, the fragments comprise nucleic acid molecules encoding the polypeptides having the sequence as shown in SEQ IDNO:28-34. The present invention further relates to polynucleotides which hybridize to the hereinabove described sequences if there is at least about 91%, at least about 92%, and at least about 95% identity between the sequences. The present invention particularly relates to polynucleotides which hybridize under stringent conditions to the hereinabove described polynucleotides. As herein used, the term “stringent conditions” means hybridization will occur only if there is at least about 95% and preferably at least about 97% identity between the sequences. For example, stringent conditions encompass overnight incubation at 42°C in a solution containing: 50% formamide, 5x SSC (150 mM NaCl, 15 mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5x Denhardt’s solution, 10% dextran sulfate, and 20 μg/ml denatured, sheared salmon sperm DNA, followed by washing the filters in 0.1x SSC at about 65°C.

[0118] Provided herein are double-stranded isolated nucleic acid molecules comprising a polynucleotide having the sequence chosen from among: (A) SEQ ID NO:1, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:19, SEQ ID NO:23 and SEQ ID NO:24; (B) polynucleotide sequence encoding polypeptides having the sequence as shown in of SEQ ID NO:10, SEQ ID NO:14, SEQ ID NO:15, and SEQ ID NO:28-34; (C) biologically active fragments thereof; and (D) a complement thereof and its complement.

[0119] In some examples, nucleic acid molecules comprise a polynucleotide sequence that hybridizes under high stringency conditions to a polynucleotide having the sequence chosen from among: (A) SEQ ID NO:1, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:19, SEQ ID NO:23 and SEQ ID NO:24; (B) polynucleotide sequences encoding polypeptides having the sequence as shown in SEQ ID NO:10, SEQ ID
NO:14, SEQ ID NO:15, and SEQ ID NO:28-34; (C) biologically active fragments thereof; and (D) a complement thereof. In other examples the nucleic acid molecules that hybridize are complementary to a polynucleotide having the sequence chosen from among: (A) SEQ ID NO:1, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:19, SEQ ID NO:23 and SEQ ID NO:24; (B) polynucleotide sequences encoding polypeptides having a sequence as shown in SEQ ID NO:10, SEQ ID NO:14, SEQ ID NO:15, and SEQ ID NO:28-34; (C) biologically active fragments thereof; and (D) a complement thereof. In some examples, the nucleic acid molecule that is complementary to a polynucleotide having the sequence chosen from among: (A) SEQ ID NO:1, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:19, SEQ ID NO:23 and SEQ ID NO:24; (B) polynucleotide sequences encoding polypeptides having a sequence as shown in SEQ ID NO:10, SEQ ID NO:14, SEQ ID NO:15, and SEQ ID NO:28-34; (C) biologically active fragments thereof; and (D) a complement thereof comprises a polynucleotide sequence chosen from among an RNAi, an anti-sense molecule, and a ribozyme.

[0120] Accordingly, provided herein are methods of determining the presence of a nucleic acid comprising a polynucleotide sequence chosen from among: (A) SEQ ID NO:1, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:19, SEQ ID NO:23 and SEQ ID NO:24; (B) polynucleotide sequences encoding polypeptides having a sequence as shown in SEQ ID NO:10, SEQ ID NO:14, SEQ ID NO:15, and SEQ ID NO:28-34; (C) biologically active fragments thereof; and (D) complements thereof or its complement comprising the steps of:

(a) providing a complement to the nucleic acid molecule comprising a polynucleotide sequence chosen from among: (A) SEQ ID NO:1, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:19, SEQ ID NO:23 and SEQ ID NO:24; (B) polynucleotide sequences encoding polypeptides having the sequence as shown in SEQ ID NO:10, SEQ ID NO:14, SEQ ID NO:15, and SEQ ID NO:28-34; (C) biologically active fragments thereof; and (D) complements thereof or the nucleic acid molecule comprising a polynucleotide sequence chosen from among: (A) SEQ ID NO:1, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:19, SEQ ID NO:23 and SEQ ID NO:24; (B) polynucleotide sequences encoding polypeptides having a sequence as shown in SEQ ID NO:10, SEQ ID NO:14, SEQ ID NO:15, and SEQ ID NO:28-34; (C) biologically active fragments thereof; and (D) complements thereof;

(b) allowing the molecules to interact; and

(c) determining whether interaction has occurred.
In some examples, the polynucleotides which hybridize to the hereinabove described polynucleotides encode polypeptides which either retain substantially the same biological function or activity as the mature polypeptide.

Alternatively, the polynucleotide may have at least 20 bases, preferably 30 bases, and more preferably at least 50 bases which hybridize to a polynucleotide of the present invention and which has an identity thereto, as hereinabove described, and which may or may not retain activity.

Thus, the present invention is directed to polynucleotides having at least about a 70% identity, at least about 90% and at least about a 95% identity to a polynucleotide which encodes the polypeptides set forth in Appendices A-C (including SEQ ID NO:10, SEQ ID NO:14 and SEQ ID NO:15) as well as fragments thereof, which fragments have at least 30 bases and preferably at least 50 bases and to polypeptides encoded by such polynucleotides.

Using the information provided herein, such as the nucleotide sequences set forth in Appendices A-C, nucleic acid molecules of the present invention encoding CD40 splice variant polypeptides may be obtained using standard cloning and screening procedures, such as those for cloning cDNAs using mRNA as starting material and by synthesis methods known by one of skill in the art. Nucleic acids of the invention are useful as hybridization probes for differential identification of the tissue(s) or cell type(s) present in a biological sample. Similarly, polypeptides and antibodies directed to those polypeptides are useful to provide immunological probes for differential identification of tissue(s) or cell type(s).

Furthermore, provided herein are methods of determining the presence of a nucleic acid comprising a polynucleotide sequence chosen from among: (A) SEQ ID NO:1, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:19, SEQ ID NO:23 and SEQ ID NO:24; (B) polynucleotide sequences encoding polypeptides having a sequence as shown in SEQ ID NO:10, SEQ ID NO:14, SEQ ID NO:15, and SEQ ID NO:28-34; (C) biologically active fragments thereof; and (D) complements thereof or its complement comprising the steps of:

(a) providing a complement to the nucleic acid molecule comprising a polynucleotide sequence chosen from among: (A) SEQ ID NO:1, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:19, SEQ ID NO:23 and SEQ ID NO:24; (B) polynucleotide sequences encoding polypeptides having a sequence as shown in SEQ ID NO:10, SEQ ID NO:14, SEQ ID NO:15, and SEQ ID NO:28-34; (C) biologically
active fragments thereof; and (D) complements thereof or the nucleic acid molecule comprising a polynucleotide sequence chosen from among: (A) SEQ ID NO:1, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:19, SEQ ID NO:23 and SEQ ID NO:24; (B) polynucleotide sequences encoding polypeptides having a sequence as shown in SEQ ID NO:10, SEQ ID NO:14, SEQ ID NO:15, and SEQ ID NO:28-34; (C) biologically active fragments thereof; and (D) complements thereof;

(b) allowing the molecules to interact; and

(c) determining whether interaction has occurred.

[0126] As described infra, polynucleotides provided herein and complements thereof, may be administered to a subject to achieve a desired affect, such as a therapeutic affect.

**Variant and Mutant Polynucleotides**

[0127] The present invention further relates to variants of the nucleic acid molecules of the present invention, which encode portions, analogs or derivatives of the disclosed CD40 splice variants. Variants may occur naturally, such as a natural allelic variant. By an “allelic variant” is intended one of several alternate forms of a gene occupying a given locus on a chromosome of an organism. Genes II, Lewin, B., ed., John Wiley & Sons, New York (1985). Non-naturally occurring variants may be produced using art-known mutagenesis techniques.

[0128] Such variants include those produced by nucleotide substitutions, deletions or additions. The substitutions, deletions or additions may involve one or more nucleotides. The variants may be altered in coding regions, non-coding regions, or both. Alterations in the coding regions may produce conservative or non-conservative amino acid substitutions, deletions or additions. In some examples, these silent substitutions, additions and deletions, which do not alter the properties and activities of the encoded CD40 splice variants proteins described herein, or portions thereof. In some examples, the alterations are conservative substitutions.

[0129] In some examples, nucleic acid molecules encode the mature proteins comprising the amino acid sequences as shown, for example, in Appendices A-C (such as, for example, SEQ ID NO:10, SEQ ID NO:14 and SEQ ID NO:15), or the CD40 splice variant polypeptides are encoded by the nucleic acid molecules of Appendices A-C (such as, for example, SEQ ID NO:1, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:19, SEQ ID NO:23 and SEQ ID NO:24). In further examples, provided herein are isolated nucleic acid molecules comprising a polynucleotide having a
nucleotide sequence at least about 93% identical, and more preferably at least about 95%, 96%, 97%, 98% or 99% identical to a polynucleotide selected from the group consisting of: (a) a nucleotide sequence encoding a CD40 splice variant polypeptide having the complete amino acid sequences as set forth in Appendices A-C (such as, for example, SEQ ID NO:10, SEQ ID NO:14 and SEQ ID NO:15); or (b) a biologically active fragment of such. In some examples, polynucleotides encode biologically active fragments that comprise that amino acid sequence SPGQWALEKA; VRPKTWLCNQRQAQTRLMLSVSPGQWALEKA; and the ECD of CD40sv2.

[0130] The present invention also provides polynucleotides encoding muteins of CD40 splice variants such as for example SEQ ID NO:95, SEQ ID NO:96, SEQ ID NO:97, SEQ ID NO:98, SEQ ID NO:99, SEQ ID NO:100, SEQ ID NO:101, SEQ ID NO:102, SEQ ID NO:103, SEQ ID NO:104, SEQ ID NO:105, SEQ ID NO:106, SEQ ID NO:107, SEQ ID NO:108, SEQ ID NO:109, SEQ ID NO:110, SEQ ID NO:111, SEQ ID NO:112, SEQ ID NO:113, SEQ ID NO:114, SEQ ID NO:115, SEQ ID NO:116, SEQ ID NO:117, SEQ ID NO:118, and SEQ ID NO:119. The present invention provides polynucleotides comprising the sequences as shown in SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49, SEQ ID NO:50, SEQ ID NO:51, SEQ ID NO:52, SEQ ID NO:53, SEQ ID NO:54, SEQ ID NO:55, SEQ ID NO:56, SEQ ID NO:57, SEQ ID NO:58, SEQ ID NO:59, SEQ ID NO:60, SEQ ID NO:61, or SEQ ID NO:62, fragments thereof and complements thereof. Such polynucleotides may be double-stranded or single stranded and include cDNA molecules, genomic DNA molecules, cRNA molecules, SiRNA molecules, RNAi molecules, mRNA molecules, anti-sense molecules and ribozymes.

[0131] By a polynucleotide having a nucleotide sequence at least about, for example, 95% "identical" to a reference nucleotide sequence encoding a CD40 splice variant polypeptide is intended that the nucleotide sequence of the polynucleotide is identical to the reference sequence except that the polynucleotide sequence may include up to five point mutations per each 100 nucleotides of the reference nucleotide sequence encoding the CD40 splice variant polypeptides. In other words, to obtain a polynucleotide having a nucleotide sequence at least 95% identical to a reference nucleotide sequence, up to 5% of the nucleotides in the reference sequence
may be deleted or substituted with another nucleotide, or a number of nucleotides up
5% of the total nucleotides in the reference sequence may be inserted into the
reference sequence. These mutations of the reference sequence may occur at the 5' or
3' terminal positions of the reference nucleotide sequence or anywhere between those
terminal positions, interspersed either individually among nucleotides in the reference
sequence or in one or more contiguous groups within the reference sequence.

[0132] As a practical matter, whether any particular nucleic acid molecule is
at least about 93%, 95%, 96%, 97%, 98% or 99% identical to, for instance, the
nucleotide sequences set forth in Appendices A-C can be determined conventionally
using known computer programs such as the Bestfit program (Wisconsin Sequence
Analysis Package, Version 8 for Unix, Genetics Computer Group, University
Research Park, 575 Science Drive, Madison, Wis. 53711). Bestfit uses the local
homology algorithm of Smith and Waterman, Advances in Applied Mathematics
2:482-489 (1981), to find the best segment of homology between two sequences.
When using Bestfit or any other sequence alignment program to determine whether a
particular sequence is, for instance, 95% identical to a reference sequence according
to the present invention, the parameters are set, of course, such that the percentage of
identity is calculated over the full length of the reference nucleotide sequence and that
gaps in homology of up to 5% of the total number of nucleotides in the reference
sequence are allowed.

[0133] The present application encompasses nucleic acid molecules having at
least about 93%, 95%, 96%, 97%, 98% or 99% identical to the nucleic acid sequences
set forth in Appendices A-C, irrespective of whether they encode a polypeptide
having CD40 activity. This is because even where a particular nucleic acid molecule
does not encode a polypeptide having CD40 activity, one of skill in the art would still
know how to use the nucleic acid molecules, for instance, as a hybridization probe or
a polymerase chain reaction (PCR) primer. Uses of the nucleic acid molecules of the
present invention that do not encode a polypeptide having CD40 splice variant
activity include, inter alia, (1) isolating the CD40 gene or allelic variants thereof in a
cDNA library; (2) in situ hybridization (e.g., "FISH") to metaphase chromosomal
spreads to provide precise chromosomal location of the CD40 genes, as described in
Verna et al., Human Chromosomes: A Manual of Basic Techniques, Pergamon Press,
New York (1988); and Northern blot analysis for detecting CD40 mRNA expression
in specific tissues.
In some examples, nucleic acid molecules comprise sequences having at least about 93%, 95%, 96%, 97%, 98% or 99% identity to the nucleic acid sequence shown in Appendices A-C which do, in fact, encode a polypeptide having CD40 polypeptide activity. By “a polypeptide having CD40 activity” is intended a polypeptide exhibiting activity similar, but not necessarily identical, to an activity of the CD40 variant polypeptides of the invention, as measured in a particular biological assay. For example, the CD40 splice variant polypeptides of the present invention may stimulate proliferation of various mammalian cells.

Of course, due to the degeneracy of the genetic code, one of ordinary skill in the art will immediately recognize that a large number of the nucleic acid molecules having a sequence at least about 93%, 95%, 96%, 97%, 98%, or 99% identical to the nucleic acid sequence of the nucleic acid sequences set forth in Appendices A-C, will encode a polypeptide “having CD40 polypeptide activity.” In fact, since degenerate variants of these nucleotide sequences all encode the same polypeptide, this will be clear to the skilled artisan even without performing the above described comparison assay. It will be further recognized in the art that, for such nucleic acid molecules that are not degenerate variants, a reasonable number will also encode a polypeptide having CD40 polypeptide activity. This is because the skilled artisan is fully aware of amino acid substitutions that are either less likely or not likely to significantly affect protein function (e.g., replacing one aliphatic amino acid with a second aliphatic amino acid), as further described below.

**Vectors and Host Cells**

The present invention also relates to vectors which include, that is comprise, the isolated nucleic acid molecules of the present invention, host cells which comprise such vectors, which are genetically engineered with the recombinant vectors, and the production of CD40 polypeptides, including the CD40 splice variants, or fragments thereof or muteins thereof by recombinant techniques. The vector may be, for example, a phage, plasmid, viral or retroviral vector. Retroviral vectors may be replication competent or replication defective. In the latter case, viral propagation generally will occur only in complementing host cells. In some examples, the vector is selected for expression in mammalian cells and in other examples, the vector is selected for expression in insect cells.
Accordingly, provided herein are vectors and host cells that comprise polynucleotides that encode the CD40 splice variants, fragments thereof and muteins thereof, and the polynucleotides disclosed herein. In some examples a vector comprising a nucleic acid molecule comprising a polynucleotide sequence chosen from among: (A) SEQ ID NO:1, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:19, SEQ ID NO:23; SEQ ID NO:24; SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49, SEQ ID NO:50, SEQ ID NO:51, SEQ ID NO:52, SEQ ID NO:53, SEQ ID NO:54, SEQ ID NO:55, SEQ ID NO:56, SEQ ID NO:57, SEQ ID NO:58, SEQ ID NO:59, SEQ ID NO:60, SEQ ID NO:61, or SEQ ID NO:62 (B) polynucleotide sequences encoding polypeptides having the sequence as shown in SEQ ID NO:10, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:28-34, SEQ ID NO:95, SEQ ID NO:96, SEQ ID NO:97, SEQ ID NO:98, SEQ ID NO:99, SEQ ID NO:100, SEQ ID NO:101, SEQ ID NO:102, SEQ ID NO:103, SEQ ID NO:104, SEQ ID NO:105, SEQ ID NO:106, SEQ ID NO:107, SEQ ID NO:108, SEQ ID NO:109, SEQ ID NO:110, SEQ ID NO:111, SEQ ID NO:112, SEQ ID NO:113, SEQ ID NO:114, SEQ ID NO:115, SEQ ID NO:116, SEQ ID NO:117, SEQ ID NO:118, or SEQ ID NO:119; (C) biologically active fragments thereof; and (D) complements thereof and a promoter that regulates the expression of the核酸 acid molecule is provided. In other examples a vector comprising a double-stranded isolated nucleic acid molecule comprising a polynucleotide sequence chosen from among: (A) SEQ ID NO:1, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:19, SEQ ID NO:23; SEQ ID NO:24; SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49, SEQ ID NO:50, SEQ ID NO:51, SEQ ID NO:52, SEQ ID NO:53, SEQ ID NO:54, SEQ ID NO:55, SEQ ID NO:56, SEQ ID NO:57, SEQ ID NO:58, SEQ ID NO:59, SEQ ID NO:60, SEQ ID NO:61, or SEQ ID NO:62; (B) polynucleotide sequences encoding polypeptides having the sequence as shown in SEQ ID NO:10, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:28-34, SEQ ID NO:95, SEQ ID NO:96, SEQ ID NO:97, SEQ ID NO:98, SEQ ID NO:99, SEQ ID NO:100, SEQ ID NO:101, SEQ ID NO:102, SEQ ID NO:103, SEQ ID NO:104, SEQ ID NO:105, SEQ ID NO:106, SEQ ID NO:107, SEQ ID NO:108, SEQ ID NO:109, SEQ ID NO:110, SEQ ID NO:111, SEQ ID NO:112, SEQ ID NO:113, SEQ ID NO:114, SEQ ID NO:115, SEQ ID NO:116, SEQ
ID NO:117, SEQ ID NO:118, or SEQ ID NO:119; (C) biologically active fragments thereof; and (D) complements thereof and its complement and a promoter that regulates the expression of the nucleic acid molecule is provided. In some examples the promoter is selected from one that is naturally contiguous to the nucleic acid molecule (homologous), one that is not naturally contiguous to the nucleic acid molecule (heterologous), an inducible promoter, a conditionally-active promoter (such as the cre-lox promoter), a constitutive promoter, and a tissue-specific promoter. Such promoters are known in the art.

[0138] The polynucleotides may be joined to a vector containing a selectable marker for propagation in a host. Generally, a plasmid vector is introduced in a precipitate, such as a calcium phosphate precipitate, or in a complex with a charged lipid. If the vector is a virus, it may be packaged in vitro using an appropriate packaging cell line and then transduced into host cells.

[0139] The DNA insert should be operatively linked to an appropriate promoter, such as the phage lambda PL promoter, the Escherichia coli lac, trp, phoA and tac promoters, the SV40 early and late promoters and promoters of retroviral LTRs, to name a few. Other suitable promoters will be known to the skilled artisan. The expression constructs will further contain sites for transcription initiation, termination and, in the transcribed region, a ribosome binding site for translation. The coding portion of the transcripts expressed by the constructs will preferably include a translation initiating codon at the beginning and a termination codon (UAA, UGA or UAG) appropriately positioned at the end of the polypeptide to be translated.

[0140] As indicated, the expression vectors will preferably include at least one selectable marker. Such markers include dihydrofolate reductase, G418 or neomycin resistance for eukaryotic cell culture and tetracycline, kanamycin or ampicillin resistance genes for culturing in E. coli and other bacteria. Representative examples of appropriate hosts include, but are not limited to, bacterial cells, such as Escherichia coli, Streptomyces and Salmonella typhimurium cells; fungal cells, such as yeast cells; insect cells such as Drosophila S2 and Spodoptera Sf9 cells; animal cells such as CHO, COS, 293 and Bowes melanoma cells; and plant cells. Appropriate culture mediums and conditions for the above-described host cells are known in the art.

Stratagene, La Jolla, CA; and ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5 available from Pharmacia (Pfizer). Among preferred eucaryotic vectors are pWLNEO, pSV2CAT, pOG44, pXT1 and pSG available from Stratagene; and pSVK3, pBPV, pMSG and pSVL available from Pharmacia. Other suitable vectors will be readily apparent to the skilled artisan. Vector sequences are known in the art and described herein.

Introduction of the construct into the host cell can be effected by calcium phosphate transfection, DEAE-dextran mediated transfection, cationic lipid-mediated transfection, electroporation, transduction, infection or other methods. Such methods are described in many standard laboratory manuals, such as Davis et al., Basic Methods In Molecular Biology (1986).

Accordingly, provided herein are recombinant host cells comprising an isolated nucleic acid comprising a polynucleotide sequence chosen from among:

(A) SEQ ID NO:1, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:19, SEQ ID NO:23; SEQ ID NO:24; SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49, SEQ ID NO:50, SEQ ID NO:51, SEQ ID NO:52, SEQ ID NO:53, SEQ ID NO:54, SEQ ID NO:55, SEQ ID NO:56, SEQ ID NO:57, SEQ ID NO:58, SEQ ID NO:59, SEQ ID NO:60, SEQ ID NO:61, or SEQ ID NO:62 (B) polynucleotide sequences encoding polypeptides having the sequence as shown in SEQ ID NO:10, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:28-34, SEQ ID NO:95, SEQ ID NO:96, SEQ ID NO:97, SEQ ID NO:98, SEQ ID NO:99, SEQ ID NO:100, SEQ ID NO:101, SEQ ID NO:102, SEQ ID NO:103, SEQ ID NO:104, SEQ ID NO:105, SEQ ID NO:106, SEQ ID NO:107, SEQ ID NO:108, SEQ ID NO:109, SEQ ID NO:110, SEQ ID NO:111, SEQ ID NO:112, SEQ ID NO:113, SEQ ID NO:114, SEQ ID NO:115, SEQ ID NO:116, SEQ ID NO:117, SEQ ID NO:118, or SEQ ID NO:119; (C) biologically active fragments thereof; and (D) complements thereof. In some examples recombinant host cells comprising an isolated polypeptide comprising an amino acid sequence chosen from among: SEQ ID NO:10, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, and SEQ ID NO:34 and active fragments thereof are provided. In other examples, recombinant host cells comprise an isolated polypeptide comprising an amino acid sequence comprising SPGQWALEKA; and VRPKTWLCNRQAQTRLMLSVVSPGQWALEKA and the
CD40sv2 extracellular amino acid domain as shown in Figure 2. In yet other examples, recombinant host cells comprise an isolated polynucleotide encoding a polypeptide comprising the muteins of CD40 splice variants as described herein.

Further provided herein are recombinant host cells comprising a vector comprising a nucleic acid molecule comprising a polynucleotide sequence chosen from among: (A) SEQ ID NO:1, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:19, SEQ ID NO:23; SEQ ID NO:24; SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49, SEQ ID NO:50, SEQ ID NO:51, SEQ ID NO:52, SEQ ID NO:53, SEQ ID NO:54, SEQ ID NO:55, SEQ ID NO:56, SEQ ID NO:57, SEQ ID NO:58, SEQ ID NO:59, SEQ ID NO:60, SEQ ID NO:61, or SEQ ID NO:62 (B) polynucleotide sequences encoding polypeptides having the sequence as shown in SEQ ID NO:10, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:28-34, SEQ ID NO:95, SEQ ID NO:96, SEQ ID NO:97, SEQ ID NO:98, SEQ ID NO:99, SEQ ID NO:100, SEQ ID NO:101, SEQ ID NO:102, SEQ ID NO:103, SEQ ID NO:104, SEQ ID NO:105, SEQ ID NO:106, SEQ ID NO:107, SEQ ID NO:108, SEQ ID NO:109, SEQ ID NO:110, SEQ ID NO:111, SEQ ID NO:112, SEQ ID NO:113, SEQ ID NO:114, SEQ ID NO:115, SEQ ID NO:116, SEQ ID NO:117, SEQ ID NO:118, or SEQ ID NO:119; (C) biologically active fragments thereof; (D) complements thereof and a promoter that regulates the expression of the nucleic acid molecule are provided. In some examples recombinant host cells comprising a vector comprising a double-stranded isolated nucleic acid molecule comprising the nucleic acid molecules comprising a polynucleotide sequence chosen from among: (A) SEQ ID NO:1, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:19, SEQ ID NO:23; SEQ ID NO:24; SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49, SEQ ID NO:50, SEQ ID NO:51, SEQ ID NO:52, SEQ ID NO:53, SEQ ID NO:54, SEQ ID NO:55, SEQ ID NO:56, SEQ ID NO:57, SEQ ID NO:58, SEQ ID NO:59, SEQ ID NO:60, SEQ ID NO:61, or SEQ ID NO:62 (B) polynucleotide sequences encoding polypeptides having the sequence as shown in SEQ ID NO:10, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:28-34, SEQ ID NO:95, SEQ ID NO:96, SEQ ID NO:97, SEQ ID NO:98, SEQ ID NO:99, SEQ ID NO:100, SEQ ID NO:101, SEQ ID NO:102, SEQ ID NO:103, SEQ ID NO:104, SEQ ID NO:105, SEQ ID NO:106, SEQ ID NO:107, SEQ
ID NO:108, SEQ ID NO:109, SEQ ID NO:110, SEQ ID NO:111, SEQ ID NO:112, SEQ ID NO:113, SEQ ID NO:114, SEQ ID NO:115, SEQ ID NO:116, SEQ ID NO:117, SEQ ID NO:118, or SEQ ID NO:119; (C) biologically active fragments thereof; (D) complements thereof and its complement and a promoter that regulates the expression of the nucleic acid molecule are provided.

[0145] In some examples the recombinant host cell is selected from a procaryotic cell and a eucaryotic cell, wherein in some examples the eucaryotic cell is selected from the group consisting of a human cell, a non-human mammalian cell, an insect cell, a fish cell, a plant cell and a fungal cell.

[0146] Provided herein is a method of producing a recombinant host cell comprising the steps of (a) providing a composition comprising a vector that comprises the nucleic acid molecule comprising a polynucleotide sequence chosen from among: (A) SEQ ID NO:1, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:19, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49, SEQ ID NO:50, SEQ ID NO:51, SEQ ID NO:52, SEQ ID NO:53, SEQ ID NO:54, SEQ ID NO:55, SEQ ID NO:56, SEQ ID NO:57, SEQ ID NO:58, SEQ ID NO:59, SEQ ID NO:60, SEQ ID NO:61, or SEQ ID NO:62 (B) polynucleotide sequences encoding polypeptides having the sequence as shown in SEQ ID NO:10, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:28-34, SEQ ID NO:95, SEQ ID NO:96, SEQ ID NO:97, SEQ ID NO:98, SEQ ID NO:99, SEQ ID NO:100, SEQ ID NO:101, SEQ ID NO:102, SEQ ID NO:103, SEQ ID NO:104, SEQ ID NO:105, SEQ ID NO:106, SEQ ID NO:107, SEQ ID NO:108, SEQ ID NO:109, SEQ ID NO:110, SEQ ID NO:111, SEQ ID NO:112, SEQ ID NO:113, SEQ ID NO:114, SEQ ID NO:115, SEQ ID NO:116, SEQ ID NO:117, SEQ ID NO:118, or SEQ ID NO:119; (C) biologically active fragments thereof; (D) complements thereof and (b) allowing a host cell to come into contact with the vector to form a recombinant host cell.

[0147] Also provided is a method of producing a polypeptide comprising the steps of:

(a) providing a composition comprising a recombinant host cell comprising an isolated nucleic acid comprising a polynucleotide sequence chosen from among: (A) SEQ ID NO:1, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:19, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID
NO:41, SEQ ID NO:42, SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49, SEQ ID NO:50, SEQ ID NO:51, SEQ ID NO:52, SEQ ID NO:53, SEQ ID NO:54, SEQ ID NO:55, SEQ ID NO:56, SEQ ID NO:57, SEQ ID NO:58, SEQ ID NO:59, SEQ ID NO:60, SEQ ID NO:61, or SEQ ID NO:62 (B) polynucleotide sequences encoding polypeptides having the sequence as shown in SEQ ID NO:10, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:28-34, SEQ ID NO:95, SEQ ID NO:96, SEQ ID NO:97, SEQ ID NO:98, SEQ ID NO:99, SEQ ID NO:100, SEQ ID NO:101, SEQ ID NO:102, SEQ ID NO:103, SEQ ID NO:104, SEQ ID NO:105, SEQ ID NO:106, SEQ ID NO:107, SEQ ID NO:108, SEQ ID NO:109, SEQ ID NO:110, SEQ ID NO:111, SEQ ID NO:112, SEQ ID NO:113, SEQ ID NO:114, SEQ ID NO:115, SEQ ID NO:116, SEQ ID NO:117, SEQ ID NO:118, or SEQ ID NO:119; (C) biologically active fragments thereof; and (D) complements thereof;

(b) culturing the recombinant host cell to produce the polypeptide.

Also provided is a method of producing a polypeptide comprising the steps of:

(a) providing a composition comprising a recombinant host cell comprising an isolated polypeptide comprising an amino acid sequence chosen from among: SEQ ID NO:10, SEQ ID NO:14, SEQ ID NO:15, and SEQ ID NO:28-34 and muteins of CD40 splice variants disclosed herein, and active fragments thereof;

(b) culturing the recombinant host cell to produce the polypeptide.

Also provided is a method of producing a polypeptide comprising the steps of:

(a) providing a composition comprising a recombinant host cell comprising a vector comprising a nucleic acid molecule comprising a polynucleotide sequence chosen from among: (A) SEQ ID NO:1, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:19, SEQ ID NO:23; SEQ ID NO:24; SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49, SEQ ID NO:50, SEQ ID NO:51, SEQ ID NO:52, SEQ ID NO:53, SEQ ID NO:54, SEQ ID NO:55, SEQ ID NO:56, SEQ ID NO:57, SEQ ID NO:58, SEQ ID NO:59, SEQ ID NO:60, SEQ ID NO:61, or SEQ ID NO:62 (B) polynucleotide sequences encoding polypeptides having the sequence as shown in SEQ ID NO:10, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:28-34, SEQ ID NO:95, SEQ ID NO:96, SEQ ID NO:97, SEQ

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ID NO:98, SEQ ID NO:99, SEQ ID NO:100, SEQ ID NO:101, SEQ ID NO:102, SEQ ID NO:103, SEQ ID NO:104, SEQ ID NO:105, SEQ ID NO:106, SEQ ID NO:107, SEQ ID NO:108, SEQ ID NO:109, SEQ ID NO:110, SEQ ID NO:111, SEQ ID NO:112, SEQ ID NO:113, SEQ ID NO:114, SEQ ID NO:115, SEQ ID NO:116, SEQ ID NO:117, SEQ ID NO:118, or SEQ ID NO:119 (C) biologically active fragments thereof; (D) complements thereof and a promoter that regulates the expression of the nucleic acid molecule;

(b) culturing the recombinant host cell to produce the polypeptide.

[0151] Also provided is a method of producing a polypeptide comprising the steps of:

(a) providing a composition comprising a recombinant host cell comprising a vector comprising a double-stranded isolated nucleic acid molecule comprising a nucleic acid molecule comprising a polynucleotide sequence chosen from among: (A) SEQ ID NO:1, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:19, SEQ ID NO:23; SEQ ID NO:24; SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49, SEQ ID NO:50, SEQ ID NO:51, SEQ ID NO:52, SEQ ID NO:53, SEQ ID NO:54, SEQ ID NO:55, SEQ ID NO:56, SEQ ID NO:57, SEQ ID NO:58, SEQ ID NO:59, SEQ ID NO:60, SEQ ID NO:61, or SEQ ID NO:62 (B) polynucleotide sequences encoding polypeptides having the sequence as shown in SEQ ID NO:10, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:28-34, SEQ ID NO:95, SEQ ID NO:96, SEQ ID NO:97, SEQ ID NO:98, SEQ ID NO:99, SEQ ID NO:100, SEQ ID NO:101, SEQ ID NO:102, SEQ ID NO:103, SEQ ID NO:104, SEQ ID NO:105, SEQ ID NO:106, SEQ ID NO:107, SEQ ID NO:108, SEQ ID NO:109, SEQ ID NO:110, SEQ ID NO:111, SEQ ID NO:112, SEQ ID NO:113, SEQ ID NO:114, SEQ ID NO:115, SEQ ID NO:116, SEQ ID NO:117, SEQ ID NO:118, or SEQ ID NO:119; (C) biologically active fragments thereof; (D) complements thereof and its complement and a promoter that regulates the expression of the nucleic acid molecule

(b) culturing the recombinant host cell to produce the polypeptide.

[0152] Provided herein is a method of producing a polypeptide comprising the steps of:

(a) providing a nucleic acid molecule comprising a polynucleotide sequence chosen from among: (A)SEQ ID NO:1, SEQ ID NO:5, SEQ ID NO:6, SEQ
ID NO:19, SEQ ID NO:23; SEQ ID NO:24; SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49, SEQ ID NO:50, SEQ ID NO:51, SEQ ID NO:52, SEQ ID NO:53, SEQ ID NO:54, SEQ ID NO:55, SEQ ID NO:56, SEQ ID NO:57, SEQ ID NO:58, SEQ ID NO:59, SEQ ID NO:60, SEQ ID NO:61, or SEQ ID NO:62 (B) polynucleotide sequences encoding polypeptides having the sequence as shown in SEQ ID NO:10, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:28-34, SEQ ID NO:95, SEQ ID NO:96, SEQ ID NO:97, SEQ ID NO:98, SEQ ID NO:99, SEQ ID NO:100, SEQ ID NO:101, SEQ ID NO:102, SEQ ID NO:103, SEQ ID NO:104, SEQ ID NO:105, SEQ ID NO:106, SEQ ID NO:107, SEQ ID NO:108, SEQ ID NO:109, SEQ ID NO:110, SEQ ID NO:111, SEQ ID NO:112, SEQ ID NO:113, SEQ ID NO:114, SEQ ID NO:115, SEQ ID NO:116, SEQ ID NO:117, SEQ ID NO:118, or SEQ ID NO:119; (C) biologically active fragments thereof; (D) complements thereof;

(b) expressing the nucleic acid molecule in a cell free expression system to produce the polypeptide.

[0153] Further provided herein are methods of producing fusion molecules as described above, methods of producing host cells comprising the fusion molecules, and methods of producing vectors comprising the fusion molecules as described above.

[0154] In some examples the cell free expression system is selected from the group consisting of a wheat germ lysate expression system, a rabbit reticulocyte expression system, and an E. coli lysate expression system.

[0155] The polypeptides may be expressed in a modified form, such as a fusion protein, and may include not only secretion signals, but also additional heterologous functional regions. For instance, a region of additional amino acids, particularly charged amino acids, may be added to the N-terminus of the polypeptide to improve stability and persistence in the host cell, during purification, or during subsequent handling and storage. Also, peptide moieties may be added to the polypeptide to facilitate purification. Such regions may be removed prior to final preparation of the polypeptide.

[0156] The addition of peptide moieties to polypeptides to engender secretion or excretion, to improve stability and to facilitate purification, among others are familiar and routine techniques in the art. In some examples, a preferred fusion
protein comprises a heterologous region from immunoglobulin that is useful to stabilize and purify proteins. For example, EP-A-O 464 533 (Canadian counterpart 2045869) discloses fusion proteins containing various portions of constant region of immunoglobulin molecules together with another human protein or part thereof. In many cases, the Fc part in a fusion protein is thoroughly advantageous for use in therapy and diagnosis and thus results, for example, in improved pharmacokinetic properties (EP-A 0232 262). On the other hand, for some uses it would be desirable to be able to delete the Fc part after the fusion protein has been expressed, detected and purified in the advantageous manner described. This is the case when the Fc portion proves to be a hindrance to use in therapy and diagnosis, for example when the fusion protein is to be used as antigen for immunizations. In drug discovery, for example, human proteins, such as hIL-5, have been fused with Fc portions for the purpose of high-throughput screening assays to identify antagonists of hIL-5. See, Bennett et al., *J. Molecular Recognition*, 8:52-58 (1995) and Johanson et al, *J. Biol. Chem.*, 270:9459-9471 (1995). Additional fusion partners are provided in the examples and include, a leucine zipper domain, a trimerization domain from tetranectin, and a trimerization domain from a mannose binding protein (also known as mannose binding lectin), such as, for example, mannose binding protein 1 (MBP1).

Accordingly, the present invention provides polynucleotide encoding fusion proteins wherein said polynucleotides comprise the sequence as shown in SEQ ID NO:64, SEQ ID NO:66, SEQ ID NO:68, SEQ ID NO:70, SEQ ID NO:71, SEQ ID NO:72, SEQ ID NO:75, SEQ ID NO:76, SEQ ID NO:79, SEQ ID NO:81, SEQ ID NO:83, SEQ ID NO:85, SEQ ID NO:87, or SEQ ID NO:89 or 2) encode the polypeptide having the sequence as shown in SEQ ID NO:121, SEQ ID NO:123, SEQ ID NO:125, SEQ ID NO:127, SEQ ID NO:128, SEQ ID NO:129, SEQ ID NO:132, SEQ ID NO:133, SEQ ID NO:136, SEQ ID NO:138, SEQ ID NO:140, SEQ ID NO:142, SEQ ID NO:144, or SEQ ID NO:146, or is a complement thereof. The present invention also provides fusion molecules comprising polypeptides having the sequence as shown in SEQ ID NO:121, SEQ ID NO:123, SEQ ID NO:125, SEQ ID NO:127, SEQ ID NO:128, SEQ ID NO:129, SEQ ID NO:132, SEQ ID NO:133, SEQ ID NO:136, SEQ ID NO:138, SEQ ID NO:140, SEQ ID NO:142, SEQ ID NO:144, or SEQ ID NO:146.

The CD40 polypeptides of the invention can be recovered and purified from recombinant cell cultures by well-known methods including ammonium sulfate
or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Most preferably, high performance liquid chromatography ("HPLC") is employed for purification. Polypeptides of the present invention include: products purified from natural sources, including bodily fluids, tissues and cells, whether directly isolated or cultured; products of chemical synthetic procedures; and products produced by recombinant techniques from a prokaryotic or eukaryotic host, including, for example, bacterial, yeast, higher plant, insect and mammalian cells as well as chemically synthesized products. Depending upon the host employed in a recombinant production procedure, the polypeptides of the present invention may be glycosylated or may be non-glycosylated. In addition, polypeptides of the invention may also include an initial modified methionine residue, in some cases as a result of host-mediated processes. Thus, it is well known in the art that the N-terminal methionine encoded by the translation initiation codon generally is removed with high efficiency from any protein after translation in all eukaryotic cells. While the N-terminal methionine on most proteins also is efficiently removed in most prokaryotes, for some proteins this prokaryotic removal process is inefficient, depending on the nature of the amino acid to which the N-terminal methionine is covalently linked.

Polypeptides and Fragments

[0159] The invention further provides isolated CD40 polypeptides comprising the amino acid sequences encoded by the nucleotide sequences set forth in Appendices A-C (such as for example, SEQ ID NO:1, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:19, SEQ ID NO:23 and SEQ ID NO:24), or the amino acid sequences set forth in Appendices A-C (such as for example, SEQ ID NO:10, SEQ ID NO:14 and SEQ ID NO:15), or a peptide or polypeptide comprising a portion of the polypeptide. Provided herein are muteins comprising an amino acid sequence having the sequences as shown in SEQ ID NO:95, SEQ ID NO:96, SEQ ID NO:97, SEQ ID NO:98, SEQ ID NO:99, SEQ ID NO:100, SEQ ID NO:101, SEQ ID NO:102, SEQ ID NO:103, SEQ ID NO:104, SEQ ID NO:105, SEQ ID NO:106, SEQ ID NO:107, SEQ ID NO:108, SEQ ID NO:109, SEQ ID NO:110, SEQ ID NO:111, SEQ ID NO:112, SEQ ID NO:113, SEQ ID NO:114, SEQ ID NO:115, SEQ ID NO:116, SEQ ID NO:117, SEQ ID NO:118, and SEQ ID NO:119, or a portion thereof, and muteins encoded by
the polynucleotide sequences set forth in the Appendices A-C. In some examples, a CD40 splice variant or mutein polypeptide is a monomer and in other examples, is a multimer, including a dimer or trimer. Multimers of the polypeptides as described herein can be produced by methods known in the art. Without being bound to theory, it is thought that a soluble CD40 splice variant, such as in the form of an entity that is a monomer or monovalent or binds to only a single CD40 ligand, such as for example CD154, will bind to either soluble or membrane bound CD40 ligand, preventing binding (ligation) of CD40 without resulting in cross-linking of CD40 ligand, especially on non-target tissue, including activated platelets. This feature may provide advantages over monoclonal antibodies to CD40 ligand known in the art.

[0160] Accordingly, provided herein are isolated polypeptides comprising an amino acid sequence, wherein the amino acid sequence is chosen from among: SEQ ID NO:10, SEQ ID NO:14, SEQ ID NO:15, and SEQ ID NO:28-34 and active fragments thereof, as well as chosen from among SEQ ID NO:95, SEQ ID NO:96, SEQ ID NO:97, SEQ ID NO:98, SEQ ID NO:99, SEQ ID NO:100, SEQ ID NO:101, SEQ ID NO:102, SEQ ID NO:103, SEQ ID NO:104, SEQ ID NO:105, SEQ ID NO:106, SEQ ID NO:107, SEQ ID NO:108, SEQ ID NO:109, SEQ ID NO:110, SEQ ID NO:111, SEQ ID NO:112, SEQ ID NO:113, SEQ ID NO:114, SEQ ID NO:115, SEQ ID NO:116, SEQ ID NO:117, SEQ ID NO:118, and SEQ ID NO:119, and active fragments thereof. In some examples isolated polypeptides are encoded by a nucleic acid molecule comprising a polynucleotide sequence chosen from among: (A) SEQ ID NO:1, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:19, SEQ ID NO:23; SEQ ID NO:24; SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49, SEQ ID NO:50, SEQ ID NO:51, SEQ ID NO:52, SEQ ID NO:53, SEQ ID NO:54, SEQ ID NO:55, SEQ ID NO:56, SEQ ID NO:57, SEQ ID NO:58, SEQ ID NO:59, SEQ ID NO:60, SEQ ID NO:61, or SEQ ID NO:62 (B) polynucleotide sequences encoding polypeptides having the sequence as shown in SEQ ID NO:10, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:28-34, SEQ ID NO:95, SEQ ID NO:96, SEQ ID NO:97, SEQ ID NO:98, SEQ ID NO:99, SEQ ID NO:100, SEQ ID NO:101, SEQ ID NO:102, SEQ ID NO:103, SEQ ID NO:104, SEQ ID NO:105, SEQ ID NO:106, SEQ ID NO:107, SEQ ID NO:108, SEQ ID NO:109, SEQ ID NO:110, SEQ ID NO:111, SEQ ID NO:112, SEQ ID NO:113, SEQ ID NO:114, SEQ ID NO:115, SEQ ID NO:116, SEQ ID NO:117, SEQ ID NO:118, or
SEQ ID NO:119; and (C) complements thereof are provided. In some examples, a polypeptide is between about 50 and about 170 amino acids in length, between about 100 and about 160 acids in length, between about 120 and about 150 amino acids in length, between about 130 and 145 amino acids in length and between about 140 and about 145 amino acids in length.

Variant and Mutant Polypeptides

[0161] To improve or alter the characteristics or properties of a CD40 polypeptide of the invention, protein engineering may be employed. Recombinant DNA technology known to those skilled in the art can be used to create novel mutant proteins or “muteins” including single or multiple amino acid substitutions, deletions, additions or fusion proteins. Such modified polypeptides can show, e.g., enhanced activity or increased stability. In addition, they may be purified in higher yields and show better solubility than the corresponding natural polypeptide, at least under certain purification and storage conditions.

N-Terminal and C-Terminal Deletion Mutants

[0162] For instance, for many proteins, including the extracellular domain ECD of a membrane associated protein or the mature form(s) of a secreted protein, (that is without a signal sequence), it is known in the art that one or more amino acids may be deleted from the N-terminus or C-terminus without substantial loss of biological function. For instance, Ron et al., J. Biol. Chem., 268:2984-2988 (1993), reported modified KGF (keratinocyte growth factor) proteins that had heparin binding activity even if 3, 8, or 27 amino-terminal amino acid residues were missing.

[0163] However, even if deletion of one or more amino acids from the N-terminus of a protein results in modification or loss of one or more biological functions of the protein, other biological activities may still be retained. Thus, the ability of the shortened protein to induce and/or bind to antibodies which recognize the complete or mature form of the protein generally will be retained when less than the majority of the residues of the complete or mature protein are removed from the N-terminus. Whether a particular polypeptide lacking N-terminal residues of a complete protein retains such immunologic activities can readily be determined by routine methods described herein and otherwise known in the art.
Accordingly, the present invention further provides polypeptides having one or more residues deleted from the amino terminus of the amino acid sequences of the CD40 splice variant molecules, and muteins thereof, as shown in Appendices A-C. In some examples, a mutein is soluble and in other examples, comprises a transmembrane domain.

Similarly, many examples of biologically functional C-terminal deletion muteins are known. For instance, interferon gamma shows up to ten times higher activity by deleting 8-10 amino acid residues from the carboxy terminus of the protein, see, for example, Dobeli et al., *J. Biotechnology*, 7:199-216 (1988).

Accordingly, provided herein are muteins of CD40 splice variant molecules and include SEQ ID NO:95, SEQ ID NO:96, SEQ ID NO:97, SEQ ID NO:98, SEQ ID NO:99, SEQ ID NO:100, SEQ ID NO:101, SEQ ID NO:102, SEQ ID NO:103, SEQ ID NO:104, SEQ ID NO:105, SEQ ID NO:106, SEQ ID NO:107, SEQ ID NO:108, SEQ ID NO:109, SEQ ID NO:110, SEQ ID NO:111, SEQ ID NO:112, SEQ ID NO:113, SEQ ID NO:114, SEQ ID NO:115, SEQ ID NO:116, SEQ ID NO:117, SEQ ID NO:118, and SEQ ID NO:119, and fragments thereof.

However, even if deletion of one or more amino acids from the C-terminus of a protein results in modification or loss of one or more biological functions of the protein, other biological activities may still be retained. Thus, the ability of the shortened protein to induce and/or bind to antibodies which recognize the complete or mature form of the protein generally will be retained when less than the majority of the residues of the complete or mature protein are removed from the C-terminus. Whether a particular polypeptide lacking C-terminal residues of a complete protein retains such immunologic activities can readily be determined by routine methods described herein and otherwise known in the art.

As described herein in the examples, CD40wt is predicted to have a disulfide bond between Cys 125 and Cys 143. With respect to CD40sv2, Cys 143 is not present and therefore, Cys 125 is likely to be unpaired. To avoid improper disulfide bond formation, it may be desirable to delete the remainder of the subdomain (that is, expand the deletion so that it starts between residue 120 and 125 rather than at residue 135) or to replace Cys 125 with another amino acid. Accordingly, in some examples, muteins are designed to maintain the cysteines found in wild-type human CD40 (SEQ ID NO:13) and in other examples, are modified with respect to the cysteines present in CD40 splice variants in order to modify naturally.
occurring disulfide bond formation or to modify improper disulfide bond formation, such as found in muteins.

Other Mutants

[0169] In addition to terminal deletion forms of the protein discussed above, it also will be recognized by one of ordinary skill in the art that some amino acid sequences of the CD40 variant polypeptides can be varied without significant effect of the structure or function of the protein. If such differences in sequence are contemplated, it should be remembered that there will be critical areas on the protein which determine activity.

[0170] Thus, the invention further includes variations of the CD40 polypeptides of the invention which show substantial CD40 polypeptide activity or which include regions of the CD40 proteins such as the protein portions discussed below. Such mutants include deletions, insertions, inversions, repeats, and type substitutions selected according to general rules known in the art so as to have little effect on activity. For example, guidance concerning how to make phenotypically silent amino acid substitutions is provided in Bowie et al., "Deciphering the Message in Protein Sequences: Tolerance to Amino Acid Substitutions," Science, 247:1306-1310 (1990), wherein the authors indicate that there are two main approaches for studying the tolerance of an amino acid sequence to change. The first method relies on the process of evolution, in which mutations are either accepted or rejected by natural selection. The second approach uses genetic engineering to introduce amino acid changes at specific positions of a cloned gene and selections or screens to identify sequences that maintain functionality.

[0171] As the authors state, these studies have revealed that proteins are surprisingly tolerant of amino acid substitutions. The authors further indicate which amino acid changes are likely to be permissive at a certain position of the protein. For example, most buried amino acid residues require nonpolar side chains, whereas few features of surface side chains are generally conserved. Other such phenotypically silent substitutions are described in Bowie, J. U. et al., supra, and the references cited therein. Typically seen as conservative substitutions are the replacements, one for another, among the aliphatic amino acids Ala, Val, Leu and Ile; interchange of the hydroxyl residues Ser and Thr, exchange of the acidic residues Asp and Glu,
substitution between the amide residues Asn and Gln, exchange of the basic residues Lys and Arg and replacements among the aromatic residues Phe and Tyr.

Thus, a fragment, derivative or analog of the polypeptides of Appendices A-C or the polypeptide encoded by the nucleic acid sequence of Appendices A-C, may be (i) one in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue) and such substituted amino acid residue may or may not be one encoded by the genetic code, or (ii) one in which one or more of the amino acid residues includes a substituent group, or (iii) one in which the mature polypeptide is fused with another compound, such as a compound to increase the half-life of the polypeptide (for example, polyethylene glycol), or (iv) one in which the additional amino acids are fused to the above form of the polypeptide, such as an IgG Fc fusion region peptide, or other fusion partners described herein and known in the art, or leader or secretory sequence or a sequence which is employed for purification of the above form of the polypeptide or a proprotein sequence. Such fragments, derivatives and analogs are deemed to be within the scope of those skilled in the art from the teachings herein.

Thus, the CD40 polypeptides of the present invention may include one or more amino acid substitutions, deletions or additions, either from natural mutations or human manipulation. As indicated, changes are preferably of a minor nature, such as conservative amino acid substitutions that do not significantly affect the folding or activity of the protein as shown below.

Conservative amino acid substitutions are considered to fall within the following groups. Aromatic amino acids include phenylalanine, tryptophan and tyrosine. Hydrophobic amino acids include leucine, isoleucine and valine. Polar amino acids include glutamine and asparagine. Basic amino acids include arginine, lysine and histidine. Acidic amino acids include aspartic acid and glutamic acid. Small amino acids include alanine, serine, threonine, methionine and glycine.

Amino acids in the CD40 polypeptides of the present invention that are essential for function can be identified by methods known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis, see, for example, Cunningham and Wells, Science, 244:1081-1085 (1989). The latter procedure introduces single alanine mutations at every residue in the molecule. The resulting mutant molecules are then tested for biological activity such as receptor binding or in vitro or in vivo
proliferative activity. For example, CD40sv1 and CD40sv2 retain all of the amino acid residues, including aspartic acid (D)84, glutamic acid (E) 114, and E117, that are reported to be required for interaction with CD40L (Singh J, et. al. 1998). One of skill in the art would be able to determine whether a particular CD40 splice variant or mutein is capable of binding a CD40 ligand using methods known in the art.

[0176] Of special interest are substitutions of charged amino acids with other charged or neutral amino acids which may produce proteins with highly desirable improved characteristics, such as less aggregation. Aggregation may not only reduce activity but also be problematic when preparing pharmaceutical formulations, because aggregates can be immunogenic, for example, Pinckard et al., Clin. Exp. Immunol., 2:331-340 (1967); Robbins et al., Diabetes, 36:838-845 (1987); Cleland et al., Crit. Rev. Therapeutic Drug Carrier Systems, 10:307-377 (1993).

[0177] Replacement of amino acids can also change the selectivity of the binding of a ligand to cell surface receptors. For example, Ostade et al., Nature, 361:266-268 (1993) describes certain mutations resulting in selective binding of TNF-α to only one of the two known types of TNF receptors. Sites that are critical for ligand-receptor binding can also be determined by structural analysis such as crystallization, X-ray crystallography, nuclear magnetic resonance or photoaffinity labeling, for example, Smith et al., J. Mol. Biol., 224:899-904 (1992) and de Vos et al., Science, 255:306-312 (1992).

[0178] The polypeptides of the present invention are preferably provided in an isolated form, and preferably are substantially purified. A recombinantly produced version of the herein described CD40 polypeptides can be substantially purified by the one-step method described in Smith and Johnson, Gene, 67:31-40 (1988). Polypeptides of the invention also can be purified from natural or recombinant sources using anti-CD40 antibodies of the invention in methods which are well known in the art of protein purification.

[0179] Further polypeptides of the present invention include polypeptides which have at least about 93% similarity, more preferably at least about 95% similarity, and still more preferably at least about 96%, 97%, 98% or 99% similarity to those described above. The polypeptides of the invention also contain those which are at least about 93% identical, more preferably at least about 94% or 95% identical, still more preferably at least 96%, 97%, 98% or 99% identical to the polypeptide encoded by the nucleic acid sequences set forth in Appendices A-C.
By "% similarity" for two polypeptides is intended a similarity score produced by comparing the amino acid sequences of the two polypeptides using the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, Wis. 53711) and the default settings for determining similarity. Bestfit uses the local homology algorithm of Smith and Waterman, Advances in Applied Mathematics 2:482-489, (1981), to find the best segment of similarity between two sequences.

By a polypeptide having an amino acid sequence at least, for example, 95% "identical" to a reference amino acid sequence of a CD40 polypeptide is intended that the amino acid sequence of the polypeptide is identical to the reference sequence except that the polypeptide sequence may include up to five amino acid alterations per each 100 amino acids of the reference amino acid of the CD40 polypeptides. In other words, to obtain a polypeptide having an amino acid sequence at least 95% identical to a reference amino acid sequence, up to 5% of the amino acid residues in the reference sequence may be deleted or substituted with another amino acid, or a number of amino acids up to 5% of the total amino acid residues in the reference sequence may be inserted into the reference sequence. These alterations of the reference sequence may occur at the amino or carboxy terminal positions of the reference amino acid sequence or anywhere between those terminal positions, interspersed either individually among residues in the reference sequence or in one or more contiguous groups within the reference sequence.

As a practical matter, whether any particular polypeptide is at least about 93%, 95%, 96%, 97%, 98% or 99% identical to, for instance, the amino acid sequences shown in Appendices A-C or to the polypeptide sequence encoded by the nucleic acid sequences set forth in Appendices A-C, can be determined conventionally using known computer programs such the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, Wis. 53711). When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set, of course, such that the percentage of identity is calculated over the full length of the reference amino acid sequence and that gaps in homology of up to 5% of the total number of amino acid residues in the reference sequence are allowed.
The polypeptide of the present invention could be used as a molecular weight marker on SDS-PAGE gels or on molecular sieve gel filtration columns using methods well known to those of skill in the art. As described in detail below, the polypeptides of the present invention can also be used to raise polyclonal and monoclonal antibodies, which are useful in assays for detecting CD40 protein expression as described below or as agonists and antagonists capable of enhancing or inhibiting CD40 protein function. Further, such polypeptides can be used in the yeast two-hybrid system to “capture” CD40 protein binding proteins which are also candidate agonists and antagonists according to the present invention. The yeast two hybrid system is described in Fields and Song, *Nature*, 340:245-246 (1989).

Muteins may be molecularly engineered to better understand which factors determine expression levels of polypeptides, such as, for example CD40ECD and CD40sv1. The muteins are likely to retain the functional properties of soluble CD40, including the ability to bind CD40L.

CD40sv1 has been observed to have lower protein expression than the extracellular domains of human CD40ECD. This decrease in protein expression may result from a number of factors, including: (1) the absence of CD40wt domain 4 (residues 146-186), (2) the presence of CD40sv1’s new domain (residues 144-166), (3) sequence differences between CD40wt and CD40sv1 domain 3 (domain 3 comprises residues 105-143; residues 136-142 are different in CD40wt and CD40sv1), and (4) sequence differences adjacent to cysteine 143, which may affect the efficiency of disulfide bond formation between residues 125 and 143. It has been demonstrated that the level of protein expression of a homologous protein, TNFR1, was improved by incorporating mutations that limit conformational flexibility in the residues adjacent to disulfide bonded cysteine residues, so long as the mutations are compatible with the native structure (Schweickhardt et.al. (2003) J. Biol. Chem. 278:28961-28967).

Accordingly, provided herein is a mutein comprising CD40wt residues 20-145 (alternatively, 20-144 or 20-143). This region is equivalent to the first three domains of CD40wt. The protein expression of this mutein may be compared to the protein expression of CD4ECD to determine the impact of domain 4. The presence of domain 4 may affect the rate or efficiency of folding for the rest of the protein. Domain 4 has two N-linked glycosylation sites (at residues 153 and 180) and these modifications may contribute to the stability or solubility of CD40 molecules.
[0187] In some examples a mutein comprising CD40sv1 residues 20-145 (alternatively, 20-144 or 20-143) is provided. This region is equivalent to the first three domains of CD40sv1. The protein expression of this mutein may be compared to the protein expression of CD40sv1 to determine the impact of the new domain in CD40sv1.

[0188] In other examples, a mutein comprising CD40wt residues 20-145 (alternatively, 20-144 or e 20-143) and CD40sv1 residues 144-166 (alternatively, 145-166 or 146-166) is provided. These regions are equivalent to the first three domains of CD40wt and the new domain of CD40sv1. The protein expression of this mutein may be compared to the protein expression of CD40sv1 to determine the impact of the sequence differences in residues 136-142.

[0189] In yet other examples, a mutein comprising CD40sv1 residues 20-143 (alternatively, 20-144 or 20-145) and CD40wt residues 144-193 (alternatively, 145-193 or 146-193) is provided. These regions are equivalent to the first three domains of CD40sv1 and domain 4 of CD40wt. The protein expression of this mutein may be compared to the protein expression of CD40sv1 to determine the impact of the sequence differences in residues 136-142.

[0190] In some examples, a mutein comprising CD40sv1 residues 20-166 is constructed with a leucine to isoleucine substitution at residue 142. Isoleucine is present in CD40wt at position 142. Isoleucine, a beta-branched amino acid, has less backbone flexibility than leucine. As a result, this substitution may reduce conformational freedom in the unfolded state, increase the rate of folding, and improve protein expression.

[0191] In other examples, a mutein comprising CD40sv1 residues 20-166 is constructed, with an asparagine to glutamic acid substitution at residue 144 and an arginine to proline substitution at residue 145. Glutamic acid is present in CD40wt at position 144, and proline is present in CD40wt at position 145. Proline has significantly less backbone flexibility than other amino acids. As a result, this substitution may reduce conformational freedom in the unfolded state, increase the rate of folding, and improve protein expression.

[0192] Relative to human CD40wt, CD40sv2 is missing residues 136-187. The missing amino acids include the last 8 residues of domain 3 through the first residue after domain 4. CD40sv2 does include the six residues immediately preceding the transmembrane domain, which are numbered 188-193. CD40wt is
predicted to have a disulfide bond between Cys 125 and Cys 143; since Cys 143 is not present in CD40sv2, Cys 125 is likely to be unpaired. To avoid improper disulfide bond formation, it may be desirable to delete the remainder of the subdomain (that is, expand the deletion so that it starts between residue 120 and 125 rather than at residue 136) or to replace Cys 125 with another amino acid.

Accordingly, provided herein is a mutein comprising CD40sv2 with residues 125-135 deleted. This removes the second subdomain in domain 3 and eliminates the unpaired cysteine at residue 125.

In some examples a mutein is provided comprising CD40sv2 with Cys 125 replaced by any other amino acid (that is, aspartic acid, glutamic acid, arginine, lysine, histidine, glutamine, asparagine, serine, threonine, alanine, glycine, proline, valine, leucine, isoleucine, methionine, phenylalanine, tyrosine, or tryptophan). Alternatively, Cys 125 may be deleted. This substitution or deletion eliminates the unpaired cysteine at residue 125 while retaining the first part of the second subdomain of domain 3. Additional muteins are disclosed herein in the Examples and Figures.

**Epitope-Bearing Portions**

In another aspect, the invention provides a peptide or polypeptide comprising an epitope-bearing portion of a polypeptide of the invention. The epitope of this polypeptide portion is an immunogenic or antigenic epitope of a polypeptide of the invention. An “immunogenic epitope” is defined as a part of a protein that elicits an antibody response when the whole protein is the immunogen. On the other hand, a region of a protein molecule to which an antibody can bind is defined as an “antigenic epitope.” The number of immunogenic epitopes of a protein generally is less than the number of antigenic epitopes. See, for instance, Geysen et al., *Proc. Natl Acad. Sci.*, USA 81:3998-4002 (1983).

As to the selection of peptides or polypeptides bearing an antigenic epitope (i.e., that contain a region of a protein molecule to which an antibody can bind), it is well known in that art that relatively short synthetic peptides that mimic part of a protein sequence are routinely capable of eliciting an antiserum that reacts with the partially mimicked protein. See, for instance, Sutcliffe et al., *Science*, 219:660-666 (1983). Peptides capable of eliciting protein-reactive sera are frequently represented in the primary sequence of a protein, can be characterized by a set of simple chemical rules, and are confined neither to immunodominant regions of intact
proteins (i.e., immunogenic epitopes) nor to the amino or carboxyl terminals. Antigenic epitope-bearing peptides and polypeptides of the invention are therefore useful to raise antibodies, including monoclonal antibodies, that bind specifically to a polypeptide of the invention. See, for instance, Wilson et al., *Cell*, 37:767-778 (1984). In some examples, antibodies specifically bind a CD40 splice variant, that is, CD40sv1, CD40sv2 or CD40sv3, or epitopes thereof, or muteins of a CD40 splice variant, or epitopes thereof, and do not specifically bind to wild-type CD40 (NP_001241 NM_001250: SEQ ID NO:13) and do not specifically bind the polypeptides having the sequence as shown in SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:150, SEQ ID NO:152, SEQ ID NO:154, SEQ ID NO:155 or SEQ ID NO:156, or CD40 polypeptides known in the art as of the filing date of the application.


[0198] Epitope-bearing peptides and polypeptides of the invention are used to induce antibodies according to methods well known in the art. See, for instance, Bittle, F. J. et al, *J. Gen. Virol.*, 66:2347-2354 (1985). Immunogenic epitope-bearing peptides of the invention, i.e., those parts of a protein that elicit an antibody response when the whole protein is the immunogen, are identified according to methods known in the art. See, for instance, U.S. Pat. No. 5,194,392 (1990) describes a general method of detecting or determining the sequence of monomers (amino acids or other compounds) which is a topological equivalent of the epitope (i.e., a "mimotope") which is complementary to a particular paratope (antigen binding site) of an antibody of interest. More generally, U.S. Pat. No. 4,433,092 (1989) describes a method of detecting or determining a sequence of monomers which is a topographical equivalent of a ligand which is complementary to the ligand binding site of a particular receptor of interest. Similarly, U.S. Pat. No. 5,480,971 (1996) on Peralkylated Oligopeptide Mixtures discloses linear C1-C7-alkyl peralkylated oligopeptides and sets and libraries of such peptides, as well as methods for using such oligopeptide sets and libraries for determining the sequence of a peralkylated oligopeptide that preferentially binds to an acceptor molecule of interest. Thus, non-peptide analogs of the epitope-bearing peptides of the invention also can be made routinely by these methods.
Fusion Molecules

[0199] As one of skill in the art will appreciate, CD40 polypeptides of the present invention and the epitope-bearing fragments thereof described above can be combined with parts of other polypeptides to produce fusion polypeptides. The parts of other polypeptides are the “fusion partner” and may comprise, for example, a constant domain of an immunoglobulin, a leucine zipper domain, or a domain from tetranectin or mannose binding protein (also known in the art as a mannose binding lectin). In some examples, a mannose binding protein is MBP1. Molecular cloning and protein engineering techniques known in the art allow for the production of chimeric polypeptides.

[0200] In some examples, to facilitate trimerization or oligomerization of CD40sv1 and CD40sv2 and increase protein stability, CD40sv1 (amino acids 1 to 166) or the extracellular domains of CD40sv2 (amino acids 1 to 141) can be fused with a Fc domain, a leucine zipper domain, or a trimerization domain derived from tetranectin (Holtet TL, 1997) or mannose binding protein (MBP1). Thus, fusion of CD40sv1 and CD40sv2 to these oligomerization domains enhances their ability to inhibit CD40:CD40L interactions.

[0201] In order to induce the dimerization of polypeptides, for example, CD40 splice variants, the Fc domain of a human immunoglobulin can be linked to the CD40 molecule. In some examples the fusion proteins can also comprise a Tobacco etch virus (TEV) protease recognition site between the CD40 molecule and the Fc domain. This would allow for release of soluble CD40 from the Fc domain after incubation with TEV protease.

[0202] Some fusion proteins facilitate purification procedures and show an increased half-life in vivo. This has been shown, e.g., for chimeric proteins consisting of the first two domains of the human CD4 polypeptide and various domains of the constant regions of the heavy or light chains of mammalian immunoglobulins, for example, EP A 394,827; Traunecker et al., Nature, 331:84-86 (1988). Fusion proteins that have a disulfide-linked dimeric structure due to an IgG part can also be more efficient in binding and neutralizing other molecules than monomeric CD40 protein or protein fragment alone, for example, Fountoulakis et al., J. Biochem., 270:3958-3964 (1995).
In order to induce trimerization of CD40 polypeptides, protein fusions comprising a leucine zipper domain as the fusion partner can be constructed. In some examples the leucine zipper domain comes from a transcription factor such as NFE2 (nuclear factor erythroid-dervied 2) or NRL (neural retina leucine zipper). In other examples the fusion partner for trimerization comprises the collagen domain of tetranectin.

In other examples, in order to induce trimerization and higher order complex formation of CD40 polypeptides, fusion proteins comprising a collagen domain of the mannose binding protein 1 (MBP1) can be constructed. MBP1 contains cysteines upstream of the collagen domain thought to participate in the formation of higher order complexes. Therefore, in some examples, the effect of cysteines in higher order complex formation are tested by generating fusion proteins comprising CD40 polypeptides and a MBP1 domain without cysteine amino acid residues.

**Compositions**

In other aspects, the present invention provides a composition containing a therapeutically effective amount of a molecule, such as a polypeptide or nucleic acid sequence, of the invention and a pharmaceutically acceptable carrier or vehicle. In some examples, the composition comprises the polypeptide in monomer form, and in other examples, comprises the polypeptide in a multimer form, such as a dimer or trimer. The compositions are suitable for veterinary or human administration. The compositions of the present invention can be in any form that allows for the composition to be administered to an animal. For example, the composition can be in the form of a solid, liquid or gas (aerosol). Typical routes of administration include, without limitation, oral, topical, parenteral, sublingual, rectal, vaginal, ocular, and intranasal. Parenteral administration includes subcutaneous injections, intravenous, intramuscular, intrasternal injection or infusion techniques. Preferably, the compositions are administered parenterally. Pharmaceutical compositions of the invention can be formulated so as to allow a molecule of the invention to be bioavailable upon administration of the composition to an animal. Compositions can take the form of one or more dosage units, where for example, a tablet can be a single dosage unit, and a container of a molecule of the invention in aerosol form can hold a plurality of dosage units.
Accordingly, provided herein are nucleic acid compositions, vector compositions and host cell compositions. In some examples the compositions comprise a nucleic acid molecule comprising a polynucleotide sequence chosen from among: (A) SEQ ID NO:1, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:19, SEQ ID NO:23; SEQ ID NO:24; SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49, SEQ ID NO:50, SEQ ID NO:51, SEQ ID NO:52, SEQ ID NO:53, SEQ ID NO:54, SEQ ID NO:55, SEQ ID NO:56, SEQ ID NO:57, SEQ ID NO:58, SEQ ID NO:59, SEQ ID NO:60, SEQ ID NO:61, or SEQ ID NO:62 (B) polynucleotide sequences encoding polypeptides having the sequence as shown in SEQ ID NO:10, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:28-34, SEQ ID NO:95, SEQ ID NO:96, SEQ ID NO:97, SEQ ID NO:98, SEQ ID NO:99, SEQ ID NO:100, SEQ ID NO:101, SEQ ID NO:102, SEQ ID NO:103, SEQ ID NO:104, SEQ ID NO:105, SEQ ID NO:106, SEQ ID NO:107, SEQ ID NO:108, SEQ ID NO:109, SEQ ID NO:110, SEQ ID NO:111, SEQ ID NO:112, SEQ ID NO:113, SEQ ID NO:114, SEQ ID NO:115, SEQ ID NO:116, SEQ ID NO:117, SEQ ID NO:118, or SEQ ID NO:119; (C) biologically active fragments thereof; (D) a complement thereof and a carrier, wherein in some examples the carrier is a pharmaceutically acceptable carrier or a buffer.

In other examples, the compositions comprise a vector comprising a nucleic acid molecule comprising a polynucleotide sequence chosen from among: (A) SEQ ID NO:1, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:19, SEQ ID NO:23; SEQ ID NO:24; SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49, SEQ ID NO:50, SEQ ID NO:51, SEQ ID NO:52, SEQ ID NO:53, SEQ ID NO:54, SEQ ID NO:55, SEQ ID NO:56, SEQ ID NO:57, SEQ ID NO:58, SEQ ID NO:59, SEQ ID NO:60, SEQ ID NO:61, or SEQ ID NO:62 (B) polynucleotide sequences encoding polypeptides having the sequence as shown in SEQ ID NO:10, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:28-34, SEQ ID NO:95, SEQ ID NO:96, SEQ ID NO:97, SEQ ID NO:98, SEQ ID NO:99, SEQ ID NO:100, SEQ ID NO:101, SEQ ID NO:102, SEQ ID NO:103, SEQ ID NO:104, SEQ ID NO:105, SEQ ID NO:106, SEQ ID NO:107, SEQ ID NO:108, SEQ ID NO:109, SEQ ID NO:110, SEQ ID NO:111, SEQ ID NO:112, SEQ ID NO:113, SEQ ID NO:114, SEQ ID NO:115, SEQ ID NO:116, SEQ ID NO:117, SEQ ID NO:118, or
SEQ ID NO:119; (C) biologically active fragments thereof; (D) complements thereof and a promoter that regulates the expression of the nucleic acid molecule and a carrier.

[0208] In yet other examples there are vector compositions comprising a double-stranded isolated nucleic acid molecule comprising a polynucleotide sequence chosen from among: (A) SEQ ID NO:1, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:19, SEQ ID NO:23; SEQ ID NO:24; SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49, SEQ ID NO:50, SEQ ID NO:51, SEQ ID NO:52, SEQ ID NO:53, SEQ ID NO:54, SEQ ID NO:55, SEQ ID NO:56, SEQ ID NO:57, SEQ ID NO:58, SEQ ID NO:59, SEQ ID NO:60, SEQ ID NO:61, or SEQ ID NO:62 (B) polynucleotide sequences encoding polypeptides having the sequence as shown in SEQ ID NO:10, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:28-34, SEQ ID NO:95, SEQ ID NO:96, SEQ ID NO:97, SEQ ID NO:98, SEQ ID NO:99, SEQ ID NO:100, SEQ ID NO:101, SEQ ID NO:102, SEQ ID NO:103, SEQ ID NO:104, SEQ ID NO:105, SEQ ID NO:106, SEQ ID NO:107, SEQ ID NO:108, SEQ ID NO:109, SEQ ID NO:110, SEQ ID NO:111, SEQ ID NO:112, SEQ ID NO:113, SEQ ID NO:114, SEQ ID NO:115, SEQ ID NO:116, SEQ ID NO:117, SEQ ID NO:118, or SEQ ID NO:119; (C) biologically active fragments thereof; (D) complements thereof and its complement and a promoter that regulates the expression of the nucleic acid molecule and a carrier. In some examples the carrier is a pharmaceutically acceptable carrier or a buffer.

[0209] Provided herein are host cell compositions comprising a recombinant host cell comprising an isolated nucleic acid comprising a polynucleotide sequence chosen from among: (A) SEQ ID NO:1, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:19, SEQ ID NO:23; SEQ ID NO:24; SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49, SEQ ID NO:50, SEQ ID NO:51, SEQ ID NO:52, SEQ ID NO:53, SEQ ID NO:54, SEQ ID NO:55, SEQ ID NO:56, SEQ ID NO:57, SEQ ID NO:58, SEQ ID NO:59, SEQ ID NO:60, SEQ ID NO:61, or SEQ ID NO:62 (B) polynucleotide sequences encoding polypeptides having the sequence as shown in SEQ ID NO:10, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:28-34, SEQ ID NO:95, SEQ ID NO:96, SEQ ID NO:97, SEQ ID NO:98, SEQ ID NO:99, SEQ ID NO:100, SEQ ID NO:101, SEQ ID NO:102, SEQ
ID NO:103, SEQ ID NO:104, SEQ ID NO:105, SEQ ID NO:106, SEQ ID NO:107, 
SEQ ID NO:108, SEQ ID NO:109, SEQ ID NO:110, SEQ ID NO:111, SEQ ID 
NO:112, SEQ ID NO:113, SEQ ID NO:114, SEQ ID NO:115, SEQ ID NO:116, SEQ 
ID NO:117, SEQ ID NO:118, or SEQ ID NO:119; (C) biologically active fragments 
thereof; and (D) complements thereof and a carrier. In other examples host cell 
compositions comprising a recombinant host cell comprising an isolated polypeptide 
comprising an amino acid sequence chosen from among: SEQ ID NO:10, SEQ ID 
NO:14, SEQ ID NO:15, and SEQ ID NO:28-34, and active fragments thereof and a 
carrier are provided. In other examples host cell compositions comprising a 
recombinant host cell comprising a vector comprising a nucleic acid molecule 
comprising a polynucleotide sequence chosen from among: (A) SEQ ID NO:1, SEQ 
ID NO:5, SEQ ID NO:6, SEQ ID NO:19, SEQ ID NO:23; SEQ ID NO:24; SEQ ID 
NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:42, SEQ ID 
NO:43, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:47, SEQ ID 
NO:48, SEQ ID NO:49, SEQ ID NO:50, SEQ ID NO:51, SEQ ID NO:52, SEQ ID 
NO:53, SEQ ID NO:54, SEQ ID NO:55, SEQ ID NO:56, SEQ ID NO:57, SEQ ID 
NO:58, SEQ ID NO:59, SEQ ID NO:60, SEQ ID NO:61, or SEQ ID NO:62 (B) 
polynucleotide sequences encoding polypeptides having the sequence as shown in 
SEQ ID NO:10, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:28-34, SEQ ID NO:95, 
SEQ ID NO:96, SEQ ID NO:97, SEQ ID NO:98, SEQ ID NO:99, SEQ ID NO:100, 
SEQ ID NO:101, SEQ ID NO:102, SEQ ID NO:103, SEQ ID NO:104, SEQ ID 
NO:105, SEQ ID NO:106, SEQ ID NO:107, SEQ ID NO:108, SEQ ID NO:109, SEQ 
ID NO:110, SEQ ID NO:111, SEQ ID NO:112, SEQ ID NO:113, SEQ ID NO:114, 
SEQ ID NO:115, SEQ ID NO:116, SEQ ID NO:117, SEQ ID NO:118, or SEQ ID 
NO:119; (C) biologically active fragments thereof; (D) complements thereof; and a 
promoter that regulates the expression of the nucleic acid molecule are provided. In 
other examples host cell compositions comprising a recombinant host cell comprising 
a vector comprising a double-stranded isolated nucleic acid molecule comprising a 
nucleic acid molecule comprising a polynucleotide sequence chosen from among: (A) 
SEQ ID NO:1, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:19, SEQ ID NO:23; SEQ 
ID NO:24; SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID 
NO:42, SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:46, SEQ ID 
NO:47, SEQ ID NO:48, SEQ ID NO:49, SEQ ID NO:50, SEQ ID NO:51, SEQ ID 
NO:52, SEQ ID NO:53, SEQ ID NO:54, SEQ ID NO:55, SEQ ID NO:56, SEQ ID
NO:57, SEQ ID NO:58, SEQ ID NO:59, SEQ ID NO:60, SEQ ID NO:61, or SEQ ID NO:62 (B) polynucleotide sequences encoding polypeptides having the sequence as shown in SEQ ID NO:10, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:28-34, SEQ ID NO:95, SEQ ID NO:96, SEQ ID NO:97, SEQ ID NO:98, SEQ ID NO:99, SEQ ID NO:100, SEQ ID NO:101, SEQ ID NO:102, SEQ ID NO:103, SEQ ID NO:104, SEQ ID NO:105, SEQ ID NO:106, SEQ ID NO:107, SEQ ID NO:108, SEQ ID NO:109, SEQ ID NO:110, SEQ ID NO:111, SEQ ID NO:112, SEQ ID NO:113, SEQ ID NO:114, SEQ ID NO:115, SEQ ID NO:116, SEQ ID NO:117, SEQ ID NO:118, or SEQ ID NO:119; (C) biologically active fragments thereof; (D) complements thereof and its complement and a promoter that regulates the expression of the nucleic acid molecule and a carrier are provided. In some examples the carrier is a pharmaceutically acceptable carrier or a buffer.

[0210] Materials used in preparing the pharmaceutical compositions can be non-toxic in the amounts used. It will be evident to those of ordinary skill in the art that the optimal dosage of the active ingredient(s) in the pharmaceutical composition will depend on a variety of factors. Relevant factors include, without limitation, the type of animal (e.g., human), the particular form of the molecule of the invention, the manner of administration, and the composition employed.

[0211] The pharmaceutically acceptable carrier or vehicle can be particulate, so that the compositions are, for example, in tablet or powder form. The carrier(s) can be liquid, with the compositions being, for example, an oral syrup or injectable liquid. In addition, the carrier(s) can be gaseous, so as to provide an aerosol composition useful in, e.g., inhalatory administration.

[0212] When intended for oral administration, the composition is preferably in solid or liquid form, where semi-solid, semi-liquid, suspension and gel forms are included within the forms considered herein as either solid or liquid. As a solid composition for oral administration, the composition can be formulated into a powder, granule, compressed tablet, pill, capsule, chewing gum, wafer or the like form. Such a solid composition typically contains one or more inert diluents. In addition, one or more of the following can be present: binders such as carboxymethylcellulose, ethyl cellulose, microcrystalline cellulose, or gelatin; excipients such as starch, lactose or dextrins, disintegrating agents such as alginic acid or sodium.
Identification of Agonists and Antagonists for Molecules of the Invention

Agonists and Antagonists—Assays and Molecules

[0213] This invention provides a method of screening compounds to identify those which modulate the action of the polypeptide of the present invention. An example of such an assay comprises combining a mammalian fibroblast cell and the polypeptide(s) of the present invention, the compound to be screened and \(^{3}\text{H}\) thymidine under cell culture conditions where the fibroblast cell would normally proliferate. A control assay may be performed in the absence of the compound to be screened and compared to the amount of fibroblast proliferation in the presence of the compound to determine if the compound stimulates proliferation by determining the uptake of \(^{3}\text{H}\) thymidine in each case. The amount of fibroblast cell proliferation is measured by liquid scintillation chromatography which measures the incorporation of \(^{3}\text{H}\) thymidine. Both agonist and antagonist compounds may be identified by this procedure.

[0214] In another method, a mammalian cell or membrane preparation expressing a receptor for a polypeptide of the present invention, as described above, is incubated with a labeled polypeptide of the present invention in the presence of the compound. The ability of the compound to enhance or block this interaction could then be measured. Alternatively, the response of a known second messenger system following interaction of a compound to be screened and the CD40 receptor is measured and the ability of the compound to bind to the receptor and elicit a second messenger response is measured to determine if the compound is a potential agonist or antagonist. Such second messenger systems include but are not limited to, cAMP guanylate cyclase, ion channels or phosphoinositide hydrolysis.

[0215] Examples of antagonist compounds include antibodies, or in some cases, oligonucleotides, which bind to the receptor for the polypeptide of the present invention but elicit no second messenger response or bind to the CD40 polypeptide itself. Alternatively, a potential antagonist may be a mutant form of the polypeptide which binds to the receptor, however, no second messenger response is elicited and, therefore, the action of the polypeptide is effectively blocked.

[0216] Another antagonist compound to the CD40 gene and gene product is an antisense construct prepared using antisense technology. Antisense technology can be used to control gene expression through triple-helix formation or antisense DNA or RNA, both of which methods are based on binding of a polynucleotide to DNA or
RNA. For example, the 5' coding portion of the polynucleotide sequence, which encodes for the mature polypeptides of the present invention, is used to design an antisense RNA oligonucleotide of from about 10 to 40 base pairs in length. A DNA oligonucleotide is designed to be complementary to a region of the gene involved in transcription, for example, triple helix—see Lee et al., *Nucl. Acids Res.*, 6:3073 (1979); Cooney et al, *Science*, 241:456 (1988); and Dervan et al., *Science*, 251: 1360 (1991), thereby preventing transcription and the production of the polypeptides of the present invention. The antisense RNA oligonucleotide hybridizes to the mRNA in vivo and blocks translation of the mRNA molecule into the polypeptide, Antisense—Okano, *J. Neurochem.*, 56:560 (1991); Oligodeoxynucleotides as Antisense Inhibitors of Gene Expression, CRC Press, Boca Raton, Fla. (1988). The oligonucleotides described above can also be delivered to cells such that the antisense RNA or DNA may be expressed in vivo to inhibit production of the polypeptide.

[0217] Potential antagonist compounds also include small molecules which bind to and occupy the binding site of the receptors thereby making the receptor inaccessible to its polypeptide such that normal biological activity is prevented. Examples of small molecules include, but are not limited to, small peptides or peptide-like molecules. Antagonist compounds may be employed to inhibit the cell growth and proliferation effects of the polypeptides of the present invention on neoplastic cells and tissues, i.e. stimulation of angiogenesis of tumors, and, therefore, retard or prevent abnormal cellular growth and proliferation, for example, in tumor formation or growth.

[0218] The antagonists may also be employed to prevent hyper-vascular diseases, and prevent the proliferation of epithelial lens cells after extracapsular cataract surgery. Prevention of the mitogenic activity of the polypeptides of the present invention may also be desirous in cases such as restenosis after balloon angioplasty. The antagonists may also be employed to prevent the growth of scar tissue during wound healing.

[0219] The present invention also provides methods for identifying agents, such as antibodies, which enhance or block the action of CD40 molecules on cells, such as its interaction with CD40-binding molecules such as receptor molecules. An agonist is a compound which increases the natural biological functions of CD40 molecules or which functions in a manner similar to CD40 molecules, while antagonists decrease or eliminate such functions.
For example, a cellular compartment, such as a membrane preparation, may be prepared from a cell that expresses a molecule that binds CD40 molecules, such as a molecule of a signaling or regulatory pathway modulated by CD40 molecules. The preparation is incubated with labeled CD40 molecules in the absence or the presence of a candidate molecule which may be a CD40 agonist or antagonist. The ability of the candidate molecule to bind the binding molecule or CD40 molecules themselves is reflected in decreased binding of the labeled ligand. Molecules which bind gratuitously, that is, without inducing the effects of CD40 molecules when bound to the CD40 binding molecules, are most likely to be good antagonists.

CD40-like effects of potential agonists and antagonists may be measured, for instance, by determining activity of a second messenger system following interaction of the candidate molecule with a cell or appropriate cell preparation, and comparing the effect with that of CD40 molecules or molecules that elicit the same effects as CD40. Second messenger systems that may be useful in this regard include but are not limited to AMP guanylate cyclase, ion channel or phosphoinositide hydrolysis second messenger systems.

Another example of an assay for CD40 antagonists is a competitive assay that combines CD40 molecules and a potential antagonist with membrane-bound CD40 receptor molecules or recombinant CD40 receptor molecules under appropriate conditions for a competitive inhibition assay. CD40 molecules can be labeled, such as by radioactivity, such that the number of CD40 molecules bound to a receptor molecule can be determined accurately to assess the effectiveness of the potential antagonist.

Potential antagonists include small organic molecules, peptides, polypeptides and antibodies that bind to a polypeptide of the invention, and thereby inhibit or extinguish its activity. Potential antagonists also may be small organic molecules, a peptide, a polypeptide such as a closely related protein or antibody that binds the same sites on a binding molecule, such as a receptor molecule, without inducing CD40-induced activities, thereby preventing the action of CD40 molecules by excluding CD40 molecules from binding. Antagonists of the invention include fragments of the CD40 molecules having the nucleic acid and amino acid sequences shown in Appendices A-C.
Other potential antagonists include antisense molecules. Antisense technology can be used to control gene expression through antisense DNA or RNA or through triple-helix formation. Antisense techniques are discussed, for example, in Okano, *J. Neurochem.*, 56:560 (1991); Oligodeoxynucleotides as Antisense Inhibitors of Gene Expression, CRC Press, Boca Raton, Fla. (1988). Triple helix formation is discussed in, for instance, Lee et al., *Nucleic Acids Research*, 6:3073 (1979); Cooney et al., *Science*, 241:456 (1988); and Dervan et al., *Science*, 251:1360 (1991). The methods are based on binding of a polynucleotide to a complementary DNA or RNA. For example, the 5′ coding portion of a polynucleotide that encodes the mature polypeptide of the present invention may be used to design an antisense RNA oligonucleotide of from about 10 to 40 base pairs in length. A DNA oligonucleotide is designed to be complementary to a region of the gene involved in transcription thereby preventing transcription and the production of CD40 molecules. The antisense RNA oligonucleotide hybridizes to the mRNA in vivo and blocks translation of the mRNA molecule into a CD40 polypeptide. The oligonucleotides described above can also be delivered to cells such that the antisense RNA or DNA may be expressed in vivo to inhibit production of CD40 molecules.

**Chromosome Assays**

In certain preferred embodiments relating to chromosomal mapping, the cDNA herein disclosed is used to clone genomic nucleic acid of the CD40 splice variants of the invention. This can be accomplished using a variety of well known techniques and libraries, which generally are available commercially. The genomic DNA then is used for in situ chromosome mapping using well known techniques for this purpose. Therefore, the nucleic acid molecules of the present invention are also valuable for chromosome identification. The sequence is specifically targeted to and can hybridize with a particular location on an individual human chromosome. Moreover, there is a current need for identifying particular sites on the chromosome. Few chromosome marking reagents based on actual sequence data (repeat polymorphisms) are presently available for marking chromosomal location. The mapping of DNAs to chromosomes according to the present invention is an important first step in correlating those sequences with genes associated with disease.

Briefly, sequences can be mapped to chromosomes by preparing PCR primers from the cDNA. Computer analysis of the 3′ untranslated region is used to
rapidly select primers that do not span more than one exon in the genomic DNA, thus complicating the amplification process. These primers are then used for PCR screening of somatic cell hybrids containing individual human chromosomes. Only those hybrids containing the human gene corresponding to the primer will yield an amplified fragment.

[0227] PCR mapping of somatic cell hybrids is a rapid procedure for assigning a particular DNA to a particular chromosome. Using the present invention with the same oligonucleotide primers, sublocalization can be achieved with panels of fragments from specific chromosomes or pools of large genomic clones in an analogous manner. Other mapping strategies that can similarly be used to map to its chromosome include in situ hybridization, prescreening with labeled flow-sorted chromosomes and preselection by hybridization to construct chromosome specific-cDNA libraries.

[0228] Fluorescence in situ hybridization (FISH) of a cDNA clone to a metaphase chromosomal spread can be used to provide a precise chromosomal location in one step. This technique can be used with cDNA as short as 50 or 60 bases. For a review of this technique, see Verma et al., Human Chromosomes: A Manual of Basic Techniques, Pergamon Press, New York (1988).

[0229] Once a sequence has been mapped to a precise chromosomal location, the physical position of the sequence on the chromosome can be correlated with genetic map data. (Such data are found, for example, in V. McKusick, Mendelian Inheritance in Man (available on line through Johns Hopkins University Welch Medical Library). The relationship between genes and diseases that have been mapped to the same chromosomal region are then identified through linkage analysis (coinheritance of physically adjacent genes).

[0230] Next, it is necessary to determine the differences in the cDNA or genomic sequence between affected and unaffected individuals. If a mutation is observed in some or all of the affected individuals but not in any normal individuals, then the mutation is likely to be the causative agent of the disease. With current resolution of physical mapping and genetic mapping techniques, a cDNA precisely localized to a chromosomal region associated with the disease could be one of between 50 and 500 potential causative genes. Note: this assumes 1 megabase mapping resolution and one gene per 20 kb.
Thrombosis Assays

[0231] Arterial thrombosis is a high-shear, platelet-dependent process. Various in vitro and in vivo models are available for assessing the effects of polypeptides, fragments, fusion proteins, antibodies, agonists and antagonists on thrombosis. Platelet adhesion, activation and aggregation can be evaluated using methods described herein or known in the art.

[0232] A platelet aggregation assay can be used to demonstrate whether a polypeptide or fragments thereof can inhibit activation of platelets thereby decreasing the possibility of thrombi formation during treatment. Briefly, the change in light transmission is measured through a stirred suspension of platelets in an aggregometer, for example, Chrono-Log 560A Whole Blood Lumi-Aggregometer (Chrono-Log Corp., Haverton, PA) according to manufacturer's instructions. Studies employing ADP are performed with platelet-rich plasma (PRP), which is obtained by low-speed centrifugation (200×g for 10 min.) of whole blood freshly drawn into trisodium citrate (at a final concentration of 11 mM). In studies using thrombin, the PRP is gel-filtered on Sepharose 2B in divalent ion-free Tyrode's solution containing 2% BSA. For all studies, the reference standard is platelet-poor plasma, which is obtained by centrifuging PRP at 1000×g for 5 min. The PRP and gel-filtered platelets are best used within three hours from the time of blood collection.

[0233] Aggregation studies are performed at 37°C. with a constantly stirred suspension of 3×10^8 platelets/ml. Platelet count can be determined with the aid of a hemacytometer. Washed platelets were allowed to stir in an aggregometer before the addition of test samples such as CD40 polynucleotides, polypeptides, agonists or antagonists and controls. Aggregation is allowed to proceed for five minutes and the amplitude of each aggregometer tracing can be calculated by, for example, Aggro-Link software.

[0234] The anti-aggregation potencies of test samples such as CD40 polynucleotides, polypeptides, agonists or antagonists can be determined from dose-responsive curves for the inhibition of the maximum aggregation responses stimulated by physiologic doses of ADP (10 μm) and thrombin (2 U/ml). The 50% inhibitory concentration of each test sample (IC_{50}) can be determined by regression analysis of these curves.

Therapeutic Uses of CD40 variants.
CD40 polynucleotides, polypeptides, agonists or antagonists of the invention may be used in developing treatments for any disorder mediated (directly or indirectly) by defective, or insufficient amounts of CD40 molecules. CD40 polypeptides, agonists or antagonists may be administered to a patient (e.g., mammal, preferably human) afflicted with such a disorder. Alternatively, a gene therapy approach may be applied to treat such disorders. Disclosure herein of CD40 nucleotide sequences permits the detection of defective CD40, and the replacement thereof with normal CD40-encoding genes. Defective genes may be detected in in vitro diagnostic assays, and by comparison of the CD40 nucleotide sequences disclosed herein with that of a CD40 gene derived from a patient suspected of harboring a defect in this gene.

Accordingly provided herein are methods for treatment of a disease or condition in a subject comprising the steps of:

(a) providing a composition comprising sCD40; and
(b) administering the composition to the subject.

In some examples, the methods for treatment of a disease or condition in a subject the composition further comprises a pharmaceutically acceptable carrier.

Provided herein are examples of methods for treatment of a disease wherein the disease is selected from the group consisting of diabetes and cardiovascular diseases. In some examples, diabetes is type II diabetes mellitus, and in other examples is type I diabetes mellitus, and in other examples, is impaired glucose tolerance or metabolic syndrome. Cardiovascular disease is selected from the group consisting of: atherosclerosis, coronary artery disease, acute coronary syndrome, myocardial infarction, unstable angina, stroke and chronic heart failure.

In some examples of the above methods for treatment of a disease, the disease or condition is restenosis and wherein the composition is administered to the subject prior to performance of percutaneous transluminal coronary angioplasty (PTCA) on the subject.

In further examples of the above methods for treatment of a disease, the disease is inflammation wherein the disease includes inflammatory bowel disease (IBD), Crohn’s disease or ulcerative colitis. In other examples, the disease is inflammation wherein the inflammation is associated with coronary stenting, coronary artery bypass surgery (CABG), balloon angioplasty, percutaneous coronary intervention, familial hypercholesterolemia or the central nervous system.
In some examples of the methods for treatment of a disease, the disease is thrombosis, acute cerebral ischemia, mixed connective tissue disease, Kawasaki disease, bleeding, graft versus host disease, pulmonary embolism, anti-cardiolipin antibody syndrome, asthma, an allergy, a CNS disease, or Alzheimer’s disease. In some examples, the disease is transplant rejection, which can be administered before, after or simultaneously with the transplant, such as for example, heart transplant, kidney transplant etc.

Also provided are methods for treatment of a disease wherein the disease is an immune cell related disease and wherein the disease is an autoimmune disease. In some examples the autoimmune disease is selected from the group consisting of: diabetes type 1, systemic lupus erythematosis, immune or idiopathic thrombocytopenic purpura, rheumatoid arthritis, multiple sclerosis, myasthenia gravis and psoriasis. In other examples the disease is an immune cell related disease wherein the disease is allograft or transplant rejection.

Provided herein are methods for treatment of a disease or condition in a subject comprising the steps of:
(a) providing a composition comprising sCD40;
(b) administering the composition to the subject; and
(c) administering another therapeutic agent to the subject. In some examples the other therapeutic agent is a statin and the disease is a cardiovascular or inflammatory disease.

Also provided are methods for treatment of a disease or condition in a subject comprising the steps of:
(a) providing a composition comprising sCD40;
(b) administering the composition to the subject; wherein the disease is cancer. In some examples the cancer is a hematological malignancy, a carcinoma, sarcoma, glioma, B-cell non-Hodgkins lymphoma, multiple myeloma and chronic lymphocytic leukemia. In other examples the carcinoma is gastric carcinoma, ovarian carcinoma, colon carcinoma, breast carcinoma and lung carcinoma. In some examples the sarcoma is osteosarcoma or Ewing sarcoma.

CD40 polypeptides of the present invention may be employed to treat lymphoproliferative disease which results in lymphadenopathy, the CD40 molecules may mediate apoptosis by stimulating clonal deletion of T-cells and may therefore, be employed to treat autoimmune disease to stimulate peripheral tolerance and cytotoxic
T-cell mediated apoptosis. The CD40 molecules may also be employed as a research tool in elucidating the biology of autoimmune disorders including systemic lupus erythematosus (SLE), Graves' disease, immunoproliferative disease lymphadenopathy (IPL), angioimmunoproliferative lymphadenopathy (AIL), immunoblastic lymphadenopathy (IBL), rheumatoid arthritis, diabetes, and multiple sclerosis, allergies and to treat graft versus host disease.

[0246] The CD40 polynucleotides, polypeptides and/or agonists or antagonists of the invention may also be used to treat, prevent, diagnose and/or prognose diseases which include, but are not limited to, autoimmune disorders, immunodeficiency disorders, and graft versus host disease.

[0247] The CD40 polypeptides of the present invention may also be employed to inhibit neoplasia, such as tumor cell growth. The CD40 polypeptides may be responsible for tumor destruction through apoptosis and cytotoxicity to certain cells.

[0248] Accordingly provided herein are methods of inducing apoptosis for treatment of cancer or inflammation in a subject comprising the steps of

(a) providing an antibody that specifically binds to or interferes with the activity of CD40 splice variants; and

(b) administering the antibody to the subject; wherein the antibody inhibits proliferation of CD40+ cells in the subject. In some examples of a method of inducing apoptosis for treatment of cancer the cancer cells are glioma cells.

[0249] Diseases associated with increased cell survival, or the inhibition of apoptosis, that may be treated, prevented, diagnosed and/or prognosed with the CD40 polynucleotides, polypeptides and/or agonists or antagonists of the invention include, but are not limited to, cancers (such as follicular lymphomas, carcinomas with p53 mutations, and hormone-dependent tumors, including, but not limited to colon cancer, cardiac tumors, pancreatic cancer, melanoma, retinoblastoma, glioblastoma, lung cancer, intestinal cancer, testicular cancer, stomach cancer, neuroblastoma, myxoma, mycoma, lymphoma, endometrioma, osteoblastoma, osteoclastoma, osteosarcoma, chondrosarcoma, adenoma, breast cancer, prostate cancer, Kaposi's sarcoma and ovarian cancer); autoimmune disorders (such as, multiple sclerosis, Sjogren's syndrome, Grave's disease, Hashimoto's thyroiditis, autoimmune diabetes, biliary cirrhosis, Behcet's disease, Crohn's disease, polymyositis, systemic lupus erythematosus and immune-related glomerulonephritis, autoimmune gastritis, autoimmune thrombocytopenic purpura, and rheumatoid arthritis), viral infections
(such as herpes viruses, pox viruses and adenoviruses), inflammation, graft vs. host disease (acute and/or chronic), acute graft rejection, and chronic graft rejection. In preferred embodiments, CD40 polynucleotides, polypeptides, agonists, or antagonists of the invention are used to inhibit growth, progression, and/or metastasis of cancers, in particular those listed above or in the paragraph that follows.

[0250] Additional diseases or conditions associated with increased cell survival, that may be treated, prevented, diagnosed and/or prognosed with the CD40 splice variants polynucleotides, polypeptides and/or agonists or antagonists of the invention include, but are not limited to, progression, and/or metastases of malignancies and related disorders such as leukemia including acute leukemias (e.g., acute lymphocytic leukemia, acute myelocytic leukemia (including myeloblastic, promyelocytic, myelomonocytic, monocytic, and erythroleukemia)) and chronic leukemias (e.g., chronic myelocytic (granulocytic) leukemia and chronic lymphocytic leukemia), polycythemia vera, lymphomas (e.g., Hodgkin's disease and non-Hodgkin's disease), multiple myeloma, Waldenstrom's macroglobulinemia, heavy chain diseases, and solid tumors including, but not limited to, sarcomas and carcinomas such as fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyo sarcoma, colon 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, Wilm's tumor, cervical cancer, testicular tumor, lung carcinoma, small cell lung carcinoma, bladder carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, menangioma, melanoma, neuroblastoma and retinoblastoma.

[0251] Diseases associated with increased apoptosis, that may be treated, prevented, diagnosed and/or prognosed with the CD40 polynucleotides, polypeptides and/or agonists or antagonists of the invention include, but are not limited to, AIDS; neurodegenerative disorders (such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, retinitis pigmentosa, cerebellar degeneration and brain
tumor or prior associated disease); autoimmune disorders (such as, multiple sclerosis, Sjogren's syndrome, Grave's disease Hashimoto's thyroiditis, autoimmune diabetes, biliary cirrhosis, Behcet's disease, Crohn's disease, polymyositis, systemic lupus erythematosis, immune-related glomerulonephritis, autoimmune gastritis, thrombocytopenic purpura, and rheumatoid arthritis), myelodysplastic syndromes (such as aplastic anemia), graft vs. host disease (acute and/or chronic), ischemic injury (such as that caused by myocardial infarction, stroke and reperfusion injury), liver injury or disease (e.g., hepatitis related liver injury, cirrhosis, ischemia/reperfusion injury, cholestasis (bile duct injury) and liver cancer); toxin-induced liver disease (such as that caused by alcohol), septic shock, ulcerative colitis, cachexia and anorexia. In preferred embodiments, CD40 polynucleotides, polypeptides, agonists, and/or antagonists are used to treat the diseases and disorders listed above.

[0252] Additionally, molecules of the invention may be employed as an agent to boost immunoresponsiveness among individuals having a temporary immune deficiency. Conditions resulting in a temporary immune deficiency that may be ameliorated or treated by administering the CD40 polypeptides or polynucleotides of the invention, or agonists thereof, include, but are not limited to, recovery from viral infections (e.g., influenza), conditions associated with malnutrition, recovery from infectious mononucleosis, or conditions associated with stress, recovery from measles, recovery from blood transfusion, and recovery from surgery.

[0253] Accordingly, provided herein are methods of stimulating or enhancing an immune response in a subject comprising the steps of:

(a) providing a modulator of CD40 TM-splice variants; and
(b) administering the modulator to the subject; wherein the modulator specifically activates the CD40+ immune cells in the subject.

[0254] CD40 polynucleotides or polypeptides of the invention, or agonists or antagonists thereof, may be used to diagnose, prognose, treat or prevent one or more of the following diseases or disorders, or conditions associated therewith: primary immunodeficiencies, immune-mediated thrombocytopenia, Kawasaki syndrome, bone marrow transplant (e.g., recent bone marrow transplant in adults or children), chronic B-cell lymphocytic leukemia, HIV infection (e.g., adult or pediatric HIV infection), chronic inflammatory demyelinating polyneuropathy, and post-transfusion purpura.
Additionally, CD40 nucleic acids or polypeptides of the invention, or agonists or antagonists thereof, may be used to diagnose, prognose, treat or prevent one or more of the following diseases, disorders, or conditions associated therewith, Guillain-Barre syndrome, anemia (e.g., anemia associated with parvovirus B19, patients with stable multiple myeloma who are at high risk for infection (e.g., recurrent infection), autoimmune hemolytic anemia (e.g., warm-type autoimmune hemolytic anemia), thrombocytopenia (e.g., neonatal thrombocytopenia), and immune-mediated neutropenia), transplantation (e.g., cytomegalovirus (CMV)-negative recipients of CMV-positive organs), hypogammaglobulinemia (e.g., hypogammaglobulinemic neonates with risk factor for infection or morbidity), epilepsy (e.g., intractable epilepsy), systemic vasculitic syndromes, myasthenia gravis (e.g., decompensation in myasthenia gravis), dermatomyositis, and polymyositis.

Autoimmune disorders and conditions associated with these disorders that may be treated, prevented, and/or diagnosed with the CD40 polynucleotides, polypeptides, and/or antagonist of the invention (e.g., anti-CD40 antibodies), include, but are not limited to, autoimmune hemolytic anemia, autoimmune neonatal thrombocytopenia, idiopathic thrombocytopenia purpura, autoimmune vasculitis, hemolytic anemia, antiphospholipid syndrome, dermatitis, allergic encephalomyelitis, myocarditis, relapsing polychondritis, rheumatic heart disease, glomerulonephritis (e.g., IgA nephropathy), Multiple Sclerosis, Neuritis, Uveitis Ophthalmia, Polyendocrinopathies, Purpura (e.g., Henoch-Schonlein purpura), Reiter's disease, Stiff-Man Syndrome, Autoimmune Pulmonary Inflammation, Guillain-Barre Syndrome, insulin dependent diabetes mellitus, and autoimmune inflammatory eye disease.

Additional autoimmune disorders that may be treated, prevented, and/or diagnosed with the compositions of the invention include, but are not limited to, autoimmune thyroiditis, hypothyroidism (i.e., Hashimoto's thyroiditis) (often characterized, e.g., by cell-mediated and humoral thyroid cytotoxicity), systemic lupus erythematosus (often characterized, e.g., by circulating and locally generated immune complexes), Goodpasture's syndrome (often characterized, e.g., by anti-basement membrane antibodies), Pemphigus (often characterized, e.g., by epidermal acantholytic antibodies), receptor autoimmunities such as, for example, (a) Graves' disease (often characterized, e.g., by TSH receptor antibodies), (b) Myasthenia Gravis (often characterized, e.g., by acetylcholine receptor antibodies), and (c) insulin
resistance (often characterized, e.g., by insulin receptor antibodies), autoimmune hemolytic anemia (often characterized, e.g., by phagocytosis of antibody-sensitized RBCs), autoimmune thrombocytopenic purpura (often characterized, e.g., by phagocytosis of antibody-sensitized platelets).

[0258] Additional autoimmune disorders that may be treated, prevented, and/or diagnosed with the compositions of the invention include, but are not limited to, rheumatoid arthritis (often characterized, e.g., by immune complexes in joints), scleroderma with anti-collagen antibodies (often characterized, e.g., by nucleolar and other nuclear antibodies), mixed connective tissue disease (often characterized, e.g., by antibodies to extractable nuclear antigens (e.g., ribonucleoprotein)), polymyositis/dermatomyositis (often characterized, e.g., by nonhistone ANA), pernicious anemia (often characterized, e.g., by antiparietal cell, microsomes, and intrinsic factor antibodies), idiopathic Addison's disease (often characterized, e.g., by humoral and cell-mediated adrenal cytotoxicity, infertility (often characterized, e.g., by antispermatozoa antibodies), glomerulonephritis (often characterized, e.g., by glomerular basement membrane antibodies or immune complexes) such as primary glomerulonephritis and IgA nephropathy, bullous pemphigoid (often characterized, e.g., by IgG and complement in basement membrane), Sjogren's syndrome (often characterized, e.g., by multiple tissue antibodies, and/or a specific nonhistone ANA (SS-B)), diabetes mellitus (often characterized, e.g., by cell-mediated and humoral islet cell antibodies), and adrenergic drug resistance (including adrenergic drug resistance with asthma or cystic fibrosis) (often characterized, e.g., by beta-adrenergic receptor antibodies).

[0259] Additional autoimmune disorders that may be treated, prevented, and/or diagnosed with the compositions of the invention include, but are not limited to, chronic active hepatitis (often characterized, e.g. by smooth muscle antibodies), primary biliary cirrhosis (often characterized, e.g., by mitochondrial antibodies), other endocrine gland failure (often characterized, e.g., by specific tissue antibodies in some cases), vitiligo (often characterized, e.g., by melanocyte antibodies), vasculitis (often characterized, e.g., by Ig and complement in vessel walls and/or low serum complement), post-MI (often characterized, e.g., by myocardial antibodies), cardiomyopathy syndrome (often characterized, e.g., by myocardial antibodies), urticaria (often characterized, e.g., by IgG and IgM antibodies to IgE), atopic dermatitis (often characterized, e.g., by IgG and IgM antibodies to IgE), asthma (often characterized,
e.g., by IgG and IgM antibodies to IgE), inflammatory myopathies, and many other inflammatory, granulomatous, degenerative, and atrophic disorders.

[0260] In an additional embodiment, CD40 polynucleotides or polypeptides, or antagonists thereof (e.g., anti-CD40 splice variants antibodies) are used to treat or prevent systemic lupus erythematosus and/or diseases, disorders or conditions associated therewith. Lupus-associated diseases, disorders, or conditions that may be treated or prevented with CD40 polynucleotides or polypeptides, or antagonists of the invention, include, but are not limited to, hematologic disorders (e.g., hemolytic anemia, leukopenia, lymphopenia, and thrombocytopenia), immunologic disorders (e.g., anti-DNA antibodies, and anti-Sm antibodies), rashes, photosensitivity, oral ulcers, arthritis, fever, fatigue, weight loss, serositis (e.g., pleuritis (pleuricy)), renal disorders (e.g., nephritis), neurological disorders (e.g., seizures, peripheral neuropathy, CNS related disorders), gastrointestinal disorders, Raynaud phenomenon, and pericarditis.

[0261] CD40 polypeptides, agonists or antagonists of the invention may be used to treat cardiovascular disorders, including peripheral artery disease, such as limb ischemia.

[0262] Cardiovascular disorders include cardiovascular abnormalities, such as arterio-arterial fistula, arteriovenous fistula, cerebral arteriovenous malformations, congenital heart defects, pulmonary atresia and Scimitar Syndrome. Congenital heart defects include aortic coarctation, cor triatriatum, coronary vessel anomalies, crisscross heart, dextrocardia, patent ductus arteriosus, Ebstein's anomaly, Eisenmenger complex, hypoplastic left heart syndrome, levocardia, tetralogy of fallot, transposition of great vessels, double outlet right ventricle, tricuspid atresia, persistent truncus arteriosus, and heart septal defects, such as aortopulmonary septal defect, endocardial cushion defects, Lutembacher's Syndrome, trilogy of Fallot, and ventricular heart septal defects.

[0263] Cardiovascular disorders also include heart disease, such as arrhythmias, carcinoid heart disease, high cardiac output, low cardiac output, cardiac tamponade, endocarditis (including bacterial), heart aneurysm, cardiac arrest, congestive heart failure, congestive cardiomyopathy, paroxysmal dyspnea, cardiac edema, heart hypertrophy, congestive cardiomyopathy, left ventricular hypertrophy, right ventricular hypertrophy, post-infarction heart rupture, ventricular septal rupture, heart valve diseases, myocardial diseases, myocardial ischemia, pericardial effusion,
pericarditis (including constrictive and tuberculous), pneumopericardium, postpericardiotomy syndrome, pulmonary heart disease, rheumatic heart disease, ventricular dysfunction, hyperemia, cardiovascular pregnancy complications, Scimitar Syndrome, cardiovascular syphilis, and cardiovascular tuberculosis.

Arrhythmias include sinus arrhythmia, atrial fibrillation, atrial flutter, bradycardia, extrasystole, Adams-Stokes Syndrome, bundle-branch block, sinoatrial block, long QT syndrome, parasystole, Lown-Ganong-Levine Syndrome, Mahaim-type pre-excitation syndrome, Wolff-Parkinson-White syndrome, sick sinus syndrome, tachycardias, and ventricular fibrillation. Tachycardias include paroxysmal tachycardia, supraventricular tachycardia, accelerated idioventricular rhythm, atrioventricular nodal reentry tachycardia, ectopic atrial tachycardia, ectopic junctional tachycardia, sinoatrial nodal reentry tachycardia, sinus tachycardia, Torsades de Pointes, and ventricular tachycardia.

Heart valve disease include aortic valve insufficiency, aortic valve stenosis, heart murmurs, aortic valve prolapse, mitral valve prolapse, tricuspid valve prolapse, mitral valve insufficiency, mitral valve stenosis, pulmonary atresia, pulmonary valve insufficiency, pulmonary valve stenosis, tricuspid atresia, tricuspid valve insufficiency, and tricuspid valve stenosis.

Myocardial diseases include alcoholic cardiomyopathy, congestive cardiomyopathy, hypertrophic cardiomyopathy, aortic subvalvular stenosis, pulmonary subvalvular stenosis, restrictive cardiomyopathy, Chagas cardiomyopathy, endocardial fibroelastosis, endomyocardial fibrosis, Kearns Syndrome, myocardial reperfusion injury, and myocarditis.

Myocardial ischemias include coronary disease, such as angina pectoris, coronary aneurysm, coronary arteriosclerosis, coronary thrombosis, coronary vasospasm, myocardial infarction and myocardial stunning.

Cardiovascular diseases also include vascular diseases such as aneurysms, angiodysplasia, angiomatosis, bacillary angiomatosis, Hippel-Lindau Disease, Klippel-Trenaunay-Weber Syndrome, Sturge-Weber Syndrome, angioneurotic edema, aortic diseases, Takayasu's Arteritis, aortitis, Leriche's Syndrome, arterial occlusive diseases, arteritis, enarteritis, polyarteritis nodosa, cerebrovascular disorders, diabetic angiopathies, diabetic retinopathy, embolisms, thrombosis, erythromelalgia, hemorrhoids, hepatic veno-occlusive disease, hypertension, hypotension, ischemia, peripheral vascular diseases, phlebitis,
pulmonary veno-occlusive disease, Raynaud's disease, CREST syndrome, retinal vein occlusion, Scimitar syndrome, superior vena cava syndrome, telangiectasia, ataciac telangiectasia, hereditary hemorrhagic telangiectasia, varicocele, varicose veins, varicose ulcer, vasculitis, and venous insufficiency.

Aneurysms include dissecting aneurysms, false aneurysms, infected aneurysms, ruptured aneurysms, aortic aneurysms, cerebral aneurysms, coronary aneurysms, heart aneurysms, and iliac aneurysms.

Arterial occlusive diseases include arteriosclerosis, intermittent claudication, carotid stenosis, fibromuscular dysplasias, mesenteric vascular occlusion, Moyamoya disease, renal artery obstruction, retinal artery occlusion, and thromboangiitis obliterans.

Cerebrovascular disorders include carotid artery diseases, cerebral amyloid angiopathy, cerebral aneurysm, cerebral anoxia, cerebral arteriosclerosis, cerebral arteriovenous malformation, cerebral artery diseases, cerebral embolism and thrombosis, carotid artery thrombosis, sinus thrombosis, Wallenberg's syndrome, cerebral hemorrhage, epidural hematoma, subdural hematoma, subarachnoid hemorrhage, cerebral infarction, cerebral ischemia (including transient), subclavian steal syndrome, periventricular leukomalacia, vascular headache, cluster headache, migraine, and vertebrobasilar insufficiency.

Embolisms include air embolisms, amniotic fluid embolisms, cholesterol embolisms, blue toe syndrome, fat embolisms, pulmonary embolisms, and thromboembolisms. Thrombosis include coronary thrombosis, hepatic vein thrombosis, retinal vein occlusion, carotid artery thrombosis, sinus thrombosis, Wallenberg's syndrome, and thrombophlebitis.

Ischemia includes cerebral ischemia, ischemic colitis, compartment syndromes, anterior compartment syndrome, myocardial ischemia, reperfusion injuries, and peripheral limb ischemia. Vasculitis includes aortitis, arteritis, Behcet's Syndrome, Churg-Strauss Syndrome, mucocutaneous lymph node syndrome, thromboangiitis obliterans, hypersensitivity vasculitis, Schoenlein-Henoch purpura, allergic cutaneous vasculitis, and Wegener's granulomatosis.

The present invention further provides for treatment of diseases or disorders associated with neovascularization by administration of the CD40 polynucleotides and/or polypeptides of the invention (including CD40 agonists and/or antagonists). Malignant and metastatic conditions which can be treated with the
polynucleotides and polypeptides of the invention include, but are not limited to those malignancies, solid tumors, and cancers described herein and otherwise known in the art (for a review of such disorders, see Fishman et al., Medicine, 2d Ed., J. B. Lippincott Co., Philadelphia (1985)).

Additionally, ocular disorders associated with neovascularization which can be treated with the CD40 polynucleotides and polypeptides of the present invention (including CD40 agonists and CD40 antagonists) include, but are not limited to: neovascular glaucoma, diabetic retinopathy, retinoblastoma, retrolental fibroplasia, uveitis, retinopathy of prematurity, macular degeneration, corneal graft neovascularization, as well as other eye inflammatory diseases, ocular tumors and diseases associated with choroidal or iris neovascularization. See, e.g., reviews by Waltman et al., Am. J. Ophthal., 85:704-710 (1978) and Gartner et al., Surv. Ophthal., 22:291-312 (1978).

Accordingly, provided herein are methods of inhibiting neoangiogenesis in a subject comprising the steps of:

(a) providing a composition comprising sCD40; and
(b) administering the composition to the subject.

Additionally, disorders which can be treated with the CD40 polynucleotides and polypeptides of the present invention include, but are not limited to, hemangioma, arthritis, psoriasis, angiofibroma, atherosclerotic plaques, delayed wound healing, granulations, hemophilic joints, hypertrophic scars, nonunion fractures, Osler-Weber syndrome, pyogenic granuloma, scleroderma, trachoma, and vascular adhesions.

Polynucleotides and/or polypeptides of the invention, and/or agonists and/or antagonists thereof, are useful in the diagnosis and treatment or prevention of a wide range of diseases and/or conditions. Such diseases and conditions include, but are not limited to, cancer (e.g., immune cell related cancers, breast cancer, prostate cancer, ovarian cancer, follicular lymphoma, cancer associated with mutation or alteration of p53, brain tumor, bladder cancer, uterocervical cancer, colon cancer, colorectal cancer, non-small cell carcinoma of the lung, small cell carcinoma of the lung, stomach cancer, etc.), lymphoproliferative disorders (e.g., lymphadenopathy), microbial (e.g., viral, bacterial, etc.) infection (e.g., HIV-1 infection, HIV-2 infection, herpesvirus infection (including, but not limited to, HSV-1, HSV-2, CMV, VZV, HHV-6, HHV-7, EBV), adenovirus infection, poxvirus infection, human papilloma
virus infection, hepatitis infection (e.g., HAV, HBV; HCV, etc.), Helicobacter pylori infection, invasive Staphylococcia, etc.), parasitic infection, nephritis, bone disease (e.g., osteoporosis), atherosclerosis, pain, cardiovascular disorders (e.g., neovascularization, hypovascularization or reduced circulation (e.g., ischemic disease (e.g., myocardial infarction, stroke, etc.)), AIDS, allergy, inflammation, neurodegenerative disease (e.g., Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, pigmentary retinitis, cerebellar degeneration, etc.), graft rejection (acute and chronic), graft vs. host disease, diseases due to osteomyelodysplasia (e.g., aplastic anemia, etc.), joint tissue destruction in rheumatism, liver disease (e.g., acute and chronic hepatitis, liver injury, and cirrhosis), autoimmune disease (e.g., multiple sclerosis, rheumatoid arthritis, systemic lupus erythematosus, immune complex glomerulonephritis, autoimmune diabetes, autoimmune thrombocytopenic purpura, Grave's disease, Hashimoto's thyroiditis, etc.), cardiomyopathy (e.g., dilated cardiomyopathy), diabetes, diabetic complications (e.g., diabetic nephropathy, diabetic neuropathy, diabetic retinopathy), influenza, asthma, psoriasis, glomerulonephritis, septic shock, and ulcerative colitis.

Accordingly, provided herein are methods of preventing bone loss induced by infection comprising the steps of:

(a) providing a composition comprising a modulator of sCD40;

and

(b) administering the composition to the subject, wherein the modulator interferes with the activity or binding of sCD40.

Polynucleotides and/or polypeptides of the invention and/or agonists and/or antagonists thereof are useful in promoting angiogenesis, wound healing (e.g., wounds, burns, and bone fractures).

Polynucleotides and/or polypeptides of the invention and/or agonists and/or antagonists thereof are also useful as an adjuvant to enhance immune responsiveness to specific antigen and/or anti-viral immune responses.

More generally, polynucleotides and/or polypeptides of the invention and/or agonists and/or antagonists thereof are useful in regulating (i.e., elevating or reducing) immune response. For example, polynucleotides and/or polypeptides of the invention may be useful in preparation or recovery from surgery, trauma, radiation therapy, chemotherapy, and transplantation, or may be used to boost immune response and/or recovery in the elderly and immunocompromised individuals. Alternatively,
polynucleotides and/or polypeptides of the invention and/or agonists and/or antagonists thereof are useful as immunosuppressive agents, for example in the treatment or prevention of autoimmune disorders. In specific embodiments, polynucleotides and/or polypeptides of the invention are used to treat or prevent chronic inflammatory, allergic or autoimmune conditions, such as those described herein or are otherwise known in the art.

[0283] Accordingly, provided herein are methods of inhibiting activation of macrophages or monocytes in a subject comprising the steps of:

(a) providing a composition comprising sCD40; and
(b) administering the composition to the subject.

[0284] The uses of the CD40 polypeptides, include but are not limited to, the treatment or prevention of viral hepatitis, Herpes viral infections, allergic reactions, adult respiratory distress syndrome, neoplasia, anaphylaxis, allergic asthma, allergen rhinitis, drug allergies (e.g., to penicillin, cephalosporins), primary central nervous system lymphoma (PCNSL), glioblastoma, chronic lymphocytic leukemia (CLL), lymphadenopathy, autoimmune disease, graft versus host disease, rheumatoid arthritis, osteoarthritis, Graves' disease, acute lymphoblastic leukemia (ALL), lymphomas (Hodgkin's disease and non-Hodgkin's lymphoma (NHL)), ophthalmopathy, uveoretinitis, the autoimmune phase of Type 1 diabetes, myasthenia gravis, glomerulonephritis, autoimmune hepato pathological disorder, autoimmune inflammatory bowel disease, and Crohn's disease. In addition, the CD40 polypeptides of the present invention may be employed to inhibit neoplasia, such as tumor cell growth.

[0285] Accordingly provided herein are methods of inhibition of activity of CD154 (CD40 ligand) in a subject comprising the steps of:

(a) providing a composition comprising sCD40; and
(b) administering the composition to the subject.

[0286] Also provided are methods of inhibiting sCD40 activity in a subject, comprising the steps of:

(a) providing a composition comprising a modulator of sCD40;

and

(b) administering the composition to the subject, wherein the modulator interferes with the activity or binding of sCD40. In some examples of the method of inhibiting sCD40 activity in a subject the modulator is an antibody.
In other examples, provided herein are methods of inhibiting binding of a CD40 ligand with a wild-type human CD40 comprising contacting the CD40 ligand with a CD40 splice variant or mutein thereof, or fusion molecule comprising said CD40 splice variant or mutein thereof, under conditions suitable for binding of said CD40 ligand to said polypeptide. In other examples, provided herein are methods for blocking wild-type human CD40 activity, comprising contacting the wild-type CD40 with a CD40 splice variant or mutein thereof, or fusion molecule comprising said CD40 splice variant or mutein thereof, in the presence of CD40 ligand and under conditions suitable to inhibit binding of the CD40 ligand with said wild-type CD40, thereby blocking wild-type CD40 activity. In some examples, a CD40 splice variant or mutein thereof is in monomer form and in other examples, is a multimer, such as a dimer or trimer. In other examples, a fusion molecule is a multimer. In some examples, the CD40 splice variant or mutein or fusion molecule is soluble.

**Antibodies**

CD40 protein specific antibodies for use in the present invention can be raised against the intact CD40 protein or an antigenic polypeptide fragment thereof, which may be presented together with a carrier protein, such as an albumin, to an animal system (such as rabbit or mouse) or, if it is long enough (at least about 25 amino acids), without a carrier.

In some examples, antibodies specifically bind a CD40 splice variant, that is, CD40sv1, CD40sv2 or CD40sv3 or muteins of a CD40 splice variant and do not specifically bind to wild-type CD40 (NP_001241 NM_001250: SEQ ID NO:13) and do not specifically bind to the polypeptides having the sequence as shown in SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:150, SEQ ID NO:152, SEQ ID NO:154, SEQ ID NO:155 or SEQ ID NO:156.

Accordingly, provided herein are methods of determining the presence of an antibody specific to an isolated polypeptide comprising an amino acid sequence chosen from among: SEQ ID NO:10, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:28-34 and active fragments thereof in a sample, comprising the steps of:

(a) providing a composition comprising the polypeptide;
(b) allowing the polypeptide to interact with the sample; and
(c) determining whether interaction has occurred between the polypeptide and the antibody.
[0291] Also provided herein are antibodies that specifically bind to or interfere with activity of an isolated polypeptide comprising an amino acid sequence chosen from among: SEQ ID NO:10, SEQ ID NO:14, SEQ ID NO:15, and SEQ ID NO:28-34 and active fragments thereof. In some examples the antibody is selected from the group consisting of: polyclonal antibodies; monoclonal antibodies; single chain antibodies; and active fragments thereof. In other examples the antibody is a fragment and the fragment is selected from the group consisting of: an antigen binding fragment, an Fc fragment, a CDR fragment, and a framework fragment.

[0292] Provided herein are isolated antibodies, wherein an antibody specifically binds to or interferes with the activity of CD40 splice variants. Also provided are fusion molecules comprising an antibody that specifically binds to or interferes with the activity of CD40 splice variants and an antigen. Furthermore, provided are methods of providing adjuvant effect to an antigen comprising the steps of:

(a) providing a fusion molecule comprising an antibody that specifically binds to or interferes with the activity of CD40 splice variants and an antigen; and

(b) immunizing an animal with the fusion molecule.

[0293] The antibodies of the present invention may be prepared by any of a variety of methods. For example, cells expressing the CD40 protein or an antigenic fragment thereof can be administered to an animal in order to induce the production of sera containing polyclonal antibodies. In a preferred method, a preparation of CD40 protein is prepared and purified to render it substantially free of natural contaminants. Such a preparation is then introduced into an animal in order to produce polyclonal antisera of greater specific activity.

[0294] In the most preferred method, the antibodies of the present invention are monoclonal antibodies, or CD40 protein binding fragments thereof. Such monoclonal antibodies can be prepared using hybridoma technology, for example, Kohler et al., Nature, 256:495 (1975); Kohler et al., Eur. J. Immunol., 6:511 (1976); Kohler et al., Eur. J. Immunol., 6:292 (1976); Hammerling et al., in: Monoclonal Antibodies and T-Cell Hybridomas, Elsevier, N.Y., (1981) pp. 563-681. In general, such procedures involve immunizing an animal (preferably a mouse) with a CD40 protein antigen or, more preferably, with a CD40 protein-expressing cell. Suitable cells can be recognized by their capacity to bind anti-CD40 protein antibody. Such
cells may be cultured in any suitable tissue culture medium; however, it is preferable to culture cells in Earle's modified Eagle's medium supplemented with 10% fetal bovine serum (inactivated at about 56°C), and supplemented with about 10 grams/liter of nonessential amino acids, about 1,000 U/ml of penicillin, and about 100 μg/ml of streptomycin. The splenocytes of such mice are extracted and fused with a suitable myeloma cell line. Any suitable myeloma cell line may be employed in accordance with the present invention; however, it is preferable to employ the parent myeloma cell line (SP20), available from the American Type Culture Collection, Manassas, Va. After fusion, the resulting hybridoma cells are selectively maintained in HAT medium, and then cloned by limiting dilution as described by Wands et al., Gastroenterology, 80:225-232 (1981).

CD40 protein antigen

Alternatively, additional antibodies capable of binding to the CD40 protein antigen may be produced in a two-step procedure through the use of anti-idiotypic antibodies. Such a method makes use of the fact that antibodies are themselves antigens, and that, therefore, it is possible to obtain an antibody which binds to a second antibody. In accordance with this method, CD40-protein specific antibodies are used to immunize an animal, preferably a mouse. The splenocytes of such an animal are then used to produce hybridoma cells, and the hybridoma cells are screened to identify clones which produce an antibody whose ability to bind to the CD40 protein-specific antibody can be blocked by the CD40 protein antigen. Such antibodies comprise anti-idiotypic antibodies to the CD40 protein-specific antibody and can be used to immunize an animal to induce formation of further CD40 protein-specific antibodies.

It will be appreciated that Fab and F(\text{ab'})_2 and other fragments of the antibodies of the present invention may be used according to the methods disclosed herein. Such fragments are typically produced by proteolytic cleavage, using enzymes such as papain (to produce Fab fragments) or pepsin (to produce F(\text{ab'})_2 fragments). Alternatively, CD40 protein-binding fragments can be produced through the application of recombinant DNA technology or through synthetic chemistry. For \textit{in vivo} use of anti-CD40 in humans, it may be preferable to use “humanized” chimeric monoclonal antibodies. Such antibodies can be produced using genetic constructs derived from hybridoma cells producing the monoclonal antibodies.

**Diagnosis**

[0297] This invention is also related to the use of the genes and polynucleotides of the present invention as part of a diagnostic assay for detecting diseases or susceptibility to diseases related to the presence of mutations in the nucleic acid sequences encoding the polypeptide of the present invention. Individuals carrying mutations in a gene of the present invention may be detected at the DNA level by a variety of techniques. Nucleic acids for diagnosis may be obtained from a patient's cells, such as from blood, urine, saliva, tissue biopsy and autopsy material. The genomic DNA may be used directly for detection or may be amplified enzymatically by using PCR, for example, Saiki et al., *Nature*, 324: 163-166 (1986), prior to analysis. RNA or cDNA may also be used for the same purpose. As an example, PCR primers complementary to the nucleic acid encoding a polypeptide of the present invention can be used to identify and analyze mutations. For example, deletions and insertions can be detected by a change in size of the amplified product in comparison to the normal genotype. Point mutations can be identified by hybridizing amplified DNA to radiolabeled RNA or alternatively, radiolabeled antisense DNA sequences. Perfectly matched sequences can be distinguished from mismatched duplexes by RNase A digestion or by differences in melting temperatures.

[0298] Genetic testing based on DNA sequence differences may be achieved by detection of alteration in electrophoretic mobility of DNA fragments in gels with or without denaturing agents. Small sequence deletions and insertions can be visualized by high resolution gel electrophoresis. DNA fragments of different sequences may be distinguished on denaturing formamide gradient gels in which the mobilities of different DNA fragments are retarded in the gel at different positions according to their specific melting or partial melting temperatures, for example, Myers et al., *Science*, 230:1242 (1985).

[0299] Sequence changes at specific locations may also be revealed by nuclease protection assays, such as RNase and S1 protection or the chemical cleavage
method as shown in Cotton et al., PNAS, USA, 85:4397-4401 (1985). Thus, the
detection of a specific DNA sequence may be achieved by methods such as
hybridization, RNase protection, chemical cleavage, direct DNA sequencing or the
use of restriction enzymes, (e.g., Restriction Fragment Length Polymorphisms
(RFLP)) and Southern blotting of genomic DNA. In addition to more conventional
gel-electrophoresis and DNA sequencing, mutations can also be detected by in situ
analysis.

[0300] The present invention also relates to a diagnostic assay for detecting
altered levels of CD40 proteins in various tissues since an over-expression of the
proteins compared to normal control tissue samples may detect the presence of
abnormal cellular proliferation, for example, a tumor. Assays used to detect levels of
protein in a sample derived from a host are well-known to those of skill in the art and
include radioimmunoassays, competitive-binding assays, Western Blot analysis,
ELISA assays and “sandwich” assays. An ELISA assay, for example, Coligan, et al.,
Current Protocols in Immunology, 1(2), Chapter 6, (1991), initially contains preparing
an antibody specific to an antigen to the polypeptides of the present invention,
preferably a monoclonal antibody.

[0301] In addition a reporter antibody is prepared against the monoclonal
antibody. To the reporter antibody is attached a detectable reagent such as
radioactivity, fluorescence or, in this example, a horseradish peroxidase enzyme. A
sample is removed from a host and incubated on a solid support, e.g. a polystyrene
dish, that binds the proteins in the sample. Any free protein binding sites on the dish
are then covered by incubating with a non-specific protein like bovine serum albumin.
Next, the monoclonal antibody is incubated in the dish during which time the
monoclonal antibodies attach to any polypeptides of the present invention attached to
the polystyrene dish. All unbound monoclonal antibody is washed out with buffer.
The reporter antibody linked to horseradish peroxidase is now placed in the dish
resulting in binding of the reporter antibody to any monoclonal antibody bound to the
protein of interest. Unattached reporter antibody is then washed out. Peroxidase
substrates are then added to the dish and the amount of color developed in a given
time period is a measurement of the amount of a polypeptide of the present invention
present in a given volume of patient sample when compared against a standard curve.

[0302] A competition assay may be employed wherein antibodies specific to a
polypeptide of the present invention are attached to a solid support and a labeled
CD40 variant and a sample derived from the host are passed over the solid support and the amount of label detected, for example by liquid scintillation chromatography, can be correlated to a quantity of a polypeptide of the present invention in the sample. A “sandwich” assay is similar to an ELISA assay. In a “sandwich” assay a polypeptide of the present invention is passed over a solid support and binds to antibody attached to a solid support. A second antibody is then bound to the polypeptide of interest. A third antibody which is labeled and specific to the second antibody is then passed over the solid support and binds to the second antibody and an amount can then be quantified.

[0303] By “assaying the expression level of the gene encoding the CD40 variant protein” is intended qualitatively or quantitatively measuring or estimating the level of the CD40 protein or the level of the mRNA encoding the CD40 protein in a first biological sample either directly, for example, by determining or estimating absolute protein level or mRNA level, or relatively, by comparing to the CD40 protein level or mRNA level in a second biological sample. Preferably, the CD40 protein level or mRNA level in the first biological sample is measured or estimated and compared to a standard CD40 protein level or mRNA level, the standard being taken from a second biological sample obtained from an individual not having the disorder or being determined by averaging levels from a population of individuals not having a disorder related to CD40 expression. As will be appreciated in the art, once a standard CD40 protein level or mRNA level is known, it can be used repeatedly as a standard for comparison.

[0304] By “biological sample” is intended any biological sample obtained from a subject, body fluid, cell line, tissue culture, or other source which contains CD40 protein or mRNA. As indicated, biological samples include body fluids (such as sera, plasma, urine, synovial fluid and spinal fluid) which contain free CD40 protein, ovarian or renal system tissue, and other tissue sources found to express complete or mature CD40 polypeptide or an CD40 receptor. Methods for obtaining tissue biopsies and body fluids from mammals are well known in the art. Where the biological sample is to include mRNA, a tissue biopsy is the preferred source.

[0305] Total cellular RNA can be isolated from a biological sample using any suitable technique such as the single-step guanidinium-thiocyanate-phenol-chloroform method described in Chomczynski and Sacchi, Anal. Biochem., 162:156-159 (1987). Levels of mRNA encoding the CD40 protein are then assayed using any appropriate
method. These include Northern blot analysis, S1 nuclease mapping, the polymerase chain reaction (PCR), reverse transcription in combination with the polymerase chain reaction (RT-PCR), and reverse transcription in combination with the ligase chain reaction (RT-LCR).

Assaying CD40 protein levels in a biological sample can occur using antibody-based techniques. For example, CD40 protein expression in tissues can be studied with classical immunohistological methods, for example, Jalkanen, M., et al., *J. Cell. Biol.*, 101:976-985 (1985); Jalkanen, M., et al., *J. Cell. Biol.*, 105:3087-3096 (1987). Other antibody-based methods useful for detecting CD40 protein gene expression include immunoassays, such as the enzyme linked immunosorbent assay (ELISA) and the radioimmunoassay (RIA). Suitable antibody assay labels are known in the art and include enzyme labels, such as, glucose oxidase, radioisotopes, and fluorescent labels, such as fluorescein and rhodamine, and biotin.

In addition to assaying CD40 protein levels in a biological sample obtained from an individual, CD40 protein can also be detected in vivo by imaging. Antibody labels or markers for in vivo imaging of CD40 protein include those detectable by X-radiography, NMR or ESR. For X-radiography, suitable labels include radioisotopes such as barium or cesium, which emit detectable radiation but are not overtly harmful to a subject. Suitable markers for NMR and ESR include those with a detectable characteristic spin, such as deuterium, which may be incorporated into the antibody by labeling of nutrients for the relevant hybridoma.

An CD40 protein-specific antibody or antibody fragment which has been labeled with an appropriate detectable imaging moiety, such as a radioisotope, a radio-opaque substance, or a material detectable by nuclear magnetic resonance, is introduced, for example, parenterally, subcutaneously or intraperitoneally, into the subject to be examined for immune system disorder. It will be understood in the art that the size of the subject and the imaging system used will determine the quantity of imaging moiety needed to produce diagnostic images. The labeled antibody or antibody fragment will then preferentially accumulate at the location of cells which contain CD40 protein. In vivo tumor imaging is described in S. W. Burchiel et al., "Immunopharmacokinetics of Radiolabeled Antibodies and Their Fragments", Chapter 13 in Tumor Imaging: The Radiochemical Detection of Cancer, S. W. Burchiel and B. A. Rhodes, eds., Masson Publishing Inc. (1982).
Formulations

[0309] CD40 polypeptide compositions will be formulated and dosed in a fashion consistent with good medical practice, taking into account the clinical condition of the individual subject, the site of delivery of the CD40 polypeptide composition, the method of administration, the scheduling of administration, and other factors known to practitioners. The "effective amount" or "therapeutically effective amount" of CD40 polypeptide for purposes herein is thus determined by such considerations.

[0310] The polypeptides, agonists and antagonists of the present invention may be employed in combination with a suitable pharmaceutical carrier to comprise a pharmaceutical composition for parenteral administration. Such compositions comprise a therapeutically effective amount of the polypeptide, agonist or antagonist and a pharmaceutically acceptable carrier or excipient. Such a carrier includes but is not limited to saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof. The formulation should suit the mode of administration.

[0311] The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration. In addition, the polypeptides, agonists and antagonists of the present invention may be employed in conjunction with other therapeutic compounds.

[0312] The pharmaceutical compositions may be administered in a convenient manner such as by the oral, topical, intravenous, intraperitoneal, intramuscular, subcutaneous, intranasal or intradermal routes. The pharmaceutical compositions are administered in an amount which is effective for treating and/or prophylaxis of the specific indication. In general, they are administered in an amount of at least about 10 \text{mu.g/kg} body weight and in most cases they will be administered in an amount not in excess of about 8 \text{mg/kg} body weight per day.

[0313] The polypeptide of the invention and agonist and antagonist compounds which are polypeptides, may also be employed in accordance with the present invention by expression of such polypeptide in vivo, which is often referred to as "gene therapy." Thus, for example, cells may be engineered with a polynucleotide
(DNA or RNA) encoding for the polypeptide ex vivo, the engineered cells are then provided to a patient to be treated with the polypeptide. Such methods are well-known in the art. For example, cells may be engineered by procedures known in the art by use of a retroviral particle containing RNA encoding for the polypeptide of the present invention.

[0314] Similarly, cells may be engineered in vivo for expression of the polypeptide in vivo, for example, by procedures known in the art. As known in the art, a producer cell for producing a retroviral particle containing RNA encoding the polypeptide of the present invention may be administered to a patient for engineering cells in vivo and expression of the polypeptide in vivo. These and other methods for administering a polypeptide of the present invention by such methods should be apparent to those skilled in the art from the teachings of the present invention. For example, the expression vehicle for engineering cells may be other than a retroviral particle, for example, an adenovirus, which may be used to engineer cells in vivo after combination with a suitable delivery vehicle.

[0315] Retroviruses from which the retroviral plasmid vectors hereinabove mentioned may be derived include, but are not limited to, Moloney Murine Leukemia Virus, spleen necrosis virus, retroviruses such as Rous Sarcoma Virus, Harvey Sarcoma Virus, avian leukemia virus, gibbon ape leukemia virus, human immunodeficiency virus, adenovirus, Myeloproliferative Sarcoma Virus, and mammary tumor virus. In one embodiment, the retroviral plasmid vector is derived from Moloney Murine Leukemia Virus.

[0316] The vector includes one or more promoters. Suitable promoters which may be employed include, but are not limited to, the retroviral LTR; the SV40 promoter; and the human cytomegalovirus (CMV) promoter described in Miller, et al., Biotechniques, Vol. 7, No. 9, 980-990 (1989), or any other promoter, for example, cellular promoters such as eukaryotic cellular promoters including, but not limited to, the histone, pol III, and β-actin promoters. Other viral promoters which may be employed include, but are not limited to, adenovirus promoters, thymidine kinase (TK) promoters, and B19 parvovirus promoters. The selection of a suitable promoter will be apparent to those skilled in the art from the teachings contained herein.

[0317] The nucleic acid sequence encoding the polypeptide of the present invention is under the control of a suitable promoter. Suitable promoters which may be employed include, but are not limited to, adenoviral promoters, such as the
adenoviral major late promoter; or heterologous promoters, such as the
cytomegalovirus (CMV) promoter; the respiratory syncytial virus (RSV) promoter;
inducible promoters, such as the MMT promoter, the metallothionein promoter; heat
shock promoters; the albumin promoter; the ApoAI promoter; human globin
promoters; viral thymidine kinase promoters, such as the Herpes Simplex thymidine
kinase promoter; retroviral LTRs (including the modified retroviral LTRs hereinabove
described); the β-actin promoter; and human growth hormone promoters. The
promoter also may be the native promoter which controls the gene encoding the
polypeptide.

[0318] The retroviral plasmid vector is employed to transduce packaging cell
lines to form producer cell lines. Examples of packaging cells which may be
transfected include, but are not limited to, the PE501, PA317, -2, -AM, PA12, T19-
14X, VT-19-17-H2, CRE, CRIP, GP+E-86, GP+envAm12, and DAN cell lines as
described in Miller, Human Gene Therapy, 1:5-14 (1990). The vector may transduce
the packaging cells through any means known in the art. Such means include, but are
not limited to, electroporation, the use of liposomes, and CaPO₄ precipitation.

[0319] In one alternative, the retroviral plasmid vector may be encapsulated
into a liposome, or coupled to a lipid, and then administered to a host.

[0320] The producer cell line generates infectious retroviral vector particles
which include the nucleic acid sequence(s) encoding the polypeptides. Such retroviral
vector particles then may be employed, to transduce eukaryotic cells, either in vitro or
in vivo. The transduced eukaryotic cells will express the nucleic acid sequence(s)
encoding the polypeptide. Eukaryotic cells which may be transduced include, but are
not limited to, embryonic stem cells, embryonic carcinoma cells, as well as
hematopoietic stem cells, hepatocytes, fibroblasts, myoblasts, keratinocytes,
endothelial cells, and bronchial epithelial cells.

Therapeutic uses of the Molecules of the Invention

[0321] The molecules of the invention are useful for treating a variety of
diseases or conditions arising from an undesirable interaction of CD40 with CD40
ligand, including, for example, cancer, an immunity disease, such as an autoimmune
disease or an inflammatory disease, an ischemic disease or an infectious disease in an
animal.
Treatment of Cancer

[0322] The molecules of the invention are useful for inhibiting the multiplication of a tumor cell or cancer cell, or for treating cancer in an animal. The molecules of the invention can be used accordingly in a variety of settings for the treatment of animal cancers. Other particular types of cancers that can be treated with molecules of the invention include, but are not limited to, those disclosed below. In some examples, the cancer is related to lymphocytic malignancy such as B cell malignancy.


and chronic leukemias including, but not limited to, lymphoblastic, myelogenous, lymphocytic, myelocytic leukemias.

Lymphomas, including but not limited to: Hodgkin's disease, non-Hodgkin's lymphoma, multiple myeloma, Waldenstrom's macroglobulinemia, heavy chain disease, polycythemia vera.

Treatment of Immunity Diseases

Molecules of the invention are useful for killing or inhibiting the replication of a cell that produces an autoimmune disease or an inflammatory disease or for treating an autoimmune disease or an inflammatory disease. Molecules of the invention can be used accordingly in a variety of settings for the treatment of an autoimmune disease or an inflammatory disease in an animal.

Particular types of autoimmune diseases that can be treated with the molecules of the invention include, but are not limited to, Th-2 lymphocyte-related disorders (e.g., atopic dermatitis, atopic asthma, rhinoconjunctivitis, allergic rhinitis, Omenn's syndrome, systemic sclerosis, and graft versus host disease); Th-1 lymphocyte-related disorders (e.g., rheumatoid arthritis, multiple sclerosis, psoriasis, Sjorgren's syndrome, Hashimoto's thyroiditis, Grave's disease, primary biliary cirrhosis, Wegener's granulomatosis, and tuberculosis); activated B lymphocyte-related disorders (e.g., systemic lupus erythematosus, Goodpasture's syndrome, rheumatoid arthritis, and type I diabetes); and those disclosed below.

Active Chronic Hepatitis, Addison's disease, allergic alveolitis, allergic reaction, allergic rhinitis, Alport's syndrome, anaphylaxis, ankylosing spondylitis, anti-phospholipid syndrome, arthritis, ascariasis, aspergillosis, atopic allergy, atopic dermatitis, atopic rhinitis, Behcet's disease, bird-fancier's lung, bronchial asthma, Caplan's syndrome, cardiomyopathy, celiac disease, Chagas' disease, chronic glomerulonephritis, Cogan's syndrome, cold agglutinin disease, congenital Rubella infection, CREST syndrome, Crohn's disease, cryoglobulinemia, Cushing's syndrome, dermatomyositis, discoid lupus, Dressler's syndrome, Eaton-Lambert syndrome, Echovirus infection, encephalomyelitis, endocrine opthalmopathy, Epstein-Barr virus infection, equine heaves, erythematosis, Evan's syndrome, Felty's syndrome, fibromyalgia, Fuch's cyclitis, gastric atrophy, gastrointestinal allergy, giant cell arteritis, glomerulonephritis, Goodpasture's syndrome, graft vs host disease, Graves'
disease, Guillain-Barre disease, Hashimoto's thyroiditis, hemolytic anemia, Henoch-Schonlein purpura, idiopathic adrenal atrophy, idiopathic pulmonary fibrosis, IgA nephropathy, inflammatory bowel diseases, insulin-dependent diabetes mellitus, juvenile arthritis, juvenile diabetes mellitus (Type I), Lambert-Eaton syndrome, laminitis, lichen planus, lupoid hepatitis, lupus, lymphopenia, Meniere's disease, mixed connective tissue disease, multiple sclerosis, myasthenia gravis, pernicious anemia, polyglandular syndromes, presenile dementia, primary agammaglobulinemia, primary biliary cirrhosis, psoriasis, psoriatic arthritis, Raynaud's phenomenon, recurrent abortion, Reiter's syndrome, rheumatic fever, rheumatoid arthritis, Sampter's syndrome, schistosomiasis, Schmidt's syndrome, scleroderma, Shulman's syndrome, Sjorgen's syndrome, Stiff-Man syndrome, sympathetic ophthalmitis, systemic lupus erythematosus, Takayasu's arteritis, temporal arteritis, thyroiditis, thrombocytopenia, thyrotoxicosis, toxic epidermal necrolysis, Type B insulin resistance, Type I diabetes mellitus, ulcerative colitis, uveitis, vitiligo, Waldenstrom's macroglobulemia, Wegener's granulomatosis.

Treatment of Infectious Diseases

[0329] Molecules of the invention are useful for killing or inhibiting the multiplication of a cell that produces an infectious disease or for treating an infectious disease. The molecules of the invention can be used accordingly in a variety of settings for the treatment of an infectious disease in an animal.

[0330] Particular types of infectious diseases that can be treated with molecules of the invention include, but are not limited to, those disclosed below.

[0331] Bacterial Diseases: Diptheria, pertussis, occult bacteremia, urinary tract infection, gastroenteritis, cellulites, epiglottitis, tracheitis, adenoid hypertrophy, retropharyngeal abscess, impetigo, eczema, pneumonia, endocarditis, septic arthritis, pneumococcal, peritonitis, bacteremia, meningitis, acute purulent meningitis, urethritis, cervicitis, proctitis, pharyngitis, salpingitis, epididymitis, gonorrhea, syphilis, listeriosis, anthrax, nocardiosis, Salmonella, typhoid fever, dysentery, conjunctivitis, sinusitis, brucellosis, tularemia, cholera, bubonic plague, tetanus, necrotizing enteritis, actinomycosis, mixed anaerobic infections, relapsing fever, leptospirosis, Lyme disease, rat bite fever, tuberculosis, lymphadenitis, leprosy, chlamydia, chlamydial pneumonia, trachoma, inclusion conjunctivitis.
Systemic fungal diseases: Histoplasmosis, coccidioidomycosis, blastomycosis, sporotrichosis, cryptococcosis, systemic candidiasis, aspergillosis, mucormycosis, mycetoma, chromomycosis.

Rickettsial Diseases: Typhus, Rocky Mountain spotted fever, ehrlichiosis, Eastern tick-borne rickettsioses, rickettsialpox, Q fever, bartonellosis.

Parasitic Diseases: Malaria, babesiosis, African sleeping sickness, Chagas' disease, leishmaniasis, Durr-Durr fever, toxoplasmosis, meningoencephalitis, keratitis, entamebiasis, Gardiasis, cryptosporidiasis, isosporiasis, cyclosporiasis, microsporidiosis, ascariasis, whipworm infection, hookworm infection, threadworm infection, ocular larva migrans, trichinosis, Guinea worm disease, lymphatic filariasis, loiasis, river blindness, canine heartworm infection, schistosomiasis, swimmer's itch, Oriental lung fluke, Oriental liver fluke, fascioliasis, fasciolopsiasis, opisthochiasis, tapeworm infections, hydatid disease, alveolar hydatid disease.

Viral Diseases: Measles, subacute sclerosing panencephalitis, common cold, mumps, Rubella, roseola, fifth disease, chickenpox, respiratory syncytial virus infection, Croup, bronchiolitis, infectious mononucleosis, poliomyelitis, herpangina, Hand-Foot-and-Mouth disease, Bornholm Disease, genital herpes, genital warts, aseptic meningitis, myocarditis, pericarditis, gastroenteritis, acquired immunodeficiency syndrome (AIDS), Reye's syndrome, Kawasaki syndrome, influenza, bronchitis, viral "walking" pneumonia, acute febrile respiratory disease, acute pharyngoconjunctival fever, epidemic keratoconjunctivitis, Herpes simplex virus 1 (HSV-1), Herpes simplex virus 2 (HSV-2), shingles, cytomegalic inclusion disease, rables, progressive multifocal leukoencephalopathy, fatal familial insomnia, Creutzfeldt-Jakob disease, Gerstmann-Straussler-Scheinker disease, tropical spastic paraparesis, Western equine encephalitis, California encephalitis, St. Louis encephalitis, yellow fever, dengue, lymphocytic choriomeningitis, Lassa fever, hemorrhagic fever, Hantavirus pulmonary syndrome, Marburg virus infections, Ebola virus infections, smallpox.

Treatment of Ischemia

Molecules of the invention are useful for killing or inhibiting the multiplication of a cell that either directly or indirectly mediates ischemic diseases by secreting agents that mediate such diseases or that produce an ischemic disease or for
treating an ischemic disease. The molecules of the invention can be used accordingly in a variety of settings for the treatment of an ischemic disease in an animal.

[0337] Particular types of ischemic diseases that can be treated with molecules of the invention include, but are not limited to, stroke, myocardial infarction, and fulminant liver failure.

CD40 “Knock-outs” and Homologous Recombination

[0338] Endogenous gene expression can also be reduced by inactivating or “knocking out” the gene and/or its promoter using targeted homologous recombination. (e.g., see Smithies et al., *Nature*, 317:230-234 (1985); Thomas & Capecchi, *Cell*, 51:503-512 (1987); Thompson et al., *Cell*, 5:313-321 (1989); each of which is incorporated by reference herein in its entirety). For example, a mutant, non-functional polynucleotide of the invention (or a completely unrelated DNA sequence) flanked by DNA homologous to the endogenous polynucleotide sequence (either the coding regions or regulatory regions of the gene) can be used, with or without a selectable marker and/or a negative selectable marker, to transfect cells that express polypeptides of the invention in vivo. In another embodiment, techniques known in the art are used to generate knockouts in cells that contain, but do not express the gene of interest. Insertion of the DNA construct, via targeted homologous recombination, results in inactivation of the targeted gene. Such approaches are particularly suited in research and agricultural fields where modifications to embryonic stem cells can be used to generate animal offspring with an inactive targeted gene (see, e.g., Thomas & Capecchi 1987 and Thompson 1989, supra). However this approach can be routinely adapted for use in humans provided the recombinant DNA constructs are directly administered or targeted to the required site in vivo using appropriate viral vectors that will be apparent to those of skill in the art.

[0339] In further embodiments of the invention, cells that are genetically engineered to express the polypeptides of the invention, or alternatively, that are genetically engineered not to express the polypeptides of the invention (e.g., knockouts) are administered to a patient in vivo. Such cells may be obtained from the patient (i.e., animal, including human) or an MHC compatible donor and can include, but are not limited to fibroblasts, bone marrow cells, blood cells (e.g., lymphocytes), adipocytes, muscle cells, endothelial cells, etc. The cells are genetically engineered in vitro using recombinant DNA techniques to introduce the coding sequence of
polypeptides of the invention into the cells, or alternatively, to disrupt the coding sequence and/or endogenous regulatory sequence associated with the polypeptides of the invention, e.g., by transduction (using viral vectors, and preferably vectors that integrate the transgene into the cell genome) or transfection procedures, including, but not limited to, the use of plasmids, cosmids, YACs, naked DNA, electroporation, liposomes, etc. The coding sequence of the polypeptides of the invention can be placed under the control of a strong constitutive or inducible promoter or promoter/enhancer to achieve expression, and preferably secretion, of the polypeptides of the invention. The engineered cells which express and preferably secrete the polypeptides of the invention can be introduced into the patient systemically, e.g., in the circulation, or intraperitoneally. Alternatively, the cells can be incorporated into a matrix and implanted in the body, e.g., genetically engineered fibroblasts can be implanted as part of a skin graft; genetically engineered endothelial cells can be implanted as part of a lymphatic or vascular graft. (See, for example, Anderson et al. U.S. Pat. No. 5,399,349; and Mulligan & Wilson, U.S. Pat. No. 5,460,959, each of which is incorporated by reference herein in its entirety).

[0340] When the cells to be administered are non-autologous or non-MHC compatible cells, they can be administered using well known techniques which prevent the development of a host immune response against the introduced cells. For example, the cells may be introduced in an encapsulated form which, while allowing for an exchange of components with the immediate extracellular environment, does not allow the introduced cells to be recognized by the host immune system.

Transgenic Non-human Animals

[0341] The polypeptides of the invention can also be expressed in transgenic animals. Animals of any species, including, but not limited to, mice, rats, rabbits, hamsters, guinea pigs, pigs, micro-pigs, goats, sheep, cows and non-human primates, e.g., baboons, monkeys, and chimpanzees may be used to generate transgenic animals. In a specific embodiment, techniques described herein or otherwise known in the art, are used to express polypeptides of the invention in humans, as part of a gene therapy protocol. Encompassed within the present invention are animals, such as mice for example, that may have been injected with nucleic acid encoding the CD40 splice variants or muteins via tail vein injection, using the hydrodynamic
method of Mirus, as disclosed in U.S. Pat. No. 6,627,616 of PCT publication WO 98/58542.

[0342] Accordingly provided herein are non-human animals injected with a nucleic acid molecule comprising a polynucleotide sequence chosen from among: (A) SEQ ID NO:1, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:19, SEQ ID NO:23; SEQ ID NO:24; SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49, SEQ ID NO:50, SEQ ID NO:51, SEQ ID NO:52, SEQ ID NO:53, SEQ ID NO:54, SEQ ID NO:55, SEQ ID NO:56, SEQ ID NO:57, SEQ ID NO:58, SEQ ID NO:59, SEQ ID NO:60, SEQ ID NO:61, or SEQ ID NO:62 (B) polynucleotide sequences encoding polypeptides having the sequence as shown in SEQ ID NO:10, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:28-34, SEQ ID NO:95, SEQ ID NO:96, SEQ ID NO:97, SEQ ID NO:98, SEQ ID NO:99, SEQ ID NO:100, SEQ ID NO:101, SEQ ID NO:102, SEQ ID NO:103, SEQ ID NO:104, SEQ ID NO:105, SEQ ID NO:106, SEQ ID NO:107, SEQ ID NO:108, SEQ ID NO:109, SEQ ID NO:110, SEQ ID NO:111, SEQ ID NO:112, SEQ ID NO:113, SEQ ID NO:114, SEQ ID NO:115, SEQ ID NO:116, SEQ ID NO:117, SEQ ID NO:118, or SEQ ID NO:119; (C) biologically active fragments thereof; or (D) a complement thereof.

[0343] Also provided are non-human animals transformed with a nucleic acid molecule comprising a polynucleotide sequence chosen from among: (A) SEQ ID NO:1, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:19, SEQ ID NO:23; SEQ ID NO:24; SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49, SEQ ID NO:50, SEQ ID NO:51, SEQ ID NO:52, SEQ ID NO:53, SEQ ID NO:54, SEQ ID NO:55, SEQ ID NO:56, SEQ ID NO:57, SEQ ID NO:58, SEQ ID NO:59, SEQ ID NO:60, SEQ ID NO:61, or SEQ ID NO:62 (B) polynucleotide sequences encoding polypeptides having the sequence as shown in SEQ ID NO:10, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:28-34, SEQ ID NO:95, SEQ ID NO:96, SEQ ID NO:97, SEQ ID NO:98, SEQ ID NO:99, SEQ ID NO:100, SEQ ID NO:101, SEQ ID NO:102, SEQ ID NO:103, SEQ ID NO:104, SEQ ID NO:105, SEQ ID NO:106, SEQ ID NO:107, SEQ ID NO:108, SEQ ID NO:109, SEQ ID NO:110, SEQ ID NO:111, SEQ ID NO:112, SEQ ID NO:113, SEQ ID NO:114, SEQ ID NO:115, SEQ ID NO:116, SEQ ID NO:117, SEQ ID NO:118, or
SEQ ID NO:119; (C) biologically active fragments thereof; or (D) a complement thereof.

[0344] Additionally, provided herein are non-human animals injected with an isolated polypeptide comprising an amino acid sequence chosen from among: SEQ ID NO:10, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:28-34 and active fragments thereof.

[0345] Any technique known in the art may be used to introduce the transgene (i.e., polynucleotides of the invention) into animals to produce the founder lines of transgenic animals. Such techniques include, but are not limited to, pronuclear microinjection (Paterson et al., Appl. Microbiol. Biotechnol. 40:691-698 (1994); Carver et al., Biotechnology (NY) 11: 1263-1270 (1993); Wright et al., Biotechnology (NY) 9:830-834 (1991); and Hoppe et al., U.S. Pat. No. 4,873,191 (1989)); retrovirus mediated gene transfer into germ lines (Van der Putten et al., Proc. Natl. Acad. Sci., USA 82:6148-6152 (1985)), blastocysts or embryos; gene targeting in embryonic stem cells (Thompson et al., Cell 56:313-321 (1989)); electroporation of cells or embryos (Lo, Mol. Cell. Biol. 3:1803-1814 (1983)); introduction of the polynucleotides of the invention using a gene gun (see, e.g., Ulmer et al., Science 259:1745 (1993); introducing nucleic acid constructs into embryonic pluripotent stem cells and transferring the stem cells back into the blastocyst; and sperm-mediated gene transfer (Lavitrano et al., Cell 57:717-723 (1989); etc. For a review of such techniques, see Gordon, "Transgenic Animals," Intl. Rev. Cytol. 115:171-229 (1989), which is incorporated by reference herein in its entirety. Further, the contents of each of the documents recited in this paragraph is herein incorporated by reference in its entirety. See also, U.S. Pat. No. 5,464,764 (Capecchi et al., Positive-Negative Selection Methods and Vectors); U.S. Pat. No. 5,631,153 (Capecchi et al., Cells and Non-Human Organisms Containing Predetermined Genomic Modifications and Positive-Negative Selection Methods and Vectors for Making Same); U.S. Pat. No. 4,736,866 (Leder et al., Transgenic Non-Human Animals); and U.S. Pat. No. 4,873,191 (Wagner et al., Genetic Transformation of Zygotes); each of which is hereby incorporated by reference in its entirety.

[0346] Any technique known in the art may be used to produce transgenic clones containing polynucleotides of the invention, for example, nuclear transfer into enucleated oocytes of nuclei from cultured embryonic, fetal, or adult cells induced to

The present invention provides for transgenic animals that carry the transgene in all their cells, as well as animals which carry the transgene in some, but not all their cells, i.e., mosaic animals or chimeric. The transgene may be integrated as a single transgene or as multiple copies such as in concatamers, e.g., head-to-head tandems or head-to-tail tandems. The transgene may also be selectively introduced into and activated in a particular cell type by following, for example, the teaching of Lasko et al. (Lasko et al., Proc. Natl. Acad. Sci. USA 89:6232-6236 (1992)). The regulatory sequences required for such a cell-type specific activation will depend upon the particular cell type of interest, and will be apparent to those of skill in the art. When it is desired that the polynucleotide transgene be integrated into the chromosomal site of the endogenous gene, gene targeting is preferred. Briefly, when such a technique is to be utilized, vectors containing some nucleotide sequences homologous to the endogenous gene are designed for the purpose of integrating, via homologous recombination with chromosomal sequences, into and disrupting the function of the nucleotide sequence of the endogenous gene. The transgene may also be selectively introduced into a particular cell type, thus inactivating the endogenous gene in only that cell type, by following, for example, the teaching of Gu et al. (Gu et al., Science 265:103-106 (1994)). The regulatory sequences required for such a cell-type specific inactivation will depend upon the particular cell type of interest, and will be apparent to those of skill in the art.

Once transgenic animals have been generated, the expression of the recombinant gene may be assayed utilizing standard techniques. Initial screening may be accomplished by Southern blot analysis or PCR techniques to analyze animal tissues to verify that integration of the transgene has taken place. The level of mRNA expression of the transgene in the tissues of the transgenic animals may also be assessed using techniques which include, but are not limited to, Northern blot analysis of tissue samples obtained from the animal, in situ hybridization analysis, and reverse transcriptase-PCR (RT-PCR). Samples of transgenic gene-expressing tissue may also be evaluated immunocytochemically or immunohistochemically using antibodies specific for the transgene product.

Once the founder animals are produced, they may be bred, inbred, outbred, or crossbred to produce colonies of the particular animal. Examples of such
breeding strategies include, but are not limited to: outbreeding of founder animals with more than one integration site in order to establish separate lines; inbreeding of separate lines in order to produce compound transgenics that express the transgene at higher levels because of the effects of additive expression of each transgene; crossing of heterozygous transgenic animals to produce animals homozygous for a given integration site in order to both augment expression and eliminate the need for screening of animals by DNA analysis; crossing of separate homozygous lines to produce compound heterozygous or homozygous lines; and breeding to place the transgene on a distinct background that is appropriate for an experimental model of interest.

[0350] Transgenic and “knock-out” animals of the invention have uses which include, but are not limited to, animal model systems useful in elaborating the biological function of CD40 polypeptides, studying conditions and/or disorders associated with aberrant CD40 expression, and in screening for compounds effective in ameliorating such conditions and/or disorders.

Kits

[0351] The present invention provides kits that can be used in the above methods. In one embodiment, a kit comprises an antibody of the invention, preferably a purified antibody, in one or more containers: In a specific embodiment, the kits of the present invention contain a substantially isolated polypeptide comprising an epitope which is specifically immunoreactive with an antibody included in the kit. Preferably, the kits of the present invention further comprise a control antibody which does not react with the polypeptide of interest. In another specific embodiment, the kits of the present invention contain a means for detecting the binding of an antibody to a polypeptide of interest (e.g., the antibody may be conjugated to a detectable substrate such as a fluorescent compound, an enzymatic substrate, a radioactive compound or a luminescent compound, or a second antibody which recognizes the first antibody may be conjugated to a detectable substrate).

[0352] Accordingly provided herein are diagnostic kits comprising a composition comprising a polynucleotide molecule that is complementary to a nucleic acid molecule comprising a polynucleotide sequence chosen from among: (A) SEQ ID NO:1, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:19, SEQ ID NO:23 and SEQ ID NO:24; (B) polynucleotide sequences encoding polypeptides of SEQ ID NO:10, SEQ
ID NO:14, SEQ ID NO:15, SEQ ID NO:28-34; (C) biologically active fragments thereof; (D) complements thereof and a vehicle.

Also provided are diagnostic kits comprising an antibody that specifically binds to an isolated polypeptide comprising an amino acid sequence, wherein the amino acid sequence is chosen from among: SEQ ID NO:10, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:28-34 and a biologically active fragment thereof.

Also provided are diagnostic kits comprising an isolated polypeptide comprising an amino acid sequence, wherein the amino acid sequences chosen from among: SEQ ID NO:10, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:28-34 and biologically active fragments thereof.

In another specific embodiment of the present invention, the kit is a diagnostic kit for use in screening serum containing antibodies specific against proliferative and/or cancerous polynucleotides and polypeptides. Such a kit may include a control antibody that does not react with the polypeptide of interest. Such a kit may include a substantially isolated polypeptide antigen comprising an epitope which is specifically immunoreactive with at least one anti-polypeptide antigen antibody. Further, such a kit includes means for detecting the binding of said antibody to the antigen (e.g., the antibody may be conjugated to a fluorescent compound such as fluorescein or rhodamine which can be detected by flow cytometry). In specific embodiments, the kit may include a recombinantly produced or chemically synthesized polypeptide antigen. The polypeptide antigen of the kit may also be attached to a solid support.

In a more specific embodiment the detecting means of the above-described kit includes a solid support to which said polypeptide antigen is attached. Such a kit may also include a non-attached reporter-labeled anti-human antibody. In this embodiment, binding of the antibody to the polypeptide antigen can be detected by binding of the said reporte-labeled antibody.

In an additional embodiment, the invention includes a diagnostic kit for use in screening serum containing antigens of the polypeptide of the invention. The diagnostic kit includes a substantially isolated antibody specifically immunoreactive with polypeptide or polynucleotide antigens, and means for detecting the binding of the polynucleotide or polypeptide antigen to the antibody. In one embodiment, the antibody is attached to a solid support. In a specific embodiment,
the antibody may be a monoclonal antibody. The detecting means of the kit may include a second, labeled monoclonal antibody. Alternatively, or in addition, the detecting means may include a labeled, competing antigen.

[0358] In one diagnostic configuration, test serum is reacted with a solid phase reagent having a surface-bound antigen obtained by the methods of the present invention. After binding with specific antigen antibody to the reagent and removing unbound serum components by washing, the reagent is reacted with reporter-labeled anti-human antibody to bind reporter to the reagent in proportion to the amount of bound anti-antigen antibody on the solid support. The reagent is again washed to remove unbound labeled antibody, and the amount of reporter associated with the reagent is determined. Typically, the reporter is an enzyme which is detected by incubating the solid phase in the presence of a suitable fluorometric, luminescent or colorimetric substrate (Sigma, St. Louis, MO.).

[0359] The solid surface reagent in the above assay is prepared by known techniques for attaching protein material to solid support material, such as polymeric beads, dip sticks, 96-well plate or filter material. These attachment methods generally include non-specific adsorption of the protein to the support or covalent attachment of the protein, typically through a free amine group, to a chemically reactive group on the solid support, such as an activated carboxyl, hydroxyl, or aldehyde group. Alternatively, streptavidin coated plates can be used in conjunction with biotinylated antigen(s).

The following Examples are provided to illustrate but not limit the present invention.

**EXAMPLES**

Example 1. Amino Acid Sequence Alignment of CD40sv1, CD40sv2, and Wild-Type CD40 and Their Structural Modules.

[0360] Wild-type CD40, designated herein as CD40wt, is a type I membrane protein that belongs to the TNF receptor (TNFR) family. The extracellular region of CD40wt contains four TNFR modules of 44 amino acids (Singh J, et. al. 1998). Screening identified at least three novel splice variants of the CD40wt molecule, including CLN00236172_5pv1.a, designated herein as CD40sv1; CLN00162416, designated herein as CD40sv2; and CLN00265506, designated herein as CD40sv3.
Nucleic acid sequences and amino acid sequences for CD40sv1, CD40sv2 and CD40sv3 are provided herein in the Appendices.

Clustal W is a general purpose multiple sequence alignment program for DNA or proteins. It produces biologically meaningful multiple sequence alignments of divergent sequences. It calculates the best match for the selected sequences, and lines them up so that the identities, similarities and differences can be seen. A polypeptide sequence alignment was done with three of the newly identified CD40 splice variants using the Clustal W program (Figure 1). A polypeptide sequence alignment was done with the three newly identified CD40 splice variants and six known CD40 molecules (Figure 2). As referenced to the CD40wt (NP_001241 NM_001250) sequence (and marked on Figure 2), the signal peptide (SP) sequence comprises about amino acids 1-19; the pfam domain (domain 1) comprises about amino acids 26-59; domain 2 comprises about amino acids 62-103; domain 3 comprises about amino acids 105 to 143; domain 4 comprises about amino acids 145-186; the transmembrane domain comprises about amino acids 193-215; and the cytoplasmic region comprises about amino acids 216 to 277 (Figure 2). An additional polypeptide alignment was done to directly compare the CD40wt sequence with the CD40sv1 and CDsv2 variants (Figure 3).

Sequence analyses showed that both CD40sv1 and CD40sv2 lacked the fourth TNFR module (Figures 4A-4B, CD40sv1 shown) which corresponds to amino acids 145 to 175 in the CD40wt polypeptide sequence (Figure 3). However, CD40sv1 and CD40sv2 retain all of the amino acid residues, including aspartic acid (D)84, glutamic acid (E) 114, and E117, that are reported to be required for interaction with CD40L (Singh J, et. al. 1998). One of skill in the art would be able to determine whether a particular CD40 splice variant or mutein is capable of binding a CD40 ligand using methods known in the art. In addition, CD40sv1 lacks the transmembrane domain (see figures) and thus encodes a natural secreted protein. The lack of the fourth TNFR module in CD40sv1 and CD40sv2 suggests that these proteins may play a role in inhibiting the CD40:CD40L interaction, thus interrupting the signaling and biological functions of the CD40:CD40L pathway. Therefore, CD40sv1 and CD40sv2 may be useful in treatment of diseases involving the CD40:CD40L pathway, such as autoimmune diseases, cancer, and transplant rejection.
Example 2. Construction of wild-type CD40 (CD40wt), CD40sv1 and CD40sv2 Clones

The wild type CD40 protein, CD40wt (SEQ ID NO:13) encodes for a type I transmembrane protein. The CD40 gene can produce several splice variants, each arising from differential splicing of different exons and each different than wild type CD40. One novel splice variant, CD40sv1 (CLN00236172), encodes a secreted protein lacking a transmembrane domain. To compare the biological activity of the novel splice variants with CD40wt, several constructs were built and these proteins were analyzed side-by-side. To make a secreted CD40wt, constructs were built with the amino acids that encode for the extracellular domains of the protein (amino acids 1 or 20 to 193), designated herein as CD40ECD. All of the constructs of CD40wt, wild-type CD40ECD, CD40sv1 and CD40sv2 were generated by standard cloning procedures as known in the art and were cloned into mammalian and insect cells. Constructs are listed in the Tables.

Example 3. Construction of CD40ECD, CD40sv1 and CD40sv2 Fusion Proteins.

To facilitate trimerization or oligomerization of CD40sv1 and CD40sv2 and increase protein stability, CD40sv1 (amino acids 1 to 166) or the extracellular domains of CD40sv2 (amino acids 1 to 141) was fused with a Fc domain, a leucine zipper domain, or a trimerization domain derived from tetranectin (Holtet TL, 1997) or mannose binding protein (MBP1). Thus, fusion of CD40sv1 and CD40sv2 to these oligomerization domains enhances their ability to modulate and in some examples, inhibit CD40:CD40L interactions.

CD40-Fc Fusion Constructs.

In order to induce the dimerization of CD40ECD and CD40sv1, a human IgG1 clone (GenBank accession number AAH19337) with annotation: “Homo sapiens immunoglobulin heavy constant gamma 1 (G1m marker), mRNA (cDNA clone MGC:12853 IMAGE:4054679)”, was selected from a lung cancer library and its Fc domain (248-464aa) was engineered into the pTT5-D and pTT5-E vectors (Figures 5A-5Bi-v and Table 6) by standard PCR and cloning techniques as known in the art. For cloning purposes a BamH1 site was engineered on the 5'-end of the Fc domain. This allowed DNA fragments encoding for amino acids 1 through 193 of CD40wt and amino acids 1 through 166 of CD40sv1 to be inserted between the EcoRI and BamH1 sites of the vector containing the Fc domain. Thus constructs encoding
for CD40ECD and CD40sv1 secreted proteins fused to a Fc domain were produced. CLN00677489 (SEQ ID NO: 120) encoded the fusion protein CD40ECD (1-193 aa)-Fc (248-464 aa) and CLN00677485 (SEQ ID NO: 120) encoded the fusion protein CD40sv1 (1-166aa)-Fc (248-464 aa) (Table 6).

[0367] To produce protein in HighFive™ insect cells, DNA fragments encoding the CD40-Fc fusion proteins were cloned into the pIB/V5His-DEST vector (Invitrogen, Carlsbad, CA) by PCR, using CD40ECD-Fc (CLN00677489) and CD40sv1-Fc (CLN00677485) as DNA templates. The following constructs were generated: CD40ECD-Fc in insect vector is CLN00783089 SEQ ID NO:122 and CD40sv1-Fc in insect vector is CLN00783084 SEQ ID NO:123.

[0368] The Fc domain can also be used to facilitate the purification of soluble CD40ECD and CD40sv1. Constructs are generated in the pIB/V5His-DEST vector in which a Tobacco Etch Virus (TEV) protease recognition site is engineered in between the CD40 and the Fc domains. CD40 fusion proteins are purified by passage over a Protein A-Sepharose column wherein the Fc domain binds to the Protein A. After elution from the column, soluble CD40 is released by incubating the purified fusion proteins with TEV protease. The seven amino acid recognition site for TEV protease is Glu-Asn-Leu-Tyr-Phe-Gln-Gly with cleavage occurring between Gln and Gly. For this purpose the following constructs were generated: CD40ECD-TEV-Fc in insect vector CLN845009 SEQ ID NO:124 and CD40sv1-TEV-Fc in insect vector CLN845008 SEQ ID NO:125.

[0369] In order to improve the secretion of the CD40ECD-Fc and the CD40sv1-Fc fusion proteins, the secretory signal peptides encoded by the first 19-20 amino acids were replaced by the 23 amino acids that encode for the signal peptide of collagen (SP) (GenBank accession number NP_001842). The following constructs were built: CLN00677491 encoding for a secreted mature CD40wt-Fc (construct SP-CD40wt(21-193aa)-Fc (SEQ ID NO:126) and CLN00677487 encoding for a secreted mature CD40sv1-Fc (construct SP-CD40sv1(21-167aa)-Fc.

[0370] CD40-leucine zipper constructs.

[0371] In order to induce trimerization of the CD40ECD and CD40sv1 proteins, fusions with a leucine zipper domain from two different transcription factors were built. The transcription factors were NFE2 (nuclear factor (erythroid-derived 2), GenBank accession number NP_006154) with a leucine zipper domain between amino acids 294 and 339, and NRL (neural retina leucine zipper, GenBank accession
number NP_006168) with a leucine zipper domain between amino acids 187 and 207-208. In addition, to improve the secretion of the CD40 constructs, the signal peptides were replaced with the collagen signal peptide and two clones for each construct were generated. Each construct was cloned into the pTT5-B vector and into the pIB/V5His-DEST vector. The constructs generated are described herein and include SEQ ID NO: 128 through SEQ ID NO:135.

[0372] CD40-tetranectin fusion constructs.

[0373] In order to induce trimerization of the CD40ECD and CD40sv1 proteins, additional fusions were constructed with the collagen domain of tetranectin (GenBank accession number NP_003269). The constructs generated are described herein and include SEQ ID NO: 136 and SEQ ID NO: 137.

[0374] CD40-mannose binding protein fusion constructs.

[0375] In order to induce trimerization and higher order complex formations of the CD40ECD and CD40sv1 proteins, fusions with the collagen domains of the secreted protein MBP1 (mannose binding protein, GenBank accession number NP_000233) were constructed. The constructs generated are described herein and include SEQ ID NO: 138 and SEQ ID NO: 139.

[0376] MBP1 contains cysteines upstream of the collagen domain thought to participate in the formation of higher order complexes. To test the effect of these cysteines in higher order complex formation with the CD40 fusion proteins, constructs containing MBP1 without cysteines were generated. The constructs generated are described herein and include SEQ ID NO: 140 and SEQ ID NO: 141.

[0377] CD40-MBP1 and CD40-tetranectin fusion constructs with N-terminus cleavable tags.

[0378] To monitor the expression and secretion of the CD40-MBP1 and CD40-tetranectin fusion constructs and for aiding in their purification, constructs were generated with N-terminal cleavable tags. Constructs were made that contained eight histidine residues, a StreTagII tag, a V5 epitope tag and a Tobacco Etch Virus protease cleavage site at the N-terminal end of the CD40 fusion proteins. Both tetranectin and mannose binding protein oligomerization domains were used, including a mannose binding protein oligomerization domain with no cysteines (Table 7, SEQ ID NOs:142-147).

Example 4. Protein Expression in Mammalian Cells.
Recombinant protein expression in mammalian cells usually maintains posttranslational modifications which might be crucial for biological function. In order to facilitate purification and expression of CD40sv1 and CD40ECD proteins, additional DNA constructs were designed adding C-terminal tags (V5H8) or a five prime signal peptide (FPSP; shown in the appendices) to the mature sequences. In some constructs a thrombin cleavage site was inserted between the CD40 portion and V5H8 tag. The FPSP was derived from a secreted protein that was found during expression studies of several hundred secreted recombinant proteins expressed in 293-T cells (data not shown). To examine the expression of the CD40 proteins in mammalian cells, five CD40sv1 constructs were used including CD40sv1, CD40sv1-V5H8, CD40sv1-Fc, FPSP-CD40sv1-Fc (SEQ ID NO:127) and FPSP-CD40sv1-V5H8. In addition, three CD40ECD constructs were used including CD40ECD-Fc (SEQ ID NO:120), CD40ECD-thrombecleavg-V5H8 (SEQ ID NO:148) and FPSP-CD40ECD-Fc (SEQ ID NO:126). The constructs are shown in Tables 6-7.

Transfection of adherent 293-T cells.

293-T cells (ATCC, Manassas, VA) were plated at 5 x 10^5 cells/well on poly-D-lysine coated 6-well plates (BD Biosciences, San Jose CA) in 2 ml complete DMEM medium (DMEM medium supplemented with 10% FBS, Mediatech, Herndon, VA) 18-24 hrs prior to transfection. The following day media was replaced by 2 ml fresh complete DMEM medium. Cells were transfected by preparing 100 µl OptiMEM (Invitrogen, Carlsbad, CA) with 3.8 µl Fugene6 (Roche, Indianapolis, IN) in a sterile reaction tube and incubated for 5 min at room temperature. In a separate tube 100 µl OptiMEM was prepared with 2 µg DNA and subsequently mixed with the medium/Fugene6 solution. The transfection complex was incubated for 15 minutes at room temperature before it was dropped into one well. Cells were incubated for 48 hrs at 37° C and 5% CO₂. After 48 hrs, medium was replaced with 2 ml serum-free production medium (HyQ PF CHO LS, HyClone, Logan, UT). Cells were incubated for another 48 hrs before cells and supernatants were harvested for further analysis.

Western blot analysis.

Protein expression in 293-T cells was evaluated using SDS-PAGE and Western blot analyses by standard methods known in the art. Supernatants were cleared of cell debris by centrifugation and subsequently mixed 4:1 with sample
buffer (XT sample buffer, Bio-Rad, Hercules, CA). Samples were denatured by heating at 99°C for 3 minutes and subsequently were either loaded on a SDS-PAGE gel (Criterion XT gel, Bio-Rad, Hercules, CA) or stored at -20°C for analysis at a later time. Cells were lysed in their respective wells by resuspension in 300 µl lysis buffer (1% NP-40; 50 mM Tris-HCl, pH 8.0; 150 mM NaCl; 1 tablet complete protease inhibitors (Roche, Indianapolis, IN)) and then transferred into separate tubes. Cell debris was pelleted by centrifugation at 14,000 rpm and the cleared lysates were transferred into fresh tubes. Cleared cell lysates were mixed 4:1 with sample buffer and prepared for SDS-PAGE as described above. Proteins in cell lysates and cell supernatants were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Cell lysates and supernatants were matched by cell number to determine secretion efficiency. The membrane was probed with a mouse monoclonal antibody specific for the extracellular domain of human CD40 (BD-Biosciences, San Jose, CA). Bound antibody was detected with a polyclonal goat-anti-mouse antibody conjugated to horseradish peroxidase (Pierce, Rockford, IL). Immunocomplexes were visualized by incubating the membrane in chemiluminescence substrate (SuperSignal West Femto, Pierce, Rockford, IL) and then exposing the membrane to light-sensitive film.

All CD40ECD proteins tested were expressed and secreted by 293-T cells, but CD40sv1 proteins were not secreted at any detectable level. About 60% of the expressed CD40ECD protein was detected in cell culture supernatant, whereas 100% of the expressed CD40sv1 proteins were retained inside the cells. Even a 10-fold increase in protein expression in 293-T cells did not lead to any detectable secreted CD40sv1 (Figure 6). Comparison of the expression levels of construct no.3 to construct no. 4 and construct no. 6 to construct no.8 showed no detectable increase in expression or secretion. Thus the presence of the signal peptide FPSP did not appear to enhance the expression of either CD40sv1 or CD40ECD proteins. However, as shown in Figures 6A-6B extension of the C-terminal sequence of CD40sv1 by fusion to a Fc domain appeared to increase expression in 293-T cells.

Transfection of suspension 293-6E cells.

As an alternative, mammalian expression in 293-6E cells was tested to produce the CD40sv1 protein. 293-6E cells stably express the EBNA1 protein and therefore are able to replicate plasmid DNA containing the oriP of Epstein Barr Virus. All of the pTT-derived vectors used herein contain the oriP of Epstein Barr Virus.
(Durocher et al. Nucleic Acids Res. 2002 Jan 15;30(2):E9). The 293-6E cells were obtained from Yves Durocher (Biotechnology Research Institute, Montreal, Canada) and were adapted to suspension to facilitate protein expression in large scale-up production.

[0387] 18-24 hours before transfection, 293-6E cells were diluted to 6 x 10^6 cells/ml in 25 ml FreeStyle medium (Invitrogen, Carlsbad, CA). Transfection complexes were prepared by adding 25 μg DNA to 1.25ml sterile PBS in a 15 ml sterile tube and 50 μl PEI (polyethylenimine dissolved in sterile water at 1 mg/ml; linear PEI 25 kD: Polysciences, Warrington, PA) to 1.25 ml sterile PBS in a separate 15 ml sterile tube. Both solutions were mixed and incubated for 1 hour at room temperature before adding to the cells. Supernatants and cells were harvested and prepared for western blot analysis as described above.

[0388] 293-6E cells were transfected with construct CD40sv1-V5H8 or CD40sv1-Fc (Table 7, constructs no. 2 and no. 3, respectively). The 293-T6 cells expressed and secreted only the CD40sv1-Fc fusion protein (Figures 7A-7B, no. 3).

[0389] A CD40sv1 construct with a C-terminal V5 epitope and His-tag was expressed, but not secreted as previously seen with 293-T cells. The secretion efficiency of 293-6E cells expressing CD40sv1-Fc fusion protein was about 30% of the total expressed protein. In contrast, the CD40ECD-Fc fusion protein was highly expressed and secreted at yields of >10 μg/ml in cell culture supernatant, confirming the extremely different expression levels of these two proteins despite their high sequence similarity.

The signal peptide, FPSP, did not improve expression or secretion of CD40sv1-Fc fusion protein in 293-6E cells. 293-6E cells were transfected with FPSP-CD40sv1-Fc (Table 7, construct no. 4), cultured for six days, harvested and prepared as described above.

[0390] Analysis of the expression kinetics showed strong intracellular expression within 4 days of culture (Fig. 8, lane 1), but secretion efficiency was around 20-30% of expressed CD40sv1-Fc (Fig. 8, lanes 5-10). The overall yield of the CD40sv1-Fc fusion protein was 30-40 ng/ml in 293-6E cell culture supernatant, based on a CD40-Fc standard (R&D Systems, Minneapolis, MN) (Fig. 8, lane 12-15) Example 5. Protein Expression in Insect cells.

[0391] CD40sv1 was highly expressed inside mammalian cells, but despite its high sequence similarity to CD40ECD, it was not as efficiently secreted as the latter,
if secreted at all. As an alternative to a mammalian expression system an insect cell system was tested. Insect cells are able to express biologically active human proteins at high levels after infection with recombinant baculovirus or transfection with a recombined expression vector. The latter method gives the advantage of being able to establish stable cell lines therefore removing the need for a transfection or an infection step for each production run.

[0392] Transfection of HighFive™ cells.

[0393] HighFive™ cells (BTI-TN-5B1-4), ExpressFive SFM medium, L-glutamine (200mM), penicillin/streptomycin, blasticidin-HCl, CellFectin and the pIB/V5-His-DEST vector for constitutive expression in insect cells were all purchased from Invitrogen (Carlsbad, CA). Transfection procedures and selection of stable cell lines were performed according to manufacturer’s recommendations. For transfection of HighFive™ cells, 12 x 10^6 cells were plated on 15 cm plates (Corning, Acton, MA) in 29 ml complete medium (ExpressFive SFM medium supplemented with 20 mM L-glutamine and pen/strep 1:200) and incubated for 1 hour at room temperature to allow cells to adhere. 20 ug of DNA was added to 500 ul complete medium in a 1.5 ml Eppendorf tube and 60 ul CellFectin was added to 500 ul complete medium in a separate 1.5 ml Eppendorf tube. Both solutions were combined in one tube and incubated for 15 min at room temperature. The transfection complex (1ml) was added to the plate and cells were incubated for 2 days.

[0394] HighFive™ insect cells were transfected with CD40sv1-Fc (construct no. 10), CD40ECD-Fc (construct no. 11) and a vector control (construct no.12) from Table 7 and cultured for 4 days as described. Cells and supernatant were harvested as described and analyzed for expression by SDS-PAGE and western blotting methods using a monoclonal mouse anti-human CD40 antibody (BD Biosciences, San Jose, CA). Both CD40sv1-Fc and CD40ECD-Fc proteins were expressed well in the HighFive™ insect cells and in addition the proteins were secreted at high levels (Figure 9A and 9B).

[0395] Selection of stably expressing insect cells.

[0396] Selection for stable cell lines expressing the CD40 proteins was performed according to manufacturer’s recommendations. Briefly, 48 hours post-transfection cells were detached by pipetting and split 1:5 to reach 20% confluency on a 15 cm plate. Cells were incubated overnight in fresh medium before blasticidin-HCl was added at 50 ug/ml final concentration. Cells were split twice a week over a
period of 2 weeks until viability was consistently over 90%. Protein expression was
determined by western blot analysis and cell lines stably expressing the desired
proteins were selected.

Adaptation of HighFive™ cells to suspension culture.

For adaptation of cells to suspension culture conditions, cells from two
15 cm plates were detached by pipetting and cell density was adjusted to 10^6 cells/ml
in complete medium containing 50 μg/ml blasticidin-HCl. A 50 ml cell suspension
was added to a 250 ml shaker flask with vent cap (Corning, Acton, MA) and
incubated at 27°C with a rocking speed of 120 rpm. Cell density was maintained at
10^6 cells/ml for 1 week until viability was above 80% and growth rate increased. The
following week cell density was reduced to 5 x 10^5 cells/ml until growth rate
increased and subsequently cells were split twice a week to 2 x 10^5 cells/ml and
maintained in complete medium containing 50 μg/ml blasticidin-HCl.

Protein expression yields from the suspension adapted HighFive™
cells were analyzed by western blot analysis and were found to be equivalent to
transiently expressing adherent HighFive™ cells (Fig. 10, lane 2 and 3 vs lane 1).
Production of CD40sv1-Fc and CD40ECD-Fc in HighFive™ insect cells was
increased approximately 100-fold when compared to expression in 293 cells.
Secretion efficiency was greater than 50% of the CD40sv1-Fc expressed from
adherent HighFive™ cells. Adaptation to suspension culture did not decrease
production yields for CD40ECDFc and specific protein yields were approximately 4-5
μg/ml for CD40ECD-Fc and approximately 2-3 μg/ml for CD40sv1-Fc, based on a
CD40-Fc standard (Fig. 10 lanes 5-8).

Large scale-up production of proteins.

For scale-up production, a 20L Wave bioreactor is seeded with 10L of
HighFive™ cell suspension at 2 x10^5 cells/ml. Culture medium is complete medium
without selection reagent. Fermentation is maintained over 5 days with constant
airflow at 0.2 L/min and constant agitation between 28 and 31 rpm. 5 days post-
inoculation, the cell suspension is harvested and pelleted at 1400 rpm (Avanti J-E
centrifuge, rotor JS-5.3, Beckman-Coulter, Fullerton, CA) to separate cells from
supernatant. A second centrifugation at 5000 rpm (Avanti J-E centrifuge, rotor JS-
5.3, Beckman-Coulter, Fullerton, CA) is required to clear cell debris from cell
supernatant. Cleared supernatant is then transferred to downstream processing and purification.

Example 6. Protein Purification

CD40sv1-Fc was obtained from HighFive™ insect cells either transiently or stably expressing the protein. The supernatant (Figure 11, Sup.) was adjusted to 1 M NaCl and concentrated 5-fold with a 10 kD molecular weight cut-off regenerated cellulose membrane (Millipore, Billerica, MA). The concentrated supernatant was diluted 3 times 1:1 with buffer A (10 mM potassium phosphate pH 6.5, 1 M NaCl) and 2-fold concentrated on the same concentration device (Figure 11, Conc. Sup.). The final conditioned and concentrated supernatant was clarified by centrifugation (Figure 11, Start) and loaded onto a Protein A-Sepharose column (Amersham, Piscataway, NJ). The column was washed with buffer A. Bound protein was eluted with a step gradient of buffer B (100 mM glycine pH 2.7; 1 M NaCl; Figure 11, Eluted Fractions). The protein was obtained at a purity >95% with a yield of 3 to 4 mg per liter cell culture supernatant.

Example 7. Inhibition of Human B cell Activity by CD40sv1-Fc Fusion Protein.

Untouched human primary B cells were purified from PBMC using the B cell isolation kit per manufacturer’s instructions (Miltenyi Biotechnology Inc., Auburn, CA). CHO cells stably transfected with human CD40L were used as a stimulator of B cells in addition to 20 ng/ml of human recombinant IL-4. 30000 purified B cells were incubated with CHO-CD40L cells in the presence of purified CD40sv1-Fc, CD40ECD-Fc, or buffer control, at the indicated concentrations (Figure 12). After four days of incubation in RPMI with 5% FBS, cell numbers were determined using the CellTiter-Glo Luminescent Cell Viability Assay Kit (Promega, Madison, WI). As shown in Figure 12, CD40sv1-Fc (squares) efficiently inhibited B cell activation. The 50% effective dose (ED$_{50}$) of CD40sv1-Fc inhibition was 2 ug/ml. In contrast, the ED$_{50}$ of the CD40ECD-Fc protein (diamonds) was 10 ug/ml. Thus CD40sv1-Fc is about 5 times as potent as the CD40ECD-Fc protein in the inhibition of B cell activation. The inhibition of B cell activation after stimulation by CD40L-CHO cells was specific to CD40sv1-Fc since CD40sv1-Fc did not have any inhibitory effect on B cells incubated with CHO cells without CD40L (Figure 13).

These data demonstrated that CD40sv1-Fc is a potent inhibitor of human B cell activation. Without being bound by theory, this inhibition appears to be
through the blockade of CD40:CD40L signaling. CD40 signaling has been associated with pathogenic processes of chronic inflammatory diseases, such as autoimmune diseases, neurodegenerative disorders, graft-versus-host disease, cancer, and atherosclerosis. Therefore, CD40sv1-Fc and other fusion proteins of CD40sv1 or CD40sv2 may be useful as antagonists in the treatment of these and other diseases involving or associated with CD40:CD40L signaling.

Example 8. CD40sv1 and Variants thereof

CD40sv1 was observed to have lower expression yields than the extracellular domains of wild type human CD40 (CD40ECD, NP_001241 residues 20-193). This decrease in expression yield may result from a number of factors, including: (1) the absence of wild type domain 4 (residues 146-186), (2) the presence of CD40sv1’s new domain (residues 144-166), (3) sequence differences between CD40wt and CD40sv1 domain 3 (domain 3 comprises residues 105-143; residues 136-142 are different in CD40wt and CD40sv1), and (4) sequence differences adjacent to cysteine 143, which may affect the efficiency of disulfide bond formation between residues 125 and 143. It has been demonstrated that the level of protein expression of a homologous protein, TNFR1, is improved by incorporating mutations that limit conformational flexibility in the residues adjacent to disulfide bonded cysteine residues, so long as the mutations are compatible with the native structure (Schweickhardt et al. (2003) J. Biol. Chem. 278:28961-28967).

Variant proteins or “muteins” may be made in order to better understand which factors determine the expression levels of CD40wt and CD40sv1 (Figure 14). All muteins are constructed using standard molecular biology techniques known in the art. The muteins are likely to retain the functional properties of soluble CD40, including the ability to bind CD40L.

Mutein 1a comprises CD40wt residues 20-145 (alternatively, 1b comprises 20-144 or 1c comprises 20-143). This region is equivalent to the first three domains of CD40wt. The expression of mutein(s) 1 may be compared to the protein expression of CD40ECD to determine the impact of domain 4 on protein expression. The presence of domain 4 may affect the rate or efficiency of folding for the rest of the protein. Domain 4 has two N-linked glycosylation sites (at residues 153 and 180) and these modifications may contribute to the stability or solubility of CD40 molecules. (Table 6, SEQ ID NO:92, 93 and 94).
[0408] Mutein 2a comprises CD40sv1 residues 20-145 (alternatively, 2b comprises 20-144 or 2c comprises 20-143). This region is equivalent to the first three domains of CD40sv1. The protein expression of mutein(s) 2 may be compared to the expression of CD40sv1 to determine the impact of the new domain in CD40sv1 on protein expression. (Table 6, SEQ ID NO:95, 96 and 97)

[0409] Muteins 3a-c comprise CD40wt residues 20-145 (alternatively, 3d-f comprise 20-144 or 3g-i comprise 20-143) and muteins 3a, d, and g comprise CD40sv1 residues 144-166 (alternatively, 3b, e and h comprise 145-166 or 3c, f, and i comprise 146-166). These regions are equivalent to the first three domains of CD40wt and the new carboxy domain of CD40sv1. The protein expression of mutein(s) 3 may be compared to the protein expression of CD40sv1 to determine the impact of the sequence differences in residues 136-142 on protein expression. Table 6, SEQ ID NO:98-106.

[0410] Muteins 4a-c comprise CD40sv1 residues 20-143 (alternatively, 4d-f comprise 20-144 or 4g-i comprise 20-145) and muteins 4a, d and g comprise CD40wt residues 144-193 (alternatively, 4b, e and h comprise 145-193 or 4c, f and i comprise 146-193). These regions are equivalent to the first three domains of CD40sv1 and domain 4 of CD40wt. The protein expression of mutein(s) 4 may be compared to the protein expression of other constructs, such as, CD40sv1, to determine the impact of the sequence differences in residues 136-142 on protein expression. Table 6, SEQ ID NO:107-115.

[0411] Mutein 5 comprises CD40sv1 residues 20-166, with a leucine to isoleucine substitution at residue 142. Isoleucine is present in CD40wt at position 142. Isoleucine, a beta-branched amino acid, has less backbone flexibility than leucine. As a result, this substitution may reduce conformational freedom in the unfolded state, increase the rate of folding, and improve protein expression. Therefore the protein expression of mutein 5 may be compared to the protein expression of CD40sv1 to determine the effect of isoleucine at residue 142 on protein production. Table 6, SEQ ID NO:116.

[0412] Mutein 6 comprises CD40sv1 residues 20-166, with an asparagine to glutamic acid at residue 144 and an arginine to proline substitution at residue 145. Glutamic acid is present in CD40wt at position 144, and proline is present in CD40wt at position 145. Proline has significantly less backbone flexibility than other amino acids. As a result, this substitution may reduce conformational freedom in the
unfolded state, increase the rate of folding, and improve protein expression. The protein expression of mutein 6 may be compared to the protein expression of CD40sv1 to determine the impact of glutamic acid at residue 144 and of proline at residue 145 on protein production. Table 6, SEQ ID NO:117.

Examples 9. CD40sv2 and Variants thereof

[0413] Relative to wild type human CD40, CD40sv2 is missing residues 135-186 (Figures 15A-15D). The missing amino acids include the last 8 residues of domain 3 through the first residue after domain 4. CD40sv2 does include the seven residues immediately preceding the transmembrane domain, which are numbered 187-193. CD40wt is predicted to have a disulfide bond between Cys 125 and Cys 143; since Cys 143 is not present in CD40sv2, Cys 125 is likely to be unpaired. To avoid improper disulfide bond formation, it may be desirable to delete the remainder of the subdomain (that is, expand the deletion so that it starts between residue 120 and 125 rather than at residue 135) or to replace Cys 125 with another amino acid.

[0414] Mutein 7 comprises CD40sv2 with residues 125-134 deleted. This removes the second subdomain in domain 3 and eliminates the unpaired cysteine at residue 125. The protein expression of mutein 7 may be compared to the protein expression of CD40sv2 to determine the impact of the unpaired cysteine residue at 125 on protein production. Table 6, SEQ ID NO: 118.

[0415] Mutein 8 comprises CD40sv2 with Cys 125 replaced by any other amino acid (that is, aspartic acid, glutamic acid, arginine, lysine, histidine, glutamine, asparagine, serine, threonine, alanine, glycine, proline, valine, leucine, isoleucine, methionine, phenylalanine, tyrosine, or tryptophan). Alternatively, Cys 125 may be deleted. This substitution or deletion eliminates the unpaired cysteine at residue 125 while retaining the first part of the second subdomain of domain 3. The protein expression of mutein 8 may be compared to the protein expression of CD40sv2 to determine the impact of the unpaired cysteine residue at 125 on protein production. Table 6, SEQ ID NO: 119.

[0416] Example 10. In vivo Models for Determining the Efficacy of CD40sv1 and CD40sv2 for Disease Treatment and Side Effects
To analyze the effect of CD40sv1 or CD40sv2 on B cell activation in vivo, DBA/2 mice are immunized with 50 μg of antigen, DNP-KLH as described by Siepmann, et al. (2001). CD40sv proteins at 0.01 μg/mouse (~0.5 μg/kg body weight), 0.1 μg (~5μg/kg), 1 μg (~50 μg/kg), 10 μg (~500 μg/kg), 100 μg (~5 mg/kg), 1000 μg (~50 mg/kg) or 4000 μg (200 mg/kg), are administrated i.m., i.v., i.p., or s.c. in mice daily for up to 21 days. Anti-CD40L monoclonal antibody, anti-CD40 monoclonal antibody, or soluble CD40sv-Fc fusion protein are used as positive controls. Mice are analyzed for antibodies against DNP for up to 21 days to evaluate the effect that administration of CD40sv has on antibody production.

To examine the effect of CD40sv on autoimmune disease, such as systemic lupus erythematosus (SLE), (SWR x NZB) F1 (SNF1) mice with established lupus nephritis are used as described by Kalled et al. (1998). CD40sv proteins at 0.01 μg/mouse (~0.5 μg/kg body weight), 0.1 μg (~5μg/kg), 1 μg (~50 μg/kg), 10 μg (~500 μg/kg), 100 μg (~5 mg/kg), 1000 μg (~50 mg/kg) or 4000 μg (200 mg/kg), are administrated i.m. i.v., i.p., or s.c. in mice daily, for up to one week per month, two weeks per month, three weeks per month, four weeks per month, every month or every other month or every third month or every fourth, fifth or sixth month for as long as necessary to achieve therapeutic effect. Anti-CD40L monoclonal antibody, anti-CD40 monoclonal antibody, or soluble CD40sv-Fc fusion protein are used as positive controls. The treated mice are evaluated daily for effects on proteinuria, inflammation, sclerosis/fibrosis, and incidence of severe nephritis as described (Kamnazz et al. 2004) for up to 300 days. Also evaluated is any increase in survival time of the treated animals as compared to control animals.

To determine the efficacy of CD40sv in treatment of transplant rejection, the pancreatic islet allograft mouse model or renal allograft primate model are used as described (Parker et al., 1995; Kirk et al. 1997). Animals in either model are treated with injection(s) of CD40sv protein. CD40sv proteins at 0.01 μg/mouse (~0.5 μg/kg body weight), 0.1 μg (~5μg/kg), 1 μg (~50 μg/kg), 10 μg (~500 μg/kg), 100 μg (~5 mg/kg), 1000 μg (~50 mg/kg) or 4000 μg (200 mg/kg), are administrated i.m., i.v., i.p., or s.c. in mice daily, for up to one week per month, two weeks per month, three weeks per month, four weeks per month, every month or every other month or every third month or every fourth, fifth or sixth month for as long as necessary to achieve therapeutic effect. Anti-CD40L monoclonal antibody, anti-CD40 monoclonal antibody, or soluble CD40sv-Fc fusion protein are used as positive
controls. Transplant graft survival are monitored daily as described (Kanmaz et al. 2004) for 1 to 300 days.

[0420] To examine side effects, such as thromboembolism, that may be caused by an inhibitor (e.g. anti-CD40L antibody) of the CD40 pathway (Kanmaz et al. 2004), rhesus monkeys are administrated doses of CD40sv proteins. CD40sv proteins at 0.01 ug/mouse (~0.5 ug/kg body weight), 0.1 ug (~5ug/kg), 1 ug (~50 ug/kg), 10 ug (~500 ug/kg), 100 ug (~5 mg/kg), 1000 ug (~50 mg/kg) or 4000 ug (200 mg/kg), are administrated i.m., i.v., i.p., or s.c. in mice daily, for up to one week per month, two weeks per month, three weeks per month, four weeks per month, every month or every other month or every third month or every fourth, fifth or sixth month for as long as necessary to achieve therapeutic effect. Anti-CD40L monoclonal antibody, anti-CD40 monoclonal antibody, or soluble CD40sv-Fc fusion protein are used as positive controls. Thromboembolic changes are examined daily as described (Kanmaz et al. 2004) for 1 to 300 days.

References:


[0428] All publications, patents and patent applications cited herein, whether supra or infra, are hereby incorporated by reference in their entirety.

[0429] It must be noted that, as used in this specification and the appended claims, the singular forms “a”, “an” and “the” include plural referents unless the content clearly dictates otherwise. Thus, for example, reference to “an antigen” includes a mixture of two or more antigens, and the like.

[0430] It will be clear that the invention may be practiced otherwise than as particularly described in the foregoing description and examples. Numerous modifications and variations of the present invention are possible in light of the above teachings and, therefore, are within the scope of the appended claims.
## TABLE 1

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**TABLE 7**
Constructs tested for expression in 293-T, 293-6E and HighFive™ insect cells.

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SEQ.ID.NO. 29  HG1019443P1 CD40_frag2
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SEQ.ID.NO. 31  HG1019445P1 CD40_frag4
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SEQ.ID.NO. 32  HG1019446P1 CD40_frag5
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SEQ.ID.NO. 34  HG1019448P1 CD40_frag7
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SEQ.ID.NO. 148 HG1019452P1 CLN00677490 MVKLPLQCVLWGCMTAVHEFPPTACREKQYLNSQCCSLCQPQQKLVDCTEFTETECLPCGESSFLDTWNRELTHCHQHKYCDPNLGRVQQKGTSBTDTICTCEGWHCTSEAESCVLHRSCSPFGVQIYAVPRKTLWCNRQAQTRLMLSVVSPGQWALEKA

150
APPENDIX D

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SEQ ID NO:150

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Ile Asn Ser Glu Cys Cys Ser Leu Cys Gln Pro Gly Gln Lys Leu Val
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Ser Asp Cys Thr Glu Phe Thr Glu Thr Glu Cys Leu Pro Cys Gly Glu
50 55 60

Ser Glu Phe Leu Asp Thr Trp Asn Arg Glu Thr His Cys His Gln His
65 70 75 80

Lys Tyr Cys Asp Pro Asn Leu Gly Leu Arg Val Gln Gln Lys Gly Thr
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Ser Glu Thr Asp Thr Ile Cys Thr Cys Glu Glu Gly Trp His Cys Thr
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Ala Gly Thr Asn Lys Thr Asp Val Val Cys Gly Glu Ser Trp Thr Met
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SEQ ID NO:151

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156
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SEQ ID NO:152

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Ile Asn Ser Gln Cys Cys Ser Leu Cys Gln Pro Gly Gln Lys Leu Val
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Ser Asp Cys Thr Glu Phe Thr Glu Thr Glu Cys Leu Pro Cys Gly Glu
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Ser Glu Phe Leu Asp Thr Trp Asn Arg Glu Thr His Cys His Gln His
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Lys Tyr Cys Asp Pro Asn Leu Gly Leu Arg Val Gln Gln Lys Gly Thr
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Ser Glu Thr Asp Thr Ile Cys Thr Cys Glu Gly Glu Gly Trp His Cys Thr
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Phe Gly Val Lys Gln Ile Ala Thr Gly Val Ser Asp Thr Ile Cys Glu
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Cys His Pro Trp Thr Ser Cys Glu Thr Lys Asp Leu Val Val Gln Gln
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SEQ ID NO:153

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SEQ ID NO:154

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Ser Asp Cys Thr Glu Phe Thr Glu Thr Glu Cys Leu Pro Cys Gly Glu
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Ser Glu Phe Leu Asp Thr Trp Asn Arg Glu Thr His Cys His Gln His
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Lys Tyr Cys Asp Pro Asn Leu Gly Leu Arg Val Gln Gln Lys Gly Thr
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Ser Glu Thr Asp Thr Ile Cys Thr Cys Glu Glu Gly Trp His Cys Thr
100 105 110

Ser Glu Ala Cys Glu Ser Cys Val Leu His Arg Ser Cys Ser Pro Gly
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Phe Gly Val Lys Gln Ile Ala Thr Gly Val Ser Asp Thr Ile Cys Glu
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Pro Cys Pro Val Gly Phe Phe Ser Asn Val Ser Ser Ala Phe Glu Lys
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Cys His Pro Trp Thr Ser Cys Glu Thr Lys Asp Leu Val Val Gln Gln
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Ala Gly Thr Asn Lys Thr Asp Val Val Cys Gly Glu Ser Trp Thr Met 180 185 190
Gly Pro Gly Glu Ser Leu Gly Arg Ser Pro Gly Ser Ala Glu Ser Pro 195 200 205
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Ala Gly Leu Tyr Gln Lys Gly Gly Gln Glu Ala Asn Gln 225 230 235

SEQ ID NO:155

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Ser Asp Cys Thr Glu Phe Thr Glu Thr Glu Cys Leu Pro Cys Gly Glu 50 55 60
Ser Glu Phe Leu Asp Thr Trp Asn Arg Glu Thr His Cys His Glu His 65 70 75 80
Lys Tyr Cys Asp Pro Asn Leu Gly Leu Arg Val Gln Gln Lys Gly Thr 85 90 95
Ser Glu Thr Asp Thr Ile Cys Thr Cys Glu Gly Glu Trp His Cys Thr 100 105 110
Ser Glu Ala Cys Glu Ser Cys Val Leu His Arg Ser Cys Ser Pro Gly 115 120 125
Phe Gly Val Lys Gln Ile Ala Val Arg Pro Lys Thr Trp Leu Cys Asn
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Arg Gln Ala Gln Thr Arg Leu Met Leu Ser Val Val
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SEQ ID NO: 156

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Claims

We claim:

1. An isolated polypeptide that is capable of binding a CD40 ligand, wherein said polypeptide is selected from the group consisting of:
   a) CD40 splice variants selected from the group consisting of CD40sv1, CD40sv2, and CD40sv3;
   b) muteins of the CD40 splice variants of a);
   c) CD40 splice variant fusion molecules comprising the CD40 splice variants of a);
   d) mutein fusion molecules comprising the muteins of b);
   e) polypeptides comprising the sequence SPGQWALEKA;
   f) polypeptides comprising the sequence VRPKTWLCLNQAQTRLMLSVVSPGQWALEKA; and
   g) polypeptides comprising the extracellular domain of CD40sv2.

2. The isolated polypeptide of claim 1 wherein said polypeptide comprises a wild-type human CD40 domain selected from the group consisting of domain 1; domain 2; domain 3; domains 1 and 2; domains 2 and 3; domains 1 and 3; and domains 1, 2 and 3 of wild-type CD40.

3. The isolated polypeptide of claim 1 wherein said polypeptide is soluble.

4. The isolated polypeptide of claim 1 wherein said polypeptide comprises part or all of a transmembrane domain.

5. The isolated polypeptide of claim 1 wherein said polypeptide is between about 100 and about 160 amino acids in length.

6. The isolated polypeptide of claim 1 wherein said polypeptide between about 130 and about 150 amino acids in length.

7. The isolated polypeptide of claim 1 wherein said polypeptide comprises the sequence for CD40sv1 as shown in SEQ ID NO:10.
8. The isolated polypeptide of claim 12 wherein said polypeptide comprises the sequence for CD40sv2 as shown in SEQ ID NO:15.

9. The isolated polypeptide of claim 12 wherein said polypeptide comprises the sequence for CD40sv3 as shown in SEQ ID NO:14.

10. The isolated polypeptide of claim 1 wherein said polypeptide is a mutein of the CD40 splice variants.

11. The isolated polypeptide of claim 10 wherein said mutein is soluble.


13. The isolated polypeptide of claim 10 wherein said mutein comprises the amino acid sequence SPGQWALEKA.

14. The isolated polypeptide of claim 10 wherein said mutein comprises the amino acid sequence VRPKTWLCNRQAQTRLMLSVVSPGQWALEKA.

15. The isolated polypeptide of claim 10 wherein said mutein comprises the extracellular domain of CD40sv2.

16. The isolated polypeptide of claim 10 wherein said mutein consists essentially of the extracellular domain of CD40sv2.

17. The isolated polypeptide of claim 10 wherein said mutein comprises a modification of the amino acid residue Cys at position 125 and/or 143 as shown in Figure 1.
18. The isolated polypeptide of claim 17 wherein said modification is a deletion of the amino acid residue Cys at position 125 and/or 143 as shown in Figure 1.

19. The isolated polypeptide of claim 18 wherein said mutein comprises SEQ ID NO:118.

20. The isolated polypeptide of claim 17 wherein said modification is a substitution of the amino acid residue Cys at position 125 and/or 143 as shown in Figure 1 for any other amino acid.

21. The isolated polypeptide of claim 20 wherein said mutein comprises SEQ ID NO:119.

22. The polypeptide of claim 1 wherein said polypeptide comprises a heterologous signal sequence.

23. The isolated polypeptide of claim 1 wherein said polypeptide is a CD40 splice variant fusion molecule.

24. The isolated polypeptide of claim 23 wherein said CD40 splice variant fusion molecule comprises a fusion partner directly linked to a CD40 splice variant.

25. The isolated polypeptide of claim 23 wherein said CD40 splice variant fusion molecule is soluble.

26. The isolated polypeptide of claim 24 wherein said fusion partner is an oligomerization domain.

27. The isolated polypeptide of claim 24 wherein said fusion partner is selected from the group consisting of a Fc domain, a leucine zipper domain, a trimerization domain from tetrancetin, and a trimerization domain from mannose binding protein 1 (MBP1).
28. The isolated polypeptide of claim 27 wherein said fusion partner is an Fc domain.

29. The isolated polypeptide of claim 28 wherein said CD40 splice variant fusion molecule has the sequence as shown in SEQ ID NO:121, SEQ ID NO:123, SEQ ID NO:125, or SEQ ID NO:127.

30. The isolated polypeptide of claim 27 wherein said fusion partner is a leucine zipper domain.

31. The isolated polypeptide of claim 30 wherein said CD40 splice variant fusion molecule has the sequence as shown in SEQ ID NO:128, SEQ ID NO:129, SEQ ID NO:132 or SEQ ID NO:133.

32. The isolated polypeptide of claim 27 wherein said fusion partner is a trimerization domain from tetranectin.

33. The isolated polypeptide of claim 32 wherein said CD40 splice variant fusion molecule has the sequence as shown in SEQ ID NO:136 or SEQ ID NO:142.

34. The isolated polypeptide of claim 27 wherein said fusion partner is a trimerization domain from MBP1.

35. The isolated polypeptide of claim 34 wherein said CD40 splice variant fusion molecule has the sequence as shown in SEQ ID NO:138, SEQ ID NO:140, SEQ ID NO:144, or SEQ ID NO:146.

36. The isolated polypeptide of claim 1 wherein said polypeptide is a mutein fusion molecule.

37. The isolated polypeptide of claim 36 wherein said mutein fusion molecule is soluble.
38. The isolated polypeptide of claim 36 wherein said mutein fusion molecule comprises a fusion partner directly linked to the mutein.

39. The isolated polypeptide of claim 38 wherein said fusion partner is an oligomerization domain.

40. The isolated polypeptide of claim 39 wherein said fusion partner is selected from the group consisting of a Fc domain, a leucine zipper domain, a trimerization domain from tetranectin, and a trimerization domain from mannose binding protein1 (MBP1).

41. The isolated polypeptide of claim 40 wherein said fusion partner is an Fc domain.

42. The isolated polypeptide of claim 40 wherein said fusion partner is a leucine zipper domain.

43. The isolated polypeptide of claim 40 wherein said fusion partner is a trimerization domain from tetranectin.

44. The isolated polypeptide of claim 40 wherein said fusion partner is a trimerization domain from MBP1.

45. The isolated polypeptide of claim 1 wherein said polypeptide comprises the sequence SPGQWALEKA.

46. The isolated polypeptide of claim 1 wherein said polypeptide comprises the sequence VRPKTWCNRQANATRLMLSVSPGQWALEKA.

47. The isolated polypeptide of claim 1 wherein said polypeptide comprises the extracellular domain of CD40 sv2.

48. A fusion molecule comprising a polypeptide of claim 1 linked to a fusion partner.
49. The fusion molecule of claim 48 wherein said fusion partner is an oligomerization domain.

50. The fusion molecule of claim 49 wherein said oligomerization domain is selected from the group consisting of Fc domain, a leucine zipper domain, a trimerization domain from tetractin, and a trimerization domain from mannose binding protein 1 (MBP1).

51. An isolated polynucleotide encoding the polypeptide or fusion protein of anyone of claims 1-50.

52. The isolated polynucleotide of claim 51 wherein said polynucleotide comprises a sequence that encodes CD40sv1.

53. The isolated polynucleotide of claim 52 wherein said polynucleotide comprises the sequences as shown in SEQ ID NO:1 or SEQ ID NO:19.

54. The isolated polynucleotide of claim 51 wherein said polynucleotide comprises a sequence that encodes CD40sv2.

55. The isolated polynucleotide of claim 54 wherein said polynucleotide comprises the sequences as shown in SEQ ID NO:6 or SEQ ID NO:24.

56. The isolated polynucleotide of claim 51 wherein said polynucleotide encodes CD40sv3.

57. The isolated polynucleotide of claim 56 wherein said polynucleotide comprises the sequences as shown in SEQ ID:5 or SEQ ID NO:23.

58. The isolated polynucleotide of claim 51 wherein said polynucleotide encodes a mutein of a CD40 splice variant, wherein the CD40 splice variant is selected from the group consisting of CD40 sv1, CD40sv2, and CD40sv3.
59. The isolated polynucleotide of claim 58 wherein said polynucleotide comprises the sequences as shown in 1) SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49, SEQ ID NO:50, SEQ ID NO:51, SEQ ID NO:52, SEQ ID NO:53, SEQ ID NO:54, SEQ ID NO:55, SEQ ID NO:56, SEQ ID NO:57, SEQ ID NO:58, SEQ ID NO:59, SEQ ID NO:60, SEQ ID NO:61, or SEQ ID NO:62, or 2) encodes the polypeptide having the sequence as shown in SEQ ID NO:95, SEQ ID NO:96, SEQ ID NO:97, SEQ ID NO:98, SEQ ID NO:99, SEQ ID NO:100, SEQ ID NO:101, SEQ ID NO:102, SEQ ID NO:103, SEQ ID NO:104, SEQ ID NO:105, SEQ ID NO:106, SEQ ID NO:107, SEQ ID NO:108, SEQ ID NO:109, SEQ ID NO:110, SEQ ID NO:111, SEQ ID NO:112, SEQ ID NO:113, SEQ ID NO:114, SEQ ID NO:115, SEQ ID NO:116, SEQ ID NO:117, SEQ ID NO:118, or SEQ ID NO:119, or 3) is a complement thereof.

60. The isolated polynucleotide of claim 51 wherein said polynucleotide encodes a CD40 splice variant fusion molecule.

61. The isolated polynucleotide of claim 60 wherein said polynucleotide 1) comprises the sequence as shown in 1) SEQ ID NO:64, SEQ ID NO:66, SEQ ID NO:68, SEQ ID NO:70, SEQ ID NO:71, SEQ ID NO:72, SEQ ID NO:75, SEQ ID NO:76, SEQ ID NO:79, SEQ ID NO:81, SEQ ID NO:83, SEQ ID NO:85, SEQ ID NO:87, or SEQ ID NO:89 or 2) encodes the polypeptide having the sequence as shown in SEQ ID NO:121, SEQ ID NO:123, SEQ ID NO:125, SEQ ID NO:127, SEQ ID NO:128, SEQ ID NO:129, SEQ ID NO:132, SEQ ID NO:133, SEQ ID NO:136, 138, SEQ ID NO:140, SEQ ID NO:142, SEQ ID NO:144, or SEQ ID NO:146, or 3) is a complement thereof.

62. The isolated polynucleotide of claim 51 wherein said polynucleotide encodes a mutein fusion molecule.

63. A composition comprising an isolated polypeptide or fusion protein of anyone of claims 1 to 50.
64. The composition of claim 63 wherein said isolated polypeptide is a CD40 splice variant.

65. The composition of claim 64 wherein said CD40 splice variant is soluble.

66. The composition of claim 63 wherein said isolated polypeptide is a mutein of a CD40 splice variant.

67. The composition of claim 66 wherein said mutein is soluble.

68. The composition of claim 63 wherein said isolated polypeptide is a CD40 splice variant fusion molecule.

69. The composition of claim 63 wherein said isolated polypeptide is a mutein fusion molecule.

70. The composition of claim 63 further comprising a buffer.

71. The composition of claim 63 further comprising a pharmaceutically acceptable buffer.

72. The isolated polypeptide or fusion molecule of any one of claims 1 to 50 that competes with wild-type CD40 for binding with a CD40 ligand.

73. A vector comprising the polynucleotide of claim 51.

74. The vector of claim 73 wherein said vector is mammalian.

75. The vector of claim 73 wherein said vector is insect.

76. A composition comprising the polynucleotide of claim 51.

77. A host cell comprising the polynucleotide of claim 51.
78. A complex comprising a polypeptide of claim 1 bound to a CD40 ligand.

79. The complex of claim 78 wherein said CD40 ligand is CD154.

80. An antibody that specifically binds a polypeptide of claim 1, or a fragment thereof wherein said antibody does not bind wild type human CD40 (SEQ ID NO:13), SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:150, SEQ ID NO:152, SEQ ID NO:154, SEQ ID NO:155 or SEQ ID NO:156.

81. The antibody of claim 80, wherein said antibody is a monoclonal antibody.

82. A method of inhibiting binding of a CD40 ligand with a wild-type human CD40 comprising contacting the CD40 ligand with the polypeptide of claim 1 under conditions suitable for binding of said CD40 ligand to said polypeptide.

83. The method of claim 82 wherein said contacting is performed in the presence of said wild-type CD40.

84. The method of claim 82 wherein said CD40 ligand is present on a cell.

85. The method of claim 82 wherein said polypeptide is a CD40 splice variant.

86. The method of claim 82 wherein said polypeptide is a mutein of a CD40 splice variant.

87. The method of claim 82 wherein said polypeptide is a CD40 splice variant fusion molecule.

88. The method of claim 82 wherein said polypeptide is a mutein fusion molecule.

89. A method for blocking wild-type human CD40 activity, comprising contacting the wild-type CD40 with the polypeptide of claim 1 in the presence of CD40 ligand
and under conditions suitable to inhibit binding of the CD40 ligand with said wild-type CD40, thereby blocking wild-type CD40 activity.

90. The method of claim 89 wherein said wild-type CD40 is present on a cell.

91. The method of claim 82 wherein said polypeptide is a CD40 splice variant.

92. The method of claim 82 wherein said polypeptide is a mutein of a CD40 splice variant.

93. The method of claim 82 wherein said polypeptide is a CD40 splice variant fusion molecule.

94. The method of claim 82 wherein said polypeptide is a mutein fusion molecule.

95. A method of producing a polypeptide of claim 1 comprising culturing a cell comprising a polynucleotide encoding a polypeptide of claim 1 under conditions suitable for production of the polypeptide and optionally recovering said polypeptide produced.

96. The method of claim 95 wherein said cell is a eukaryotic cell or a prokaryotic cell.

97. The method of claim 96 wherein said cell is an insect cell.

98. The method of claim 96 wherein said cell is a mammalian cell.

99. The method of claim 95 wherein said polypeptide is a CD40 splice variant.

100. The method of claim 95 wherein said polypeptide is a mutein of a CD40 splice variant.

101. The method of claim 95 wherein said polypeptide is a CD40 splice variant fusion molecule.
102. The method of claim 95 wherein said polypeptide is a mutein fusion molecule.

103. A method of treatment of a disease or condition in a subject comprising administering the polypeptide of claim 1 to said subject.

104. The method of claim 103 wherein the disease is selected from the group consisting of diseases arising from undesirable interaction between wild type human CD40 and CD40 ligand; autoimmune diseases; cardiovascular diseases, and oncological disease.

105. A method of treatment of an immune cell related disease in a subject comprising administering the polypeptide of claim 1 to said subject, wherein immune cell proliferation in said subject is inhibited.

106. The method of claim 105 wherein said immune cell related disease is autoimmune disease.

107. A kit comprising a polypeptide of claim 1.

108. A kit comprising an antibody of claim 80.

109. The use of a polypeptide or fusion molecule of anyone of claims 1 to 50 in the manufacture of a medicament for ameliorating the symptoms of a disease in a subject.

110. The use of a polypeptide or fusion molecule of anyone of claims 1 to 50 in the manufacture of a medicament for inhibiting immune cell proliferation in a subject.
FIGURE 1

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### FIG. 3

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FIGS. 4A-4B

FIG. 4A

FIG. 4B
FIGS. 7A-7B

FIG. 7A

FIG. 7B
FIGS. 9A-9B

FIG. 9A

FIG. 9B
FIG. 11

Eluted Fractions

Sup Perm. Conc. Sup Start FT Wash
FIG. 13

- sCD40wt-Fc
- sCD40sv-Fc
- Dialysis buffer

(Chart showing RLU against protein concentration in ng/ml.)
CD40sv2 and CD40sv2 Muteins

FIG. 15A
CD40wt

FIG. 15B
CD40sv2

FIG. 15C
mutant 7

FIG. 15D
mutant 8

Deletion of residues 125-135

Replace Cys143 with any other residue or delete it

LEGEND

Wt

disulfide bond
gap