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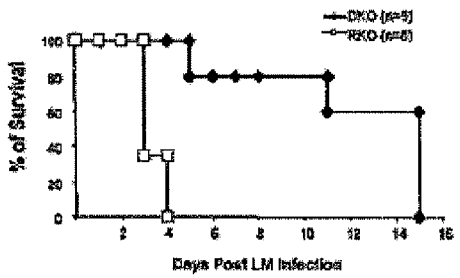

(57) Abstract: Compositions and methods for the stimulating, enhancing or promoting an immune response in a host are provided. One embodiment provides methods and compositions for stimulating, enhancing, or promoting an immune response in a host by administering an effective amount of a B7-H4 antagonist, preferably sH4 or variant thereof. Compositions of sH4 can be used to enhance an immune response to treat infection, cancer, or as part of a vaccine.

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COMPOSITIONS AND METHODS FOR STIMULATING AN IMMUNE RESPONSE

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims benefit of and priority to U.S.S.N. 60/877,319 filed on December 27, 2006 and U.S.S.N. 60/949,742 filed on July 13, 2007, both of which are incorporated by reference in their entirety where permissible.

GOVERNMENT SUPPORT

Funding for the work described herein was provided in part by grant number R01 CA98731 awarded by the National Institutes of Health to Lieping Chen. The federal government may have certain rights in the invention.

TECHNICAL FIELD

This invention relates to compositions and methods for enhancing immune responses in a host, in particular to compositions and methods for treating or inhibiting cancer or infection.

BACKGROUND

Modulating immune responses is an important treatment for many diseases and disorders. For example, it would be advantageous to enhance an immune response in patients suffering from cancer or infection. Current cancer therapies are based on the use of drugs and/or radiation which kills replicating cells indiscriminately. The idea is to kill cancer cells faster than killing the patient's normal cells. Surgery is used to reduce tumor bulk, but

has little impact once the cancer has metastasized. Radiation is effective only in a localized area.

Cancer treatments can in themselves kill or maim the patient, even with maintenance therapy. For example, for some types of cancer, bone marrow transplants have been used to maintain the patient following treatment with otherwise fatal amounts of chemotherapy. Efficacy has not been proven for treatment of solid tumors, however. "Cocktails" of different chemotherapeutic agents and combinations of very high doses of chemotherapy with restorative agents, for example, granulocyte macrophage colony stimulating factor ("GM-CSF"), erythropoietin, thrombopoetin, granulocyte stimulating factor, ("G-CSF"), macrophage colony stimulating factor ("M-CSF") and stem cell factor ("SCF") to restore platelet and white cell levels, have been used to treat aggressive cancers. Even with the supportive or restrictive therapy, side effects are severe.

Other treatments have been tried in an attempt to decrease mortality and morbidity. Vaccines to stimulate the patient's immune system have been attempted, but not with great success. Various cytokines, such as tumor necrosis factor, interferon gamma, and interleukin-2 ("IL-2"), alone or in combination, have been used to kill cancers, but have not produced cures. More recently, anti-angiogenic compounds such as thalidomide have been tried in compassionate use cases and shown to cause tumor remission. In animal studies, compounds inducing a procoagulant state, such as an inhibitor of protein C, have been used to cause tumor remission. New studies have shown that inhibitors of cytokine receptors, such as tumor necrosis factor receptors ("TNFRs") which are released in a soluble form from tumor

cells, in high concentrations relative to normal cells, may restore the immune system's attack on the tumor cells (Jablonska and Peitruska, *Arch. Immunol. Ther. Exp.* (Warsz) 1997, 45(5-6), 449-453; Chen, et al., *J. Neuropathol. Exp. Neurol.* 1997, 56(5), 541-550).

Studies in both mice and humans have involved the i.p. or intravenous (i.v.) administration of cytokine proteins as more specific activators of the immune response (Adachi, et al, *Cancer Immunol. Immunother.* 37: 1-6, (1993); Lissoni, et al, *Tumori.* 78: 118-20 (1992)). Treating murine ovarian tumors with a combination of recombinant IL-2 and GM-CSF proteins had some beneficial effect in inhibiting ascites production; however, IL-2 was only effective if it was combined with GM-CSF (Kikuchi, et al., *Cancer Immunol. Immunother.*, 43: 257-261 (1996)). Similarly, a combination of IL-2 and lymphokine-activated killer (LAK) cells was able to reduce i.p. sarcomas in mice, while IL-2 protein alone was not as effective (Ottow, et al., *Cellular Immunology*, 104: 366-376 (1987)). Human clinical trials evaluating IL-2 protein therapy of ovarian cancer patients indicated some antitumor effects (Chapman et al., *Investigational New Drugs*, 6:179-188. (1988); Lissoni et al., *Tumori*, 78:118-120 (1992); Sparano et al., *J. of Immunotherapy*, 16:216-223 (1994); Freedman et al., *J. of Immunotherapy,* 16:198-210 (1994); Edwards et al., *J. Clin. Oncol.*, 15:3399-3407 (1997)).

Other studies in mice have involved the injection of DNA constructs encoding "suicide" genes followed by treatment with prodrugs. This approach has successfully caused regression of some small tumors but has been less successful on larger tumor masses. (Szala, et al., *Gene Therapy*, 3:

1025-1031 (1996); Sugaya, et al., *Hum Gene Ther*, 7: 223-230 (1996)). In another study, liposome-mediated E1A gene therapy for mice bearing ovarian cancers that overexpress HER-2/neu resulted in reduced mortality among these tumor bearing mice. (Yu, et al., *Oncogene*, 11: 1383-1388 (1995)). Similarly, the successful treatment of murine ovarian carcinoma (MOT) has been demonstrated using cisplatin-induced gene transfer of DNA constructs encoding IFN-gamma via i.p. injection. (Son, *Cancer Gene Therapy*, 4: 391-396 (1997)). However, this study demonstrated that tumors were poorly responsive to either the IFN-gamma gene or cisplatin alone, suggesting that the effectiveness of the cisplatin-based gene therapy protocol was mainly due to enhanced sensitization of cisplatin-exposed tumor cells to transfection by the IFN-gene. (Son, *Cancer Gene Therapy*, 4: 391-396 (1997)).

Therefore, it is object of the invention to provide compositions and methods for enhancing or promoting an immune response in a host.

It is another object to provide methods and compositions for enhancing an immune response in a host to treat infection or cancer.

SUMMARY

Compositions and methods for the stimulating, enhancing or promoting an immune response in a host by administering an effective amount of a B7-H4 antagonist, preferably sH4 or variant thereof, have been developed. Compositions of sH4 can be used to enhance an immune response to treat infection, cancer, or as part of a vaccine. Other suitable B7-H4 antagonists include, but are not limited to, antibodies specific for B7-H4 or B7-H4 receptor, inhibitory nucleic acids specific for B7-H4 or

B7-H4 receptor, and other molecules that inhibit or interfere with B7-H4 activity in vivo.

In one embodiment, cancer is treated by administering to a host an effective amount of a B7-H4 antagonist to stimulate, enhance, or promote an immune response in the host. Representative cancers that can be treated include, but are not limited to, bladder, brain, breast, cervical, colo-rectal, esophageal, kidney, liver, lung, nasopharangeal, pancreatic, prostate, skin, stomach, uterine, ovarian, and testicular cancer. Still another embodiment provides compositions and methods for treating or inhibiting an infection by administering to a host an effective amount of a B7-H4 antagonist to stimulate, enhance, or promote an immune response in the host. Suitable infections that can be treated include, but are not limited to, bacterial, viral, fungal, and protozoan infections.

Another embodiment provides vaccine compositions including a B7-H4 antagonist, preferably sH4. The vaccine also includes an antigen source. The antigen source can provide viral antigens, bacterial antigens, fungal antigens, or protozoan antigens. In a preferred embodiment, the antigen is a tumor specific antigen. The vaccine composition optionally includes an adjuvant.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic diagram showing the disruption of the B7-H4 gene. A 4.7 kb DNA fragment containing exons encoding the IgV and IgC domains of murine B7-H4 gene is substituted by a 1.7 kb fragment encoding the neomycin resistant (Neo) gene. Closed boxes represent B7-H4 coding

exons. Lines between exons represent intron sequences. Open boxes represent untranslated exons. The Neo is represented by a shaded box.

Figure 2a is a line graph of percent survival versus days post *Listeria monocytogenes* (LM) infection in wildtype mice (♦) or B7-H4KO mice (□). **Figure 2b** is a graph of CFU/g of spleen (x 10^8) on day 2 or day 3 for wildtype mice (○) or B7-H4KO mice (▲) infected with LM. **Figure 2c** is a line graph percent spleen granulocytes versus days post LM infection in wildtype mice (♦) or B7-H4KO mice (□) infected with LM. **Figure 2d** is a bar graph of CFU/g of liver x 10^4 in three B7-H4 KO mice or littermate control i.p. injected with 150 pg Gr-1 mAb or control rat IgG (LPS-free) 24 hours prior to Listeria infection. Mice were then i.p. injected with 3 x 10^6 CFU of Listeria. Twenty-four hours post infection, mice were terminated and Listeria in liver was counted.

Figure 3 is a bar graph of LM CFU/granulocyte versus hours post LM infection in wildtype mice (♦) or B7-H4KO mice (□).

Figure 4 is a line graph of percent survival versus days post LM infection in RKO mice (♦) or B7-H4KO mice (□).

Figure 5a is a line graph of CPM versus G-CSF (ng/ml) in two x 10^6 bone marrow cells of wildtype mice (♦) or B7-H4KO mice (□) plated with the indicated concentration of recombinant G-CSF for 3 days. The cultures were pulsed with ^{3}HTdR for18 hrs before the end of culture, harvested and counted by a scintillation counter. **Figure 5b** is a panel of line graphs of the dilution of CFSE in gated Gr-1+CD11b+ granulocytes analyzed by flow cytometry. Two x 10^6 of bone marrow cell from the indicated mice were

labeled with CFSE and cultured for 5 days. Cells were harvested and doubly stained with Gr-1/CD11b mAb.

Figure 6 is a line graph of CPM versus days. Two x 10^6 of bone marrow cells from normal B6 mice were plated in the 96-well plates coated with 20 μg/ml of recombinant murine B7-H4Ig (△) or murine Ig control protein (▲) in the absence (A) or presence of 0.1 ng/ml (B) or 1 ng/ml (C) of recombinant murine G-CSF. Cells were harvested on day 2-5 days as indicated. The cultures were pulsed with ^{3}HTdR for18 hrs before the end of culture, harvested and counted by a scintillation counter. *P<0.05.

Detailed Description of the Invention

I. Definitions

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. Although methods and materials similar or equivalent to those described herein can be used to practice the invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety where permissible. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

The term "effective amount" or "therapeutically effective amount" means a dosage sufficient to stimulate or enhance an immune response in a host or to otherwise provide a desired pharmacologic and/or physiologic effect. The precise dosage will vary according to a variety of factors such as

subject-dependent variables (e.g., age, immune system health, etc.), the disease, and the treatment being effected.

A "fragment" of a sH4 polypeptide is a fragment of the sH4 polypeptide that is shorter than the full-length sH4 polypeptide. Generally, fragments will be five or more amino acids in length. An antigenic fragment has the ability to be recognized and bound by an antibody.

The terms "individual," "host," "subject," and "patient" are used interchangeably herein, and refer to a mammal, including, but not limited to, murines, simians, humans, mammalian farm animals, mammalian sport animals, and mammalian pets.

As used herein, "operably linked" means incorporated into a genetic construct so that expression control sequences effectively control expression of a coding sequence of interest.

The terms "polypeptide" and "protein" are used interchangeably and mean any peptide-linked chain of amino acids, regardless of length or post-translational modification. Embodiments include sH4 polypeptides with conservative substitutions. Conservative substitutions typically include substitutions within the following groups: glycine and alanine; valine, isoleucine, and leucine; aspartic acid and glutamic acid; asparagine, glutamine, serine and threonine; lysine, histidine and arginine; and phenylalanine and tyrosine.

The terms “sH4” or “sH4 polypeptide” refers to soluble fragments of B7-H4 including biologically active fragments of the extracellular domain of B7-H4. Soluble fragments generally lack some or all of the intracellular and/or transmembrane domains.

As used herein, the term "treating" includes alleviating, preventing and/or eliminating one or more symptoms associated with the disease to be treated.

The term "B7-H4" antagonist refers to compound that interferes or inhibits a biological activity of B7-H4, reduces or inhibits the expression or bioavailability of B7-H4, or inhibits the interaction of B7-H4 with its natural ligand/receptor.

II. Compositions

Compositions including one or more B7-H4 antagonists are provided. Suitable B7-H4 antagonists include, but are not limited to, sH4, antibodies to B7-H4 and antigen binding fragments thereof, antibodies to B7-H4 receptor and antigen binding fragments thereof, inhibitory nucleic acids that down regulate expression of B7-H4, and inhibitory nucleic acids that down regulate expression of B7-H4 receptor.

A. Soluble H4 (sH4)

It has been discovered that soluble H4 (sH4) promotes immune responses. It is believed that sH4 binds to B7-H4 receptors *in vivo*, causing the interaction of B7-H4 with the B7-H4 receptor to be reduced or inhibited. Soluble H4 is derived from B7-H4. B7-H4 is expressed in cells as a transmembrane protein, with an intracellular domain, a single transmembrane-spanning domain, and an extracellular domain. Compositions including sH4 are provided herein.

One embodiment provides sH4 polypeptides that reduce the binding of B7-H4 to B7-H4 receptor(s) by at least 20%, more preferably by at least 30%, more preferably by at least 40%, 50%, 60%, 70%, 80%, 90%, 95%,

96%, 97%, 98%, 99% or more compared to a control. Another embodiment provides sH4 compositions that do not activate B7-H4 receptor activity or produce a detectable amount of B7-H4 activity in a cell expressing a B7-H4 receptor on its surface. In some embodiments, sH4 compositions are capable of reducing or inhibiting one or more activities of a B7-H4 receptor in a cell expressing a B7-H4 receptor on its surface. In still other embodiments, the cell is a lymphocyte, a T cell, a CD4+ T cell, a CD8+ T cell, a T_h1 cell, a B cell, a plasma cell, a macrophage, a neutrophil or an NK cell.

sH4 polypeptides can include the entire extracellular domain of B7-H4 or fragments thereof. In other embodiments, sH4 polypeptides include fragments of the extracellular domain of B7-H4. sH4 polypeptides can be derived from any species of origin. In a preferred embodiment the sH4 polypeptides are of human origin.

The sH4 polypeptides disclosed herein include variant polypeptides. As used herein, a "variant" polypeptide contains at least one amino acid sequence alteration as compared to the amino acid sequence of the corresponding wild-type polypeptide. An amino acid sequence alteration can be, for example, a substitution, a deletion, or an insertion of one or more amino acids.

A variant sH4 polypeptide can have any combination of amino acid substitutions, deletions or insertions. In one embodiment, isolated sH4 variant polypeptides have an integer number of amino acid alterations such that their amino acid sequence shares at least 60, 70, 80, 85, 90, 95, 97, 98, 99, 99.5 or 100% identity with an amino acid sequence of a corresponding wild type amino acid sequence. In a preferred embodiment, sH4 variant

polypeptides have an amino acid sequence sharing at least 60, 70, 80, 85, 90, 95, 97, 98, 99, 99.5 or 100% identity with the amino acid sequence of a corresponding wild type polypeptide.

Percent sequence identity can be calculated using computer programs or direct sequence comparison. Preferred computer program methods to determine identity between two sequences include, but are not limited to, the GCG program package, FASTA, BLASTP, and TBLASTN (see, e.g., D. W. Mount, 2001, Bioinformatics: Sequence and Genome Analysis, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.). The BLASTP and TBLASTN programs are publicly available from NCBI and other sources. The well-known Smith Waterman algorithm may also be used to determine identity.

Exemplary parameters for amino acid sequence comparison include the following: 1) algorithm from Needleman and Wunsch (*J. Mol. Biol.*, 48:443-453 (1970)); 2) BLOSSUM62 comparison matrix from Hentikoff and Hentikoff (*Proc. Natl. Acad. Sci. U.S.A.*, 89:10915-10919 (1992)) 3) gap penalty = 12; and 4) gap length penalty = 4. A program useful with these parameters is publicly available as the "gap" program (Genetics Computer Group, Madison, Wis.). The aforementioned parameters are the default parameters for polypeptide comparisons (with no penalty for end gaps).

Alternatively, polypeptide sequence identity can be calculated using the following equation: % identity = (the number of identical residues)/(alignment length in amino acid residues)*100. For this calculation, alignment length includes internal gaps but does not include terminal gaps.

Amino acid substitutions in sH4 variant polypeptides may be "conservative" or "non-conservative". As used herein, "conservative" amino acid substitutions are substitutions wherein the substituted amino acid has similar structural or chemical properties, and "non-conservative" amino acid substitutions are those in which the charge, hydrophobicity, or bulk of the substituted amino acid is significantly altered. Non-conservative substitutions will differ more significantly in their effect on maintaining (a) the structure of the peptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain.

Examples of conservative amino acid substitutions include those in which the substitution is within one of the five following groups: 1) small aliphatic, nonpolar or slightly polar residues (Ala, Ser, Thr, Pro, Gly); 2) polar, negatively charged residues and their amides (Asp, Asn, Glu, Gln); polar, positively charged residues (His, Arg, Lys); large aliphatic, nonpolar residues (Met, Leu, Ile, Val, Cys); and large aromatic resides (Phe, Tyr, Trp). Examples of non-conservative amino acid substitutions are those where 1) a hydrophilic residue, e.g., seryl or threonyl, is substituted for (or by) a hydrophobic residue, e.g., leucyl, isoleucyl, phenylalanyl, valyl, or alanyl; 2) a cysteine or proline is substituted for (or by) any other residue; 3) a residue having an electropositive side chain, e.g., lysyl, arginyl, or histidyl, is substituted for (or by) an electronegative residue, e.g., glutamyl or aspartyl; or 4) a residue having a bulky side chain, e.g., phenylalanine, is substituted for (or by) a residue that does not have a side chain, e.g., glycine.

sH4 polypeptides may be modified by chemical moieties that may be present in polypeptides in a normal cellular environment, for example, phosphorylation, methylation, amidation, sulfation, acylation, glycosylation, sumoylation and ubiquitylation. sH4 variant polypeptides may also be modified with a label capable of providing a detectable signal, either directly or indirectly, including, but not limited to, radioisotopes and fluorescent compounds.

sH4 polypeptides may also be modified by chemical moieties that are not normally added to polypeptides in a cellular environment. Such modifications may be introduced into the molecule by reacting targeted amino acid residues of the polypeptide with an organic derivatizing agent that is capable of reacting with selected side chains or terminal residues. Another modification is cyclization of the protein.

Examples of chemical derivatives of the polypeptides include lysinyl and amino terminal residues derivatized with succinic or other carboxylic acid anhydrides. Derivatization with a cyclic carboxylic anhydride has the effect of reversing the charge of the lysinyl residues. Other suitable reagents for derivatizing amino-containing residues include imidoesters such as methyl picolinimidate; pyridoxal phosphate; pyridoxal; chloroborohydride; trinitrobenzenesulfonic acid; *O*-methylisourea; 2,4 pentanedione; and transaminase-catalyzed reaction with glyoxylate. Carboxyl side groups, aspartyl or glutamyl, may be selectively modified by reaction with carbodiimides (R—N=C=N--R') such as 1-cyclohexyl-3-(2-morpholinyl-(4-ethyl)carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl) carbodiimide. Furthermore,

aspartyl and glutamyl residues can be converted to asparaginyl and glutaminyl residues by reaction with ammonia. Polypeptides may also include one or more D-amino acids that are substituted for one or more L-amino acids.

The sH4 polypeptides may be coupled to other polypeptides to form fusion proteins. Provided are sH4 polypeptides having a first fusion partner comprising all or a part of a sH4 polypeptide fused (i) directly to a second polypeptide or, (ii) optionally, fused to a linker peptide sequence that is fused to the second polypeptide. The presence of the fusion partner can alter the solubility, affinity and/or valency of the sH4 polypeptide. As used herein, "valency" refers to the number of binding sites available per molecule. sH4 fusion proteins described herein include any combination of amino acid alteration (i.e. substitution, deletion or insertion), fragment, and/or modification as described above.

The second polypeptide binding partner may be N-terminal or C-terminal relative to the sH4 polypeptide. In a preferred embodiment, the second polypeptide is C-terminal to the sH4 polypeptide.

A large number of polypeptide sequences that are routinely used as fusion protein binding partners are well known in the art. Examples of useful polypeptide binding partners include, but are not limited to, green fluorescent protein (GFP), glutathione S-transferase (GST), polyhistidine, myc, hemagglutinin, Flag™ tag (Kodak, New Haven, CT), maltose E binding protein, protein A, and one or more domains of an Ig heavy chain constant region, preferably having an amino acid sequence corresponding to the hinge, C_{H2} and C_{H3} regions of a human immunoglobulin Cγ chain.

B. Antibodies to B7-H4 and B7-H4 Receptors

Antibodies to B7-H4 or B7-H4 receptors can be used as B7-H4 antagonists. Antibodies or antibody fragments that specifically bind to B7-H4 or B7-H4 receptor can be used to reduce or inhibit the binding of B7-H4 to a receptor on T cells. Methods of producing antibodies are well known and within the ability of one of ordinary skill in the art and are described in more detail below.

The antibodies disclosed herein specifically bind to a B7-H4 or B7-H4 receptor and are capable of reducing or inhibiting the binding of B7-H4. These antibodies are defined as "blocking", "function-blocking" or "antagonistic" antibodies. In preferred embodiments the antagonistic antibodies specifically bind to a portion of the extracellular domain of B7-H4.

The immunogen used to generate the antibody may be any immunogenic portion of B7-H4 or B7-H4 receptor. Preferred immunogens include all or a part of the extracellular domain of human B7-H4, where these residues contain the post-translation modifications, such as glycosylation, found on native B7-H4 or B7-H4 receptor. Immunogens including the extracellular domain or immunogenic fragments thereof are produced in a variety of ways known in the art, e.g., expression of cloned genes using conventional recombinant methods, isolation from cells of origin, cell populations expressing high levels of B7-H4 or B7-H4 receptor.

The antibodies disclosed herein are capable of binding to a B7-H4 or B7-H4 receptor polypeptide having at least about 70%, more preferably 75%, 80%, 85%, 90%, 95% identity to human B7-H4, GENBANK Accession Number AAP37283.

The antibodies may be polyclonal or monoclonal antibodies. The antibodies may be xenogeneic, allogeneic, syngeneic, or modified forms thereof, such as humanized or chimeric antibodies. The antibodies may also be anti-idiotypic antibodies. Antibodies, as used herein, also includes antibody fragments including Fab and $F(ab)_2$ fragments, and antibodies produced as a single chain antibody or scFv instead of the normal multimeric structure. The antibodies may be an IgG such as IgG1, IgG2, IgG3 or IgG4; or IgM, IgA, IgE or IgD isotype. The constant domain of the antibody heavy chain maybe selected depending on the effector function desired. The light chain constant domain may be a kappa or lambda constant domain.

C. Inhibitory Nucleic Acids Specific for B7-H4 or B7-H4 Receptors.

In another embodiment, B7-H4 or B7-H4 receptor expression is downregulated by providing one or more inhibitory nucleic acids including, but not limited to, ribozymes, triplex-forming oligonucleotides (TFOs), antisense DNA, siRNA, and microRNA specific for nucleic acids encoding B7-H4 or B7-H4 receptor. B7-H4 or B7-H4 receptor antagonists can also be provided in combination with other immunomodulatory agents, such as those described above.

The term "siRNA" means a small interfering RNA that is a short-length double-stranded RNA that is not toxic. Generally, there is no particular limitation in the length of siRNA as long as it does not show toxicity. "siRNAs" can be, for example, 15 to 49 bp, preferably 15 to 35 bp, and more preferably 21 to 30 bp long. Alternatively, the double-stranded RNA portion of a final transcription product of siRNA to be expressed can be,

for example, 15 to 49 bp, preferably 15 to 35 bp, and more preferably 21 to 30 bp long. The double-stranded RNA portions of siRNAs in which two RNA strands pair up are not limited to the completely paired ones, and may contain nonpairing portions due to mismatch (the corresponding nucleotides are not complementary), bulge (lacking in the corresponding complementary nucleotide on one strand), and the like. Nonpairing portions can be contained to the extent that they do not interfere with siRNA formation. The "bulge" used herein preferably comprise 1 to 2 nonpairing nucleotides, and the double-stranded RNA region of siRNAs in which two RNA strands pair up contains preferably 1 to 7, more preferably 1 to 5 bulges. In addition, the "mismatch" used herein is contained in the double-stranded RNA region of siRNAs in which two RNA strands pair up, preferably 1 to 7, more preferably 1 to 5, in number. In a preferable mismatch, one of the nucleotides is guanine, and the other is uracil. Such a mismatch is due to a mutation from C to T, G to A, or mixtures thereof in DNA coding for sense RNA, but not particularly limited to them. Furthermore, in certain embodiments the double-stranded RNA region of siRNAs in which two RNA strands pair up may contain both bulge and mismatched, which sum up to, preferably 1 to 7, more preferably 1 to 5 in number.

The terminal structure of siRNA may be either blunt or cohesive (overhanging) as long as siRNA can silence, reduce, or inhibit the target gene expression due to its RNAi effect. The cohesive (overhanging) end structure is not limited only to the 3' overhang, and the 5' overhanging structure may be included as long as it is capable of inducing the RNAi effect. In addition, the number of overhanging nucleotide is not limited to the already reported 2

or 3, but can be any numbers as long as the overhang is capable of inducing the RNAi effect. For example, the overhang consists of 1 to 8, preferably 2 to 4 nucleotides. Herein, the total length of siRNA having cohesive end structure is expressed as the sum of the length of the paired double-stranded portion and that of a pair comprising overhanging single-strands at both ends. For example, in the case of 19 bp double-stranded RNA portion with 4 nucleotide overhangs at both ends, the total length is expressed as 23 bp. Furthermore, since this overhanging sequence has low specificity to a target gene, it is not necessarily complementary (antisense) or identical (sense) to the target gene sequence. Furthermore, as long as siRNA is able to maintain its gene silencing effect on the target gene, siRNA may contain a low molecular weight RNA (which may be a natural RNA molecule such as tRNA, rRNA or viral RNA, or an artificial RNA molecule), for example, in the overhanging portion at its one end.

In addition, the terminal structure of the siRNA is not necessarily the cut off structure at both ends as described above, and may have a stem-loop structure in which ends of one side of double-stranded RNA are connected by a linker RNA. The length of the double-stranded RNA region (stem-loop portion) can be, for example, 15 to 49 bp, preferably 15 to 35 bp, and more preferably 21 to 30 bp long. Alternatively, the length of the double-stranded RNA region that is a final transcription product of siRNAs to be expressed is, for example, 15 to 49 bp, preferably 15 to 35 bp, and more preferably 21 to 30 bp long. Furthermore, there is no particular limitation in the length of the linker as long as it has a length so as not to hinder the pairing of the stem portion. For example, for stable pairing of the stem portion and suppression

of the recombination between DNAs coding for the portion, the linker portion may have a clover-leaf tRNA structure. Even though the linker has a length that hinders pairing of the stem portion, it is possible, for example, to construct the linker portion to include introns so that the introns are excised during processing of precursor RNA into mature RNA, thereby allowing pairing of the stem portion. In the case of a stem-loop siRNA, either end (head or tail) of RNA with no loop structure may have a low molecular weight RNA. As described above, this low molecular weight RNA may be a natural RNA molecule such as tRNA, rRNA or viral RNA, or an artificial RNA molecule.

MiRNAs are produced by the cleavage of short stem-loop precursors by Dicer-like enzymes; whereas, siRNAs are produced by the cleavage of long double-stranded RNA molecules. MiRNAs are single-stranded, whereas siRNAs are double-stranded.

Methods for producing siRNA are known in the art. Because the sequence for B7-H4 is know, one of skill in the art could readily produce siRNAs that downregulate B7-H4 expression in host using the information that is publicly available.

D. Vaccines Containing sH4

sH4 and/or nucleic acids encoding the same may be administered alone or in combination with any other suitable treatment to vaccinate a host. In one embodiment, sH4 and/or nucleic acids encoding the same may be administered in conjunction with, or as a component of, a vaccine composition. Suitable components of vaccine compositions include an antigen and optionally an adjuvant. sH4 compositions described herein can

be administered prior to, concurrently with, or after the administration of a vaccine or component thereof. In one embodiment the sH4 composition is administered at the same time as administration of a vaccine. Because sH4 increases immune responses, it is believed that sH4 can increase the effectiveness of a vaccine by promoting an immune response to the specific antigen of the vaccine.

The sH4 compositions described herein may be administered in conjunction with prophylactic vaccines, which confer resistance in a subject to subsequent exposure to infectious agents, or in conjunction with therapeutic vaccines, which can be used to initiate or enhance a subject's immune response to a pre-existing antigen, such as a tumor antigen in a subject with cancer, or a viral antigen in a subject infected with a virus.

The desired outcome of a prophylactic, therapeutic or de-sensitized immune response may vary according to the disease, according to principles well known in the art. For example, an immune response against an infectious agent may completely prevent colonization and replication of an infectious agent, affecting "sterile immunity" and the absence of any disease symptoms. However, a vaccine against infectious agents may be considered effective if it reduces the number, severity or duration of symptoms; if it reduces the number of individuals in a population with symptoms; or reduces the transmission of an infectious agent. Similarly, immune responses against cancer, allergens or infectious agents may completely treat a disease, may alleviate symptoms, or may be one facet in an overall therapeutic intervention against a disease. For example, the stimulation of an immune response against a cancer may be coupled with surgical, chemotherapeutic,

radiologic, hormonal and other immunologic approaches in order to affect treatment.

1. Antigens

Antigens that can be used in the vaccine compositions can be peptides, proteins, polysaccharides, saccharides, lipids, nucleic acids, or combinations thereof. The antigen can be derived from a virus, bacterium, parasite, plant, protozoan, fungus, tissue or transformed cell such as a cancer or leukemic cell and can be a whole cell or immunogenic component thereof, e.g., cell wall components or molecular components thereof.

Suitable antigens are known in the art and are available from commercial government and scientific sources. In one embodiment, the antigens are whole inactivated or attenuated organisms. These organisms may be infectious organisms, such as viruses, parasites and bacteria. These organisms may also be tumor cells. The antigens may be purified or partially purified polypeptides derived from tumors or viral or bacterial sources. The antigens can be recombinant polypeptides produced by expressing DNA encoding the polypeptide antigen in a heterologous expression system. The antigens can be DNA encoding all or part of an antigenic protein. The DNA may be in the form of vector DNA such as plasmid DNA.

Antigens may be provided as single antigens or may be provided in combination. Antigens may also be provided as complex mixtures of polypeptides or nucleic acids.

i. Viral antigens

A viral antigen can be isolated from, or encoded by nucleic acid from, any virus including, but not limited to, a virus from any of the following viral families: *Arenaviridae, Arterivirus, Astroviridae, Baculoviridae, Badnavirus, Barnaviridae, Birnaviridae, Bromoviridae, Bunyaviridae, Caliciviridae, Capillovirus, Carlavirus, Caulimovirus, Circoviridae, Closterovirus, Comoviridae, Coronaviridae (e.g., Coronavirus*, such as severe acute respiratory syndrome (SARS) virus), *Corticoviridae, Cystoviridae, Deltavirus, Dianthovirus, Enamovirus, Filoviridae* (e.g., Marburg virus and Ebola virus (e.g., Zaire, Reston, Ivory Coast, or Sudan strain)), *Flaviviridae,* (e.g., Hepatitis C virus, Dengue virus 1, Dengue virus 2, Dengue virus 3, and Dengue virus 4), *Hepadnaviridae, Herpesviridae (e.g.*, Human herpesvirus 1, 3, 4, 5, and 6, and Cytomegalovirus), *Hypoviridae, Iridoviridae, Leviviridae, Lipothrixviridae, Microviridae, Orthomyxoviridae* (e.g., Influenzavirus A and B and C), *Papovaviridae, Paramyxoviridae (e.g.*, measles, mumps, and human respiratory syncytial virus), *Parvoviridae, Picornaviridae (e.g.*, poliovirus, rhinovirus, hepatovirus, and aphthovirus), *Poxviridae (e.g.*, vaccinia and smallpox virus), *Reoviridae* (e.g., rotavirus), *Retroviridae (e.g.*, lentivirus, such as human immunodeficiency virus (HIV) 1 and HIV 2), *Rhabdoviridae* (for example, rabies virus, measles virus, respiratory syncytial virus, etc.), *Togaviridae* (for example, rubella virus, dengue virus, etc.), and *Totiviridae*. Suitable viral antigens also include all or part of Dengue protein M, Dengue protein E, Dengue D1NS1, Dengue D1NS2, and Dengue D1NS3.

Viral antigens may be derived from a particular strain such as a papilloma virus, a herpes virus, i.e. herpes simplex 1 and 2; a hepatitis virus, for example, hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis C virus (HCV), the delta hepatitis D virus (HDV), hepatitis E virus (HEV) and hepatitis G virus (HGV), the tick-borne encephalitis viruses; parainfluenza, varicella-zoster, cytomeglavirus, Epstein-Barr, rotavirus, rhinovirus, adenovirus, coxsackieviruses, equine encephalitis, Japanese encephalitis, yellow fever, Rift Valley fever,and lymphocytic choriomeningitis.

ii. Bacterial antigens

Bacterial antigens can originate from, or encoded by nucleic acid from, any bacteria including, but not limited to, *Actinomyces, Anabaena, Bacillus, Bacteroides, Bdellovibrio, Bordetella, Borrelia, Campylobacter, Caulobacter, Chlamydia, Chlorobium, Chromatium, Clostridium, Corynebacterium, Cytophaga, Deinococcus, Escherichia, Francisella, Halobacterium, Heliobacter, Haemophilus, Hemophilus influenza type B (HIB), Hyphomicrobium, Legionella, Leptspirosis, Listeria, Meningococcus A, B and C, Methanobacterium, Micrococcus, Myobacterium, Mycoplasma, Myxococcus, Neisseria, Nitrobacter, Oscillatoria, Prochloron, Proteus, Pseudomonas, Phodospirillum, Rickettsia, Salmonella, Shigella, Spirillum, Spirochaeta, Staphylococcus, Streptococcus, Streptomyces, Sulfolobus, Thermoplasma, Thiobacillus, and Treponema, Vibrio*, and *Yersinia*.

iii. Parasite antigens

Parasite antigens can be obtained from, or encoded by nucleic acid encoded by, parasites such as, but not limited to, an antigen derived from *Cryptococcus neoformans, Histoplasma capsulatum, Candida albicans,*

Candida tropicalis, Nocardia asteroides, Rickettsia ricketsii, Rickettsia typhi, Mycoplasma pneumoniae, Chlamydial psittaci, Chlamydial trachomatis, Plasmodium falciparum, Trypanosoma brucei, Entamoeba histolytica, Toxoplasma gondii, Trichomonas vaginalis and *Schistosoma mansoni.*

These include Sporozoan antigens, Plasmodian antigens, such as all or part of a Circumsporozoite protein, a Sporozoite surface protein, a liver stage antigen, an apical membrane associated protein, or a Merozoite surface protein.

iv. Allergens and environmental antigens

The antigen can be an allergen or environmental antigen, such as, but not limited to, an antigen derived from naturally occurring allergens such as pollen allergens (tree-, herb, weed-, and grass pollen allergens), insect allergens (inhalant, saliva and venom allergens), animal hair and dandruff allergens, and food allergens. Important pollen allergens from trees, grasses and herbs originate from the taxonomic orders of *Fagales, Oleales, Pinales* and *platanaceae* including i.a. birch (*Betula*), alder (*Alnus*), hazel (*Corylus*), hornbeam (*Carpinus*) and olive (*Olea*), cedar (*Cryptomeriaand Juniperus*), Plane tree (*Platanus*), the order of Poales including i.e. grasses of the genera *Lolium, Phleum, Poa, Cynodon, Dactylis, Holcus, Phalaris, Secale*, and Sorghum, the orders of *Asterales* and *Urticales* including i.a. herbs of the genera Ambrosia, Artemisia, and Parietaria. Other allergen antigens that may be used include allergens from house dust mites of the genus *Dermatophagoides* and *Euroglyphus*, storage mite e.g *Lepidoglyphys, Glycyphagus* and *Tyrophagus*, those from cockroaches, midges and fleas *e.g. Blatella, Periplaneta, Chironomus* and *Ctenocepphalides*, those from

mammals such as cat, dog and horse, birds, venom allergens including such originating from stinging or biting insects such as those from the taxonomic order of *Hymenoptera* including bees (superfamily *Apidae*), wasps (superfamily *Vespidea*), and ants (superfamily *Formicoidae*). Still other allergen antigens that may be used include inhalation allergens from fungi such as from the genera *Alternaria* and *Cladosporium*.

v. Tumor antigens

In a preferred embodiment, the antigen can be a tumor antigen, including a tumor-associated or tumor-specific antigen, such as, but not limited to, alpha-actinin-4, Bcr-Abl fusion protein, Casp-8, beta-catenin, cdc27, cdk4, cdkn2a, coa-1, dek-can fusion protein, EF2, ETV6-AML1 fusion protein, LDLR-fucosyltransferaseAS fusion protein, HLA-A2, HLA-A11, hsp70-2, KIAAO205, Mart2, Mum-1, 2, and 3, neo-PAP, myosin class I, OS-9, pml-RARα fusion protein, PTPRK, K-ras, N-ras, Triosephosphate isomeras, Bage-1, Gage 3,4,5,6,7, GnTV, Herv-K-mel, Lage-1, Mage-A1,2,3,4,6,10,12, Mage-C2, NA-88, NY-Eso-1/Lage-2, SP17, SSX-2, and TRP2-Int2, MelanA (MART-I), gp100 (Pmel 17), tyrosinase, TRP-1, TRP-2, MAGE-1, MAGE-3, BAGE, GAGE-1, GAGE-2, p15(58), CEA, RAGE, NY-ESO (LAGE), SCP-1, Hom/Mel-40, PRAME, p53, H-Ras, HER-2/neu, BCR-ABL, E2A-PRL, H4-RET, IGH-IGK, MYL-RAR, Epstein Barr virus antigens, EBNA, human papillomavirus (HPV) antigens E6 and E7, TSP-180, MAGE-4, MAGE-5, MAGE-6, p185erbB2, p180erbB-3, c-met, nm-23H1, PSA, TAG-72-4, CA 19-9, CA 72-4, CAM 17.1, NuMa, K-ras, β-Catenin, CDK4, Mum-1, p16, TAGE, PSMA, PSCA, CT7, telomerase, 43-9F, 5T4, 791Tgp72, α-fetoprotein, 13HCG, BCA225, BTAA, CA 125, CA

15-3 (CA 27.29/BCAA), CA 195, CA 242, CA-50, CAM43, CD68/KP1, CO-029, FGF-5, G250, Ga733 (EpCAM), HTgp-175, M344, MA-50, MG7-Ag, MOV18, NB/70K, NY-CO-1, RCAS1, SDCCAG16, TA-90 (Mac-2 binding protein/cyclophilin C-associated protein), TAAL6, TAG72, TLP, and TPS.

2. Adjuvants

Optionally, the vaccines may include one or more adjuvants. These can be incorporated into, administered with, or administered separately from, the vaccine compositions. The adjuvant can be, but is not limited to, one or more of the following: oil emulsions (e.g., Freund's adjuvant); saponin formulations; virosomes and viral-like particles; bacterial and microbial derivatives; immunostimulatory oligonucleotides; ADP-ribosylating toxins and detoxified derivatives; alum; BCG; mineral-containing compositions (e.g., mineral salts, such as aluminium salts and calcium salts, hydroxides, phosphates, sulfates, etc.); bioadhesives and/or mucoadhesives; microparticles; liposomes; polyoxyethylene ether and polyoxyethylene ester formulations; polyphosphazene; muramyl peptides; imidazoquinolone compounds; and surface active substances (e.g. lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, and dinitrophenol).

Adjuvants may also include immunomodulators such as cytokines, interleukins (e.g., IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-12, etc.), interferons (e.g., interferon-.gamma.), macrophage colony stimulating factor, and tumor necrosis factor; and co-stimulatory molecules, such as those of the B7 family.

Such proteinaceous adjuvants may be provided as the full-length polypeptide or an active fragment thereof, or in the form of DNA, such as plasmid DNA.

III. Methods for Stimulating an Immune Response

An immune response can be stimulated or enhanced in a host, preferably a human host, by interfering with the biological activity of B7-H4 in the host. sH4 competes with B7-H4 for B7-H4 ligands. Thus, an immune response can be enhanced or promoted by administering a therapeutically effective amount of sH4 or variant thereof to a host.

In another embodiment, a B7-H4 antagonist is administered to host in an amount effective to decrease the amount of or bioavailability of B7-H4 in the host. Representative antagonists of B7-H4 include, but are not limited to antibodies specific to B7-H4 or B7-H4 receptors and inhibitory nucleic acids specific for nucleic acids encoding B7-H4 or a B7-H4 receptor.

A. Methods for Treating Infections

In one embodiment, an immune response is stimulated or enhanced in a host by administering an amount of sH4 effective to inhibit B7-H4 activity in the host. It will be appreciated that other antagonists of B7-H4 can also be used alone or in combination with sH4. It is believed that inhibiting B7-H4 with sH4 promotes or enhances an immune response in the host. Inhibition of B7-H4 activity is typically compared to an appropriate control or predetermined amount of activity using conventional methods. For example, threshold B7-H4 activity in a host can be determined prior to administration of sH4. B7-H4 activity after administration of sH4 that is lower than the threshold B7-H4 activity demonstrates an inhibition of B7-H4 activity and stimulation or enhancement of an immune response.

Stimulating an immune response in a host is desirable, for example, when the host suffers from a viral infection, bacterial infection, fungal, protozoa infection, or hyperproliferative condition such as cancer. Thus, one embodiment provides a method for treating infection by administering an amount of sH4 effective to inhibit B7-H4 activity in the host.

Representative infections that can be treated, include but are not limited to infections cause by microoganisms including, but not limited to, *Actinomyces, Anabaena, Bacillus, Bacteroides, Bdellovibrio, Bordetella, Borrelia, Campylobacter, Caulobacter, Chlamydia, Chlorobium, Chromatium, Clostridium, Corynebacterium, Cytophaga, Deinococcus, Escherichia, Francisella, Halobacterium, Heliobacter, Haemophilus, Hemophilus influenza type B (HIB), Hyphomicrobium, Legionella, Leptspirosis, Listeria, Meningococcus A, B and C, Methanobacterium, Micrococcus, Myobacterium, Mycoplasma, Myxococcus, Neisseria, Nitrobacter, Oscillatoria, Prochloron, Proteus, Pseudomonas, Phodospirillum, Rickettsia, Salmonella, Shigella, Spirillum, Spirochaeta, Staphylococcus, Streptococcus, Streptomyces, Sulfolobus, Thermoplasma, Thiobacillus, and Treponema, Vibrio, Yersinia, Cryptococcus neoformans, Histoplasma capsulatum, Candida albicans, Candida tropicalis, Nocardia asteroides, Rickettsia ricketsii, Rickettsia typhi, Mycoplasma pneumoniae, Chlamydial psittaci, Chlamydial trachomatis, Plasmodium falciparum, Trypanosoma brucei, Entamoeba histolytica, Toxoplasma gondii, Trichomonas vaginalis* and *Schistosoma mansoni*.

B. Methods for Treating Cancer

Another embodiment provides methods and compositions for stimulating or enhancing an immune response in host for treating cancer by

administering an amount of sH4 effective to inhibit B7-H4 activity in the host. It will be appreciated that other antagonists of B7-H4 can also be used alone or in combination with sH4. The types of cancer that can be treated with the provided compositions and methods include, but are not limited to, the following: bladder, brain, breast, cervical, colo-rectal, esophageal, kidney, liver, lung, nasopharangeal, pancreatic, prostate, skin, stomach, uterine, ovarian, testicular and the like. Administration is not limited to the treatment of an existing tumor or infectious disease but can also be used to prevent or lower the risk of developing such diseases in an individual, i.e., for prophylactic use. Potential candidates for prophylactic vaccination include individuals with a high risk of developing cancer, i.e., with a personal or familial history of certain types of cancer.

Malignant tumors which may be treated are classified herein according to the embryonic origin of the tissue from which the tumor is derived. Carcinomas are tumors arising from endodermal or ectodermal tissues such as skin or the epithelial lining of internal organs and glands. Sarcomas, which arise less frequently, are derived from mesodermal connective tissues such as bone, fat, and cartilage. The leukemias and lymphomas are malignant tumors of hematopoietic cells of the bone marrow. Leukemias proliferate as single cells, whereas lymphomas tend to grow as tumor masses. Malignant tumors may show up at numerous organs or tissues of the body to establish a cancer.

Another embodiment provides a method for treating infection in host by administering an amount of sH4 effective to inhibit B7-H4 activity in a host. The infection can be viral, bacterial, or protozoan. Without being bound

by one theory, it is believed that interfering with the activity of B7-H4, for example binding its natural ligand, will prevent or reduce the inhibition of T cell response by B7-H4. By inhibiting the activity of B7-H4, an immune response can progress and increase the ability to fight infection.

C. Gene Therapy

Nucleic acids encoding sH4 antagonists can be administered to an individual in need thereof in an amount effective to treat an inflammatory response or autoimmune disease. DNA delivery involves introduction of a "foreign" DNA into a cell and ultimately, into a live animal. Gene delivery can be achieved using viral vectors or non-viral vectors. One approach includes nucleic acid transfer into primary cells in culture followed by autologous transplantation of the *ex vivo* transformed cells into the individual, either systemically or into a particular organ or tissue.

Nucleic acid therapy can be accomplished by direct transfer of a functionally active DNA into mammalian somatic tissue or organ *in vivo*. DNA transfer can be achieved using a number of approaches described below. These systems can be tested for successful expression *in vitro* by use of a selectable marker (e.g., G418 resistance) to select transfected clones expressing the DNA, followed by detection of the presence of the B7-H4 expression product (after treatment with the inducer in the case of an inducible system) using an antibody to the product in an appropriate immunoassay. Efficiency of the procedure, including DNA uptake, plasmid integration and stability of integrated plasmids, can be improved by linearizing the plasmid DNA using known methods, and co-transfection using high molecular weight mammalian DNA as a "carrier".

Retroviral-mediated human therapy utilizes amphotrophic, replication-deficient retrovirus systems (Weiss and Taylor, *Cell,* 82:531-533 (1995)). Such vectors have been used to introduce functional DNA into human cells or tissues, for example, the adenosine deaminase gene into lymphocytes, the NPT-II gene and the gene for tumor necrosis factor into tumor infiltrating lymphocytes. Retrovirus-mediated gene delivery generally requires target cell proliferation for gene transfer (Bordignon et al. *Science* 270:470-475 (1995)). This condition is met by certain of the preferred target cells into which the present DNA molecules are to be introduced, i.e., actively growing tumor cells. Gene therapy of cystic fibrosis using transfection by plasmids using any of a number of methods and by retroviral vectors has been described by U.S. Patent No. 5,240,846 to Collins et al.

The DNA molecules encoding the B7-H4 polypeptides or fusion proteins may be packaged into retrovirus vectors using packaging cell lines that produce replication-defective retroviruses, as is well-known in the art (see, for example Stone, D. et al. *J. Endocrinology,* 164:103-118 (2000)). Additional viruses for gene delivery are described in Reynolds et al. Molecular Medicine Today, 5:25-31 (1999)).

Other virus vectors may also be used, including recombinant adenoviruses (Murphy et al. *Proc Natl Acad Scii* 94:13921-13926 (1997)), herpes simplex virus (HSV) for neuron-specific delivery and persistence (Lowenstein et al. Brain Res. Molec. Brain Res, 30:169-175 (1995)). Advantages of adenovirus vectors for human gene therapy include the fact that recombination is rare, no human malignancies are known to be associated with such viruses, the adenovirus genome is double stranded

DNA which can be manipulated to accept foreign genes of up to 7.5 kb in size, and live adenovirus is a safe human vaccine organisms. Adeno-associated virus is also useful for human therapy (Samulski, R. J. et al., EMBO J. 10:3941 (1991).

Another vector which can express the disclosed DNA molecule and is useful in the present therapeutic setting, particularly in humans, is vaccinia virus, which can be rendered non-replicating (Peplinkski, G.R. et al. Surgical Oncology Clinics of North America, 7575-588 1998)). Descriptions of recombinant vaccinia viruses and other viruses containing heterologous DNA and their uses in immunization and DNA therapy are reviewed in: Moss, B., *Curr. Opin. Genet. Dev.* 3:86-90 (1993); Moss, B. *Biotechnology* 20: 345-362 (1992); Moss, B., *Curr Top Microbiol Immunol* 158:25-38 (1992); Moss, B., *Science* 252:1662-1667 (1991); Piccini, A et al., *Adv. Virus Res.* 34:43-64 (1988); Moss, B. et al., *Gene Amplif Anal* 3:201-213 (1983).

In addition to naked DNA or RNA, or viral vectors, engineered bacteria may be used as vectors. A number of bacterial strains including *Salmonella*, BCG and *Listeria monocytogenes* (LM) (Hoiseth & Stocker, *Nature* 291, 238-239 (1981); Poirier, T P et al. J. Exp. Med. 168, 25-32 (1988); (Sadoff, J. C., et al., *Science* 240, 336-338 (1988); Stover, C. K., et al., *Nature* 351, 456-460 (1991); Aldovini, A. et al., *Nature* 351, 479-482 (1991); Schafer, R., et al., *J. Immunol.* 149, 53-59 (1992); Ikonomidis, G. et al., *J. Exp. Med.* 180, 2209-2218 (1994)). These organisms display two promising characteristics for use as vaccine vectors: (1) enteric routes of

infection, providing the possibility of oral vaccine delivery; and (2) infection of monocytes/macrophages thereby targeting antigens to professional APCs.

In addition to virus-mediated gene transfer *in vivo*, physical means well-known in the art can be used for direct transfer of DNA, including administration of plasmid DNA (Wolff et al., *Science*, 247:1465-1468 (1990); Hickman, M.A, et al. *Hum. Gene Ther.*, 5:1477-1483 (1994)) and particle-bombardment mediated gene transfer (O'Brien, J. et al. *Brain Res Brain Res Protco*, 10:12-15 (2002)). Furthermore, electroporation, a well-known means to transfer genes into cell *in vitro*, can be used to transfer DNA molecules to tissues in vivo (Titomirov, A. V. et al., Biochim. Biophys. Acta 1088:131 ((1991)).

"Carrier mediated gene transfer" has also been described (Wu, C. H. et al., J. Biol. Chem. 264:16985 (1989); Wu, G. Y. et al., J. Biol. Chem. 263:14621 (1988); Soriano, P. et al., Proc. Natl. Acad. Sci. USA 80:7128 (1983); Wang, C-Y. et al., Proc. Natl. Acad. Sci. USA 84:7851 (1982); Wilson, J. M. et al., J. Biol. Chem. 267:963 (1992)). Preferred carriers are targeted liposomes (Liu et al. *Curr Med Chem,* 10:1307-1315 (2003)) such as immunoliposomes, which can incorporate acylated mAbs into the lipid bilayer. Polycations such as asialoglycoprotein/polylysine (Wu et al., 1989, supra) may be used, where the conjugate includes a molecule which recognizes the target tissue (e.g., asialoorosomucoid for liver) and a DNA binding compound to bind to the DNA to be transfected. Polylysine is an example of a DNA binding molecule which binds DNA without damaging it. This conjugate is then complexed with plasmid DNA for transfer.

Plasmid DNA used for transfection or microinjection may be prepared using methods well-known in the art, for example using the Quiagen procedure (Quiagen), followed by DNA purification using known methods, such as the methods exemplified herein.

D. Combination Therapy

The disclosed compositions can be administered alone or in combination with one or more additional therapeutic agents. For example, the disclosed compositions can be administered with an antibody or antigen binding fragment thereof specific for a growth factor receptors or tumor specific antigens. Representative growth factors receptors include, but are not limited to, epidermal growth factor receptor (EGFR; HER1); c-erbB2 (HER2); c-erbB3 (HER3); c-erbB4 (HER4); insulin receptor; insulin-like growth factor receptor 1 (IGF-1R); insulin-like growth factor receptor 2/Mannose–6-phosphate receptor (IGF-II R/M-6-P receptor); insulin receptor related kinase (IRRK); platelet-derived growth factor receptor (PDGFR); colony-stimulating factor–1receptor (CSF-1R) (c-Fms); steel receptor (c-Kit); Flk2/Flt3; fibroblast growth factor receptor 1 (Flg/Cek1); fibroblast growth factor receptor 2 (Bek/Cek3/K-Sam); Fibroblast growth factor receptor 3; Fibroblast growth factor eceptor 4; nerve growth factor receptor (NGFR) (TrkA); BDNF receptor (TrkB); NT-3-receptor (TrkC); vascular endothelial growth factor receptor 1 (Flt1); vascular endothelial growth factor receptor 2/Flk1/KDR; hepatocyte growth factor receptor (HGF-R/Met); Eph; Eck; Eek; Cek4/Mek4/HEK; Cek5; Elk/Cek6; Cek7; Sek/Cek8; Cek9; Cek10; HEK11; 9 Ror1; Ror2; Ret; Axl; RYK; DDR; and Tie.

Additional therapeutic agents include conventional cancer therapeutics such as chemotherapeutic agents, cytokines, chemokines, and radiation therapy. The majority of chemotherapeutic drugs can be divided in to: alkylating agents, antimetabolites, anthracyclines, plant alkaloids, topoisomerase inhibitors, and other antitumour agents. All of these drugs affect cell division or DNA synthesis and function in some way. Additional therapeutics include monoclonal antibodies and the new tyrosine kinase inhibitors e.g. imatinib mesylate (GLEEVEC® or GLIVEC®), which directly targets a molecular abnormality in certain types of cancer (chronic myelogenous leukemia, gastrointestinal stromal tumors).

Representative chemotherapeutic agents include, but are not limited to cisplatin, carboplatin, oxaliplatin, mechlorethamine, cyclophosphamide, chlorambucil, vincristine, vinblastine, vinorelbine, vindesine, taxol and derivatives thereof, irinotecan, topotecan, amsacrine, etoposide, etoposide phosphate, teniposide, epipodophyllotoxins, trastuzumab (HERCEPTIN®), cetuximab, and rituximab (RITUXAN® or MABTHERA®), bevacizumab (AVASTIN®), and combinations thereof.

IV. Pharmaceutical Compositions

C. Pharmaceutical compositions

Pharmaceutical compositions including B7-H4 antagonists, and vectors containing the same are provided. The pharmaceutical compositions may be for administration by oral, parenteral (intramuscular, intraperitoneal, intravenous (IV) or subcutaneous injection), transdermal (either passively or using iontophoresis or electroporation), transmucosal (nasal, vaginal, rectal,

or sublingual) routes of administration or using bioerodible inserts and can be formulated in dosage forms appropriate for each route of administration.

A. Formulations for Parenteral Administration

In a preferred embodiment, the peptides are administered in an aqueous solution, by parenteral injection. The formulation may also be in the form of a suspension or emulsion. In general, pharmaceutical compositions are provided including effective amounts of a B7-H4 antagonist, or derivative products, and optionally include pharmaceutically acceptable diluents, preservatives, solubilizers, emulsifiers, adjuvants and/or carriers. Such compositions include diluents sterile water, buffered saline of various buffer content (e.g., Tris-HCl, acetate, phosphate), pH and ionic strength; and optionally, additives such as detergents and solubilizing agents (e.g., TWEEN 20, TWEEN 80, Polysorbate 80), anti-oxidants (e.g., ascorbic acid, sodium metabisulfite), and preservatives (e.g., Thimersol, benzyl alcohol) and bulking substances (e.g., lactose, mannitol). Examples of non-aqueous solvents or vehicles are propylene glycol, polyethylene glycol, vegetable oils, such as olive oil and corn oil, gelatin, and injectable organic esters such as ethyl oleate. The formulations may be lyophilized and redissolved/resuspended immediately before use. The formulation may be sterilized by, for example, filtration through a bacteria retaining filter, by incorporating sterilizing agents into the compositions, by irradiating the compositions, or by heating the compositions.

B. Formulations for Enteral Administration

B7-H4 antagonists can be formulated for oral delivery. Oral solid dosage forms are described generally in Remington's Pharmaceutical

Sciences, 18th Ed. 1990 (Mack Publishing Co. Easton Pa. 18042) at Chapter 89. Solid dosage forms include tablets, capsules, pills, troches or lozenges, cachets, pellets, powders, or granules or incorporation of the material into particulate preparations of polymeric compounds such as polylactic acid, polyglycolic acid, etc. or into liposomes. Such compositions may influence the physical state, stability, rate of *in vivo* release, and rate of *in vivo* clearance of the present proteins and derivatives. See, e.g., Remington's Pharmaceutical Sciences, 18th Ed. (1990, Mack Publishing Co., Easton, Pa. 18042) pages 1435-1712 which are herein incorporated by reference where permissible. The compositions may be prepared in liquid form, or may be in dried powder (e.g., lyophilized) form. Liposomal or proteinoid encapsulation may be used to formulate the compositions (as, for example, proteinoid microspheres reported in U.S. Patent No. 4,925,673). Liposomal encapsulation may be used and the liposomes may be derivatized with various polymers (e.g., U.S. Patent No. 5,013,556). See also Marshall, K. In: Modern Pharmaceutics Edited by G. S. Banker and C. T. Rhodes Chapter 10, 1979. In general, the formulation will include the peptide (or chemically modified forms thereof) and inert ingredients which protect peptide in the stomach environment, and release of the biologically active material in the intestine.

The polypeptide antagonists may be chemically modified so that oral delivery of the derivative is efficacious. Generally, the chemical modification contemplated is the attachment of at least one moiety to the component molecule itself, where said moiety permits (a) inhibition of proteolysis; and (b) uptake into the blood stream from the stomach or intestine. Also desired is the increase in overall stability of the component or components and increase

in circulation time in the body. PEGylation is a preferred chemical modification for pharmaceutical usage. Other moieties that may be used include: propylene glycol, copolymers of ethylene glycol and propylene glycol, carboxymethyl cellulose, dextran, polyvinyl alcohol, polyvinyl pyrrolidone, polyproline, poly-1,3-dioxolane and poly-1,3,6-tioxocane [see, e.g., Abuchowski and Davis (1981) "Soluble Polymer-Enzyme Adducts," in Enzymes as Drugs. Hocenberg and Roberts, eds. (Wiley-Interscience: New York, N.Y.) pp. 367-383; and Newmark, et al. (1982) J. Appl. Biochem. 4:185-189].

Another embodiment provides liquid dosage forms for oral administration, including pharmaceutically acceptable emulsions, solutions, suspensions, and syrups, which may contain other components including inert diluents; adjuvants such as wetting agents, emulsifying and suspending agents; and sweetening, flavoring, and perfuming agents.

Controlled release oral formulations may be desirable. The B7-H4 antagonists can be incorporated into an inert matrix which permits release by either diffusion or leaching mechanisms, e.g., gums. Slowly degenerating matrices may also be incorporated into the formulation. Another form of a controlled release is based on the Oros therapeutic system (Alza Corp.), i.e. the drug is enclosed in a semipermeable membrane which allows water to enter and push drug out through a single small opening due to osmotic effects. For oral formulations, the location of release may be the stomach, the small intestine (the duodenum, the jejunem, or the ileum), or the large intestine. Preferably, the release will avoid the deleterious effects of the stomach environment, either by protection of the peptide (or derivative) or by release of

the peptide (or derivative) beyond the stomach environment, such as in the intestine. To ensure full gastric resistance a coating impermeable to at least pH 5.0 is essential. Examples of the more common inert ingredients that are used as enteric coatings are cellulose acetate trimellitate (CAT), hydroxypropylmethylcellulose phthalate (HPMCP), HPMCP 50, HPMCP 55, polyvinyl acetate phthalate (PVAP), Eudragit L30D, Aquateric, cellulose acetate phthalate (CAP), Eudragit L, Eudragit S, and Shellac. These coatings may be used as mixed films.

C. Topical Delivery Formulations

Compositions can be applied topically. This does not work well for most peptide formulations, although it can be effective especially if applied to the lungs, nasal, oral (sublingual, buccal), vaginal, or rectal mucosa.

The B7-H4 antagonists can be delivered to the lungs while inhaling and traverses across the lung epithelial lining to the blood stream when delivered either as an aerosol or spray dried particles having an aerodynamic diameter of less than about 5 microns.

A wide range of mechanical devices designed for pulmonary delivery of therapeutic products can be used, including but not limited to nebulizers, metered dose inhalers, and powder inhalers, all of which are familiar to those skilled in the art. Some specific examples of commercially available devices are the Ultravent nebulizer (Mallinckrodt Inc., St. Louis, Mo.); the Acorn II nebulizer (Marquest Medical Products, Englewood, Colo.); the Ventolin metered dose inhaler (Glaxo Inc., Research Triangle Park, N.C.); and the Spinhaler powder inhaler (Fisons Corp., Bedford, Mass.). Nektar, Alkermes and Mannkind all have inhalable insulin powder preparations approved or in

clinical trials where the technology could be applied to the formulations described herein.

Formulations for administration to the mucosa will typically be spray dried drug particles, which may be incorporated into a tablet, gel, capsule, suspension or emulsion. Standard pharmaceutical excipients are available from any formulator. Oral formulations may be in the form of chewing gum, gel strips, tablets or lozenges.

Transdermal formulations may also be prepared. These will typically be ointments, lotions, sprays, or patches, all of which can be prepared using standard technology. Transdermal formulations will require the inclusion of penetration enhancers.

D. Controlled Delivery Polymeric Matrices

Controlled release polymeric devices can be made for long term release systemically following implantation of a polymeric device (rod, cylinder, film, disk) or injection (microparticles). The matrix can be in the form of microparticles such as microspheres, where peptides are dispersed within a solid polymeric matrix or microcapsules, where the core is of a different material than the polymeric shell, and the peptide is dispersed or suspended in the core, which may be liquid or solid in nature. Unless specifically defined herein, microparticles, microspheres, and microcapsules are used interchangeably. Alternatively, the polymer may be cast as a thin slab or film, ranging from nanometers to four centimeters, a powder produced by grinding or other standard techniques, or even a gel such as a hydrogel.

Either non-biodegradable and biodegradable matrices can be used for delivery of B7-H4 antagonists, although biodegradable matrices are preferred.

These may be natural or synthetic polymers, although synthetic polymers are preferred due to the better characterization of degradation and release profiles. The polymer is selected based on the period over which release is desired. In some cases linear release may be most useful, although in others a pulse release or "bulk release" may provide more effective results. The polymer may be in the form of a hydrogel (typically in absorbing up to about 90% by weight of water), and can optionally be crosslinked with multivalent ions or polymers.

The matrices can be formed by solvent evaporation, spray drying, solvent extraction and other methods known to those skilled in the art. Bioerodible microspheres can be prepared using any of the methods developed for making microspheres for drug delivery, for example, as described by Mathiowitz and Langer, J. Controlled Release 5,13-22 (1987); Mathiowitz, et al., Reactive Polymers 6, 275-283 (1987); and Mathiowitz, et al., J. Appl. Polymer Sci. 35, 755-774 (1988).

The devices can be formulated for local release to treat the area of implantation or injection – which will typically deliver a dosage that is much less than the dosage for treatment of an entire body – or systemic delivery. These can be implanted or injected subcutaneously, into the muscle, fat, or swallowed.

IV. Methods of Manufacture

As discussed above polypeptide sH4, nucleic acid constructs encoding sH4 or variants thereof, and inhibitory nucleic acids specific for B7-H4 and B7-H4 receptor can be produced using standard molecular biology protocols known in the art. See for example, Molecular Cloning: A

Laboratory Manual (Sambrook and Russel eds. 3rd ed.) Cold Spring Harbor, New York (2001). Alternatively, sH4 or variants there of can be isolated and purified from a host expressing them using conventional biochemical techniques.

A. Methods for producing polypeptides

Isolated polypeptides can be obtained by, for example, chemical synthesis or by recombinant production in a host cell. To recombinantly produce a costimulatory polypeptide, a nucleic acid containing a nucleotide sequence encoding the polypeptide can be used to transform, transduce, or transfect a bacterial or eukaryotic host cell (e.g., an insect, yeast, or mammalian cell). In general, nucleic acid constructs include a regulatory sequence operably linked to a nucleotide sequence encoding a costimulatory polypeptide. Regulatory sequences (also referred to herein as expression control sequences) typically do not encode a gene product, but instead affect the expression of the nucleic acid sequences to which they are operably linked.

Useful prokaryotic and eukaryotic systems for expressing and producing polypeptides are well know in the art include, for example, *Escherichia coli* strains such as BL-21, and cultured mammalian cells such as CHO cells.

In eukaryotic host cells, a number of viral-based expression systems can be utilized to express polypeptides. Viral based expression systems are well known in the art and include, but are not limited to, baculoviral, SV40, retroviral, or vaccinia based viral vectors.

Mammalian cell lines that stably express variant costimulatory polypeptides can be produced using expression vectors with appropriate control elements and a selectable marker. For example, the eukaryotic expression vectors pCR3.1 (Invitrogen Life Technologies) and p91023(B) (see Wong *et al.* (1985) *Science* 228:810-815) are suitable for expression of variant costimulatory polypeptides in, for example, Chinese hamster ovary (CHO) cells, COS-1 cells, human embryonic kidney 293 cells, NIH3T3 cells, BHK21 cells, MDCK cells, and human vascular endothelial cells (HUVEC). Following introduction of an expression vector by electroporation, lipofection, calcium phosphate, or calcium chloride co-precipitation, DEAE dextran, or other suitable transfection method, stable cell lines can be selected (e.g., by antibiotic resistance to G418, kanamycin, or hygromycin). The transfected cells can be cultured such that the polypeptide of interest is expressed, and the polypeptide can be recovered from, for example, the cell culture supernatant or from lysed cells. Alternatively, polypeptides can be produced by (a) ligating amplified sequences into a mammalian expression vector such as pcDNA3 (Invitrogen Life Technologies), and (b) transcribing and translating *in vitro* using wheat germ extract or rabbit reticulocyte lysate.

Polypeptides can be isolated using, for example, chromatographic methods such as DEAE ion exchange, gel filtration, and hydroxylapatite chromatography. For example, a polypeptide in a cell culture supernatant or a cytoplasmic extract can be isolated using a protein G column. In some embodiments, polypeptides can be "engineered" to contain an amino acid sequence that allows the polypeptides to be captured onto an affinity matrix. For example, a tag such as c-myc, hemagglutinin, polyhistidine, or Flag™

(Kodak) can be used to aid polypeptide purification. Such tags can be inserted anywhere within the polypeptide, including at either the carboxyl or amino terminus. Other fusions that can be useful include enzymes that aid in the detection of the polypeptide, such as alkaline phosphatase. Immunoaffinity chromatography also can be used to purify polypeptides.

B. Methods for producing isolated nucleic acid molecules

Isolated nucleic acid molecules can be produced by standard techniques, including, without limitation, common molecular cloning and chemical nucleic acid synthesis techniques. For example, polymerase chain reaction (PCR) techniques can be used to obtain an isolated nucleic acid encoding a variant costimulatory polypeptide. PCR is a technique in which target nucleic acids are enzymatically amplified. Typically, sequence information from the ends of the region of interest or beyond can be employed to design oligonucleotide primers that are identical in sequence to opposite strands of the template to be amplified. PCR can be used to amplify specific sequences from DNA as well as RNA, including sequences from total genomic DNA or total cellular RNA. Primers typically are 14 to 40 nucleotides in length, but can range from 10 nucleotides to hundreds of nucleotides in length. General PCR techniques are described, for example in PCR Primer: A Laboratory Manual, ed. by Dieffenbach and Dveksler, Cold Spring Harbor Laboratory Press, 1995. When using RNA as a source of template, reverse transcriptase can be used to synthesize a complementary DNA (cDNA) strand. Ligase chain reaction, strand displacement amplification, self-sustained sequence replication or nucleic acid sequence-based amplification also can be used to obtain isolated nucleic

acids. See, for example, Lewis (1992) *Genetic Engineering News* 12:1; Guatelli *et al.* (1990) *Proc. Natl. Acad. Sci. USA* 87:1874-1878; and Weiss (1991) *Science* 254:1292-1293.

Isolated nucleic acids can be chemically synthesized, either as a single nucleic acid molecule or as a series of oligonucleotides (e.g., using phosphoramidite technology for automated DNA synthesis in the 3' to 5' direction). For example, one or more pairs of long oligonucleotides (e.g., >100 nucleotides) can be synthesized that contain the desired sequence, with each pair containing a short segment of complementarity (e.g., about 15 nucleotides) such that a duplex is formed when the oligonucleotide pair is annealed. DNA polymerase can be used to extend the oligonucleotides, resulting in a single, double-stranded nucleic acid molecule per oligonucleotide pair, which then can be ligated into a vector. Isolated nucleic acids can also obtained by mutagenesis. Nucleic acids can be mutated using standard techniques, including oligonucleotide-directed mutagenesis and/or site-directed mutagenesis through PCR. See, Short Protocols in Molecular Biology. Chapter 8, Green Publishing Associates and John Wiley & Sons, edited by Ausubel *et al,* 1992. Examples of amino acid positions that can be modified include those described herein.

C. Methods for producing antibodies

The basic antibody structural unit is known to comprise a tetramer of subunits. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one "light" (about 25 kDa) and one "heavy" chain (about 50-70 kDa). The amino-terminal portion of each chain includes a variable region of about 100 to 110 or more amino acids primarily responsible

for antigen recognition. The carboxy-terminal portion of each chain defines a constant region primarily responsible for effector function.

Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta; or epsilon, and define the antibody's isotype as IgG, IgM, IgA, IgD and IgE, respectively. Within light and heavy chains, the variable and constant regions are joined by a "J" region of about 12 or more amino acids, with the heavy chain also including a "D" region of about 10 more amino acids. (See generally, Fundamental Immunology, Paul, W., ed., 2nd ed. Raven Press, N.Y., 1989, Ch. 7).

The variable regions of each light/heavy chain pair form the antibody binding site. Thus, an intact antibody has two binding sites. Except in bifunctional or bispecific antibodies, the two binding sites are the same. The chains all exhibit the same general structure of relatively conserved framework regions (FR) joined by three hypervariable regions, also called complementarity determining regions or CDRs. The CDRs from the two chains of each pair are aligned by the framework regions, enabling binding to a specific epitope. From N-terminal to C-terminal, both light and heavy chains comprise the domains FR1, CDR1, FR2, CDR2, FR3, CDR3 and FR4.

1. Production of polyclonal antibodies

Polyclonal antibodies are obtained as sera from immunized animals such as rabbits, goats, rodents, etc. and may be used directly without further treatment or may be subjected to conventional enrichment or purification methods such as ammonium sulfate precipitation, ion exchange chromatography, and affinity chromatography.

2. Production of monoclonal antibodies

Monoclonal antibodies may be produced using conventional hybridoma technology, such as the procedures introduced by Kohler and Milstein (Nature, 256:495-97 (1975)), and modifications thereof (see above references). An animal, preferably a mouse is primed by immunization with an immunogen as above to elicit the desired antibody response in the primed animal. B lymphocytes from the lymph nodes, spleens or peripheral blood of a primed, animal are fused with myeloma cells, generally in the presence of a fusion promoting agent such as polyethylene glycol (PEG). Any of a number of murine myeloma cell lines are available for such use: the P3-NS1/1-Ag4-1, P3-x63-k0Ag8.653, Sp2/0-Ag14, or HL1-653 myeloma lines (available from the ATCC, Rockville, Md.). Subsequent steps include growth in selective medium so that unfused parental myeloma cells and donor lymphocyte cells eventually die while only the hybridoma cells survive. These are cloned and grown and their supernatants screened for the presence of antibody of the desired specificity, e.g. by immunoassay techniques. Positive clones are subcloned, e.g., by limiting dilution, and the monoclonal antibodies are isolated.

Hybridomas produced according to these methods can be propagated *in vitro* or *in vivo* (in ascites fluid) using techniques known in the art (see generally Fink et al., Prog. Clin. Pathol., 9:121-33 (1984)). Generally, the individual cell line is propagated in culture and the culture medium containing high concentrations of a single monoclonal antibody can be harvested by decantation, filtration, or centrifugation.

a. Production of chimeric and humanized monoclonal antibodies

Chimeric and humanized antibodies have the same or similar binding specificity and affinity as a mouse or other nonhuman antibody that provides the starting material for construction of a chimeric or humanized antibody. Chimeric antibodies are antibodies whose light and heavy chain genes have been constructed, typically by genetic engineering, from immunoglobulin gene segments belonging to different species. For example, the variable (V) segments of the genes from a mouse monoclonal antibody may be joined to human constant (C) segments, such as IgG1 and IgG4. Human isotype IgG1 is preferred. In some methods, the isotype of the antibody is human IgG1. IgM antibodies can also be used in some methods. A typical chimeric antibody is thus a hybrid protein consisting of the V or antigen-binding domain from a mouse antibody and the C or effector domain from a human antibody.

Humanized antibodies have variable region framework residues substantially from a human antibody (termed an acceptor antibody) and complementarity determining regions substantially from a mouse-antibody, (referred to as the donor immunoglobulin). See, Queen et al., Proc. Natl. Acad. Sci. USA 86:10029-10033 (1989), WO 90/07861, U.S. Patent No. 5,693,762, U.S. Patent No. 5,693,761, U.S. Patent No. 5,585,089, U.S. Patent No. 5,530,101, and Winter, U.S. Patent No. 5,225,539). The constant region(s), if present, are also substantially or entirely from a human immunoglobulin. The human variable domains are usually chosen from human antibodies whose framework sequences exhibit a high degree of sequence identity with

the murine variable region domains from which the CDRs were derived. The heavy and light chain variable region framework residues can be derived from the same or different human antibody sequences. The human antibody sequences can be the sequences of naturally occurring human antibodies or can be consensus sequences of several human antibodies. Certain amino acids from the human variable region framework residues are selected for substitution based on their possible influence on CDR conformation and/or binding to antigen. Investigation of such possible influences is by modeling, examination of the characteristics of the amino acids at particular locations, or empirical observation of the effects of substitution or mutagenesis of particular amino acids.

For example, when an amino acid differs between a murine variable region framework residue and a selected human variable region framework residue, the human framework amino acid should usually be substituted by the equivalent framework amino acid from the mouse antibody when it is reasonably expected that the amino acid:

(1) noncovalently binds antigen directly,

(2) is adjacent to a CDR region,

(3) otherwise interacts with a CDR region (e.g. is within about 6 A of a CDR region), or

(4) participates in the VL-VH interface.

Other candidates for substitution are acceptor human framework amino acids that are unusual for a human immunoglobulin at that position. These amino acids can be substituted with amino acids from the equivalent position of the mouse donor antibody or from the equivalent positions of more

typical human immunoglobulins. Other candidates for substitution are acceptor human framework amino acids that are unusual for a human immunoglobulin at that position. The variable region frameworks of humanized immunoglobulins usually show at least 85% sequence identity to a human variable region framework sequence or consensus of such sequences.

b. Production of human monoclonal antibodies

Human antibodies against B7-H4 or B7-H4 receptor are provided by a variety of techniques described below. Some human antibodies are selected by competitive binding experiments, or otherwise, to have the same epitope specificity as a particular mouse antibody. Human antibodies preferably have isotype specificity human IgG1.

One method for producing human monoclonal antibodies is the trioma methodology. The basic approach and an exemplary cell fusion partner, SPAZ-4, for use in this approach have been described by Oestberg et al., Hybridoma 2:361-367 (1983); Oestberg, U.S. Patent No. 4,634,664; and Engleman et al., U.S. Patent No. 4,634,666). The antibody-producing cell lines obtained by this method are called triomas, because they are descended from three cells-two human and one mouse. Initially, a mouse myeloma line is fused with a human B-lymphocyte to obtain a non-antibody-producing xenogeneic hybrid cell, such as the SPAZ-4 cell line. The xenogeneic cell is then fused with an immunized human B-lymphocyte to obtain an antibody-producing trioma cell line. Triomas have been found to produce antibody more stably than ordinary hybridomas made from human cells.

The immunized B-lymphocytes are obtained from the blood, spleen, lymph nodes or bone marrow of a human donor. If antibodies against a

specific antigen or epitope are desired, it is preferable to use that antigen or epitope thereof for immunization. Immunization can be either *in vivo* or *in vitro*. For *in vivo* immunization, B cells are typically isolated from a human immunized with B7-H4 or B7-H4 receptor. In some methods, B cells are isolated from the same patient who is ultimately to be administered antibody therapy. For *in vitro* immunization, B-lymphocytes are typically exposed to antigen for a period of 7-14 days in a media such as RPMI-1640 supplemented with 10% human plasma.

The immunized B-lymphocytes are fused to a xenogeneic hybrid cell such as SPAZ-4 by well known methods. For example, the cells are treated with 40-50% polyethylene glycol of MW 1000-4000, at about 37° C, for about 5-10 min. Cells are separated from the fusion mixture and propagated in media selective for the desired hybrids (e.g., HAT or AH). Clones secreting antibodies having the required binding specificity are identified by assaying the trioma culture medium for the ability to bind to B7-H4 or B7-H4 receptor. Triomas producing human antibodies having the desired specificity are subcloned by the limiting dilution technique and grown in vitro in culture medium. The trioma cell lines obtained are then tested for the ability to bind B7-H4 or B7-H4 receptor.

Although triomas are genetically stable they do not produce antibodies at very high levels. Expression levels can be increased by cloning antibody genes from the trioma into one or more expression vectors, and transforming the vector into standard mammalian, bacterial or yeast cell lines.

Human antibodies against B7-H4 or B7-H4 receptor can also be produced from non-human transgenic mammals having transgenes encoding

at least a segment of the human immunoglobulin locus. Usually, the endogenous immunoglobulin locus of such transgenic mammals is functionally inactivated. Preferably, the segment of the human immunoglobulin locus includes unrearranged sequences of heavy and light chain components. Both inactivation of endogenous immunoglobulin genes and introduction of exogenous immunoglobulin genes can be achieved by targeted homologous recombination, or by introduction of YAC chromosomes. The transgenic mammals resulting from this process are capable of functionally rearranging the immunoglobulin component sequences, and expressing a repertoire of antibodies of various isotypes encoded by human immunoglobulin genes, without expressing endogenous immunoglobulin genes. The production and properties of mammals having these properties are described in detail by, e.g., Lonberg et al., WO93/1222, U.S. Patent No. 5,877,397, U.S. Patent No. 5,874,299, U.S. Patent No. 5,814,318, U.S. Patent No. 5,789,650, U.S. Patent No. 5,770,429, U.S. Patent No. 5,661,016, U.S. Patent No. 5,633,425, U.S. Patent No. 5,625,126, U.S. Patent No. 5,569,825, U.S. Patent No. 5,545,806, Nature 148, 1547-1553 (1994), Nature Biotechnology 14, 826 (1996), Kucherlapati, WO 91/10741.

Transgenic mice are particularly suitable. Anti-B7-H4 and anti-B7-H4 receptor antibodies are obtained by immunizing a transgenic nonhuman mammal with polypeptides corresponding to full length B7-H4 or B7-H4 receptor polypeptides or immunogenic fragments thereof. Monoclonal antibodies are prepared by, e.g., fusing B-cells from such mammals to suitable myeloma cell lines using conventional Kohler-Milstein technology. Human polyclonal antibodies can also be provided in the form

of serum from humans immunized with an immunogenic agent. Optionally, such polyclonal antibodies can be concentrated by affinity purification using B7-H4 or B7-H4 receptor polypeptides or fragments thereof as an affinity reagent.

A further approach for obtaining human anti-B7-H4 and anti-B7-H4 receptor antibodies is to screen a DNA library from human B cells according to the general protocol outlined by Huse et al., Science 246:1275-1281 (1989). As described for trioma methodology, such B cells can be obtained from a human immunized with full length B7-H4 or B7-H4 receptor polypeptides or immunogenic fragments thereof. Optionally, such B cells are obtained from a patient who is ultimately to receive antibody treatment. Antibodies binding to B7-H4, B7-H4 receptor, or fragments thereof are selected. Sequences encoding such antibodies (or binding fragments) are then cloned and amplified. The protocol described by Huse is rendered more efficient in combination with phage-display technology. See, e.g., WO 91/17271 by Dower et al., WO 92/01047 by McCafferty et al., U.S. Patent No. 5,877,218, U.S. Patent No. 5,871,907, U.S. Patent No. 5,858,657, U.S. Patent No. 5,837,242, U.S. Patent No. 5,733,743 and U.S. Patent No. 5,565,332). In these methods, libraries of phage are produced in which members display different antibodies on their outer surfaces. Antibodies are usually displayed as Fv or Fab fragments. Phage displaying antibodies with a desired specificity are selected by affinity enrichment to a B7-H4 or B7-H4 receptor polypeptide or fragment thereof.

In a variation of the phage-display method, human antibodies having the binding specificity of a selected murine antibody can be produced (WO

92/20791 by Winter). In this method, either the heavy or light chain variable region of the selected murine antibody is used as a starting material. If, for example, a light chain variable region is selected as the starting material, a phage library is constructed in which members display the same light chain variable region (i.e., the murine starting material) and a different heavy chain variable region. The heavy chain variable regions are obtained from a library of rearranged human heavy chain variable regions. A phage showing strong specific binding for αSyn (e.g., at least 10^8 and preferably at least $10^9 M^{-1}$) is selected. The human heavy chain variable region from this phage then serves as a starting material for constructing a further phage library. In this library, each phage displays the same heavy chain variable region (i.e., the region identified from the first display library) and a different light chain variable region. The light chain variable regions are obtained from a library of rearranged human variable light chain regions. Again, phage showing strong specific binding for B7-H4 or B7-H4 receptor are selected. These phage display the variable regions of completely human anti-B7-H4 or anti-B7-H4 receptor antibodies. These antibodies usually have the same or similar epitope specificity as the murine starting material.

The heavy and light chain variable regions of chimeric, humanized, or human antibodies can be linked to at least a portion of a human constant region. The choice of constant region depends, in part, whether antibody-dependent complement and/or cellular mediated toxicity is desired. For example, isotopes IgG1 and IgG3 have complement activity and isotypes IgG2 and IgG4 do not. Choice of isotype can also affect passage of antibody into the brain. Human isotype IgG 1 is preferred. Light chain constant

regions can be lambda or kappa. Antibodies can be expressed as tetramers containing two light and two heavy chains, as separate heavy chains, light chains, as Fab, Fab' F(ab')2, and Fv, or as single chain antibodies in which heavy and light chain variable domains are linked through a spacer.

3. Expression of recombinant antibodies

Chimeric, humanized and human antibodies are typically produced by recombinant expression. Recombinant polynucleotide constructs typically include an expression control sequence operably linked to the coding sequences of antibody chains, including naturally associated or heterologous promoter regions. Preferably, the expression control sequences are eukaryotic promoter systems in vectors capable of transforming or transfecting eukaryotic host cells. Once the vector has been incorporated into the appropriate host, the host is maintained under conditions suitable for high level expression of the nucleotide sequences, and the collection and purification of the crossreacting antibodies. These expression vectors are typically replicable in the host organisms either as episomes or as an integral part of the host chromosomal DNA. Commonly, expression vectors contain selection markers, e.g., ampicillin-resistance or hygromycin-resistance, to permit detection of those cells transformed with the desired DNA sequences.

E. coli is one prokaryotic host particularly useful for cloning DNA sequences. Microbes, such as yeast are also useful for expression. *Saccharomyces* is a preferred yeast host, with suitable vectors having expression control sequences, an origin of replication, termination sequences and the like as desired. Typical promoters include 3-phosphoglycerate kinase and other glycolytic enzymes. Inducible yeast promoters include,

among others, promoters from alcohol dehydrogenase, isocytochrome C, and enzymes responsible for maltose and galactose utilization.

Mammalian cells are a preferred host for expressing nucleotide segments encoding immunoglobulins or fragments thereof (Winnacker, From Genes to Clones, VCH Publishers, NY, 1987). A number of suitable host cell lines capable of secreting intact heterologous proteins have been developed in the art, and include CHO cell lines, various COS cell lines, HeLa cells, L cells, human embryonic kidney cell, and myeloma cell lines. Preferably, the cells are nonhuman. Expression vectors for these cells can include expression control sequences, such as an origin of replication, a promoter, an enhancer (Queen et al., Immunol. Rev. 89:49 (1986)), and necessary processing information sites, such as ribosome binding sites, RNA splice sites, polyadenylation sites, and transcriptional terminator sequences. Preferred expression control sequences are promoters derived from endogenous genes including cytomegalovirus, SV40, adenovirus, bovine papillomavirus (Co et al., *J. Immunol.* 148:1149 (1992).

Alternatively, antibody coding sequences can be incorporated in transgenes for introduction into the genome of a transgenic animal and subsequent expression in the milk of the transgenic animal (see, e.g., U.S. Patent No. 5,741,957, U.S. Patent No. 5,304,489, U.S. Patent No. 5,849,992). Suitable transgenes include coding sequences for light and/or heavy chains in operable linkage with a promoter and enhancer from a mammary gland specific gene, such as casein or beta lactoglobulin.

The vectors containing the DNA segments of interest can be transferred into the host cell by well-known methods, depending on the type of

cellular host. For example, calcium chloride transfection is commonly utilized for prokaryotic cells, whereas calcium phosphate treatment, electroporation, lipofection, biolistics or viral-based transfection can be used for other cellular hosts. Other methods used to transform mammalian cells include the use of polybrene, protoplast fusion, liposomes, electroporation, and microinjection (see generally, Sambrook et al., supra). For production of transgenic animals, transgenes can be microinjected into fertilized oocytes, or can be incorporated into the genome of embryonic stem cells, and the nuclei of such cells transferred into enucleated oocytes.

Once expressed, antibodies can be purified according to standard procedures of the art, including HPLC purification, column chromatography, gel electrophoresis and the like (see generally, Scopes, Protein Purification (Springer-Verlag, NY, 1982)).

Polypeptide immunogens disclosed herein can also be linked to a suitable carrier molecule to form a conjugate which helps elicit an immune response. Suitable carriers include serum albumins, keyhole limpet hemocyanin, immunoglobulin molecules, thyroglobulin, ovalbumin, tetanus toxoid, or a toxoid from other pathogenic bacteria, such as diphtheria, *E. coli*, *cholera*, or *H. pylori*, or an attenuated toxin derivative. T cell epitopes are also suitable carrier molecules. Some conjugates can be formed by linking agents to an immunostimulatory polymer molecule (e.g., tripalmitoyl-S-glycerine cysteine (Pam_3Cys), mannan (a manose polymer), or glucan (a beta 1→2 polymer)), cytokines (e.g., IL-1, IL-1 alpha and beta peptides, IL-2, gamma-INF, IL-10, GM-CSF), and chemokines (e.g., MIP1alpha and beta, and RANTES). Immunogenic agents can also be linked

to peptides that enhance transport across tissues, as described in WO 97/17613 and WO 97/17614 toO'Mahony. Immunogens may be linked to the carries with or with out spacers amino acids (e.g., gly-gly).

Some conjugates can be formed by linking agents to at least one T cell epitope. Some T cell epitopes are promiscuous while other T cell epitopes are universal. Promiscuous T cell epitopes are capable of enhancing the induction of T cell immunity in a wide variety of subjects displaying various HLA types. In contrast to promiscuous T cell epitopes, universal T cell epitopes are capable of enhancing the induction of T cell immunity in a large percentage, e.g., at least 75%, of subjects displaying various HLA molecules encoded by different HLA-DR alleles.

A large number of naturally occurring T-cell epitopes exist, such as, tetanus toxoid (e.g., the P2 and P30 epitopes), Hepatitis B surface antigen, pertussis, toxoid, measles virus F protein, *Chlamydia trachomitis* major outer membrane protein, diphtheria toxoid, *Plasmodium falciparum* circumsporozite T, *Plasmodium falciparum* CS antigen, *Schistosoma mansoni* triose phosphate isomersae, *Escherichia coli* TraT, and Influenza virus hemagluttinin (HA). The immunogenic peptides can also be conjugated to the T-cell epitopes described in Sinigaglia F. et al., Nature, 336:778-780 (1988); Chicz R. M. et al., J. Exp. Med., 178:27-47 (1993); Hammer J. et al., Cell 74:197-203 (1993); Falk K. et al., Immunogenetics, 39:230-242 (1994); WO 98/23635; and, Southwood S. et al. J. Immunology, 160:3363-3373 (1998).

Alternatively, the conjugates can be formed by linking agents to at least one artificial T-cell epitope capable of binding a large proportion of

MHC Class II molecules., such as the pan DR epitope ("PADRE"). PADRE is described in U.S. Patent No. 5,736,142, WO 95/07707, and Alexander J et al., Immunity, 1:751-761 (1994). A preferred PADRE peptide is AKXVAAWTLKAAA (SEQ ID NO:1), wherein X is preferably cyclohexylalanine, tyrosine or phenylalanine, with cyclohexylalanine being most preferred.

Immunogenic agents can be linked to carriers by chemical crosslinking. Techniques for linking an immunogen to a carrier include the formation of disulfide linkages using N-succinimidyl-3-(2-pyridyl-thio)propionate (SPDP) and succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) (if the peptide lacks a sulfhydryl group, this can be provided by addition of a cysteine residue). These reagents create a disulfide linkage between themselves and peptide cysteine resides on one protein and an amide linkage through the epsilon-amino on a lysine, or other free amino group in other amino acids. A variety of such disulfide/amide-forming agents are described by Immun. Rev. 62, 185 (1982). Other bifunctional coupling agents form a thioether rather than a disulfide linkage. Many of these thio-ether-forming agents are commercially available and include reactive esters of 6-maleimidocaproic acid, 2-bromoacetic acid, and 2-iodoacetic acid, 4-(*N*-maleimido-methyl)cyclohexane-1-carboxylic acid. The carboxyl groups can be activated by combining them with succinimide or 1-hydroxyl-2-nitro-4-sulfonic acid, sodium salt.

Immunogenicity can be improved through the addition of spacer residues (e.g., Gly-Gly) between the T_h epitope and the peptide immunogen.

In addition to physically separating the T_h epitope from the B cell epitope (i.e., the peptide immunogen), the glycine residues can disrupt any artificial secondary structures created by the joining of the T_h epitope with the peptide immunogen, and thereby eliminate interference between the T and/or B cell responses. The conformational separation between the helper epitope and the antibody eliciting domain thus permits more efficient interactions between the presented immunogen and the appropriate T_h and B cells.

To enhance the induction of T cell immunity in a large percentage of subjects displaying various HLA types to a disclosed agent, a mixture of conjugates with different T_h cell epitopes can be prepared. The mixture may contain a mixture of at least two conjugates with different T_h cell epitopes, a mixture of at least three conjugates with different T_h cell epitopes, or a mixture of at least four conjugates with different T_h cell epitopes. The mixture may be administered with an adjuvant.

Immunogenic peptides can also be expressed as fusion proteins with carriers (i.e., heterologous peptides). The immunogenic peptide can be linked at its amino terminus, its carboxyl terminus, or both to a carrier. Optionally, multiple repeats of the immunogenic peptide can be present in the fusion protein. Optionally, an immunogenic peptide can be linked to multiple copies of a heterologous peptide, for example, at both the N and C termini of the peptide. Some carrier peptides serve to induce a helper T-cell response against the carrier peptide. The induced helper T-cells in turn induce a B-cell response against the immunogenic peptide linked to the carrier peptide.

The present invention will be further understood by reference to the following non-limiting examples.

EXAMPLES

Example 1: Generation of B7-H4KO mice.

Mice

6-8-week-old C57BL16 (B6) mice were obtained from the Jackson Laboratory. RAG-1 KO mice were purchased from Taconic Farms. Both female and male mice were used for the experiments. All mice were housed under specific pathogen-free conditions in the Johns Hopkins Animal Facility with all protocols approved by the Institutional Animal Care and Use Committee. The general strategy to generate gene KO mice by homologous recombination was previously described (Dong, H. et al., *Immunity* 20:327-336 (2004); Tamada, K. et al., *J Immunol.*, 168, 4832-4835 (2002).). To generate B7-H4 KO mice, a 5.09 kb DNA fragment upstream of the IgV domain (exon 3) of the murine B7-H4 genomic DNA was PCR amplified from a 129SvJ bacterial artificial chromosome (BAC) library (Invitrogen, Carlsbad, CA) and was cloned into the 5'-arm position of the pKOscrambler vector NTKV-1907 (Stratagene, La Jolla, CA). A 5.57 kb DNA fragment downstream of the IgC domain (exon 4) of B7-H4 genomic DNA was PCR amplified from the same library and was cloned into the 3'-arm position of the same vector to generate a targeting plasmid resulting in removing IgV and IgC domains from the B7-H4 gene (Fig. 1A). The targeting fragment containing the 5'- arm and the 3'- arm sequences of the B7-H4 gene, a positive selection marker NEO, and a negative selection marker TK was transfected into 129SvIE embryonic stem (ES) cells. ES cell transfectants

underwent neomycin drug selection. The targeted clones were identified by Southern blot analysis using a 3' external probe. Chimeric mice were produced by injection of targeted ES cells into blastocysts of B6 hosts. Heterozygous B7-H4 (+I-) mice were obtained from breeding chimeric mice with B6 mice. PCR analysis was employed to distinguish the wildtype and deficient B7-H4 allele. The sequences of the three PCR primers are:

(1) 5'-GTTAGATAGGGTCTCACTGGGTAGC (SEQ ID NO:2),

(2) 5'-CCTACAGCCTTCAGTATGCCAGAGA (SEQ ID NO:3),

(3) 5'-AGACTAGTGAGACGTGCTACTTCCA (SEQ ID NO:4).

Homozygous mice were produced by back-crossing to B6 for more than ten generations before use for further analysis. B7-H4 KO/RAG-1 KO mice were obtained by backcrossing B7-H4 KO and RAG-1 KO mice.

B7-H4KO mice were generated by homologous recombination in 129 ES cells by deleting the entire Ig V and Ig C regions of the B7-H4 gene to completely eliminate their interaction with its potential receptor. Exons encoding both the Ig V and Ig *C* domains of B7-H4 gene were replaced with a Neo gene cassette (Fig. 1). Targeted recombination of ES cells was confirmed by Southern blot analysis and the data from 4 independent ES clones is shown.

B7-H4+ allele is predicted to have a 12.25 kb Spe1 fragment and B7-H4- allele has an 8.9 kb Spel fragment. The clones (2 and 3) with both fragments indicate a recombination. Chimeric male mice were derived from these ES clones by standard procedures. They were backcrossed to C57BLl6 (B6) females and heterozygous mutant mice were established from two independently targeted ES clones. Heterozygous or homozygous B7-H4

mutant mice were then identified by PCR analysis of genomic DNA isolated from tail biopsies. Southern blot analysis confirmed the replacement of genomic DNA. RT-PCR analysis demonstrated B7-H4 mRNA was not expressed in livers of B7-H4-deficient mice. B7-H4KO mice develop normally and give normal litter numbers. These mice were backcrossed to the B6 background for 10 generations before they were used in studies described below.

Example 2: B7-H4KO mice have enhanced granulocyte-mediated resistance to Listeria infection.

Antibodies, recombinant protein and flow cytometry analysis

Primary and secondary antibodies against murine Gr-1 and CD11b, which are directly conjugated with FITC, PE, or APC, were purchased from BD Pharmingen (San Diego, CA) or eBiosciences (San Diego, CA). Non-conjugated primary antibodies were purified from hybridoma culture supernatant. B7-H4Ig fusion protein was prepared as described by Sica, G. L. et al., *Immunity*, 18:849-861 (2003). All cells were stained using standard protocols and were analyzed on a FACSCalibur flow cytometry (id). The data was analyzed with Software CellQuest (BD) or FlowJo (Tree Star, Inc., Ashland, OR). For in *vivo* studies, mAbs were prepared and purified as previously described (id). Anti-NK1.1 hybridoma (PK136) and anti-IFN-γ hybridoma (R4-6A2) were purchased from ATCC. Anti-Gr-1 hybridoma (RB6-8C5) was a generous gift from Dr. Hans Schreiber in University of Chicago. Control mouse IgG, rat IgG, and hamster IgG were purchased from Sigma (St. Louis, MO) and further purified as previously described (id). Carrageenan was purchased from Sigma. All cell culture media and

antibiotics were purchased from Cellgro (Herndon, VA). Fetal bovine serum (FBS) was from Hyclone (Logan, UT).

Listeria infection and colony counting

Listeria monocytogenes strain DP-L4056 was kindly provided by Dr. Thomas W. Dubensky Jr. from Cerus Corp. To prepare Listeria stock, Listeria cells were grown in DIFCO Listeria Enrichment Broth (Becton Dickinson Co., Sparks, MD) to 0.8-1 at OD600 nm. Culture was harvested by centrifugation and was washed twice with PBS. Pellets were then re-suspended in stock solution (PBS with 15-20% glycerol) and aliquoted to 200 µl per microtube for storage at -80°C. The colony-forming units (CFU) of Listeria stock were determined by counting colonies of series dilutions of the aliquots growing on BBL CHROMagar Listeria plates (Becton Dickinson Co., Sparks, MD). Prior to infection, Listeria stock was thawed and diluted in PBS to appropriate concentration of CFU/ml and applied to mice or cells as indicated. Mice 6-8 weeks old were infected by intraperitoneal (i.p.) or intravenous (i.v.) injection of indicated CFU of Listeria. At indicated time points post-infection, a piece of mouse liver or spleen was cut, weighed, and ground in PBS. The liver suspension was plated on BBL CHROMagar Listeria plates or on agar plates of Listeria Enrichment Broth. Colonies were counted 2 days post plating, and adjusted to CFU/g of liver or spleen.

Listeria infection of granulocytes in vitro.

Granulocytes were isolated similar to the methods described by Chen, L. Y. et al., *Hum. Mol. Genet.*, 12:2547-2558 (2003). Briefly, mice were injected i.p. with 3% thioglycollate broth. Four to five hours post injection, peritoneal cavities of each mouse were washed with 5 ml PBS and cells were

harvested by centrifugation. By this method, > 90% harvested cells are Gr-1$^+$CD11b+ granulocyte. 1 x 10^6 granulocytes were incubated with 1 x 10^8 CFU of LM for 10 min at 37°C. The cultures were terminated by adding Penicillin-Streptomycin (Cellgro). Subsequently, cells were harvested by centrifugation, plated in 96-well plates. The plates were incubated at 37'C and harvested at indicated time points. Cells were lysed immediately by resuspending in 1 ml of sterile water. Cell lysates or diluted cell lysates were plated on agar plates of Listeria Enrichment Broth for colony counting.

Respiratory burst and phagocytosis of granulocytes.

Granulocyte phagocytic activity and oxidative burst activity were measured as described by Radsak, M. P., et al., *J. Immunol.*, 172:4956-4963 (2004); Radsak, M. P. et al., *Blood*, 101:2810-281 5 (2003). Briefly, 1 x 10^6 granulocyte were incubated with 5 x 10^7 of red-fluorescent micro-beads (Fluoresbrite Polychromatic Red 1.0 Micron Microspheres, Polysciences, Inc. Warrington, PA) and 25 μM of DCFH-DA (2',7',-dihydrochlorofluoresein diacetate, Sigma-Aldrich) for 30-60 min at 37°C. Cells were washed twice with FACS buffer (1 % FBS in PBS) and fixed in 1% paraformaldehyde in PBS. Analysis was performed by flow cytometry.

Pathology

The method for tissue processing and staining was described by Dong, H. et al, *Nature Med.* 8:793-800 (2002). Briefly, spleen specimens of 6-8 week old mice were embedded in OCT compound (Sakura Finetek USA, Torrance, CA) and frozen at -80°C. Frozen tissues were sliced, mounted and stained with 5 μg/ml Gr-1-biotin antibody. ABC peroxidase (Vector laboratories, Inc., Burlingame, CA) and DAB peroxidase substrate

(Sigma-Aldrich, St. Louis, MO) were then applied to slides according to the company protocols. Finally, hematoxylin solution was used to stain Gr-1 negative cells.

Results

B7-H4KO mice display normal numbers and ratios of T, B, NK, NKT cells, and macrophages. In addition, there are no obvious alterations in T cell responses, judged by in *vitro* proliferation of purified T cells by CD3 cross-linking, allogeneic antigen stimulation, or cytolytic T cell response to alloantigens. These results indicate that polyclonal T cell responses to antigens are not impaired in B7-H4KO mice. Consistent with these *in vitro* findings, it was also found that B7-H4KO mice have normal responses to Con-A induced hepatitis (Dong, H. et al., *Immunity*, 20327-336 (2004))., hapten-induced hypersensitivity (Tsushima, F. et al. *Eur. J. Immunol.*, 33:2773-2782 (2003).), and OVA-induced airway inflammation (Kamata, T. et al, *J. Clin. Invest.*, 111:109-119 (2003)). B7-H4-deficient mice were also found to be comparable to wild-type mice in OT-I and OT-II cell expansion to OVA proteins (Sica, G. L. et al., *Immunity*, 18849-861 (2003)), CD4-Vβ8.118.2 T cell expansion to superantigens (Tamada, K. et al., *J Immunol.*, 168:4832-4835 (2002)), and CTL activities to allogeneic antigens *in vivo* (Tamada, K. et al, *Nature Med.*, 6:283-289 (2000)). Normal B cell responses were also observed after immunization by TNP-KLH (Tamura, H. et al., *Blood* 97:1809-1 816 (2001)). Finally, B7-H4KO mice do not develop spontaneous autoimmune diseases up to 1.5 years in SPF condition.

While the data indicates that B7-H4 plays a minimal role in antigen-driven T and B cell responses in assays, these responses were

conducted in the absence of active infection, which usually requires a much more sophisticated coordination between innate and adaptive immunity. To test this possibility, the effect of B7-H4 ablation was evaluated in mice infected with *Listeria monocytogenes* (LM) to examine whether B7-H4 contributes to immunity against infection. Mice were challenged with an intra-peritoneal dose (i.p.) (2×10^6 CFU) of LM sufficient to induce lethality. The survival of these mice was then subsequently evaluated. B7-H4KO mice were significantly more resistant to LM infection: B7-H4KO mice survived much longer than their wild-type (WT) littermate and up to 40% of mice cleared bacteria and lived indefinitely, while all littermates died around day 9 (Fig. 2a). This effect is correlated with decreased Listeria numbers in the spleens (Fig. 2b) and liver in B7-H4KO mice. Interestingly, the majority of mice were dead within 3-4 days, time points at which adaptive immunity is usually not yet developed. The results thus suggest a role of B7-H4 in altering the context of the innate immune response.

To address mechanisms of this resistance, the cell compositions of both innate and adaptive immunity were examined. The mice were infected with Listeria and T, B, NK, macrophages and granulocytes in peripheral blood and in lymphoid organs were examined by specific mAb. Although there were no significant differences in NK, macrophages, T cells, and B cells within the first 3 days after LM infection, significantly more granulocytes in spleens were found from LM-infected B7-H4KO mice than identically infected WT littermates at day 3 upon infection (Fig. 2c). Similar results were also obtained in granulocytes isolated from livers and in peripheral blood after infection. In uninfected B7-H4KO mice, however,

granulocyte numbers were within normal range of WT controls. The results indicate that the role of B7-H4 is to inhibit granulocyte responses during LM infection.

To determine if granulocytes are required for the resistance of LM infection in B7-H4KO mice, granulocytes were depleted by inoculation of Gr-1 mAb. Injection of Gr-I mAb led to rapid decline of granulocytes to undetectable levels at day 2 in spleens. Depletion of Gr-I + granulocytes led to a significant increase of LM load in livers from B7-KO mice, in comparison with those treated with either PBS or isotype-matched control mAb (Fig. 2d). Depletion of NK cells by NKI .I mAb did not affect colony formation of LM in liver, while depletion of macrophages by carrageenan increased LM colonies to a moderate but less significant level as compared to Gr-I cell depletion. The results thus support the notion that Gr-I+ granulocytes play a critical role in the resistance to LM infection in the absence of B7-H4.

Whether B7-H4-deficient granulocytes have modified functionalities was determined by co-culture of purified granulocytes and LM. B7-H4-deficient granulocytes display normal uptake and growth inhibition of LM in culture system (Fig. 3). In addition, respiratory burst and phagocytosis by B7-H4KO granulocytes are also normal, indicating B7-H4KO granulocytes are functionally indifferent from WT granulocytes. Therefore, increased resistance to LM infection in B7-H4KO mice is likely caused by an increased number, not increased functional capacity of granulocytes.

Example 3: Granulocyte-mediated innate resistance in B7-H4KO mice is independent of adaptive immunity.

Activated and memory T cells are important components in the immunity against LM (Nathan, C. *Nature Rev.Immunol.*, 6:173-1 82 (2006)). While the data supports that resistance of B7-H4KO mice to LM infection requires granulocytes, it is unknown whether adaptive immunity also contributes to this resistance. Because increased granulocyte numbers post-LM infection was a major phenotype found in B7-H4KO mice, the responses of B7-H4KO mice to LM infection were explored in the absence of adaptive immunity. B7-H4KO mice were backcrossed to the RAG-1 KO background to eliminate T and B cells.

Results

Unlike RAG-1 KO (RKO) mice, which possess small spleens, B7-H4/RAG-1 double KO (DKO) mice display enlarged spleens. The spleen sizes of DKO mice are similar to those of WT and B7-H4KO mice in B6 background. Further analysis of cell components in spleen, peripheral blood, liver, and bone marrow revealed that Grl+CD11b+ granulocytes increased dramatically.

RKO and DKO mice were challenged by administration of a lethal dose of LM to examine their innate resistance. Infection of RKO mice by LM led to exponential growth of LM in liver and 100% mortality by day 4 (Fig. 4). In sharp contrast, DKO mice have significantly less bacterial load in the liver at day 2 and the majority of the mice were able to survive more than 10 days LM challenge (Fig. 4). Similar exponential growth of LM in other organs including spleens were observed indicating a dissemination of LM

infection. In contrast to long-term survival of a significant fraction of infected B6 background B7-H4KO mice (Fig. 2a), all DKO mice eventually died of infection at day 15, support an important role of adaptive immunity (Fig. 4). Combined together with rapid clearance of LM from liver and other organs in DKO mice as early as day 2, the results suggest that lack of B7-H4 confers enhanced innate immunity against LM infection, which is largely mediated through increased granulocytes.

Example 4: B7-H4 directly inhibits proliferation of granulocytes.

Bone marrow cell culture and granulocyte growth and inhibition assay

Bone marrow cells were aspirated and prepared as described by Wilcox, R. A. et al., *Blood*, 103:177-1 84 (2004). For B7-H4-mediated growth inhibition, B7-H4Ig or control murine Ig were coated in 96-well plates overnight. After extensive washing, BM cells were plated 2 x 10^6/well in 24-well plates with or without recombinant murine G-CSF (Pepro Tech Inc., Rocky Hill, NJ) at indicated concentrations. Cells were harvested at indicated time points and cell numbers were counted with Beckman Coulter Counter (Beckman, Fullerton, CA). To examine cell growth, 2 x 10^5/well of BM cells were plated in 96-well plates with G-CSF. After being pulsed with ^{3}HTdR, cells were harvested with FilterMate cell harvester (Perkin Elmer, Shelton, CT) 16 hours post ^{3}HTdR pulse. The incorporated ^{3}HTdR was detected by Trilux Liquid Scintillation and Luminescence Counter (Wallac, Turku, Finland). For cell division assay, BM cells were first labeled with 2 μM of carboxfluorescein diacetate succinimidyl ester (CFSE, Invitrogen, Carlsbad, CA) and then were added to and cultured in 96- or 24-well plates.

Cells were harvested at indicated time points, stained with mAb Gr-1 and CD11b and subjected to flow cytometry analysis for CFSE content (2) at different time points.

Results

Increased granulocytes in B7-H4KO mice indicates that B7-H4 play a role in delivering an inhibitory growth signal to granulocytes. Granulocytes from B7-H4 KO mice were examined to determine whether they have better growth potential than WT granulocytes. To do so, bone marrow (BM) cells, which contain large numbers of granulocyte precursors, were prepared and cultured from WT or B7-H4 KO mice in the presence or absence of G-CSF for 3 days to facilitate differentiation of granulocyte/neutrophil. The proliferation of BM cells was subsequently determined by ^{3}HTdR incorporation. Fig. 5A shows that while BM cells respond to G-CSF by proliferating in a dose-dependent fashion, proliferation of BM cells from B7-H4KO mice was significantly higher than those from WT mice. Flow cytometry analysis of BM cells which respond to G-CSF in the end of culture, shows that more than 95% of survived cells are CD11b+Gr-1+ granulocytes.

While this data is consistent with an inhibitory effect of B7-H4 in granulocytes, other cellular components in BM cells may also contribute to proliferation. To precisely exclude this possibility, BM cells were labeled with CFSE and after stimulation with G-CSF for 3 days, the cells were stained with anti-Gr-1 +/CD11b+ mAbs to monitor granulocytes for cell division. Fig. 5b shows that 70% Gr-1+CD11b+ granulocytes from B7-H4KO mice (B6) divide at least once whereas only 56% granulocytes

from WT B6 mice had diluted CSFE. Similar, but more significant differences were found in mice with the RAG-1 KO background: 86% granulocytes from DKO mice entered division whereas only 64.8% granulocytes from RKO mice had diluted CSFE. The results thus indicate that lack of B7-H4 on BM cells increase proliferation of BM-derived granulocytes.

Considering that the lack of B7-H4 could result in increased proliferation of BM-derived granulocytes, whether B7-H4 could directly inhibit their proliferation was determined. To test this, WT BM-derived granulocytes were cultured in the presence of recombinant B7-H4Ig fusion protein and examined proliferation of granulocytes. Proliferation of WT BM cells was significantly inhibited by B7-H4Ig, a fusion protein of B7-H4 extracellular portion and immunoglobulin Fc. The inhibition was evident at day 3 of the culture and became more significant at day 4 and 5 (Fig. 6a). Addition of 0.1 ng/ml of G-CSF in the culture, albeit moderately increasing proliferation of BM cells, did not significantly overcome B7-H4Ig mediated suppression (Fig. 6b). Increasing G-CSF to 1 ng/ml in the culture, however, could recover B7-H4Ig-mediated growth inhibition of BM cells in large degree (Fig. 6c). Similar inhibition was also observed in B7-H4 deficient granulocytes. Combined together, the results provide further evidence that B7-H4 is inhibitory for the proliferation of granulocytes, which could be reversed by G-CSF.

It has been unexpectedly discovered that B7-H4 can negatively regulate innate immunity against Listeria infection. It is believed that the effect of B7-H4 is mediated through growth suppression of granulocytes. In

the context of broad expression pattern of B7-H4 in peripheral tissue, the data supports B7-H4 as an important regulatory molecule in the control of innate immunity in peripheral tissues, in addition to the previously described role of B7-H4 in the inhibition of T cell responses.

In B7-H4KO mice, the majority of the extracellular portion of B7-H4 protein is deleted to assure complete elimination of interaction between endogenous B7-H4 and its putative receptor. Ablation of this gene, however, does not have a profound effect on T cell responses to polyclonal and allogeneic antigen stimulation in *vitro.* Similar observations have been made in a recent study (Suh, W. K. et al. *Mol. Cell. Biol.*, 26:6403-641 1 (2006)). While these findings indicate that B7-H4 does not substantially influence the inhibition of strong polyclonal T cell responses to CD3 cross-linking or allogeneic antigens, it is possible that B7-H4 affects more selective steps during cascade of T cell responses. For example, a recent study shows that although B7-H4KO mice responded normally to several types of airway inflammatory responses as well as LCMV and influenza infection, the mice have slightly enhanced T-cell immune responses to *Leishmania major* infection. Responses of granulocytes in this knock-out system, however, were not examined. The experiments indicate that a dominant role of B7-H4 in Listeria infection is to suppress granulocyte-mediated innate immunity and this effect could also be observed in RAG-1 KO mice in the absence of adaptive immune system. Therefore, in addition to inhibition of T cell immunity as reported previously, B7-H4 may play a critical role in negative regulation of innate immunity against bacterial infection.

Although there is slightly increased granulocytes in the spleens of B61B7-H4KO mice, dramatic increase of granulocytes occur upon LM infection (Fig. 2). This increase, however, is not simply due to increased recruitment by LM-induced inflammation. B7-H4 KO mice in B6 background have a small increase of granulocytes in blood, bone marrow and spleen without infection. A more dramatic elevation of granulocytes is observed in RAG-1 KO background. In addition, bone marrow cells from B7-H4KO mice produce more granulocytes in the presence of G-CSF stimulation. Finally, inclusion of B7-H4 protein in culture significantly inhibits growth of bone marrow-derived granulocytes. Interestingly, the role of B7-H4 in the inhibition of granulocytes could be reversed, at least partially, by addition of higher concentrations of G-CSF in culture. G-CSF is a critical factor for growth and homeostasis of granulocyte *in vivo.* The result suggests that B7-H4 may serve as a negative regulator to antagonize the role of G-CSF *in vivo.* Combined together, the results support that B7-H4 provides an inhibitory signal for responsiveness of granulocytes to G-CSF, a foremost growth factor for granulocytes, and thus may regulate homeostasis of granulocytes.

The mechanism of B7-H4-mediated growth inhibition of granulocytes is yet to be elucidated. It has been shown that B7-H4, upon binding to its putative receptor, inhibits cell cycle progression on T cells (Sica, G. L. et al., *Immunity* 18:849-861 (2003); Kryczek, I. et al. *J Eicp Med,* 203:871-881 (2006)). In the cell culture system, dilution of CFSE and incorporation of ^{3}HTdR are clearly inhibited (Fig. 6a). Bone marrow cells were observed to undergo proliferation (Fig. 6a) and cell division (Fig. 5g) in

the absence of exogenously supplied G-CSF, a key growth factor for granulocytes. It is possible that endogenous G-CSF is produced by bone marrow cells and maintains basal level of proliferation *in vitro*. This suppression could be largely reversed by adding G-CSF (Fig. 6c). During the culture, significant increases of cell apoptosis was not observed for up to 5 days. Therefore, growth inhibition may be a dominant mechanism in granulocytes by B7-H4 ligation. B7-H4 mRNA is widely expressed by various cells while its cell surface expression could be largely contained in cytoplasm as observed in ovarian cancer and infiltrating macrophages (Kryczek, I. et al., *J Eicp Med*, 203:871-881 (2006)). Surface expression of B7-H4 could be regulated by cytokines within the bone marrow microenvironment to inhibit granulocyte growth.

Granulocytes, including neutrophils, are one of the earliest cells to arrive at the site of an infection and are the first line of host defense against infection through their capacity to phagocytose (Nathan, C. *Nature Rev. Immunol.*, 6:173-1 82 (2006)). The findings showing an increased resistance to Listeria infection in B7-H4KO mice implicates a new approach to enhance innate immunity against infection by Listeria and possibly other pathogens. It is also interesting that B7-H4 KO mice in the RAG-1 background have a more profound increase in the number of granulocytes and are more resistant to early phase LM infection in comparison with B7-H4 KO mice in B6 background. These data implicate a possible suppressive role of adaptive immunity components including T and B cells in granulocyte homeostasis and response to Listeria infection. Therefore, the method to selective blockade of B7-H4 expression such as neutralizing mAb

or appropriately engineered B7-H4 protein with antagonistic activity may represent a new approach to increase granulocytes and enhanced innate immunity against pathogen infection.

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments described herein. Such equivalents are intended to be encompassed by the following claims.

We claim:

1. A pharmaceutical composition dosage unit comprising an effective amount of a B7-H4 antagonist to inhibit B7-H4 activity in an individual in need thereof, and a pharmaceutically acceptable carrier.

2. The pharmaceutical composition of claim 1 wherein the B7-H4 antagonist is sH4 or a variant thereof.

3. The pharmaceutical composition of claim 1 wherein the B7-H4 antagonist is selected from the group consisting of anti-B7-H4 antibody, anti-B7-H4 receptor antibody, inhibitory nucleic acid specific for B7-H4 or B7-H4 receptor, nucleic acid encoding sH4, and combinations thereof.

4. The pharmaceutical composition of claim 3 wherein the inhibitory nucleic acid is selected from the group consisting of antisense DNA, antisense RNA, siRNA, and microRNA.

5. The pharmaceutical composition of claim 1 wherein the B7-H4 activity is B7-H4 binding to cell-surface receptor.

6. The pharmaceutical composition of claim 5 wherein the cell-surface receptor is a receptor on a T cell.

7. The pharmaceutical composition of claim 1 wherein the B7-H4 activity is inhibition of T cell activity.

8. The composition of claim 1 further comprising a second therapeutic agent.

9. The composition of claim 8 wherein the second therapeutic agent is selected from the group consisting of an antibody or antigen binding fragment thereof specific for a growth factor receptor or tumor specific antigen, cytokines, chemokines, proliferation inhibitors and cytotoxic agents.

10. A method for stimulating or enhancing an immune response in an individual comprising

administering to the individual an effective amount of a B7-H4 antagonist to inhibit B7-H4 activity in the individual.

11. The method of claim 8 wherein the B7-H4 antagonist is sH4 or a variant thereof.

12. The method of claim 8 wherein the B7-H4 antagonist is selected from the group consisting of anti-B7-H4 antibody, anti-B7-H4 receptor antibody, inhibitory nucleic acid specific for B7-H4 or B7-H4 receptor, nucleic acid encoding sH4, and combinations thereof.

13. The method of claim 12 wherein the inhibitory nucleic acid is selected from the group consisting of antisense DNA, antisense RNA, siRNA, and microRNA.

14. The method of claim 10 wherein the B7-H4 activity is B7-H4 binding to cell-surface receptor.

15. The method of claim 14 wherein the cell-surface receptor is a receptor on a T cell.

16. The method of claim 10 wherein the B7-H4 activity is inhibition of T cell activity.

17. The method of claim 10 wherein the method is used to treat an infection or cancer in the individual.

18. The method of claim 17 for treating a cancer wherein the cancer is selected from the group consisting of bladder, brain, breast, cervical, colo-rectal, esophageal, kidney, liver, lung, nasopharangeal, pancreatic, prostate, skin, stomach, uterine, ovarian, and testicular cancers.

19. The method of claim 17 for treating an infection wherein the infection is due to a virus, bacteria, fungus, and protozoa.

20. A vaccine composition comprising

an antigen and a B7-H4 antagonist in an amount effective to inhibit B7-H4 activity in a individual.

21. The vaccine composition of claim 20 wherein the antigen is an expression vector encoding the antigen.

22. The vaccine composition of claim 20 wherein the antigen comprises an antigenic polypeptide.

23. The vaccine composition of claim 22 wherein the antigenic polypeptide is viral, fungal, bacterial, or protozoan.

24. The vaccine composition of claim 22 wherein the antigenic polypeptide comprising a tumor specific antigen.

25. A method for treating cancer comprising

administering to a subject an effective amount of a B7-H4 antagonist to bind to a B7-H4 molecule on a tumor cell to inhibit the B7-H4 molecule from binding to a B7-H4 receptor on an immune cell.

26. The method of claim 25 wherein the B7-H4 antagonist is selected from the group consisting of an antibody or antigen binding fragment thereof and sH4.

27. The method of claim 25 further comprising administering to the subject a second therapeutic agent.

28. The method of claim 27, wherein the second therapeutic agent is selected from the group consisting of an antibody or antigen binding fragment thereof specific for a growth factor receptor or tumor specific antigen, cytokines, chemokines,proliferation inhibitors and cytotoxic agents.

29. The method of claim 25 further comprising administering an effective amount of radiation.

Figure 1

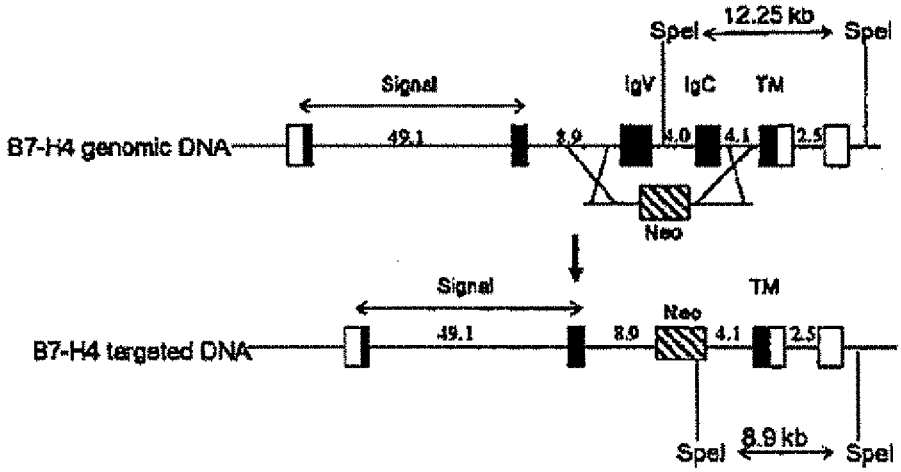

Figure 2a

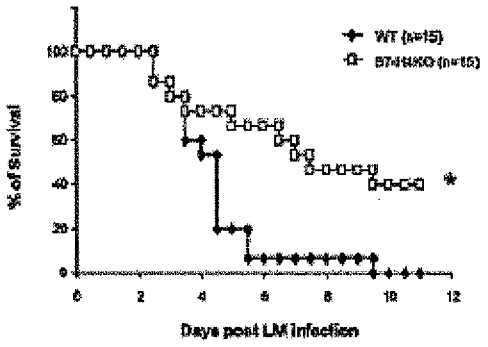

Figure 2b

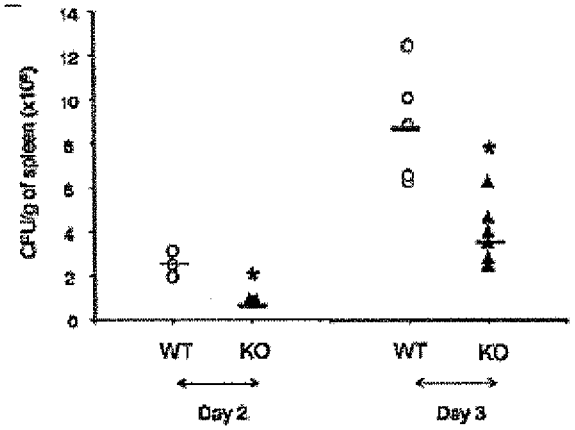

Figure 2c

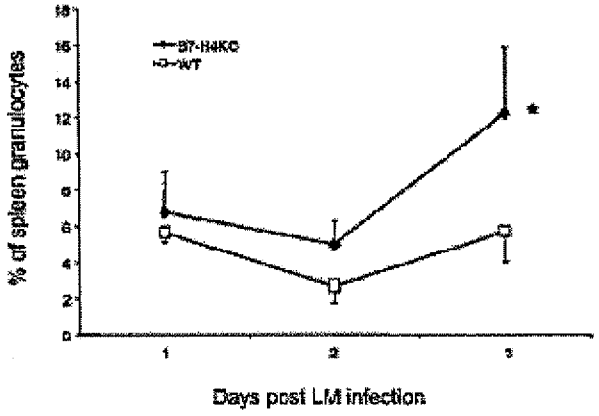

Figure 2d

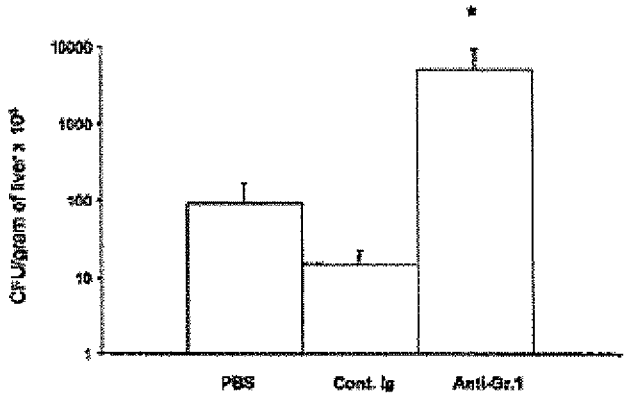

Figure 3

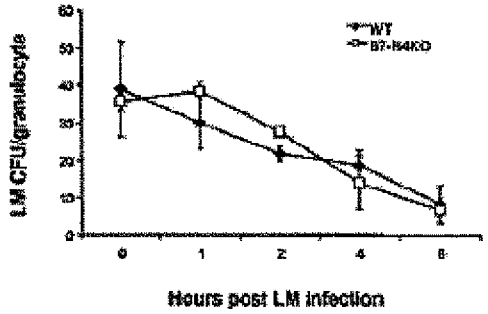

Figure 4

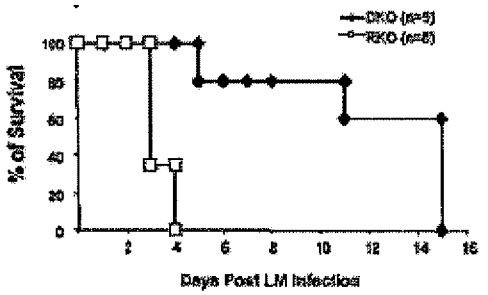

Figure 5a

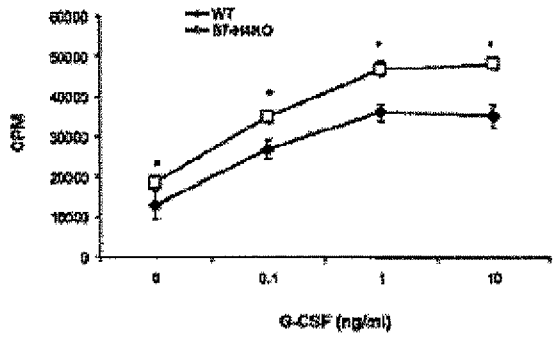

Figure 5b

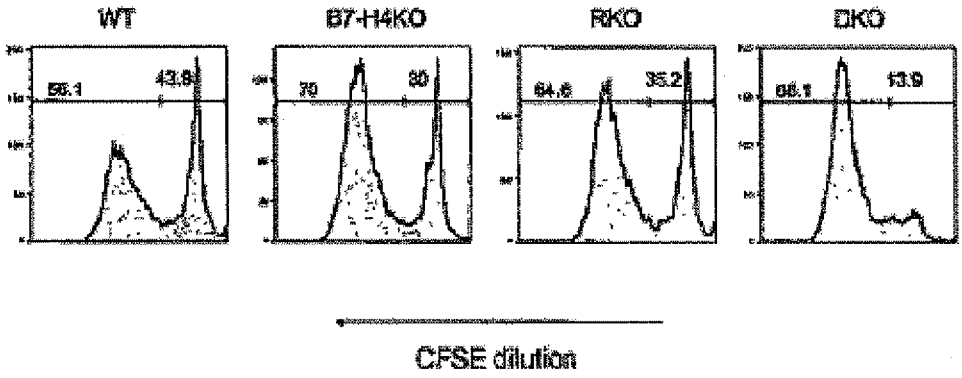

Figure 6