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COMPOSITIONS AND METHODS FOR WT1 SPECIFIC IMMUNOTHERAPY

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BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates generally to the immunotherapy of malignant diseases such as leukemia and cancers. The invention is more specifically related to compositions for generating or enhancing an immune response to WT1, and to the use of such compositions for preventing and/or treating malignant diseases.

Description of the Related Art

Cancer and leukemia are significant health problems in the United States and throughout the world. Although advances have been made in detection and treatment of such diseases, no vaccine or other universally successful method for prevention or treatment of cancer and leukemia is currently available. Management of the diseases currently relies on a combination of early diagnosis and aggressive treatment, which may include one or more of a variety of treatments such as surgery, radiotherapy, chemotherapy and hormone therapy. The course of treatment for a particular cancer is often selected based on a variety of prognostic parameters, including an analysis of specific tumor markers. However, the use of established markers often leads to a result that is difficult to interpret, and the high mortality continues to be observed in many cancer patients.

Immunotherapies have the potential to substantially improve cancer and leukemia treatment and survival. Recent data demonstrate that

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leukemia can be cured by immunotherapy in the context of bone marrow transplantation (*e.g.*, donor lymphocyte infusions). Such therapies may involve the generation or enhancement of an immune response to a tumor-associated antigen (TAA). However, to date relatively few TAAs are known and the generation of an immune response against such antigens has, with rare exception, not been shown to be therapeutically beneficial.

Accordingly, there is a need in the art for improved methods for leukemia and cancer prevention and therapy. The present invention fulfills these needs and further provides other related advantages.

BRIEF SUMMARY OF THE INVENTION

Briefly stated, this invention provides compositions and methods for the diagnosis and therapy of diseases such as leukemia and cancer. In one aspect, the present invention provides polypeptides comprising an immunogenic portion of a native WT1, or a variant thereof that differs in one or more substitutions, deletions, additions and/or insertions such that the ability of the variant to react with antigen-specific antisera and/or T-cell lines or clones is not substantially diminished. Within certain embodiments of the present invention, the polypeptide comprises at least an immunogenic portion of WT1 wherein the immunogenic portion is contained within amino acids 2-281 of WT1. Within certain embodiments, the polypeptide comprises no more than 16 consecutive amino acid residues of a native WT1 polypeptide. Within other embodiments, the polypeptide comprises an immunogenic portion of amino acid residues 1 - 174 of a native WT1 polypeptide or a variant thereof, wherein the polypeptide comprises no more than 16 consecutive amino acid residues present within amino acids 175 to 449 of the native WT1 polypeptide. The immunogenic portion preferably binds to an MHC class I and/or class II molecule. Within certain embodiments, the polypeptide comprises a sequence selected from the group consisting of (a) sequences recited in any one or more of Tables II - XLVI, (b) variants of the foregoing sequences that differ in one or more substitutions, deletions, additions and/or insertions such that the ability of

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the variant to react with antigen-specific antisera and/or T-cell lines or clones is not substantially diminished and (c) mimetics of the polypeptides recited above, such that the ability of the mimetic to react with antigen-specific antisera and/or T cell lines or clones is not substantially diminished.

Within other embodiments, the polypeptide comprises a sequence selected from the group consisting of (a) ALLPAVPSL (SEQ ID NO:34), GATLKGVAA (SEQ ID NO:88), CMTWNQMNL (SEQ ID NOs: 49 and 258), SCLESQPTI (SEQ ID NOs: 199 and 296), SCLESQPAI (SEQ ID NO:198), NLYQMTSQL (SEQ ID NOs: 147 and 284), ALLPAVSSL (SEQ ID NOs: 35 and 255), RMFPNAPYL (SEQ ID NOs: 185 and 293), VLDFAPPGA (SEQ ID NO:241), VLDFAPPGAS (SEQ ID NO:411), SEQ ID NOs: 414-450, ALLPAVPSL (SEQ ID NO:451) (b) variants of the foregoing sequences that differ in one or more substitutions, deletions, additions and/or insertions such that the ability of the variant to react with antigen-specific antisera and/or T-cell lines or clones is not substantially diminished and (c) mimetics of the polypeptides recited above, such that the ability of the mimetic to react with antigen-specific antisera and/or T cell lines or clones is not substantially diminished. Mimetics may comprises amino acids in combination with one or more amino acid mimetics or may be entirely nonpeptide mimetics.

Within further aspects, the present invention provides polypeptides comprising a variant of an immunogenic portion of a WT1 protein, wherein the variant differs from the immunogenic portion due to substitutions at between 1 and 3 amino acid positions within the immunogenic portion such that the ability of the variant to react with antigen-specific antisera and/or T-cell lines or clones is not substantially reduced or is enhanced relative to a native WT1 protein.

The present invention further provides WT1 polynucleotides that encode a WT1 polypeptide as described above.

Within other aspects, the present invention provides pharmaceutical compositions and vaccines. Pharmaceutical compositions may comprise a polypeptide or mimetic as described above and/or one or more of (i)

a WT1 polynucleotide; (ii) an antibody or antigen-binding fragment thereof that specifically binds to a WT1 polypeptide; (iii) a T cell that specifically reacts with a WT1 polypeptide or (iv) an antigen-presenting cell that expresses a WT1 polypeptide, in combination with a pharmaceutically acceptable carrier or excipient. Vaccines comprise a polypeptide as described above and/or one or more of (i) a WT1 polynucleotide, (ii) an antigen-presenting cell that expresses a WT1 polypeptide or (iii) an anti-idiotypic antibody, and a non-specific immune response enhancer. Within certain embodiments, less than 23 consecutive amino acid residues, preferably less than 17 amino acid residues, of a native WT1 polypeptide are present within a WT1 polypeptide employed within such pharmaceutical compositions and vaccines. The immune response enhancer may be an adjuvant. Preferably, an immune response enhancer enhances a T cell response.

The present invention further provides methods for enhancing or inducing an immune response in a patient, comprising administering to a patient a pharmaceutical composition or vaccine as described above. In certain embodiments, the patient is a human.

The present invention further provides methods for inhibiting the development of a malignant disease in a patient, comprising administering to a patient a pharmaceutical composition or vaccine as described above. Malignant diseases include, but are not limited to leukemias (*e.g.*, acute myeloid, acute lymphocytic and chronic myeloid) and cancers (*e.g.*, breast, lung, thyroid or gastrointestinal cancer or a melanoma). The patient may, but need not, be afflicted with the malignant disease, and the administration of the pharmaceutical composition or vaccine may inhibit the onset of such a disease, or may inhibit progression and/or metastasis of an existing disease.

The present invention further provides, within other aspects, methods for removing cells expressing WT1 from bone marrow and/or peripheral blood or fractions thereof, comprising contacting bone marrow, peripheral blood or a fraction of bone marrow or peripheral blood with T cells that specifically react with a WT1 polypeptide, wherein the step of contacting is

performed under conditions and for a time sufficient to permit the removal of WT1 positive cells to less than 10%, preferably less than 5% and more preferably less than 1%, of the number of myeloid or lymphatic cells in the bone marrow, peripheral blood or fraction. Bone marrow, peripheral blood and fractions may be obtained from a patient afflicted with a disease associated with WT1 expression, or may be obtained from a human or non-human mammal not afflicted with such a disease.

Within related aspects, the present invention provides methods for inhibiting the development of a malignant disease in a patient, comprising administering to a patient bone marrow, peripheral blood or a fraction of bone marrow or peripheral blood prepared as described above. Such bone marrow, peripheral blood or fractions may be autologous, or may be derived from a related or unrelated human or non-human animal (*e.g.*, syngeneic or allogeneic).

In other aspects, the present invention provides methods for stimulating (or priming) and/or expanding T cells, comprising contacting T cells with a WT1 polypeptide under conditions and for a time sufficient to permit the stimulation and/or expansion of T cells. Such T cells may be autologous, allogeneic, syngeneic or unrelated WT1-specific T cells, and may be stimulated *in vitro* or *in vivo*. Expanded T cells may, within certain embodiments, be present within bone marrow, peripheral blood or a fraction of bone marrow or peripheral blood, and may (but need not) be clonal. Within certain embodiments, T cells may be present in a mammal during stimulation and/or expansion. WT1-specific T cells may be used, for example, within donor lymphocyte infusions.

Within related aspects, methods are provided for inhibiting the development of a malignant disease in a patient, comprising administering to a patient T cells prepared as described above. Such T cells may, within certain embodiments, be autologous, syngeneic or allogeneic.

The present invention further provides, within other aspects, methods for monitoring the effectiveness of an immunization or therapy for a

malignant disease associated with WT1 expression in a patient. Such methods are based on monitoring antibody, CD4+ T cell and/or CD8+ T cell responses in the patient. Within certain such aspects, a method may comprise the steps of: (a) incubating a first biological sample with one or more of: (i) a WT1 polypeptide; (ii) a polynucleotide encoding a WT1 polypeptide; or (iii) an antigen presenting cell that expresses a WT1 polypeptide, wherein the first biological sample is obtained from a patient prior to a therapy or immunization, and wherein the incubation is performed under conditions and for a time sufficient to allow immunocomplexes to form; (b) detecting immunocomplexes formed between the WT1 polypeptide and antibodies in the biological sample that specifically bind to the WT1 polypeptide; (c) repeating steps (a) and (b) using a second biological sample obtained from the same patient following therapy or immunization; and (d) comparing the number of immunocomplexes detected in the first and second biological samples, and therefrom monitoring the effectiveness of the therapy or immunization in the patient.

Within certain embodiments of the above methods, the step of detecting comprises (a) incubating the immunocomplexes with a detection reagent that is capable of binding to the immunocomplexes, wherein the detection reagent comprises a reporter group, (b) removing unbound detection reagent, and (c) detecting the presence or absence of the reporter group. The detection reagent may comprise, for example, a second antibody, or antigen-binding fragment thereof, capable of binding to the antibodies that specifically bind to the WT1 polypeptide or a molecule such as Protein A. Within other embodiments, a reporter group is bound to the WT1 polypeptide, and the step of detecting comprises removing unbound WT1 polypeptide and subsequently detecting the presence or absence of the reporter group.

Within further aspects, methods for monitoring the effectiveness of an immunization or therapy for a malignant disease associated with WT1 expression in a patient may comprise the steps of: (a) incubating a first biological sample with one or more of: (i) a WT1 polypeptide; (ii) a polynucleotide encoding a WT1 polypeptide; or (iii) an antigen presenting cell

that expresses a WT1 polypeptide, wherein the biological sample comprises CD4+ and/or CD8+ T cells and is obtained from a patient prior to a therapy or immunization, and wherein the incubation is performed under conditions and for a time sufficient to allow specific activation, proliferation and/or lysis of T cells; (b) detecting an amount of activation, proliferation and/or lysis of the T cells; (c) repeating steps (a) and (b) using a second biological sample comprising CD4+ and/or CD8+ T cells, wherein the second biological sample is obtained from the same patient following therapy or immunization; and (d) comparing the amount of activation, proliferation and/or lysis of T cells in the first and second biological samples, and therefrom monitoring the effectiveness of the therapy or immunization in the patient.

The present invention further provides methods for inhibiting the development of a malignant disease associated with WT1 expression in a patient, comprising the steps of: (a) incubating $CD4^+$ and/or CD8+ T cells isolated from a patient with one or more of: (i) a WT1 polypeptide; (ii) a polynucleotide encoding a WT1 polypeptide; or (iii) an antigen presenting cell that expresses a WT1 polypeptide, such that the T cells proliferate; and (b) administering to the patient an effective amount of the proliferated T cells, and therefrom inhibiting the development of a malignant disease in the patient. Within certain embodiments, the step of incubating the T cells may be repeated one or more times.

Within other aspects, the present invention provides methods for inhibiting the development of a malignant disease associated with WT1 expression in a patient, comprising the steps of: (a) incubating $CD4^+$ and/or CD8+ T cells isolated from a patient with one or more of: (i) a WT1 polypeptide; (ii) a polynucleotide encoding a WT1 polypeptide; or (iii) an antigen presenting cell that expresses a WT1 polypeptide, such that the T cells proliferate; (b) cloning one or more cells that proliferated; and (c) administering to the patient an effective amount of the cloned T cells.

Within other aspects, methods are provided for determining the presence or absence of a malignant disease associated with WT1 expression

in a patient, comprising the steps of: (a) incubating CD4+ and/or CD8+ T cells isolated from a patient with one or more of: (i) a WT1 polypeptide; (ii) a polynucleotide encoding a WT1 polypeptide; or (iii) an antigen presenting cell that expresses a WT1 polypeptide; and (b) detecting the presence or absence of specific activation of the T cells, therefrom determining the presence or absence of a malignant disease associated with WT1 expression. Within certain embodiments, the step of detecting comprises detecting the presence or absence of proliferation of the T cells.

Within further aspects, the present invention provides methods for determining the presence or absence of a malignant disease associated with WT1 expression in a patient, comprising the steps of: (a) incubating a biological sample obtained from a patient with one or more of: (i) a WT1 polypeptide; (ii) a polynucleotide encoding a WT1 polypeptide; or (iii) an antigen presenting cell that expresses a WT1 polypeptide, wherein the incubation is performed under conditions and for a time sufficient to allow immunocomplexes to form; and (b) detecting immunocomplexes formed between the WT1 polypeptide and antibodies in the biological sample that specifically bind to the WT1 polypeptide; and therefrom determining the presence or absence of a malignant disease associated with WT1 expression.

These and other aspects of the present invention will become apparent upon reference to the following detailed description and attached drawings. All references disclosed herein are hereby incorporated by reference in their entirety as if each was incorporated individually.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts a comparison of the mouse (MO) and human (HU) WT1 protein sequences (SEQ ID NOS: 320 and 319 respectively).

Figure 2 is a Western blot illustrating the detection of WT1 specific antibodies in patients with hematological malignancy (AML). Lane 1 shows molecular weight markers; lane 2 shows a positive control (WT1 positive human leukemia cell line immunoprecipitated with a WT1 specific antibody);

lane 3 shows a negative control (WT1 positive cell line immunoprecipitated with mouse sera); and lane 4 shows a WT1 positive cell line immunoprecipitated with sera of a patient with AML. For lanes 2-4, the immunoprecipitate was separated by gel electrophoresis and probed with a WT1 specific antibody.

Figure 3 is a Western blot illustrating the detection of a WT1 specific antibody response in B6 mice immunized with TRAMP-C, a WT1 positive tumor cell line. Lanes 1, 3 and 5 show molecular weight markers, and lanes 2, 4 and 6 show a WT1 specific positive control (N180, Santa Cruz Biotechnology, polypeptide spanning 180 amino acids of the N-terminal region of the WT1 protein, migrating on the Western blot at 52 kD). The primary antibody used was WT180 in lane 2, sera of non-immunized B6 mice in lane 4 and sera of the immunized B6 mice in lane 6.

Figure 4 is a Western blot illustrating the detection of WT1 specific antibodies in mice immunized with representative WT1 peptides. Lanes 1, 3 and 5 show molecular weight markers and lanes 2, 4 and 6 show a WT1 specific positive control (N180, Santa Cruz Biotechnology, polypeptide spanning 180 amino acids of the N-terminal region of the WT1 protein, migrating on the Western blot at 52 kD). The primary antibody used was WT180 in lane 2, sera of non-immunized B6 mice in lane 4 and sera of the immunized B6 mice in lane 6.

Figures 5A to 5C are graphs illustrating the stimulation of proliferative T cell responses in mice immunized with representative WT1 peptides. Thymidine incorporation assays were performed using one T cell line and two different clones, as indicated, and results were expressed as cpm. Controls indicated on the x axis were no antigen (No Ag) and B6/media; antigens used were p6-22 human (p1), p117-139 (p2) or p244-262 human (p3).

Figure 6A and 6B are histograms illustrating the stimulation of proliferative T cell responses in mice immunized with representative WT1 peptides. Three weeks after the third immunization, spleen cells of mice that had been inoculated with Vaccine A or Vaccine B were cultured with medium alone (medium) or spleen cells and medium (B6/no antigen), B6 spleen cells

pulsed with the peptides p6-22 (p6), p117-139 (p117), p244-262 (p244) (Vaccine A; Figure 6A) or p287-301 (p287), p299-313 (p299), p421-435 (p421) (Vaccine B; Figure 6B) and spleen cells pulsed with an irrelevant control peptide (irrelevant peptide) at 25ug/ml and were assayed after 96hr for proliferation by (^{3}H) thymidine incorporation. Bars represent the stimulation index (SI), which is calculated as the mean of the experimental wells divided by the mean of the control (B6 spleen cells with no antigen).

Figures 7A-7D are histograms illustrating the generation of proliferative T-cell lines and clones specific for p117-139 and p6-22. Following *in vivo* immunization, the initial three *in vitro* stimulations (IVS) were carried out using all three peptides of Vaccine A or B, respectively. Subsequent IVS were carried out as single peptide stimulations using only the two relevant peptides p117-139 and p6-22. Clones were derived from both the p6-22 and p117-139 specific T cell lines, as indicated. T cells were cultured with medium alone (medium) or spleen cells and medium (B6/no antigen), B6 spleen cells pulsed with the peptides p6-22 (p6), p117-139 (p117) or an irrelevant control peptide (irrelevant peptide) at 25ug/ml and were assayed after 96hr for proliferation by (^{3}H) thymidine incorporation. Bars represent the stimulation index (SI), which is calculated as the mean of the experimental wells divided by the mean of the control (B6 spleen cells with no antigen).

Figures 8A and 8B present the results of TSITES Analysis of human WT1 (SEQ ID NO:319) for peptides that have the potential to elicit Th responses. Regions indicated by "A" are AMPHI midpoints of blocks, "R" indicates residues matching the Rothbard/Taylor motif, "D" indicates residues matching the IAd motif, and 'd' indicates residues matching the IEd motif.

Figures 9A and 9B are graphs illustrating the elicitation of WT1 peptide-specific CTL in mice immunized with WT1 peptides. Figure 9A illustrates the lysis of target cells by allogeneic cell lines and Figure 9B shows the lysis of peptide coated cell lines. In each case, the % lysis (as determined by standard chromium release assays) is shown at three indicated effector:target ratios. Results are provided for lymphoma cells (LSTRA and

E10), as well as E10 + p235-243 (E10+P235). E10 cells are also referred to herein as EL-4 cells.

Figures 10A-10D are graphs illustrating the elicitation of WT1 specific CTL, which kill WT1 positive tumor cell lines but do not kill WT1 negative cell lines, following vaccination of B6 mice with WT1 peptide P117. Figure 10A illustrates that T-cells of non-immunized B6 mice do not kill WT1 positive tumor cell lines. Figure 10B illustrates the lysis of the target cells by allogeneic cell lines. Figures 10C and 10D demonstrate the lysis of WT1 positive tumor cell lines, as compared to WT1 negative cell lines in two different experiments. In addition, Figures 10C and 10D show the lysis of peptide-coated cell lines (WT1 negative cell line E10 coated with the relevant WT1 peptide P117) In each case, the % lysis (as determined by standard chromium release assays) is shown at three indicated effector:target ratios. Results are provided for lymphoma cells (E10), prostate cancer cells (TRAMP-C), a transformed fibroblast cell line (BLK-SV40), as well as E10+p117.

Figures 11A and 11B are histograms illustrating the ability of representative peptide P117-139 specific CTL to lyse WT1 positive tumor cells. Three weeks after the third immunization, spleen cells of mice that had been inoculated with the peptides p235-243 or p117-139 were stimulated *in vitro* with the relevant peptide and tested for ability to lyse targets incubated with WT1 peptides as well as WT1 positive and negative tumor cells. The bars represent the mean % specific lysis in chromium release assays performed in triplicate with an E:T ratio of 25:1. Figure 11A shows the cytotoxic activity of the p235-243 specific T cell line against the WT1 negative cell line EL-4 (EL-4, WT1 negative); EL-4 pulsed with the relevant (used for immunization as well as for restimulation) peptide p235-243 (EL-4+p235); EL-4 pulsed with the irrelevant peptides p117-139 (EL-4+p117), p126-134 (EL-4+p126) or p130-138 (EL-4+p130) and the WT1 positive tumor cells BLK-SV40 (BLK-SV40, WT1 positive) and TRAMP-C (TRAMP-C, WT1 positive), as indicated. Figure 11B shows cytotoxic activity of the p117-139 specific T cell line against EL-4; EL-4 pulsed with the relevant peptide P117-139 (EL-4+p117) and EL-4 pulsed with

the irrelevant peptides p123-131 (EL-4+p123), or p128-136 (EL-4+p128); BLK-SV40 and TRAMP-C, as indicated.

Figures 12A and 12B are histograms illustrating the specificity of lysis of WT1 positive tumor cells, as demonstrated by cold target inhibition. The bars represent the mean % specific lysis in chromium release assays performed in triplicate with an E:T ratio of 25:1. Figure 12A shows the cytotoxic activity of the p117-139 specific T cell line against the WT1 negative cell line EL-4 (EL-4, WT1 negative); the WT1 positive tumor cell line TRAMP-C (TRAMP-C, WT1 positive); TRAMP-C cells incubated with a ten-fold excess (compared to the hot target) of EL-4 cells pulsed with the relevant peptide p117-139 (TRAMP-C + p117 cold target) without ^{51}Cr labeling and TRAMP-C cells incubated with EL-4 pulsed with an irrelevant peptide without ^{51}Cr labeling (TRAMP-C + irrelevant cold target), as indicated. Figure 12B shows the cytotoxic activity of the p117-139 specific T cell line against the WT1 negative cell line EL-4 (EL-4, WT1 negative); the WT1 positive tumor cell line BLK-SV40 (BLK-SV40, WT1 positive); BLK-SV40 cells incubated with the relevant cold target (BLK-SV40 + p117 cold target) and BLK-SV40 cells incubated with the irrelevant cold target (BLK-SV40 + irrelevant cold target), as indicated.

Figures 13A-13C are histograms depicting an evaluation of the 9mer CTL epitope within p117-139. The p117-139 tumor specific CTL line was tested against peptides within aa117-139 containing or lacking an appropriate H-2^b class I binding motif and following restimulation with p126-134 or p130-138. The bars represent the mean % specific lysis in chromium release assays performed in triplicate with an E:T ratio of 25:1. Figure 13A shows the cytotoxic activity of the p117-139 specific T cell line against the WT1 negative cell line EL-4 (EL-4, WT1 negative) and EL-4 cells pulsed with the peptides p117-139 (EL-4 + p117), p119-127 (EL-4 + p119), p120-128 (EL-4 + p120), p123-131 (EL-4 + p123), p126-134 (EL-4 + p126), p128-136 (EL-4 + p128), and p130-138 (EL-4 + p130). Figure 13B shows the cytotoxic activity of the CTL line after restimulation with p126-134 against the WT1 negative cell line EL-4, EL-4 cells pulsed with p117-139 (EL-4 + p117), p126-134 (EL-4 + p126) and the WT1

positive tumor cell line TRAMP-C. Figure 13C shows the cytotoxic activity of the CTL line after restimulation with p130-138 against EL-4, EL-4 cells pulsed with p117-139 (EL-4 + p117), p130-138 (EL-4 + p130) and the WT1 positive tumor cell line TRAMP-C.

Figure 14 depicts serum antibody reactivity to WT1 in 63 patients with AML. Reactivity of serum antibody to WT1/N-terminus protein was evaluated by ELISA in patients with AML. The first and second lanes represent the positive and negative controls, respectively. The first and second lanes represent the ositive and negative controls, respectively. Commercially obtained WT1 specific antibody WT180 was used for the positive control. The next 63 lanes represent results using sera from each individual patient. The OD values depicted were from ELISA using a 1:500 serum dilution. The figure includes cumulative data from 3 separate experiments.

Figure 15 depicts serum antibody reactivity to WT1 proteins and control proteins in 2 patients with AML. Reactivity of serum antibody to WT1/full-length, WT1N-terminus, TRX and Ra12 proteins was evaluated by ELISA in 2 patients with AML. The OD values depicted were from ELISA using a 1:500 serum dilution. AML-1 and AML-2 denote serum from 2 of the individual patients in Figure 1 with demonstrated antibody reactivity to WT1/full-length. The WT1 full-length protein was expressed as a fusion protein with Ra12. The WT1/N-terminus protein was expressed as a fusion protein with TRX. The control Ra12 and TRX proteins were purified in a similar manner. The results confirm that the serum antibody reactivity against the WT1 fusion proteins is directed against the WT1 portions of the protein.

Figure 16 depicts serum antibody reactivity to WT1 in 81 patients with CML. Reactivity of serum antibody to WT1/full-length protein was evaluated by ELISA in patients with AML. The first and second lanes represent the positive and negative controls, respectively. Commercially obtained WT1 specific antibody WT180 was used for the positive control. The next 81 lanes represent results using sera from each individual patient. The OD values

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depicted were from ELISA using a 1:500 serum dilution. The figure includes cumulative data from 3 separate experiments.

Figure 17 depicts serum antibody reactivity to WT1 proteins and control proteins in 2 patients with CML. Reactivity of serum antibody to WT1/full-length, WT1/N-terminus, TRX and Ra12 proteins was evaluated by ELISA in 2 patients with CML. The OD values depicted were from ELISA using a 1:500 serum dilution. CML-1 and CML-2 denote serum from 2 of the individual patients in Figure 3 with demonstrated antibody reactivity to WT1/full-length. The WT1/full-length protein was expressed as a fusion protein with Ra12. The WT1/N-terminus protein was expressed as a fusion protein with TRX. The control Ra12 and TRX proteins were purified in a similar manner. The results confirm that the serum antibody reactivity against the WT1 fusion proteins is directed against the WT1 portions of the protein.

Figure 18 provides the characteristics of the recombinant WT1 proteins used for serological analysis.

Figure 19A-19E is a bar graph depicting the antibody responses in mice elicited by vaccination with different doses of WT1 protein.

Figure 20 is a bar graph of the proliferative T-cell responses in mice immunized with WT1 protein.

Figure 21 is a photograph of human DC, examined by fluorescent microscopy, expressing WT1 following adeno WT1 and Vaccinia WT1 infection.

Figure 22 is a photograph that demonstrates that WT1 expression in human DC is reproducible following adeno WT1 infection and is not induced by a control Adeno infection.

Figure 23 is a graph of an IFN-gamma ELISPOT assay showing that WT1 whole gene *in vitro* priming elicits WT1 specific T-cell responses.

Figure 24 shows amino acids 2-281 (SEQ ID NO:461) of the WT1 protein and the cDNA encoding these amino acid residues (SEQ ID NO:460). This truncated WT1 protein is referred to as WT1-F.

DETAILED DESCRIPTION OF THE INVENTION

U.S. patents, U.S. patent application publications, U.S. patent applications, foreign patents, foreign patent applications and non-patent publications referred to in this specification and/or listed in the Application Data Sheet, are incorporated herein by reference, in their entirety.

As noted above, the present invention is generally directed to compositions and methods for the immunotherapy and diagnosis of malignant diseases. The compositions described herein may include WT1 polypeptides, WT1 polynucleotides, antigen-presenting cells (APC, *e.g.,* dendritic cells) that express a WT1 polypeptide, agents such as antibodies that bind to a WT1 polypeptide and/or immune system cells (*e.g.,* T cells) specific for WT1. WT1 Polypeptides of the present invention generally comprise at least a portion of a Wilms Tumor gene product (WT1) or a variant thereof. Nucleic acid sequences of the subject invention generally comprise a DNA or RNA sequence that encodes all or a portion of such a polypeptide, or that is complementary to such a sequence. Antibodies are generally immune system proteins, or antigen-binding fragments thereof, that are capable of binding to a portion of a WT1 polypeptide. T cells that may be employed within such compositions are generally T cells (*e.g.,* $CD4^+$ and/or $CD8^+$) that are specific for a WT1 polypeptide. Certain methods described herein further employ antigen-presenting cells that express a WT1 polypeptide as provided herein.

The present invention is based on the discovery that an immune response raised against a Wilms Tumor (WT) gene product (*e.g.,* WT1) can provide prophylactic and/or therapeutic benefit for patients afflicted with malignant diseases characterized by increased WT1 gene expression. Such diseases include, but are not limited to, leukemias (*e.g.,* acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphocytic leukemia (ALL) and childhood ALL), as well as many cancers such as lung, breast, thyroid and gastrointestinal cancers and melanomas. The WT1 gene was originally identified and isolated on the basis of a cytogenetic deletion at chromosome 11p13 in patients with Wilms' tumor (*see* Call et al., U.S. Patent

No. 5,350,840). The gene consists of 10 exons and encodes a zinc finger transcription factor, and sequences of mouse and human WT1 proteins are provided in Figure 1 and SEQ ID NOs: 319 and 320.

WT1 Polypeptides

Within the context of the present invention, a WT1 polypeptide is a polypeptide that comprises at least an immunogenic portion of a native WT1 (*i.e.*, a WT1 protein expressed by an organism that is not genetically modified), or a variant thereof, as described herein. A WT1 polypeptide may be of any length, provided that it comprises at least an immunogenic portion of a native protein or a variant thereof. In other words, a WT1 polypeptide may be an oligopeptide (*i.e.*, consisting of a relatively small number of amino acid residues, such as 8-10 residues, joined by peptide bonds), a full length WT1 protein (*e.g.*, present within a human or non-human animal, such as a mouse) or a polypeptide of intermediate size. Within certain embodiments, the use of WT1 polypeptides that contain a small number of consecutive amino acid residues of a native WT1 polypeptide is preferred. Such polypeptides are preferred for certain uses in which the generation of a T cell response is desired. For example, such a WT1 polypeptide may contain less than 23, preferably no more than 18, and more preferably no more than 15 consecutive amino acid residues, of a native WT1 polypeptide. Polypeptides comprising nine consecutive amino acid residues of a native WT1 polypeptide are generally suitable for such purposes. Additional sequences derived from the native protein and/or heterologous sequences may be present within any WT1 polypeptide, and such sequences may (but need not) possess further immunogenic or antigenic properties. Polypeptides as provided herein may further be associated (covalently or noncovalently) with other polypeptide or non-polypeptide compounds.

An "immunogenic portion," as used herein is a portion of a polypeptide that is recognized (*i.e.*, specifically bound) by a B-cell and/or T-cell surface antigen receptor. Certain preferred immunogenic portions bind to an

MHC class I or class II molecule. As used herein, an immunogenic portion is said to "bind to" an MHC class I or class II molecule if such binding is detectable using any assay known in the art. For example, the ability of a polypeptide to bind to MHC class I may be evaluated indirectly by monitoring the ability to promote incorporation of ^{125}I labeled β2-microglobulin (β2m) into MHC class I/β2m/peptide heterotrimeric complexes (*see* Parker et al., *J. Immunol. 152*:163, 1994). Alternatively, functional peptide competition assays that are known in the art may be employed. Certain immunogenic portions have one or more of the sequences recited within one or more of Tables II - XIV. Representative immunogenic portions include, but are not limited to, RDLNALLPAVPSLGGGG (human WT1 residues 6-22; SEQ ID NO:1), PSQASSGQARMFPNAPYLPSCLE (human and mouse WT1 residues 117-139; SEQ ID NOs: 2 and 3 respectively), GATLKGVAAGSSSSVKWTE (human WT1 residues 244-262; SEQ ID NO:4), GATLKGVAA (human WT1 residues 244-252; SEQ ID NO:88), CMTWNQMNL (human and mouse WT1 residues 235-243; SEQ ID NOs: 49 and 258 respectively), SCLESQPTI (mouse WT1 residues 136-144; SEQ ID NO:296), SCLESQPAI (human WT1 residues 136-144; SEQ ID NO:198), NLYQMTSQL (human and mouse WT1 residues 225-233; SEQ ID NOs: 147 and 284 respectively); ALLPAVSSL (mouse WT1 residues 10-18; SEQ ID NO:255); RMFPNAPYL (human and mouse WT1 residues 126-134; SEQ ID NOs: 185 and 293 respectively), VLDFAPPGA (human WT1 residues 37-45; SEQ ID NO:241), or VLDFAPPGAS (human WT1 residues 37-46; SEQ ID NO:411). Further immunogenic portions are provided in SEQ ID NOs:414-451. Further immunogenic portions are provided herein, and others may generally be identified using well known techniques, such as those summarized in Paul, *Fundamental Immunology*, 3rd ed., 243-247 (Raven Press, 1993) and references cited therein. Representative techniques for identifying immunogenic portions include screening polypeptides for the ability to react with antigen-specific antisera and/or T-cell lines or clones. An immunogenic portion of a native WT1 polypeptide is a portion that reacts with such antisera and/or T-cells at a level that is not substantially less than the

reactivity of the full length WT1 (*e.g.*, in an ELISA and/or T-cell reactivity assay). In other words, an immunogenic portion may react within such assays at a level that is similar to or greater than the reactivity of the full length polypeptide. Such screens may generally be performed using methods well known to those of ordinary skill in the art, such as those described in Harlow and Lane, *Antibodies: A Laboratory Manual,* Cold Spring Harbor Laboratory, 1988.

Alternatively, immunogenic portions may be identified using computer analysis, such as the Tsites program (*see* Rothbard and Taylor, *EMBO J.* 7:93-100, 1988; Deavin et al., *Mol. Immunol.* 33:145-155, 1996), which searches for peptide motifs that have the potential to elicit Th responses. CTL peptides with motifs appropriate for binding to murine and human class I or class II MHC may be identified according to BIMAS (Parker et al., *J. Immunol.* 152:163, 1994) and other HLA peptide binding prediction analyses. Alternatively, immungenic portions that bind to a particular MHC molecule can be identified by using defined peptide binding motifs such as those described in Rammensee et al., Immunogenetics 41:178-228, 1995. To confirm peptide binding to murine and human class I or class II MHC molecules, peptide binding assays known in the art may be used. To confirm immunogenicity, a peptide may be tested using an HLA A2 or other transgenic mouse model and/or an *in vitro* stimulation assay using dendritic cells, fibroblasts or peripheral blood cells.

As noted above, a composition may comprise a variant of a native WT1 protein. A polypeptide "variant," as used herein, is a polypeptide that differs from a native polypeptide in one or more substitutions, deletions, additions and/or insertions, such that the immunogenicity of the polypeptide is retained (*i.e.*, the ability of the variant to react with antigen-specific antisera and/or T-cell lines or clones is not substantially diminished relative to the native polypeptide). In other words, the ability of a variant to react with antigen-specific antisera and/or T-cell lines or clones may be enhanced or unchanged, relative to the native polypeptide, or may be diminished by less than 50%, and

preferably less than 20%, relative to the native polypeptide. In one embodiment the the ability of a variant to react with antigen-specific antisera and/or T-cell lines or clones may be diminished by less than 10%, 5%, 4%, 3%, 2%, 1%, or 0.5 %, relative to the native polypeptide. Such variants may generally be identified by modifying one of the above polypeptide sequences and evaluating the reactivity of the modified polypeptide with antisera and/or T-cells as described herein. In one embodiment of the present invention, a variant may be identified by evaluating its ability to bind to a human or a muring HLA molecule. In one preferred embodiment, a variant polypeptide has a modification such that the ability of the variant polypeptide to bind to a class I or class II MHC molecule, for example HLA-A2, HLA-A24, HLA-A1, HLA-A3, HLA-A68, HLA-A11, HLA-A31, HLA-A33, HLA-B14, HLA-B40, HLA-B60, HLA-B62, HLA-B7, HLA-B8, HLA-B27, HLA-B3501, HLA-B37, HLA-B38, HLA-B39, HLA-B44, HLA-B51, HLA-B52, HLA-B58, HLA-CW03, HLA-CW04, HLA-CW06, or HLA-CW07, is increased relative to that of a wild type (unmodified) WT1 polypeptide. In a further embodiment, the N-terminal portion of WT1 from amino acids 1-281 is modified such that the ability of any number of polypeptides therein that can bind to HLA-A2, HLA-A24, HLA-A1, HLA-A3, HLA-A68, HLA-A11, HLA-A31, HLA-A33, HLA-B14, HLA-B40, HLA-B60, HLA-B62, HLA-B7, HLA-B8, HLA-B27, HLA-B3501, HLA-B37, HLA-B38, HLA-B39, HLA-B44, HLA-B51, HLA-B52, HLA-B58, HLA-CW03, HLA-CW04, HLA-CW06, or HLA-CW07 is increased relative to that of the wild type(unmodified) WT1 polypeptide sequence. In a further embodiment, a polypeptide comprising at least one immunogenic portion (epitope) is modified such that its ability to bind to an MHC molecule, such as HLA-A2, HLA-A24, HLA-A1, HLA-A3, HLA-A68, HLA-A11, HLA-A31, HLA-A33, HLA-B14, HLA-B40, HLA-B60, HLA-B62, HLA-B7, HLA-B8, HLA-B27, HLA-B3501, HLA-B37, HLA-B38, HLA-B39, HLA-B44, HLA-B51, HLA-B52, HLA-B58, HLA-CW03, HLA-CW04, HLA-CW06, or HLA-CW07 is increased. Example 30 describes illustrative variants that can be generated. The skilled artisan would readily recognize that these are illustrative variants and that other variants can be generated in a similar manner for

peptides that bind to HLA molecules other than HLA-A2. In a further embodiment, the ability of the variant polypeptide to bind to a HLA molecule is increased by at least 2 fold, preferably at least 3 fold, 4 fold, or 5 fold relative to that of a native WT1 polypeptide. It has been found, within the context of the present invention, that a relatively small number of substitutions (*e.g.*, 1 to 3) within an immunogenic portion of a WT1 polypeptide may serve to enhance the ability of the polypeptide to elicit an immune response. Suitable substitutions may generally be identified by using computer programs, as described above, and the effect confirmed based on the reactivity of the modified polypeptide with antisera and/or T-cells as described herein. Accordingly, within certain preferred embodiments, a WT1 polypeptide comprises a variant in which 1 to 3 amino acid resides within an immunogenic portion are substituted such that the ability to react with antigen-specific antisera and/or T-cell lines or clones is statistically greater than that for the unmodified polypeptide. Such substitutions are preferably located within an MHC binding site of the polypeptide, which may be identified as described above. Preferred substitutions allow increased binding to MHC class I or class II molecules.

Certain variants contain conservative substitutions. A "conservative substitution" is one in which an amino acid is substituted for another amino acid that has similar properties, such that one skilled in the art of peptide chemistry would expect the secondary structure and hydropathic nature of the polypeptide to be substantially unchanged. Amino acid substitutions may generally be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity and/or the amphipathic nature of the residues. For example, negatively charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine and arginine; and amino acids with uncharged polar head groups having similar hydrophilicity values include leucine, isoleucine and valine; glycine and alanine; asparagine and glutamine; and serine, threonine, phenylalanine and tyrosine. Other groups of amino acids that may represent conservative changes include: (1) ala, pro, gly, glu, asp, gln, asn, ser, thr; (2) cys, ser, tyr, thr; (3) val, ile, leu, met, ala,

phe; (4) lys, arg, his; and (5) phe, tyr, trp, his. A variant may also, or alternatively, contain nonconservative changes. Variants may also (or alternatively) be modified by, for example, the deletion or addition of amino acids that have minimal influence on the immunogenicity, secondary structure and hydropathic nature of the polypeptide.

In a preferred embodiment, a variant polypeptide of the WT1 N-terminus (amino acids 1-249) is constructed, wherein the variant polypeptide is capable of binding to an antibody that recognizes full-length WT1 and/or WT1 N-terminus polypeptide. A non-limiting example of an antibody is anti WT1 antibody WT180 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

As noted above, WT1 polypeptides may be conjugated to a signal (or leader) sequence at the N-terminal end of the protein which co-translationally or post-translationally directs transfer of the protein. A polypeptide may also, or alternatively, be conjugated to a linker or other sequence for ease of synthesis, purification or identification of the polypeptide (*e.g.*, poly-His), or to enhance binding of the polypeptide to a solid support. For example, a polypeptide may be conjugated to an immunoglobulin Fc region.

WT1 polypeptides may be prepared using any of a variety of well known techniques. Recombinant polypeptides encoded by a WT1 polynucleotide as described herein may be readily prepared from the polynucleotide. In general, any of a variety of expression vectors known to those of ordinary skill in the art may be employed to express recombinant WT1 polypeptides. Expression may be achieved in any appropriate host cell that has been transformed or transfected with an expression vector containing a DNA molecule that encodes a recombinant polypeptide. Suitable host cells include prokaryotes, yeast and higher eukaryotic cells. Preferably, the host cells employed are *E. coli*, yeast or a mammalian cell line such as COS or CHO. Supernatants from suitable host/vector systems which secrete recombinant protein or polypeptide into culture media may be first concentrated using a commercially available filter. The concentrate may then be applied to a suitable purification matrix such as an affinity matrix or an ion exchange resin.

Finally, one or more reverse phase HPLC steps can be employed to further purify a recombinant polypeptide. Such techniques may be used to prepare native polypeptides or variants thereof. For example, polynucleotides that encode a variant of a native polypeptide may generally be prepared using standard mutagenesis techniques, such as oligonucleotide-directed site-specific mutagenesis, and sections of the DNA sequence may be removed to permit preparation of truncated polypeptides.

Certain portions and other variants may also be generated by synthetic means, using techniques well known to those of ordinary skill in the art. For example, polypeptides having fewer than about 500 amino acids, preferably fewer than about 100 amino acids, and more preferably fewer than about 50 amino acids, may be synthesized. Polypeptides may be synthesized using any of the commercially available solid-phase techniques, such as the Merrifield solid-phase synthesis method, where amino acids are sequentially added to a growing amino acid chain. *See* Merrifield, *J. Am. Chem. Soc.* *85*:2149-2146, 1963. Equipment for automated synthesis of polypeptides is commercially available from suppliers such as Applied BioSystems, Inc. (Foster City, CA), and may be operated according to the manufacturer's instructions.

In general, polypeptides and polynucleotides as described herein are isolated. An "isolated" polypeptide or polynucleotide is one that is removed from its original environment. For example, a naturally-occurring protein is isolated if it is separated from some or all of the coexisting materials in the natural system. Preferably, such polypeptides are at least about 90% pure, more preferably at least about 95% pure and most preferably at least about 99% pure. A polynucleotide is considered to be isolated if, for example, it is cloned into a vector that is not a part of the natural environment.

Within further aspects, the present invention provides mimetics of WT1 polypeptides. Such mimetics may comprise amino acids linked to one or more amino acid mimetics (*i.e.*, one or more amino acids within the WT1 protein may be replaced by an amino acid mimetic) or may be entirely nonpeptide mimetics. An amino acid mimetic is a compound that is

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conformationally similar to an amino acid such that it can be substituted for an amino acid within a WT1 polypeptide without substantially diminishing the ability to react with antigen-specific antisera and/or T cell lines or clones. A nonpeptide mimetic is a compound that does not contain amino acids, and that has an overall conformation that is similar to a WT1 polypeptide such that the ability of the mimetic to react with WT1-specific antisera and/or T cell lines or clones is not substantially diminished relative to the ability of a WT1 polypeptide. Such mimetics may be designed based on standard techniques (*e.g.*, nuclear magnetic resonance and computational techniques) that evaluate the three dimensional structure of a peptide sequence. Mimetics may be designed where one or more of the side chain functionalities of the WT1 polypeptide are replaced by groups that do not necessarily have the same size or volume, but have similar chemical and/or physical properties which produce similar biological responses. It should be understood that, within embodiments described herein, a mimetic may be substituted for a WT1 polypeptide.

Within other illustrative embodiments, a polypeptide may be a fusion polypeptide that comprises multiple polypeptides as described herein, or that comprises at least one polypeptide as described herein and an unrelated sequence, such as a known tumor protein. A fusion partner may, for example, assist in providing T helper epitopes (an immunological fusion partner), preferably T helper epitopes recognized by humans, or may assist in expressing the protein (an expression enhancer) at higher yields than the native recombinant protein. Certain preferred fusion partners are both immunological and expression enhancing fusion partners. Other fusion partners may be selected so as to increase the solubility of the polypeptide or to enable the polypeptide to be targeted to desired intracellular compartments. Still further fusion partners include affinity tags, which facilitate purification of the polypeptide.

Fusion polypeptides may generally be prepared using standard techniques, including chemical conjugation. Preferably, a fusion polypeptide is expressed as a recombinant polypeptide, allowing the production of increased

levels, relative to a non-fused polypeptide, in an expression system. Briefly, DNA sequences encoding the polypeptide components may be assembled separately, and ligated into an appropriate expression vector. The 3' end of the DNA sequence encoding one polypeptide component is ligated, with or without a peptide linker, to the 5' end of a DNA sequence encoding the second polypeptide component so that the reading frames of the sequences are in phase. This permits translation into a single fusion polypeptide that retains the biological activity of both component polypeptides.

A peptide linker sequence may be employed to separate the first and second polypeptide components by a distance sufficient to ensure that each polypeptide folds into its secondary and tertiary structures. Such a peptide linker sequence is incorporated into the fusion polypeptide using standard techniques well known in the art. Suitable peptide linker sequences may be chosen based on the following factors: (1) their ability to adopt a flexible extended conformation; (2) their inability to adopt a secondary structure that could interact with functional epitopes on the first and second polypeptides; and (3) the lack of hydrophobic or charged residues that might react with the polypeptide functional epitopes. Preferred peptide linker sequences contain Gly, Asn and Ser residues. Other near neutral amino acids, such as Thr and Ala may also be used in the linker sequence. Amino acid sequences which may be usefully employed as linkers include those disclosed in Maratea et al., *Gene 40*:39-46, 1985; Murphy et al., *Proc. Natl. Acad. Sci. USA 83*:8258-8262, 1986; U.S. Patent No. 4,935,233 and U.S. Patent No. 4,751,180. The linker sequence may generally be from 1 to about 50 amino acids in length. Linker sequences are not required when the first and second polypeptides have non-essential N-terminal amino acid regions that can be used to separate the functional domains and prevent steric interference.

The ligated DNA sequences are operably linked to suitable transcriptional or translational regulatory elements. The regulatory elements responsible for expression of DNA are located only 5' to the DNA sequence encoding the first polypeptides. Similarly, stop codons required to end

translation and transcription termination signals are only present 3' to the DNA sequence encoding the second polypeptide.

The fusion polypeptide can comprise a polypeptide as described herein together with an unrelated immunogenic protein, such as an immunogenic protein capable of eliciting a recall response. Examples of such proteins include tetanus, tuberculosis and hepatitis proteins (*see*, for example, Stoute et al. *New Engl. J. Med.*, *336*:86-91, 1997).

In one preferred embodiment, the immunological fusion partner is derived from a Mycobacterium sp., such as a Mycobacterium tuberculosis-derived Ra12 fragment. Ra12 compositions and methods for their use in enhancing the expression and/or immunogenicity of heterologous polynucleotide/polypeptide sequences is described in U.S. Patent Application 60/158,585, the disclosure of which is incorporated herein by reference in its entirety. Briefly, Ra12 refers to a polynucleotide region that is a subsequence of a *Mycobacterium tuberculosis* MTB32A nucleic acid. MTB32A is a serine protease of 32 KD molecular weight encoded by a gene in virulent and avirulent strains of *M. tuberculosis*. The nucleotide sequence and amino acid sequence of MTB32A have been described (for example, U.S. Patent Application 60/158,585; see also, Skeiky *et al.*, *Infection and Immun.* (1999) 67:3998-4007, incorporated herein by reference). C-terminal fragments of the MTB32A coding sequence express at high levels and remain as soluble polypeptides throughout the purification process. Moreover, Ra12 may enhance the immunogenicity of heterologous immunogenic polypeptides with which it is fused. One preferred Ra12 fusion polypeptide comprises a 14 KD C-terminal fragment corresponding to amino acid residues 192 to 323 of MTB32A. Other preferred Ra12 polynucleotides generally comprise at least about 15 consecutive nucleotides, at least about 30 nucleotides, at least about 60 nucleotides, at least about 100 nucleotides, at least about 200 nucleotides, or at least about 300 nucleotides that encode a portion of a Ra12 polypeptide. Ra12 polynucleotides may comprise a native sequence (*i.e.*, an endogenous sequence that encodes a Ra12 polypeptide or a portion thereof) or may

comprise a variant of such a sequence. Ra12 polynucleotide variants may contain one or more substitutions, additions, deletions and/or insertions such that the biological activity of the encoded fusion polypeptide is not substantially diminished, relative to a fusion polypeptide comprising a native Ra12 polypeptide. Variants preferably exhibit at least about 70% identity, more preferably at least about 80% identity and most preferably at least about 90% identity to a polynucleotide sequence that encodes a native Ra12 polypeptide or a portion thereof.

Within other preferred embodiments, an immunological fusion partner is derived from protein D, a surface protein of the gram-negative bacterium Haemophilus influenza B (WO 91/18926). Preferably, a protein D derivative comprises approximately the first third of the protein (*e.g.*, the first N-terminal 100-110 amino acids), and a protein D derivative may be lipidated. Within certain preferred embodiments, the first 109 residues of a Lipoprotein D fusion partner is included on the N-terminus to provide the polypeptide with additional exogenous T-cell epitopes and to increase the expression level in *E. coli* (thus functioning as an expression enhancer). The lipid tail ensures optimal presentation of the antigen to antigen presenting cells. Other fusion partners include the non-structural protein from influenzae virus, NS1 (hemaglutinin). Typically, the N-terminal 81 amino acids are used, although different fragments that include T-helper epitopes may be used.

In another embodiment, the immunological fusion partner is the protein known as LYTA, or a portion thereof (preferably a C-terminal portion). LYTA is derived from *Streptococcus pneumoniae*, which synthesizes an N-acetyl-L-alanine amidase known as amidase LYTA (encoded by the LytA gene; *Gene 43*:265-292, 1986). LYTA is an autolysin that specifically degrades certain bonds in the peptidoglycan backbone. The C-terminal domain of the LYTA protein is responsible for the affinity to the choline or to some choline analogues such as DEAE. This property has been exploited for the development of *E. coli* C-LYTA expressing plasmids useful for expression of fusion proteins. Purification of hybrid proteins containing the C-LYTA fragment

at the amino terminus has been described (*see Biotechnology 10*:795-798, 1992). Within a preferred embodiment, a repeat portion of LYTA may be incorporated into a fusion polypeptide. A repeat portion is found in the C-terminal region starting at residue 178. A particularly preferred repeat portion incorporates residues 188-305.

Within another illustrative embodiment the fusion partner comprises a twin arginine translocation (TAT) signal peptide from the TorA signal peptide in *E. coli* on the N-terminus; see J. Mol. Microbiol. (2000) 2(2): 179-189; Journal of Bacteriology, Jan 2001 p604-610 Vol 183, No 2; Journal of Biochemistry Vol 276, March 16 2001 pp 8159-8164), hereby incorporated herein in its entirety.

Yet another illustrative embodiment involves fusion polypeptides, and the polynucleotides encoding them, wherein the fusion partner comprises a targeting signal capable of directing a polypeptide to the endosomal/lysosomal compartment, as described in U.S. Patent No. 5,633,234. An immunogenic polypeptide of the invention, when fused with this targeting signal, will associate more efficiently with MHC class II molecules and thereby provide enhanced in vivo stimulation of $CD4^+$ T-cells specific for the polypeptide.

The invention provides truncated forms of WT1 polypeptides that can be recombinantly expressed in *E. coli* without the addition of a fusion partner. Examples of these truncated forms are shown in SEQ ID NOs:342-346, and are encoded by polynucleotides shown in SEQ ID NOs:337-341. In variations of these truncations, the first 76 amino acids of WT1 can be fused to the C-terminus of the protein, creating a recombinant protein that is easier to express in *E. coli*. Other hosts in addition to *E. coli* can also be used, such as, for example, *B. megaterium*. The protein can further be prepared without a histidine tag.

In other embodiments, different subunits can be made and fused together in an order which differs from that of native WT1. In addition, fusions can be made with, for example, Ra12. Exemplary fusion proteins are shown in

SEQ ID NOs: 332-336 and can be encoded by polynucleotides shown in SEQ ID NOs: 327-331.

WT1 Polynucleotides

Any polynucleotide that encodes a WT1 polypeptide as described herein is a WT1 polynucleotide encompassed by the present invention. Such polynucleotides may be single-stranded (coding or antisense) or double-stranded, and may be DNA (genomic, cDNA or synthetic) or RNA molecules. Additional coding or non-coding sequences may, but need not, be present within a polynucleotide of the present invention, and a polynucleotide may, but need not, be linked to other molecules and/or support materials.

WT1 polynucleotides may encode a native WT1 protein, or may encode a variant of WT1 as described herein. Polynucleotide variants may contain one or more substitutions, additions, deletions and/or insertions such that the immunogenicity of the encoded polypeptide is not diminished, relative to a native WT1 protein. The effect on the immunogenicity of the encoded polypeptide may generally be assessed as described herein. Preferred variants contain nucleotide substitutions, deletions, insertions and/or additions at no more than 20%, preferably at no more than 10%, of the nucleotide positions that encode an immunogenic portion of a native WT1 sequence. Certain variants are substantially homologous to a native gene, or a portion thereof. Such polynucleotide variants are capable of hybridizing under moderately stringent conditions to a naturally occurring DNA sequence encoding a WT1 polypeptide (or a complementary sequence). Suitable moderately stringent conditions include prewashing in a solution of 5 X SSC, 0.5% SDS, 1.0 mM EDTA (pH 8.0); hybridizing at 50°C-65°C, 5 X SSC, overnight; followed by washing twice at 65°C for 20 minutes with each of 2X, 0.5X and 0.2X SSC containing 0.1% SDS). Such hybridizing DNA sequences are also within the scope of this invention.

It will be appreciated by those of ordinary skill in the art that, as a result of the degeneracy of the genetic code, there are many nucleotide

sequences that encode a WT1 polypeptide. Some of these polynucleotides bear minimal homology to the nucleotide sequence of any native gene. Nonetheless, polynucleotides that vary due to differences in codon usage are specifically contemplated by the present invention.

Therefore, according to another aspect of the present invention, polynucleotide compositions are provided that comprise some or all of a polynucleotide sequence set forth herein, complements of a polynucleotide sequence set forth herein, and degenerate variants of a polynucleotide sequence set forth herein. In certain preferred embodiments, the polynucleotide sequences set forth herein encode immunogenic polypeptides, as described above.

Once an immunogenic portion of WT1 is identified, as described above, a WT1 polynucleotide may be prepared using any of a variety of techniques. For example, a WT1 polynucleotide may be amplified from cDNA prepared from cells that express WT1. Such polynucleotides may be amplified via polymerase chain reaction (PCR). For this approach, sequence-specific primers may be designed based on the sequence of the immunogenic portion and may be purchased or synthesized. For example, suitable primers for PCR amplification of a human WT1 gene include: first step - P118: 1434-1414: 5' GAG AGT CAG ACT TGA AAG CAGT 3' (SEQ ID NO:5) and P135: 5' CTG AGC CTC AGC AAA TGG GC 3' (SEQ ID NO:6); second step - P136: 5' GAG CAT GCA TGG GCT CCG ACG TGC GGG 3' (SEQ ID NO:7) and P137: 5' GGG GTA CCC ACT GAA CGG TCC CCG A 3' (SEQ ID NO:8). Primers for PCR amplification of a mouse WT1 gene include: first step - P138: 5' TCC GAG CCG CAC CTC ATG 3' (SEQ ID NO:9) and P139: 5' GCC TGG GAT GCT GGA CTG 3' (SEQ ID NO:10), second step - P140: 5' GAG CAT GCG ATG GGT TCC GAC GTG CGG 3' (SEQ ID NO:11) and P141: 5' GGG GTA CCT CAA AGC GCC ACG TGG AGT TT 3' (SEQ ID NO:12).

An amplified portion may then be used to isolate a full length gene from a human genomic DNA library or from a suitable cDNA library, using well known techniques. Alternatively, a full length gene can be constructed

from multiple PCR fragments. WT1 polynucleotides may also be prepared by synthesizing oligonucleotide components, and ligating components together to generate the complete polynucleotide.

WT1 polynucleotides may also be synthesized by any method known in the art, including chemical synthesis (*e.g.*, solid phase phosphoramidite chemical synthesis). Modifications in a polynucleotide sequence may also be introduced using standard mutagenesis techniques, such as oligonucleotide-directed site-specific mutagenesis (*see* Adelman et al., *DNA* 2:183, 1983). Alternatively, RNA molecules may be generated by *in vitro* or *in vivo* transcription of DNA sequences encoding a WT1 polypeptide, provided that the DNA is incorporated into a vector with a suitable RNA polymerase promoter (such as T7 or SP6). Certain portions may be used to prepare an encoded polypeptide, as described herein. In addition, or alternatively, a portion may be administered to a patient such that the encoded polypeptide is generated *in vivo* (*e.g.*, by transfecting antigen-presenting cells such as dendritic cells with a cDNA construct encoding a WT1 polypeptide, and administering the transfected cells to the patient).

Polynucleotides that encode a WT1 polypeptide may generally be used for production of the polypeptide, *in vitro* or *in vivo*. WT1 polynucleotides that are complementary to a coding sequence (*i.e.*, antisense polynucleotides) may also be used as a probe or to inhibit WT1 expression. cDNA constructs that can be transcribed into antisense RNA may also be introduced into cells of tissues to facilitate the production of antisense RNA.

Any polynucleotide may be further modified to increase stability *in vivo*. Possible modifications include, but are not limited to, the addition of flanking sequences at the 5' and/or 3' ends; the use of phosphorothioate or 2' O-methyl rather than phosphodiesterase linkages in the backbone; and/or the inclusion of nontraditional bases such as inosine, queosine and wybutosine, as well as acetyl- methyl-, thio- and other modified forms of adenine, cytidine, guanine, thymine and uridine.

Nucleotide sequences as described herein may be joined to a variety of other nucleotide sequences using established recombinant DNA techniques. For example, a polynucleotide may be cloned into any of a variety of cloning vectors, including plasmids, phagemids, lambda phage derivatives and cosmids. Vectors of particular interest include expression vectors, replication vectors, probe generation vectors and sequencing vectors. In general, a vector will contain an origin of replication functional in at least one organism, convenient restriction endonuclease sites and one or more selectable markers. Other elements will depend upon the desired use, and will be apparent to those of ordinary skill in the art. In particular, one embodiment of the invention comprises expression vectors which incorporate the nucleic acid molecules of the invention, in operable linkage (i.e., "operably linked") to an expression control sequence (promoter). Construction of such vectors, such as viral (*e.g.*, adenovirus or Vaccinia virus) or attenuated viral vectors is well within the skill of the art, as is the transformation or transfection of cells, to produce eukaryotic cell lines, or prokaryotic cell strains which encode the molecule of interest. Exemplary of the host cells which can be employed in this fashion are COS cells, CHO cells, yeast cells, insect cells (e.g., Spodoptera frugiperda or Sf-9 cells), NIH 3T3 cells, and so forth. Prokaryotic cells, such as *E. coli* and other bacteria may also be used.

Within certain embodiments, polynucleotides may be formulated so as to permit entry into a cell of a mammal, and expression therein. Such formulations are particularly useful for therapeutic purposes, as described below. Those of ordinary skill in the art will appreciate that there are many ways to achieve expression of a polynucleotide in a target cell, and any suitable method may be employed. For example, a polynucleotide may be incorporated into a viral vector such as, but not limited to, adenovirus, adeno-associated virus, retrovirus, or vaccinia or other pox virus (*e.g.*, avian pox virus). Techniques for incorporating DNA into such vectors are well known to those of ordinary skill in the art. A retroviral vector may additionally transfer or incorporate a gene for a selectable marker (to aid in the identification or

selection of transduced cells) and/or a targeting moiety, such as a gene that encodes a ligand for a receptor on a specific target cell, to render the vector target specific. Targeting may also be accomplished using an antibody, by methods known to those of ordinary skill in the art. cDNA constructs within such a vector may be used, for example, to transfect human or animal cell lines for use in establishing WT1 positive tumor models which may be used to perform tumor protection and adoptive immunotherapy experiments to demonstrate tumor or leukemia-growth inhibition or lysis of such cells.

Other therapeutic formulations for polynucleotides include colloidal dispersion systems, such as macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. A preferred colloidal system for use as a delivery vehicle *in vitro* and *in vivo* is a liposome (*i.e.*, an artificial membrane vesicle). The preparation and use of such systems is well known in the art.

Antibody Compositions, Fragments Thereof and Other Binding Agents

According to another aspect, the present invention further provides binding agents, such as antibodies and antigen-binding fragments thereof, that exhibit immunological binding to a WT1 polypeptide disclosed herein, or to a portion, variant or derivative thereof. An antibody, or antigen-binding fragment thereof, is said to "specifically bind," "immunogically bind," and/or is "immunologically reactive" to a WT1 polypeptide of the invention if it reacts at a detectable level (within, for example, an ELISA assay) with the polypeptide, and does not react detectably with unrelated polypeptides under similar conditions.

Immunological binding, as used in this context, generally refers to the non-covalent interactions of the type which occur between an immunoglobulin molecule and an antigen for which the immunoglobulin is specific. The strength, or affinity of immunological binding interactions can be expressed in terms of the dissociation constant (K_d) of the interaction, wherein

a smaller K_d represents a greater affinity. Immunological binding properties of selected polypeptides can be quantified using methods well known in the art. One such method entails measuring the rates of antigen-binding site/antigen complex formation and dissociation, wherein those rates depend on the concentrations of the complex partners, the affinity of the interaction, and on geometric parameters that equally influence the rate in both directions. Thus, both the "on rate constant" (K_{on}) and the "off rate constant" (K_{off}) can be determined by calculation of the concentrations and the actual rates of association and dissociation. The ratio of K_{off} /K_{on} enables cancellation of all parameters not related to affinity, and is thus equal to the dissociation constant K_d. See, generally, Davies et al. (1990) Annual Rev. Biochem. 59:439-473.

An "antigen-binding site," or "binding portion" of an antibody refers to the part of the immunoglobulin molecule that participates in antigen binding. The antigen binding site is formed by amino acid residues of the N-terminal variable ("V") regions of the heavy ("H") and light ("L") chains. Three highly divergent stretches within the V regions of the heavy and light chains are referred to as "hypervariable regions" which are interposed between more conserved flanking stretches known as "framework regions," or "FRs". Thus the term "FR" refers to amino acid sequences which are naturally found between and adjacent to hypervariable regions in immunoglobulins. In an antibody molecule, the three hypervariable regions of a light chain and the three hypervariable regions of a heavy chain are disposed relative to each other in three dimensional space to form an antigen-binding surface. The antigen-binding surface is complementary to the three-dimensional surface of a bound antigen, and the three hypervariable regions of each of the heavy and light chains are referred to as "complementarity-determining regions," or "CDRs."

Binding agents may be further capable of differentiating between patients with and without a WT1-associated cancer, using the representative assays provided herein. For example, antibodies or other binding agents that bind to a tumor protein will preferably generate a signal indicating the presence of a cancer in at least about 20% of patients with the disease, more preferably

at least about 30% of patients. Alternatively, or in addition, the antibody will generate a negative signal indicating the absence of the disease in at least about 90% of individuals without the cancer. To determine whether a binding agent satisfies this requirement, biological samples (*e.g.*, blood, sera, sputum, urine and/or tumor biopsies) from patients with and without a cancer (as determined using standard clinical tests) may be assayed as described herein for the presence of polypeptides that bind to the binding agent. Preferably, a statistically significant number of samples with and without the disease will be assayed. Each binding agent should satisfy the above criteria; however, those of ordinary skill in the art will recognize that binding agents may be used in combination to improve sensitivity.

Any agent that satisfies the above requirements may be a binding agent. For example, a binding agent may be a ribosome, with or without a peptide component, an RNA molecule or a polypeptide. In a preferred embodiment, a binding agent is an antibody or an antigen-binding fragment thereof. Antibodies may be prepared by any of a variety of techniques known to those of ordinary skill in the art. *See, e.g.*, Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, 1988. In general, antibodies can be produced by cell culture techniques, including the generation of monoclonal antibodies as described herein, or via transfection of antibody genes into suitable bacterial or mammalian cell hosts, in order to allow for the production of recombinant antibodies. In one technique, an immunogen comprising the polypeptide is initially injected into any of a wide variety of mammals (*e.g.*, mice, rats, rabbits, sheep or goats). In this step, the polypeptides of this invention may serve as the immunogen without modification. Alternatively, particularly for relatively short polypeptides, a superior immune response may be elicited if the polypeptide is joined to a carrier protein, such as bovine serum albumin or keyhole limpet hemocyanin. The immunogen is injected into the animal host, preferably according to a predetermined schedule incorporating one or more booster immunizations, and the animals are bled periodically. Polyclonal antibodies specific for the

polypeptide may then be purified from such antisera by, for example, affinity chromatography using the polypeptide coupled to a suitable solid support.

Monoclonal antibodies specific for an antigenic polypeptide of interest may be prepared, for example, using the technique of Kohler and Milstein, *Eur. J. Immunol.* 6:511-519, 1976, and improvements thereto. Briefly, these methods involve the preparation of immortal cell lines capable of producing antibodies having the desired specificity (*i.e.*, reactivity with the polypeptide of interest). Such cell lines may be produced, for example, from spleen cells obtained from an animal immunized as described above. The spleen cells are then immortalized by, for example, fusion with a myeloma cell fusion partner, preferably one that is syngeneic with the immunized animal. A variety of fusion techniques may be employed. For example, the spleen cells and myeloma cells may be combined with a nonionic detergent for a few minutes and then plated at low density on a selective medium that supports the growth of hybrid cells, but not myeloma cells. A preferred selection technique uses HAT (hypoxanthine, aminopterin, thymidine) selection. After a sufficient time, usually about 1 to 2 weeks, colonies of hybrids are observed. Single colonies are selected and their culture supernatants tested for binding activity against the polypeptide. Hybridomas having high reactivity and specificity are preferred.

Monoclonal antibodies may be isolated from the supernatants of growing hybridoma colonies. In addition, various techniques may be employed to enhance the yield, such as injection of the hybridoma cell line into the peritoneal cavity of a suitable vertebrate host, such as a mouse. Monoclonal antibodies may then be harvested from the ascites fluid or the blood. Contaminants may be removed from the antibodies by conventional techniques, such as chromatography, gel filtration, precipitation, and extraction. The polypeptides of this invention may be used in the purification process in, for example, an affinity chromatography step.

A number of therapeutically useful molecules are known in the art which comprise antigen-binding sites that are capable of exhibiting

immunological binding properties of an antibody molecule. The proteolytic enzyme papain preferentially cleaves IgG molecules to yield several fragments, two of which (the "F(ab)" fragments) each comprise a covalent heterodimer that includes an intact antigen-binding site. The enzyme pepsin is able to cleave IgG molecules to provide several fragments, including the "$F(ab')_2$" fragment which comprises both antigen-binding sites. An "Fv" fragment can be produced by preferential proteolytic cleavage of an IgM, and on rare occasions IgG or IgA immunoglobulin molecule. Fv fragments are, however, more commonly derived using recombinant techniques known in the art. The Fv fragment includes a non-covalent $V_H::V_L$ heterodimer including an antigen-binding site which retains much of the antigen recognition and binding capabilities of the native antibody molecule. Inbar et al. (1972) Proc. Nat. Acad. Sci. USA 69:2659-2662; Hochman et al. (1976) Biochem 15:2706-2710; and Ehrlich et al. (1980) Biochem 19:4091-4096.

A single chain Fv ("sFv") polypeptide is a covalently linked $V_H::V_L$ heterodimer which is expressed from a gene fusion including V_H- and V_L-encoding genes linked by a peptide-encoding linker. Huston et al. (1988) Proc. Nat. Acad. Sci. USA 85(16):5879-5883. A number of methods have been described to discern chemical structures for converting the naturally aggregated--but chemically separated--light and heavy polypeptide chains from an antibody V region into an sFv molecule which will fold into a three dimensional structure substantially similar to the structure of an antigen-binding site. See, *e.g.*, U.S. Pat. Nos. 5,091,513 and 5,132,405, to Huston et al.; and U.S. Pat. No. 4,946,778, to Ladner et al.

Each of the above-described molecules includes a heavy chain and a light chain CDR set, respectively interposed between a heavy chain and a light chain FR set which provide support to the CDRS and define the spatial relationship of the CDRs relative to each other. As used herein, the term "CDR set" refers to the three hypervariable regions of a heavy or light chain V region. Proceeding from the N-terminus of a heavy or light chain, these regions are denoted as "CDR1," "CDR2," and "CDR3" respectively. An antigen-binding site,

therefore, includes six CDRs, comprising the CDR set from each of a heavy and a light chain V region. A polypeptide comprising a single CDR, (*e.g.*, a CDR1, CDR2 or CDR3) is referred to herein as a "molecular recognition unit." Crystallographic analysis of a number of antigen-antibody complexes has demonstrated that the amino acid residues of CDRs form extensive contact with bound antigen, wherein the most extensive antigen contact is with the heavy chain CDR3. Thus, the molecular recognition units are primarily responsible for the specificity of an antigen-binding site.

As used herein, the term "FR set" refers to the four flanking amino acid sequences which frame the CDRs of a CDR set of a heavy or light chain V region. Some FR residues may contact bound antigen; however, FRs are primarily responsible for folding the V region into the antigen-binding site, particularly the FR residues directly adjacent to the CDRS. Within FRs, certain amino residues and certain structural features are very highly conserved. In this regard, all V region sequences contain an internal disulfide loop of around 90 amino acid residues. When the V regions fold into a binding-site, the CDRs are displayed as projecting loop motifs which form an antigen-binding surface. It is generally recognized that there are conserved structural regions of FRs which influence the folded shape of the CDR loops into certain "canonical" structures--regardless of the precise CDR amino acid sequence. Further, certain FR residues are known to participate in non-covalent interdomain contacts which stabilize the interaction of the antibody heavy and light chains.

A number of "humanized" antibody molecules comprising an antigen-binding site derived from a non-human immunoglobulin have been described, including chimeric antibodies having rodent V regions and their associated CDRs fused to human constant domains (Winter et al. (1991) Nature 349:293-299; Lobuglio et al. (1989) Proc. Nat. Acad. Sci. USA 86:4220-4224; Shaw et al. (1987) J Immunol. 138:4534-4538; and Brown et al. (1987) Cancer Res. 47:3577-3583), rodent CDRs grafted into a human supporting FR prior to fusion with an appropriate human antibody constant domain (Riechmann et al. (1988) Nature 332:323-327; Verhoeyen et al. (1988) Science

239:1534-1536; and Jones et al. (1986) Nature 321:522-525), and rodent CDRs supported by recombinantly veneered rodent FRs (European Patent Publication No. 519,596, published Dec. 23, 1992). These "humanized" molecules are designed to minimize unwanted immunological response toward rodent antihuman antibody molecules which limits the duration and effectiveness of therapeutic applications of those moieties in human recipients.

As used herein, the terms "veneered FRs" and "recombinantly veneered FRs" refer to the selective replacement of FR residues from, *e.g.*, a rodent heavy or light chain V region, with human FR residues in order to provide a xenogeneic molecule comprising an antigen-binding site which retains substantially all of the native FR polypeptide folding structure. Veneering techniques are based on the understanding that the ligand binding characteristics of an antigen-binding site are determined primarily by the structure and relative disposition of the heavy and light chain CDR sets within the antigen-binding surface. Davies et al. (1990) Ann. Rev. Biochem. 59:439-473. Thus, antigen binding specificity can be preserved in a humanized antibody only wherein the CDR structures, their interaction with each other, and their interaction with the rest of the V region domains are carefully maintained. By using veneering techniques, exterior (*e.g.*, solvent-accessible) FR residues which are readily encountered by the immune system are selectively replaced with human residues to provide a hybrid molecule that comprises either a weakly immunogenic, or substantially non-immunogenic veneered surface.

The process of veneering makes use of the available sequence data for human antibody variable domains compiled by Kabat et al., in Sequences of Proteins of Immunological Interest, 4th ed., (U.S. Dept. of Health and Human Services, U.S. Government Printing Office, 1987), updates to the Kabat database, and other accessible U.S. and foreign databases (both nucleic acid and protein). Solvent accessibilities of V region amino acids can be deduced from the known three-dimensional structure for human and murine antibody fragments. There are two general steps in veneering a murine antigen-binding site. Initially, the FRs of the variable domains of an antibody

molecule of interest are compared with corresponding FR sequences of human variable domains obtained from the above-identified sources. The most homologous human V regions are then compared residue by residue to corresponding murine amino acids. The residues in the murine FR which differ from the human counterpart are replaced by the residues present in the human moiety using recombinant techniques well known in the art. Residue switching is only carried out with moieties which are at least partially exposed (solvent accessible), and care is exercised in the replacement of amino acid residues which may have a significant effect on the tertiary structure of V region domains, such as proline, glycine and charged amino acids.

In this manner, the resultant "veneered" murine antigen-binding sites are thus designed to retain the murine CDR residues, the residues substantially adjacent to the CDRs, the residues identified as buried or mostly buried (solvent inaccessible), the residues believed to participate in non-covalent (*e.g.*, electrostatic and hydrophobic) contacts between heavy and light chain domains, and the residues from conserved structural regions of the FRs which are believed to influence the "canonical" tertiary structures of the CDR loops. These design criteria are then used to prepare recombinant nucleotide sequences which combine the CDRs of both the heavy and light chain of a murine antigen-binding site into human-appearing FRs that can be used to transfect mammalian cells for the expression of recombinant human antibodies which exhibit the antigen specificity of the murine antibody molecule.

In another embodiment of the invention, monoclonal antibodies of the present invention may be coupled to one or more therapeutic agents. Suitable agents in this regard include radionuclides, differentiation inducers, drugs, toxins, and derivatives thereof. Preferred radionuclides include ^{90}Y, ^{123}I, ^{125}I, ^{131}I, ^{186}Re, ^{188}Re, ^{211}At, and ^{212}Bi. Preferred drugs include methotrexate, and pyrimidine and purine analogs. Preferred differentiation inducers include phorbol esters and butyric acid. Preferred toxins include ricin, abrin, diptheria toxin, cholera toxin, gelonin, Pseudomonas exotoxin, Shigella toxin, and pokeweed antiviral protein.

A therapeutic agent may be coupled (*e.g.*, covalently bonded) to a suitable monoclonal antibody either directly or indirectly (*e.g.*, via a linker group). A direct reaction between an agent and an antibody is possible when each possesses a substituent capable of reacting with the other. For example, a nucleophilic group, such as an amino or sulfhydryl group, on one may be capable of reacting with a carbonyl-containing group, such as an anhydride or an acid halide, or with an alkyl group containing a good leaving group (*e.g.*, a halide) on the other.

Alternatively, it may be desirable to couple a therapeutic agent and an antibody via a linker group. A linker group can function as a spacer to distance an antibody from an agent in order to avoid interference with binding capabilities. A linker group can also serve to increase the chemical reactivity of a substituent on an agent or an antibody, and thus increase the coupling efficiency. An increase in chemical reactivity may also facilitate the use of agents, or functional groups on agents, which otherwise would not be possible.

It will be evident to those skilled in the art that a variety of bifunctional or polyfunctional reagents, both homo- and hetero-functional (such as those described in the catalog of the Pierce Chemical Co., Rockford, IL), may be employed as the linker group. Coupling may be effected, for example, through amino groups, carboxyl groups, sulfhydryl groups or oxidized carbohydrate residues. There are numerous references describing such methodology, *e.g.*, U.S. Patent No. 4,671,958, to Rodwell et al.

Where a therapeutic agent is more potent when free from the antibody portion of the immunoconjugates of the present invention, it may be desirable to use a linker group which is cleavable during or upon internalization into a cell. A number of different cleavable linker groups have been described. The mechanisms for the intracellular release of an agent from these linker groups include cleavage by reduction of a disulfide bond (*e.g.*, U.S. Patent No. 4,489,710, to Spitler), by irradiation of a photolabile bond (*e.g.*, U.S. Patent No. 4,625,014, to Senter et al.), by hydrolysis of derivatized amino acid side chains (*e.g.*, U.S. Patent No. 4,638,045, to Kohn et al.), by serum complement-

mediated hydrolysis (*e.g.*, U.S. Patent No. 4,671,958, to Rodwell et al.), and acid-catalyzed hydrolysis (*e.g.*, U.S. Patent No. 4,569,789, to Blattler et al.).

It may be desirable to couple more than one agent to an antibody. In one embodiment, multiple molecules of an agent are coupled to one antibody molecule. In another embodiment, more than one type of agent may be coupled to one antibody. Regardless of the particular embodiment, immunoconjugates with more than one agent may be prepared in a variety of ways. For example, more than one agent may be coupled directly to an antibody molecule, or linkers that provide multiple sites for attachment can be used. Alternatively, a carrier can be used.

A carrier may bear the agents in a variety of ways, including covalent bonding either directly or via a linker group. Suitable carriers include proteins such as albumins (*e.g.*, U.S. Patent No. 4,507,234, to Kato et al.), peptides and polysaccharides such as aminodextran (*e.g.*, U.S. Patent No. 4,699,784, to Shih et al.). A carrier may also bear an agent by noncovalent bonding or by encapsulation, such as within a liposome vesicle (*e.g.*, U.S. Patent Nos. 4,429,008 and 4,873,088). Carriers specific for radionuclide agents include radiohalogenated small molecules and chelating compounds. For example, U.S. Patent No. 4,735,792 discloses representative radiohalogenated small molecules and their synthesis. A radionuclide chelate may be formed from chelating compounds that include those containing nitrogen and sulfur atoms as the donor atoms for binding the metal, or metal oxide, radionuclide. For example, U.S. Patent No. 4,673,562, to Davison et al. discloses representative chelating compounds and their synthesis.

T Cells

Immunotherapeutic compositions may also, or alternatively, comprise T cells specific for WT1. Such cells may generally be prepared *in vitro* or *ex vivo*, using standard procedures. For example, T cells may be present within (or isolated from) bone marrow, peripheral blood or a fraction of bone marrow or peripheral blood of a mammal, such as a patient, using a

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commercially available cell separation system, such as the CEPRATE™ system, available from CellPro Inc., Bothell WA (see also U.S. Patent No. 5,240,856; U.S. Patent No. 5,215,926; WO 89/06280; WO 91/16116 and WO 92/07243). Alternatively, T cells may be derived from related or unrelated humans, non-human animals, cell lines or cultures.

T cells may be stimulated with WT1 polypeptide, polynucleotide encoding a WT1 polypeptide and/or an antigen presenting cell (APC) that expresses a WT1 polypeptide. Such stimulation is performed under conditions and for a time sufficient to permit the generation of T cells that are specific for the WT1 polypeptide. Preferably, a WT1 polypeptide or polynucleotide is present within a delivery vehicle, such as a microsphere, to facilitate the generation of antigen-specific T cells. Briefly, T cells, which may be isolated from a patient or a related or unrelated donor by routine techniques (such as by Ficoll/Hypaque density gradient centrifugation of peripheral blood lymphocytes), are incubated with WT1 polypeptide. For example, T cells may be incubated *in vitro* for 2-9 days (typically 4 days) at 37°C with WT1 polypeptide (*e.g.*, 5 to 25 μg/ml) or cells synthesizing a comparable amount of WT1 polypeptide. It may be desirable to incubate a separate aliquot of a T cell sample in the absence of WT1 polypeptide to serve as a control.

T cells are considered to be specific for a WT1 polypeptide if the T cells kill target cells coated with a WT1 polypeptide or expressing a gene encoding such a polypeptide. T cell specificity may be evaluated using any of a variety of standard techniques. For example, within a chromium release assay or proliferation assay, a stimulation index of more than two fold increase in lysis and/or proliferation, compared to negative controls, indicates T cell specificity. Such assays may be performed, for example, as described in Chen et al., *Cancer Res. 54*:1065-1070, 1994. Alternatively, detection of the proliferation of T cells may be accomplished by a variety of known techniques. For example, T cell proliferation can be detected by measuring an increased rate of DNA synthesis (*e.g.*, by pulse-labeling cultures of T cells with tritiated thymidine and measuring the amount of tritiated thymidine incorporated into DNA). Other

ways to detect T cell proliferation include measuring increases in interleukin-2 (IL-2) production, Ca^{2+} flux, or dye uptake, such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium. Alternatively, synthesis of lymphokines (such as interferon-gamma) can be measured or the relative number of T cells that can respond to a WT1 polypeptide may be quantified. Contact with a WT1 polypeptide (200 ng/ml - 100 μg/ml, preferably 100 ng/ml - 25 μg/ml) for 3 - 7 days should result in at least a two fold increase in proliferation of the T cells and/or contact as described above for 2-3 hours should result in activation of the T cells, as measured using standard cytokine assays in which a two fold increase in the level of cytokine release (*e.g.*, TNF or IFN-γ) is indicative of T cell activation (*see* Coligan et al., Current Protocols in Immunology, vol. 1, Wiley Interscience (Greene 1998). WT1 specific T cells may be expanded using standard techniques. Within preferred embodiments, the T cells are derived from a patient or a related or unrelated donor and are administered to the patient following stimulation and expansion.

T cells that have been activated in response to a WT1 polypeptide, polynucleotide or WT1-expressing APC may be $CD4^+$ and/or $CD8^+$. Specific activation of $CD4^+$ or $CD8^+$ T cells may be detected in a variety of ways. Methods for detecting specific T cell activation include detecting the proliferation of T cells, the production of cytokines (*e.g.*, lymphokines), or the generation of cytolytic activity (*i.e.*, generation of cytotoxic T cells specific for WT1). For $CD4^+$ T cells, a preferred method for detecting specific T cell activation is the detection of the proliferation of T cells. For $CD8^+$ T cells, a preferred method for detecting specific T cell activation is the detection of the generation of cytolytic activity.

For therapeutic purposes, $CD4^+$ or $CD8^+$ T cells that proliferate in response to the WT1 polypeptide, polynucleotide or APC can be expanded in number either *in vitro* or *in vivo*. Proliferation of such T cells *in vitro* may be accomplished in a variety of ways. For example, the T cells can be re-exposed to WT1 polypeptide, with or without the addition of T cell growth factors, such as interleukin-2, and/or stimulator cells that synthesize a WT1 polypeptide.

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The addition of stimulator cells is preferred where generating $CD8^+$ T cell responses. T cells can be grown to large numbers *in vitro* with retention of specificity in response to intermittent restimulation with WT1 polypeptide. Briefly, for the primary *in vitro* stimulation (IVS), large numbers of lymphocytes (*e.g.*, greater than 4×10^7) may be placed in flasks with media containing human serum. WT1 polypeptide (*e.g.*, peptide at 10 μg/ml) may be added directly, along with tetanus toxoid (*e.g.*, 5 μg/ml). The flasks may then be incubated (*e.g.*, 37°C for 7 days). For a second IVS, T cells are then harvested and placed in new flasks with $2\text{-}3 \times 10^7$ irradiated peripheral blood mononuclear cells. WT1 polypeptide (*e.g.*, 10 μg/ml) is added directly. The flasks are incubated at 37°C for 7 days. On day 2 and day 4 after the second IVS, 2-5 units of interleukin-2 (IL-2) may be added. For a third IVS, the T cells may be placed in wells and stimulated with the individual's own EBV transformed B cells coated with the peptide. IL-2 may be added on days 2 and 4 of each cycle. As soon as the cells are shown to be specific cytotoxic T cells, they may be expanded using a 10 day stimulation cycle with higher IL-2 (20 units) on days 2, 4 and 6.

Alternatively, one or more T cells that proliferate in the presence of WT1 polypeptide can be expanded in number by cloning. Methods for cloning cells are well known in the art, and include limiting dilution. Responder T cells may be purified from the peripheral blood of sensitized patients by density gradient centrifugation and sheep red cell rosetting and established in culture by stimulating with the nominal antigen in the presence of irradiated autologous filler cells. In order to generate $CD4^+$ T cell lines, WT1 polypeptide is used as the antigenic stimulus and autologous peripheral blood lymphocytes (PBL) or lymphoblastoid cell lines (LCL) immortalized by infection with Epstein Barr virus are used as antigen presenting cells. In order to generate $CD8^+$ T cell lines, autologous antigen-presenting cells transfected with an expression vector which produces WT1 polypeptide may be used as stimulator cells. Established T cell lines may be cloned 2-4 days following antigen stimulation by plating stimulated T cells at a frequency of 0.5 cells per well in 96-well flat-

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bottom plates with 1 x 10^6 irradiated PBL or LCL cells and recombinant interleukin-2 (rIL2) (50 U/ml). Wells with established clonal growth may be identified at approximately 2-3 weeks after initial plating and restimulated with appropriate antigen in the presence of autologous antigen-presenting cells, then subsequently expanded by the addition of low doses of rIL2 (10 U/ml) 2-3 days following antigen stimulation. T cell clones may be maintained in 24-well plates by periodic restimulation with antigen and rIL2 approximately every two weeks.

Within certain embodiments, allogeneic T-cells may be primed (*i.e.,* sensitized to WT1) *in vivo* and/or *in vitro*. Such priming may be achieved by contacting T cells with a WT1 polypeptide, a polynucleotide encoding such a polypeptide or a cell producing such a polypeptide under conditions and for a time sufficient to permit the priming of T cells. In general, T cells are considered to be primed if, for example, contact with a WT1 polypeptide results in proliferation and/or activation of the T cells, as measured by standard proliferation, chromium release and/or cytokine release assays as described herein. A stimulation index of more than two fold increase in proliferation or lysis, and more than three fold increase in the level of cytokine, compared to negative controls, indicates T-cell specificity. Cells primed *in vitro* may be employed, for example, within a bone marrow transplantation or as donor lymphocyte infusion.

T cells specific for WT1 can kill cells that express WT1 protein. Introduction of genes encoding T-cell receptor (TCR) chains for WT1 are used as a means to quantitatively and qualitatively improve responses to WT1 bearing leukemia and cancer cells. Vaccines to increase the number of T cells that can react to WT1 positive cells are one method of targeting WT1 bearing cells. T cell therapy with T cells specific for WT1 is another method. An alternative method is to introduce the TCR chains specific for WT1 into T cells or other cells with lytic potential. In a suitable embodiment, the TCR alpha and beta chains are cloned out from a WT1 specific T cell line and used for

adoptive T cell therapy, such as described in WO96/30516, incorporated herein by reference.

T Cell Receptor Compositions

The T cell receptor (TCR) consists of 2 different, highly variable polypeptide chains, termed the T-cell receptor α and β chains, that are linked by a disulfide bond (Janeway, Travers, Walport. Immunobiology. Fourth Ed., 148-159. Elsevier Science Ltd/Garland Publishing. 1999). The α/β heterodimer complexes with the invariant CD3 chains at the cell membrane. This complex recognizes specific antigenic peptides bound to MHC molecules. The enormous diversity of TCR specificities is generated much like immunoglobulin diversity, through somatic gene rearrangement. The β chain genes contain over 50 variable (V), 2 diversity (D), over 10 joining (J) segments, and 2 constant region segments (C). The α chain genes contain over 70 V segments, and over 60 J segments but no D segments, as well as one C segment. During T cell development in the thymus, the D to J gene rearrangement of the β chain occurs, followed by the V gene segment rearrangement to the DJ. This functional VDJβ exon is transcribed and spliced to join to a Cβ. For the α chain, a Vα gene segment rearranges to a Jα gene segment to create the functional exon that is then transcribed and spliced to the Cα. Diversity is further increased during the recombination process by the random addition of P and N-nucleotides between the V, D, and J segments of the β chain and between the V and J segments in the α chain (Janeway, Travers, Walport. Immunobiology. Fourth Ed., 98 and 150. Elsevier Science Ltd/Garland Publishing. 1999).

The present invention, in another aspect, provides TCRs specific for a polypeptide disclosed herein, or for a variant or derivative thereof. In accordance with the present invention, polynucleotide and amino acid sequences are provided for the V-J or V-D-J junctional regions or parts thereof for the alpha and beta chains of the T-cell receptor which recognize tumor polypeptides described herein. In general, this aspect of the invention relates

to T-cell receptors which recognize or bind tumor polypeptides presented in the context of MHC. In a preferred embodiment the tumor antigens recognized by the T-cell receptors comprise a polypeptide of the present invention. For example, cDNA encoding a TCR specific for a WT1 peptide can be isolated from T cells specific for a tumor polypeptide using standard molecular biological and recombinant DNA techniques.

This invention further includes the T-cell receptors or analogs thereof having substantially the same function or activity as the T-cell receptors of this invention which recognize or bind tumor polypeptides. Such receptors include, but are not limited to, a fragment of the receptor, or a substitution, addition or deletion mutant of a T-cell receptor provided herein. This invention also encompasses polypeptides or peptides that are substantially homologous to the T-cell receptors provided herein or that retain substantially the same activity. The term "analog" includes any protein or polypeptide having an amino acid residue sequence substantially identical to the T-cell receptors provided herein in which one or more residues, preferably no more than 5 residues, more preferably no more than 25 residues have been conservatively substituted with a functionally similar residue and which displays the functional aspects of the T-cell receptor as described herein.

The present invention further provides for suitable mammalian host cells, for example, non-specific T cells, that are transfected with a polynucleotide encoding TCRs specific for a polypeptide described herein, thereby rendering the host cell specific for the polypeptide. The α and β chains of the TCR may be contained on separate expression vectors or alternatively, on a single expression vector that also contains an internal ribosome entry site (IRES) for cap-independent translation of the gene downstream of the IRES. Said host cells expressing TCRs specific for the polypeptide may be used, for example, for adoptive immunotherapy of WT1-associated cancer as discussed further below.

In further aspects of the present invention, cloned TCRs specific for a polypeptide recited herein may be used in a kit for the diagnosis of WT1-

associated cancer. For example, the nucleic acid sequence or portions thereof, of tumor-specific TCRs can be used as probes or primers for the detection of expression of the rearranged genes encoding the specific TCR in a biological sample. Therefore, the present invention further provides for an assay for detecting messenger RNA or DNA encoding the TCR specific for a polypeptide.

Peptide-MHC Tetrameric Complexes

The present invention, in another aspect, provides peptide-MHC tetrameric complexes (tetramers) specific for T cells that recognize a polypeptide disclosed herein, or for a variant or derivative thereof. In one embodiment, tetramers may be used in the detection of WT1 specific T-cells. Tetramers may be used in monitoring WT1 specific immune responses, early detection of WT1 associated malignancies and for monitoring minimal residual disease. Tetramer staining is typically carried out with flow cytometric analysis and can be used to identify groups within a patient population suffering from a WT1 asssociated disease at a higher risk for relapse or disease progression.

Pharmaceutical Compositions

In additional embodiments, the present invention concerns formulation of one or more of the polynucleotide, polypeptide, T-cell, TCR, and/or antibody compositions disclosed herein in pharmaceutically-acceptable carriers for administration to a cell or an animal, either alone, or in combination with one or more other modalities of therapy.

It will be understood that, if desired, a composition as disclosed herein may be administered in combination with other agents as well, such as, *e.g.*, other proteins or polypeptides or various pharmaceutically-active agents. In fact, there is virtually no limit to other components that may also be included, given that the additional agents do not cause a significant adverse effect upon contact with the target cells or host tissues. The compositions may thus be delivered along with various other agents as required in the particular instance. Such compositions may be purified from host cells or other biological sources,

or alternatively may be chemically synthesized as described herein. Likewise, such compositions may further comprise substituted or derivatized RNA or DNA compositions.

Therefore, in another aspect of the present invention, pharmaceutical compositions are provided comprising one or more of the polynucleotide, polypeptide, antibody, TCR, and/or T-cell compositions described herein in combination with a physiologically acceptable carrier. In certain preferred embodiments, the pharmaceutical compositions of the invention comprise immunogenic polynucleotide and/or polypeptide compositions of the invention for use in prophylactic and theraputic vaccine applications. Vaccine preparation is generally described in, for example, M.F. Powell and M.J. Newman, eds., "Vaccine Design (the subunit and adjuvant approach)," Plenum Press (NY, 1995). Generally, such compositions will comprise one or more polynucleotide and/or polypeptide compositions of the present invention in combination with one or more immunostimulants.

It will be apparent that any of the pharmaceutical compositions described herein can contain pharmaceutically acceptable salts of the polynucleotides and polypeptides of the invention. Such salts can be prepared, for example, from pharmaceutically acceptable non-toxic bases, including organic bases (*e.g.*, salts of primary, secondary and tertiary amines and basic amino acids) and inorganic bases (*e.g.*, sodium, potassium, lithium, ammonium, calcium and magnesium salts).

In another embodiment, illustrative immunogenic compositions, *e.g.*, vaccine compositions, of the present invention comprise DNA encoding one or more of the polypeptides as described above, such that the polypeptide is generated *in situ*. As noted above, the polynucleotide may be administered within any of a variety of delivery systems known to those of ordinary skill in the art. Indeed, numerous gene delivery techniques are well known in the art, such as those described by Rolland, *Crit. Rev. Therap. Drug Carrier Systems* *15*:143-198, 1998, and references cited therein. Appropriate polynucleotide expression systems will, of course, contain the necessary regulatory DNA

regulatory sequences for expression in a patient (such as a suitable promoter and terminating signal). Alternatively, bacterial delivery systems may involve the administration of a bacterium (such as *Bacillus-Calmette-Guerrin*) that expresses an immunogenic portion of the polypeptide on its cell surface or secretes such an epitope.

Therefore, in certain embodiments, polynucleotides encoding immunogenic polypeptides described herein are introduced into suitable mammalian host cells for expression using any of a number of known viral-based systems. In one illustrative embodiment, retroviruses provide a convenient and effective platform for gene delivery systems. A selected nucleotide sequence encoding a polypeptide of the present invention can be inserted into a vector and packaged in retroviral particles using techniques known in the art. The recombinant virus can then be isolated and delivered to a subject. A number of illustrative retroviral systems have been described (*e.g.*, U.S. Pat. No. 5,219,740; Miller and Rosman (1989) BioTechniques 7:980-990; Miller, A. D. (1990) Human Gene Therapy 1:5-14; Scarpa et al. (1991) Virology 180:849-852; Burns et al. (1993) Proc. Natl. Acad. Sci. USA 90:8033-8037; and Boris-Lawrie and Temin (1993) Cur. Opin. Genet. Develop. 3:102-109.

In addition, a number of illustrative adenovirus-based systems have also been described. Unlike retroviruses which integrate into the host genome, adenoviruses persist extrachromosomally thus minimizing the risks associated with insertional mutagenesis (Haj-Ahmad and Graham (1986) J. Virol. 57:267-274; Bett et al. (1993) J. Virol. 67:5911-5921; Mittereder et al. (1994) Human Gene Therapy 5:717-729; Seth et al. (1994) J. Virol. 68:933-940; Barr et al. (1994) Gene Therapy 1:51-58; Berkner, K. L. (1988) BioTechniques 6:616-629; and Rich et al. (1993) Human Gene Therapy 4:461-476).

Various adeno-associated virus (AAV) vector systems have also been developed for polynucleotide delivery. AAV vectors can be readily constructed using techniques well known in the art. See, *e.g.*, U.S. Pat. Nos. 5,173,414 and 5,139,941; International Publication Nos. WO 92/01070 and

WO 93/03769; Lebkowski et al. (1988) Molec. Cell. Biol. 8:3988-3996; Vincent et al. (1990) Vaccines 90 (Cold Spring Harbor Laboratory Press); Carter, B. J. (1992) Current Opinion in Biotechnology 3:533-539; Muzyczka, N. (1992) Current Topics in Microbiol. and Immunol. 158:97-129; Kotin, R. M. (1994) Human Gene Therapy 5:793-801; Shelling and Smith (1994) Gene Therapy 1:165-169; and Zhou et al. (1994) J. Exp. Med. 179:1867-1875.

Additional viral vectors useful for delivering the polynucleotides encoding polypeptides of the present invention by gene transfer include those derived from the pox family of viruses, such as vaccinia virus and avian poxvirus. By way of example, vaccinia virus recombinants expressing the novel molecules can be constructed as follows. The DNA encoding a polypeptide is first inserted into an appropriate vector so that it is adjacent to a vaccinia promoter and flanking vaccinia DNA sequences, such as the sequence encoding thymidine kinase (TK). This vector is then used to transfect cells which are simultaneously infected with vaccinia. Homologous recombination serves to insert the vaccinia promoter plus the gene encoding the polypeptide of interest into the viral genome. The resulting TK.sup.(-) recombinant can be selected by culturing the cells in the presence of 5-bromodeoxyuridine and picking viral plaques resistant thereto.

A vaccinia-based infection/transfection system can be conveniently used to provide for inducible, transient expression or coexpression of one or more polypeptides described herein in host cells of an organism. In this particular system, cells are first infected in vitro with a vaccinia virus recombinant that encodes the bacteriophage T7 RNA polymerase. This polymerase displays exquisite specificity in that it only transcribes templates bearing T7 promoters. Following infection, cells are transfected with the polynucleotide or polynucleotides of interest, driven by a T7 promoter. The polymerase expressed in the cytoplasm from the vaccinia virus recombinant transcribes the transfected DNA into RNA which is then translated into polypeptide by the host translational machinery. The method provides for high level, transient, cytoplasmic production of large quantities of RNA and its

translation products. See, *e.g.*, Elroy-Stein and Moss, Proc. Natl. Acad. Sci. USA (1990) 87:6743-6747; Fuerst et al. Proc. Natl. Acad. Sci. USA (1986) 83:8122-8126.

Alternatively, avipoxviruses, such as the fowlpox and canarypox viruses, can also be used to deliver the coding sequences of interest. Recombinant avipox viruses, expressing immunogens from mammalian pathogens, are known to confer protective immunity when administered to non-avian species. The use of an Avipox vector is particularly desirable in human and other mammalian species since members of the Avipox genus can only productively replicate in susceptible avian species and therefore are not infective in mammalian cells. Methods for producing recombinant Avipoxviruses are known in the art and employ genetic recombination, as described above with respect to the production of vaccinia viruses. See, *e.g.*, WO 91/12882; WO 89/03429; and WO 92/03545. A number of pox viruses have been developed as live viral vectors for the expression of heterologous proteins (Cepko et al., Cell 37:1053-1062 (1984); Morin et al., Proc. Natl. Acad. Sci. USA 84:4626-4630 (1987); Lowe et al., Proc. Natl. Acad. Sci. USA, 84:3896-3900 (1987); Panicali & Paoletti, Proc. Natl. Acad. Sci. USA, 79:4927-4931 (1982); Machett et al., Proc. Natl. Acad. Sci. USA, 79:7415-7419 (1982)). Representative fowlpox and swinepox virus are available through the ATCC under accession numbers VR-229 and VR-363, respectively. A recombinant vaccinia--CEA is available through the ATCC under accession number VR2323. Other illustrative viral vectors also include, but are not limited to, those described by Therion Biologics (Cambridge, MA, USA), for example, in U.S. Patent Nos. 6,051,410, 5,858,726, 5,656,465, 5,804,196, 5,747,324, 6,319,496, 6,165,460.

Any of a number of alphavirus vectors can also be used for delivery of polynucleotide compositions of the present invention, such as those vectors described in U.S. Patent Nos. 5,843,723; 6,015,686; 6,008,035 and 6,015,694. Certain vectors based on Venezuelan Equine Encephalitis (VEE)

can also be used, illustrative examples of which can be found in U.S. Patent Nos. 5,505,947 and 5,643,576.

Moreover, molecular conjugate vectors, such as the adenovirus chimeric vectors described in Michael et al. J. Biol. Chem. (1993) 268:6866-6869 and Wagner et al. Proc. Natl. Acad. Sci. USA (1992) 89:6099-6103, can also be used for gene delivery under the invention.

Additional illustrative information on these and other known viral-based delivery systems can be found, for example, in Fisher-Hoch et al., *Proc. Natl. Acad. Sci. USA 86*:317-321, 1989; Flexner et al., *Ann. N.Y. Acad. Sci. 569*:86-103, 1989; Flexner et al., *Vaccine 8*:17-21, 1990; U.S. Patent Nos. 4,603,112, 4,769,330, and 5,017,487; WO 89/01973; U.S. Patent No. 4,777,127; GB 2,200,651; EP 0,345,242; WO 91/02805; Berkner, *Biotechniques 6*:616-627, 1988; Rosenfeld et al., *Science 252*:431-434, 1991; Kolls et al., *Proc. Natl. Acad. Sci. USA 91*:215-219, 1994; Kass-Eisler et al., *Proc. Natl. Acad. Sci. USA 90*:11498-11502, 1993; Guzman et al., *Circulation 88*:2838-2848, 1993; and Guzman et al., *Cir. Res. 73*:1202-1207, 1993.

As would be readily appreciated by the skilled artisan, any number of additional components may be present in a DNA or retroviral vector expressing a WT1 polypeptide or any portion thereof, as described herein. For example, an expression vector for delivery of a polynucleotide or peptide of the present invention may include any number of a variety of costimulatory molecules, including, but not limited to CD28, B7-1, ICAM-1, and LFA-3. A delivery vector may also include any number of cytokines, for example IFN-γ, GM-CSF, or IL-2. In one illustrative embodiment, a recombinant viral vector, *e.g.* a vaccinia or fowlpox vector, includes B7-1, ICAM-1, and LFA-3.

The present invention also comprises the use of any combination of the DNA and/or viral vectors described herein for use in the treatment of malignancies associated with the expression of WT1. In one illustrative embodiment, a recombinant vaccinia viral vector is administered to an animal or human patient afflicted with a WT1-associated malignancy, followed by administration of a recombinant fowlpox vector. In another embodiment of the

present invention, the recombinant fowlpox is adminstered twice following administration of the vaccinia vector (e.g. a prime/boost/boost vaccination regimen).

In certain embodiments, a polynucleotide may be integrated into the genome of a target cell. This integration may be in the specific location and orientation *via* homologous recombination (gene replacement) or it may be integrated in a random, non-specific location (gene augmentation). In yet further embodiments, the polynucleotide may be stably maintained in the cell as a separate, episomal segment of DNA. Such polynucleotide segments or "episomes" encode sequences sufficient to permit maintenance and replication independent of or in synchronization with the host cell cycle. The manner in which the expression construct is delivered to a cell and where in the cell the polynucleotide remains is dependent on the type of expression construct employed.

In another embodiment of the invention, a polynucleotide is administered/delivered as "naked" DNA, for example as described in Ulmer et al., *Science 259*:1745-1749, 1993 and reviewed by Cohen, *Science 259*:1691-1692, 1993. The uptake of naked DNA may be increased by coating the DNA onto biodegradable beads, which are efficiently transported into the cells.

In still another embodiment, a composition of the present invention can be delivered via a particle bombardment approach, many of which have been described. In one illustrative example, gas-driven particle acceleration can be achieved with devices such as those manufactured by Powderject Pharmaceuticals PLC (Oxford, UK) and Powderject Vaccines Inc. (Madison, WI), some examples of which are described in U.S. Patent Nos. 5,846,796; 6,010,478; 5,865,796; 5,584,807; and EP Patent No. 0500 799. This approach offers a needle-free delivery approach wherein a dry powder formulation of microscopic particles, such as polynucleotide or polypeptide particles, are accelerated to high speed within a helium gas jet generated by a hand held device, propelling the particles into a target tissue of interest.

In a related embodiment, other devices and methods that may be useful for gas-driven needle-less injection of compositions of the present invention include those provided by Bioject, Inc. (Portland, OR), some examples of which are described in U.S. Patent Nos. 4,790,824; 5,064,413; 5,312,335; 5,383,851; 5,399,163; 5,520,639 and 5,993,412.

According to another embodiment, the pharmaceutical compositions described herein will comprise one or more immunostimulants in addition to the immunogenic polynucleotide, polypeptide, antibody, T-cell, TCR, and/or APC compositions of this invention. An immunostimulant refers to essentially any substance that enhances or potentiates an immune response (antibody and/or cell-mediated) to an exogenous antigen. One preferred type of immunostimulant comprises an adjuvant. Many adjuvants contain a substance designed to protect the antigen from rapid catabolism, such as aluminum hydroxide or mineral oil, and a stimulator of immune responses, such as lipid A, *Bortadella pertussis* or *Mycobacterium tuberculosis* derived proteins. Certain adjuvants are commercially available as, for example, Freund's Incomplete Adjuvant and Complete Adjuvant (Difco Laboratories, Detroit, MI); Merck Adjuvant 65 (Merck and Company, Inc., Rahway, NJ); AS-2 (SmithKline Beecham, Philadelphia, PA); aluminum salts such as aluminum hydroxide gel (alum) or aluminum phosphate; salts of calcium, iron or zinc; an insoluble suspension of acylated tyrosine; acylated sugars; cationically or anionically derivatized polysaccharides; polyphosphazenes; biodegradable microspheres; monophosphoryl lipid A and quil A. Cytokines, such as GM-CSF, interleukin-2, -7, -12, and other like growth factors, may also be used as adjuvants.

Within certain embodiments of the invention, the adjuvant composition is preferably one that induces an immune response predominantly of the Th1 type. High levels of Th1-type cytokines (*e.g.*, IFN-γ, TNFα, IL-2 and IL-12) tend to favor the induction of cell mediated immune responses to an administered antigen. In contrast, high levels of Th2-type cytokines (*e.g.*, IL-4, IL-5, IL-6 and IL-10) tend to favor the induction of humoral immune responses. Following application of a vaccine as provided herein, a patient will support an

immune response that includes Th1- and Th2-type responses. Within a preferred embodiment, in which a response is predominantly Th1-type, the level of Th1-type cytokines will increase to a greater extent than the level of Th2-type cytokines. The levels of these cytokines may be readily assessed using standard assays. For a review of the families of cytokines, see Mosmann and Coffman, Ann. Rev. Immunol. 7:145-173, 1989.

Certain preferred adjuvants for eliciting a predominantly Th1-type response include, for example, a combination of monophosphoryl lipid A, preferably 3-de-O-acylated monophosphoryl lipid A, together with an aluminum salt. MPL® adjuvants are available from Corixa Corporation (Seattle, WA; *see*, for example, US Patent Nos. 4,436,727; 4,877,611; 4,866,034 and 4,912,094). CpG-containing oligonucleotides (in which the CpG dinucleotide is unmethylated) also induce a predominantly Th1 response. Such oligonucleotides are well known and are described, for example, in WO 96/02555, WO 99/33488 and U.S. Patent Nos. 6,008,200 and 5,856,462. Immunostimulatory DNA sequences are also described, for example, by Sato et al., *Science* 273:352, 1996. Another preferred adjuvant comprises a saponin, such as Quil A, or derivatives thereof, including QS21 and QS7 (Aquila Biopharmaceuticals Inc., Framingham, MA); Escin; Digitonin; or *Gypsophila* or *Chenopodium quinoa* saponins . Other preferred formulations include more than one saponin in the adjuvant combinations of the present invention, for example combinations of at least two of the following group comprising QS21, QS7, Quil A, β-escin, or digitonin.

Alternatively the saponin formulations may be combined with vaccine vehicles composed of chitosan or other polycationic polymers, polylactide and polylactide-co-glycolide particles, poly-N-acetyl glucosamine-based polymer matrix, particles composed of polysaccharides or chemically modified polysaccharides, liposomes and lipid-based particles, particles composed of glycerol monoesters, etc. The saponins may also be formulated in the presence of cholesterol to form particulate structures such as liposomes or ISCOMs. Furthermore, the saponins may be formulated together with a

polyoxyethylene ether or ester, in either a non-particulate solution or suspension, or in a particulate structure such as a paucilamelar liposome or ISCOM. The saponins may also be formulated with excipients such as Carbopol^{R} to increase viscosity, or may be formulated in a dry powder form with a powder excipient such as lactose.

In one preferred embodiment, the adjuvant system includes the combination of a monophosphoryl lipid A and a saponin derivative, such as the combination of QS21 and 3D-MPL® adjuvant, as described in WO 94/00153, or a less reactogenic composition where the QS21 is quenched with cholesterol, as described in WO 96/33739. Other preferred formulations comprise an oil-in-water emulsion and tocopherol. Another particularly preferred adjuvant formulation employing QS21, 3D-MPL® adjuvant and tocopherol in an oil-in-water emulsion is described in WO 95/17210.

Another enhanced adjuvant system involves the combination of a CpG-containing oligonucleotide and a saponin derivative particularly the combination of CpG and QS21 is disclosed in WO 00/09159. Preferably the formulation additionally comprises an oil in water emulsion and tocopherol.

Additional illustrative adjuvants for use in the pharmaceutical compositions of the invention include Montanide ISA 720 (Seppic, France), SAF (Chiron, California, United States), ISCOMS (CSL), MF-59 (Chiron), the SBAS series of adjuvants (*e.g.*, SBAS-2 or SBAS-4, available from SmithKline Beecham, Rixensart, Belgium), Detox (Enhanzyn®) (Corixa, Hamilton, MT), RC-529 (Corixa, Hamilton, MT) and other aminoalkyl glucosaminide 4-phosphates (AGPs), such as those described in pending U.S. Patent Application Serial Nos. 08/853,826 and 09/074,720, and in U.S. Patent Nos. 6,303,347 the disclosures of which are incorporated herein by reference in their entireties, and polyoxyethylene ether adjuvants such as those described in WO 99/52549A1. Additional illustrative adjuvants for use in the pharmaceutical compositions of the invention include isotucerosol.

Other preferred adjuvants include adjuvant molecules of the general formula

(I): $HO(CH_2CH_2O)_n$-A-R,

wherein, n is 1-50, A is a bond or –C(O)-, R is C_{1-50} alkyl or Phenyl C_{1-50} alkyl.

One embodiment of the present invention consists of a vaccine formulation comprising a polyoxyethylene ether of general formula (I), wherein *n* is between 1 and 50, preferably 4-24, most preferably 9; the *R* component is C_{1-50}, preferably C_4-C_{20} alkyl and most preferably C_{12} alkyl, and *A* is a bond. The concentration of the polyoxyethylene ethers should be in the range 0.1-20%, preferably from 0.1-10%, and most preferably in the range 0.1-1%. Preferred polyoxyethylene ethers are selected from the following group: polyoxyethylene-9-lauryl ether, polyoxyethylene-9-steoryl ether, polyoxyethylene-8-steoryl ether, polyoxyethylene-4-lauryl ether, polyoxyethylene-35-lauryl ether, and polyoxyethylene-23-lauryl ether. Polyoxyethylene ethers such as polyoxyethylene lauryl ether are described in the Merck index (12^{th} edition: entry 7717). These adjuvant molecules are described in WO 99/52549.

The polyoxyethylene ether according to the general formula (I) above may, if desired, be combined with another adjuvant. For example, a preferred adjuvant combination is preferably with CpG as described in the pending UK patent application GB 9820956.2.

According to another embodiment of this invention, an immunogenic composition described herein is delivered to a host via antigen presenting cells (APCs), such as dendritic cells, macrophages, B cells, monocytes and other cells that may be engineered to be efficient APCs. Such cells may, but need not, be genetically modified to increase the capacity for presenting the antigen, to improve activation and/or maintenance of the T cell response, to have anti-tumor effects *per se* and/or to be immunologically compatible with the receiver (*i.e.*, matched HLA haplotype). APCs may generally be isolated from any of a variety of biological fluids and organs, including tumor and peritumoral tissues, and may be autologous, allogeneic, syngeneic or xenogeneic cells.

Certain preferred embodiments of the present invention use dendritic cells or progenitors thereof as antigen-presenting cells. Dendritic cells are highly potent APCs (Banchereau and Steinman, *Nature* 392:245-251, 1998) and have been shown to be effective as a physiological adjuvant for eliciting prophylactic or therapeutic antitumor immunity (*see* Timmerman and Levy, *Ann. Rev. Med. 50*:507-529, 1999). In general, dendritic cells may be identified based on their typical shape (stellate *in situ*, with marked cytoplasmic processes (dendrites) visible *in vitro*), their ability to take up, process and present antigens with high efficiency and their ability to activate naïve T cell responses. Dendritic cells may, of course, be engineered to express specific cell-surface receptors or ligands that are not commonly found on dendritic cells *in vivo* or *ex vivo*, and such modified dendritic cells are contemplated by the present invention. As an alternative to dendritic cells, secreted vesicles antigen-loaded dendritic cells (called exosomes) may be used within a vaccine (*see* Zitvogel et al., *Nature Med. 4*:594-600, 1998).

Dendritic cells and progenitors may be obtained from peripheral blood, bone marrow, tumor-infiltrating cells, peritumoral tissues-infiltrating cells, lymph nodes, spleen, skin, umbilical cord blood or any other suitable tissue or fluid. For example, dendritic cells may be differentiated *ex vivo* by adding a combination of cytokines such as GM-CSF, IL-4, IL-13 and/or TNFα to cultures of monocytes harvested from peripheral blood. Alternatively, CD34 positive cells harvested from peripheral blood, umbilical cord blood or bone marrow may be differentiated into dendritic cells by adding to the culture medium combinations of GM-CSF, IL-3, TNFα, CD40 ligand, LPS, flt3 ligand and/or other compound(s) that induce differentiation, maturation and proliferation of dendritic cells.

Dendritic cells are conveniently categorized as "immature" and "mature" cells, which allows a simple way to discriminate between two well characterized phenotypes. However, this nomenclature should not be construed to exclude all possible intermediate stages of differentiation. Immature dendritic cells are characterized as APC with a high capacity for

antigen uptake and processing, which correlates with the high expression of Fcγ receptor and mannose receptor. The mature phenotype is typically characterized by a lower expression of these markers, but a high expression of cell surface molecules responsible for T cell activation such as class I and class II MHC, adhesion molecules (*e.g.,* CD54 and CD11) and costimulatory molecules (*e.g.,* CD40, CD80, CD86 and 4-1BB).

APCs may generally be transfected with a polynucleotide of the invention (or portion or other variant thereof) such that the encoded polypeptide, or an immunogenic portion thereof, is expressed on the cell surface. Such transfection may take place *ex vivo*, and a pharmaceutical composition comprising such transfected cells may then be used for therapeutic purposes, as described herein. Alternatively, a gene delivery vehicle that targets a dendritic or other antigen presenting cell may be administered to a patient, resulting in transfection that occurs *in vivo*. *In vivo* and *ex vivo* transfection of dendritic cells, for example, may generally be performed using any methods known in the art, such as those described in WO 97/24447, or the gene gun approach described by Mahvi et al., *Immunology and cell Biology* 75:456-460, 1997. Antigen loading of dendritic cells may be achieved by incubating dendritic cells or progenitor cells with the tumor polypeptide, DNA (naked or within a plasmid vector) or RNA; or with antigen-expressing recombinant bacterium or viruses (*e.g.,* vaccinia, fowlpox, adenovirus or lentivirus vectors). Prior to loading, the polypeptide may be covalently conjugated to an immunological partner that provides T cell help (*e.g.,* a carrier molecule). Alternatively, a dendritic cell may be pulsed with a non-conjugated immunological partner, separately or in the presence of the polypeptide.

While any suitable carrier known to those of ordinary skill in the art may be employed in the pharmaceutical compositions of this invention, the type of carrier will typically vary depending on the mode of administration. Compositions of the present invention may be formulated for any appropriate manner of administration, including for example, topical, oral, nasal, mucosal,

intravenous, intracranial, intraperitoneal, subcutaneous and intramuscular administration.

Carriers for use within such pharmaceutical compositions are biocompatible, and may also be biodegradable. In certain embodiments, the formulation preferably provides a relatively constant level of active component release. In other embodiments, however, a more rapid rate of release immediately upon administration may be desired. The formulation of such compositions is well within the level of ordinary skill in the art using known techniques. Illustrative carriers useful in this regard include microparticles of poly(lactide-co-glycolide), polyacrylate, latex, starch, cellulose, dextran and the like. Other illustrative delayed-release carriers include supramolecular biovectors, which comprise a non-liquid hydrophilic core (*e.g.*, a cross-linked polysaccharide or oligosaccharide) and, optionally, an external layer comprising an amphiphilic compound, such as a phospholipid (*see e.g.*, U.S. Patent No. 5,151,254 and PCT applications WO 94/20078, WO/94/23701 and WO 96/06638). The amount of active compound contained within a sustained release formulation depends upon the site of implantation, the rate and expected duration of release and the nature of the condition to be treated or prevented.

In another illustrative embodiment, biodegradable microspheres (*e.g.*, polylactate polyglycolate) are employed as carriers for the compositions of this invention. Suitable biodegradable microspheres are disclosed, for example, in U.S. Patent Nos. 4,897,268; 5,075,109; 5,928,647; 5,811,128; 5,820,883; 5,853,763; 5,814,344, 5,407,609 and 5,942,252. Modified hepatitis B core protein carrier systems. such as described in WO/99 40934, and references cited therein, will also be useful for many applications. Another illustrative carrier/delivery system employs a carrier comprising particulate-protein complexes, such as those described in U.S. Patent No. 5,928,647, which are capable of inducing a class I-restricted cytotoxic T lymphocyte responses in a host.

In another illustrative embodiment, calcium phosphate core particles are employed as carriers, vaccine adjuvants, or as controlled release matrices for the compositions of this invention. Exemplary calcium phosphate particles are disclosed, for example, in published patent application No. WO/0046147.

The pharmaceutical compositions of the invention will often further comprise one or more buffers (*e.g.*, neutral buffered saline or phosphate buffered saline), carbohydrates (*e.g.*, glucose, mannose, sucrose or dextrans), mannitol, proteins, polypeptides or amino acids such as glycine, antioxidants, bacteriostats, chelating agents such as EDTA or glutathione, adjuvants (*e.g.*, aluminum hydroxide), solutes that render the formulation isotonic, hypotonic or weakly hypertonic with the blood of a recipient, suspending agents, thickening agents and/or preservatives. Alternatively, compositions of the present invention may be formulated as a lyophilizate.

The pharmaceutical compositions described herein may be presented in unit-dose or multi-dose containers, such as sealed ampoules or vials. Such containers are typically sealed in such a way to preserve the sterility and stability of the formulation until use. In general, formulations may be stored as suspensions, solutions or emulsions in oily or aqueous vehicles. Alternatively, a pharmaceutical composition may be stored in a freeze-dried condition requiring only the addition of a sterile liquid carrier immediately prior to use.

The development of suitable dosing and treatment regimens for using the particular compositions described herein in a variety of treatment regimens, including *e.g.*, oral, parenteral, intravenous, intranasal, and intramuscular administration and formulation, is well known in the art, some of which are briefly discussed below for general purposes of illustration.

In certain applications, the pharmaceutical compositions disclosed herein may be delivered *via* oral administration to an animal. As such, these compositions may be formulated with an inert diluent or with an assimilable edible carrier, or they may be enclosed in hard- or soft-shell gelatin

capsule, or they may be compressed into tablets, or they may be incorporated directly with the food of the diet.

The active compounds may even be incorporated with excipients and used in the form of ingestible tablets, buccal tables, troches, capsules, elixirs, suspensions, syrups, wafers, and the like (see, for example, Mathiowitz *et al.*, Nature 1997 Mar 27;386(6623):410-4; Hwang *et al.*, Crit Rev Ther Drug Carrier Syst 1998;15(3):243-84; U. S. Patent 5,641,515; U. S. Patent 5,580,579 and U. S. Patent 5,792,451). Tablets, troches, pills, capsules and the like may also contain any of a variety of additional components, for example, a binder, such as gum tragacanth, acacia, cornstarch, or gelatin; excipients, such as dicalcium phosphate; a disintegrating agent, such as corn starch, potato starch, alginic acid and the like; a lubricant, such as magnesium stearate; and a sweetening agent, such as sucrose, lactose or saccharin may be added or a flavoring agent, such as peppermint, oil of wintergreen, or cherry flavoring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar, or both. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active compounds may be incorporated into sustained-release preparation and formulations.

Typically, these formulations will contain at least about 0.1% of the active compound or more, although the percentage of the active ingredient(s) may, of course, be varied and may conveniently be between about 1 or 2% and about 60% or 70% or more of the weight or volume of the total formulation. Naturally, the amount of active compound(s) in each therapeutically useful composition may be prepared is such a way that a suitable dosage will be obtained in any given unit dose of the compound. Factors such as solubility, bioavailability, biological half-life, route of administration, product shelf life, as well as other pharmacological

considerations will be contemplated by one skilled in the art of preparing such pharmaceutical formulations, and as such, a variety of dosages and treatment regimens may be desirable.

For oral administration the compositions of the present invention may alternatively be incorporated with one or more excipients in the form of a mouthwash, dentifrice, buccal tablet, oral spray, or sublingual orally-administered formulation. Alternatively, the active ingredient may be incorporated into an oral solution such as one containing sodium borate, glycerin and potassium bicarbonate, or dispersed in a dentifrice, or added in a therapeutically-effective amount to a composition that may include water, binders, abrasives, flavoring agents, foaming agents, and humectants. Alternatively the compositions may be fashioned into a tablet or solution form that may be placed under the tongue or otherwise dissolved in the mouth.

In certain circumstances it will be desirable to deliver the pharmaceutical compositions disclosed herein parenterally, intravenously, intramuscularly, or even intraperitoneally. Such approaches are well known to the skilled artisan, some of which are further described, for example, in U. S. Patent 5,543,158; U. S. Patent 5,641,515 and U. S. Patent 5,399,363. In certain embodiments, solutions of the active compounds as free base or pharmacologically acceptable salts may be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions may also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations generally will contain a preservative to prevent the growth of microorganisms.

Illustrative pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions (for example, see U. S. Patent 5,466,468). In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and

fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (*e.g.*, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and/or vegetable oils. Proper fluidity may be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and/or by the use of surfactants. The prevention of the action of microorganisms can be facilitated by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

In one embodiment, for parenteral administration in an aqueous solution, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, a sterile aqueous medium that can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage may be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. Moreover, for human administration, preparations will of course preferably meet sterility, pyrogenicity, and the general safety and purity standards as required by FDA Office of Biologics standards.

In another embodiment of the invention, the compositions disclosed herein may be formulated in a neutral or salt form. Illustrative pharmaceutically-acceptable salts include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids

such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like. Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective.

The carriers can further comprise any and all solvents, dispersion media, vehicles, coatings, diluents, antibacterial and antifungal agents, isotonic and absorption delaying agents, buffers, carrier solutions, suspensions, colloids, and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions. The phrase "pharmaceutically-acceptable" refers to molecular entities and compositions that do not produce an allergic or similar untoward reaction when administered to a human.

In certain embodiments, the pharmaceutical compositions may be delivered by intranasal sprays, inhalation, and/or other aerosol delivery vehicles. Methods for delivering genes, nucleic acids, and peptide compositions directly to the lungs *via* nasal aerosol sprays has been described, *e.g.*, in U. S. Patent 5,756,353 and U. S. Patent 5,804,212. Likewise, the delivery of drugs using intranasal microparticle resins (Takenaga *et al.*, J Controlled Release 1998 Mar 2;52(1-2):81-7) and lysophosphatidyl-glycerol compounds (U. S. Patent 5,725,871) are also well-known in the pharmaceutical arts. Likewise, illustrative transmucosal drug delivery in the form of a polytetrafluoroetheylene support matrix is described in U. S. Patent 5,780,045.

In certain embodiments, liposomes, nanocapsules, microparticles, lipid particles, vesicles, and the like, are used for the introduction of the

compositions of the present invention into suitable host cells/organisms. In particular, the compositions of the present invention may be formulated for delivery either encapsulated in a lipid particle, a liposome, a vesicle, a nanosphere, or a nanoparticle or the like. Alternatively, compositions of the present invention can be bound, either covalently or non-covalently, to the surface of such carrier vehicles.

The formation and use of liposome and liposome-like preparations as potential drug carriers is generally known to those of skill in the art (see for example, Lasic, Trends Biotechnol 1998 Jul;16(7):307-21; Takakura, Nippon Rinsho 1998 Mar;56(3):691-5; Chandran *et al.*, Indian J Exp Biol. 1997 Aug;35(8):801-9; Margalit, Crit Rev Ther Drug Carrier Syst. 1995;12(2-3):233-61; U.S. Patent 5,567,434; U.S. Patent 5,552,157; U.S. Patent 5,565,213; U.S. Patent 5,738,868 and U.S. Patent 5,795,587, each specifically incorporated herein by reference in its entirety).

Liposomes have been used successfully with a number of cell types that are normally difficult to transfect by other procedures, including T cell suspensions, primary hepatocyte cultures and PC 12 cells (Renneisen *et al.*, J Biol Chem. 1990 Sep 25;265(27):16337-42; Muller *et al.*, DNA Cell Biol. 1990 Apr;9(3):221-9). In addition, liposomes are free of the DNA length constraints that are typical of viral-based delivery systems. Liposomes have been used effectively to introduce genes, various drugs, radiotherapeutic agents, enzymes, viruses, transcription factors, allosteric effectors and the like, into a variety of cultured cell lines and animals. Furthermore, he use of liposomes does not appear to be associated with autoimmune responses or unacceptable toxicity after systemic delivery.

In certain embodiments, liposomes are formed from phospholipids that are dispersed in an aqueous medium and spontaneously form multilamellar concentric bilayer vesicles (also termed multilamellar vesicles (MLVs).

Alternatively, in other embodiments, the invention provides for pharmaceutically-acceptable nanocapsule formulations of the compositions of

the present invention. Nanocapsules can generally entrap compounds in a stable and reproducible way (see, for example, Quintanar-Guerrero *et al.*, Drug Dev Ind Pharm. 1998 Dec;24(12):1113-28). To avoid side effects due to intracellular polymeric overloading, such ultrafine particles (sized around 0.1 μm) may be designed using polymers able to be degraded *in vivo*. Such particles can be made as described, for example, by Couvreur *et al.*, Crit Rev Ther Drug Carrier Syst. 1988;5(1):1-20; zur Muhlen *et al.*, Eur J Pharm Biopharm. 1998 Mar;45(2):149-55; Zambaux *et al.* J Controlled Release. 1998 Jan 2;50(1-3):31-40; and U. S. Patent 5,145,684.

Therapy of Malignant Diseases

Immunologic approaches to cancer therapy are based on the recognition that cancer cells can often evade the body's defenses against aberrant or foreign cells and molecules, and that these defenses might be therapeutically stimulated to regain the lost ground, e.g. pgs. 623-648 in Klein, Immunology (Wiley-Interscience, New York, 1982). Numerous recent observations that various immune effectors can directly or indirectly inhibit growth of tumors has led to renewed interest in this approach to cancer therapy, e.g. Jager, et al., Oncology 2001;60(1):1-7; Renner, et al., Ann Hematol 2000 Dec;79(12):651-9.

Four-basic cell types whose function has been associated with antitumor cell immunity and the elimination of tumor cells from the body are: i) B-lymphocytes which secrete immunoglobulins into the blood plasma for identifying and labeling the nonself invader cells; ii) monocytes which secrete the complement proteins that are responsible for lysing and processing the immunoglobulin-coated target invader cells; iii) natural killer lymphocytes having two mechanisms for the destruction of tumor cells, antibody-dependent cellular cytotoxicity and natural killing; and iv) T-lymphocytes possessing antigen-specific receptors and having the capacity to recognize a tumor cell carrying complementary marker molecules (Schreiber, H., 1989, in Fundamental Immunology (ed). W. E. Paul, pp. 923-955).

Cancer immunotherapy generally focuses on inducing humoral immune responses, cellular immune responses, or both. Moreover, it is well established that induction of $CD4^+$ T helper cells is necessary in order to secondarily induce either antibodies or cytotoxic $CD8^+$ T cells. Polypeptide antigens that are selective or ideally specific for cancer cells, particularly cancer cells associated with WT1 expression, offer a powerful approach for inducing immune responses against cancer associated with WT1 expression, and are an important aspect of the present invention.

In further aspects of the present invention, the compositions and vaccines described herein may be used to inhibit the development of malignant diseases (*e.g.*, progressive or metastatic diseases or diseases characterized by small tumor burden such as minimal residual disease). In general, such methods may be used to prevent, delay or treat a disease associated with WT1 expression. In other words, therapeutic methods provided herein may be used to treat an existing WT1-associated disease, or may be used to prevent or delay the onset of such a disease in a patient who is free of disease or who is afflicted with a disease that is not yet associated with WT1 expression.

As used herein, a disease is "associated with WT1 expression" if diseased cells (*e.g.*, tumor cells) at some time during the course of the disease generate detectably higher levels of a WT1 polypeptide than normal cells of the same tissue. Association of WT1 expression with a malignant disease does not require that WT1 be present on a tumor. For example, overexpression of WT1 may be involved with initiation of a tumor, but the protein expression may subsequently be lost. Alternatively, a malignant disease that is not characterized by an increase in WT1 expression may, at a later time, progress to a disease that is characterized by increased WT1 expression. Accordingly, any malignant disease in which diseased cells formerly expressed, currently express or are expected to subsequently express increased levels of WT1 is considered to be "associated with WT1 expression."

Immunotherapy may be performed using any of a variety of techniques, in which compounds or cells provided herein function to remove

WT1-expressing cells from a patient. Such removal may take place as a result of enhancing or inducing an immune response in a patient specific for WT1 or a cell expressing WT1. Alternatively, WT1-expressing cells may be removed *ex vivo* (*e.g.*, by treatment of autologous bone marrow, peripheral blood or a fraction of bone marrow or peripheral blood). Fractions of bone marrow or peripheral blood may be obtained using any standard technique in the art.

Within such methods, pharmaceutical compositions and vaccines may be administered to a patient. As used herein, a "patient" refers to any warm-blooded animal, preferably a human. A patient may or may not be afflicted with a malignant disease. Accordingly, the above pharmaceutical compositions and vaccines may be used to prevent the onset of a disease (*i.e.*, prophylactically) or to treat a patient afflicted with a disease (*e.g.*, to prevent or delay progression and/or metastasis of an existing disease). A patient afflicted with a disease may have a minimal residual disease (*e.g.*, a low tumor burden in a leukemia patient in complete or partial remission or a cancer patient following reduction of the tumor burden after surgery radiotherapy and/or chemotherapy). Such a patient may be immunized to inhibit a relapse (*i.e.*, prevent or delay the relapse, or decrease the severity of a relapse). Within certain preferred embodiments, the patient is afflicted with a leukemia (*e.g.*, AML, CML, ALL or childhood ALL), a myelodysplastic syndrome (MDS) or a cancer (*e.g.*, gastrointestinal, lung, thyroid or breast cancer or a melanoma), where the cancer or leukemia is WT1 positive (*i.e.*, reacts detectably with an anti-WT1 antibody, as provided herein or expresses WT1 mRNA at a level detectable by RT-PCR, as described herein) or suffers from an autoimmune disease directed against WT1-expressing cells.

Other diseases associated with WT1 overexpression include kidney cancer (such as renal cell carcinoma, or Wilms tumor), as described in Satoh F., et al., *Pathol. Int.* 50(6):458-71(2000), and Campbell C. E. et al., *Int. J. Cancer* 78(2):182-8 (1998); and mesothelioma, as described in Amin, K.M. et al., *Am. J. Pathol.* 146(2):344-56 (1995). Harada et al. (*Mol. Urol.* 3(4):357-364 (1999) describe WT1 gene expression in human testicular germ-cell tumors.

Nonomura et al. *Hinyokika Kiyo* 45(8):593-7 (1999) describe molecular staging of testicular cancer using polymerase chain reaction of the testicular cancer-specific genes. Shimizu et al., *Int. J. Gynecol. Pathol.* 19(2):158-63 (2000) describe the immunohistochemical detection of the Wilms' tumor gene (WT1) in epithelial ovarian tumors.

WT1 overexpression was also described in desmoplastic small round cell tumors, by Barnoud, R. et al., *Am. J. Surg. Pathol.* 24(6):830-6 (2000); and *Pathol. Res. Pract.* 194(10):693-700 (1998). WT1 overexpression in glioblastoma and other cancer was described by Menssen, H.D. et al., *J. Cancer Res. Clin. Oncol.* 126(4):226-32 (2000), "Wilms' tumor gene (WT1) expression in lung cancer, colon cancer and glioblastoma cell lines compared to freshly isolated tumor specimens." Other diseases showing WT1 overexpression include EBV associated diseases, such as Burkitt's lymphoma and nasopharyngeal cancer (Spinsanti P. et al., *Leuk. Lymphoma* 38(5-6):611-9 (2000), "Wilms' tumor gene expression by normal and malignant human B lymphocytes."

In *Leukemia* 14(9):1634-4 (2000), Pan et al., describe *in vitro* IL-12 treatment of peripheral blood mononuclear cells from patients with leukemia or myelodysplastic syndromes, and reported an increase in cytotoxicity and reduction in WT1 gene expression. In *Leukemia* 13(6):891-900 (1999), Patmasiriwat et al. reported WT1 and GATA1 expression in myelodysplastic syndrome and acute leukemia. In *Leukemia* 13(3):393-9 (1999), Tamaki et al. reported that the Wilms' tumor gene WT1 is a good marker for diagnosis of disease progression of myelodysplastic syndromes. Expression of the Wilms' tumor gene WT1 in solid tumors, and its involvement in tumor cell growth, was discussed in relation to gastric cancer, colon cancer, lung cancer, breast cancer cell lines, germ cell tumor cell line, ovarian cancer, the uterine cancer, thyroid cancer cell line, hepatocellular carcinoma, in Oji et al., *Jpn. J. Cancer Res.* 90(2):194-204 (1999).

The compositions provided herein may be used alone or in combination with conventional therapeutic regimens such as surgery,

irradiation, chemotherapy and/or bone marrow transplantation (autologous, syngeneic, allogeneic or unrelated). As discussed in greater detail below, binding agents and T cells as provided herein may be used for purging of autologous stem cells. Such purging may be beneficial prior to, for example, bone marrow transplantation or transfusion of blood or components thereof. Binding agents, T cells, antigen presenting cells (APC) and compositions provided herein may further be used for expanding and stimulating (or priming) autologous, allogeneic, syngeneic or unrelated WT1-specific T-cells *in vitro* and/or *in vivo*. Such WT1-specific T cells may be used, for example, within donor lymphocyte infusions.

Routes and frequency of administration, as well as dosage, will vary from individual to individual, and may be readily established using standard techniques. In general, the pharmaceutical compositions and vaccines may be administered by injection (*e.g.*, intracutaneous, intramuscular, intravenous or subcutaneous), intranasally (*e.g.*, by aspiration) or orally. In some tumors, pharmaceutical compositions or vaccines may be administered locally (by, for example, rectocoloscopy, gastroscopy, videoendoscopy, angiography or other methods known in the art). Preferably, between 1 and 10 doses may be administered over a 52 week period. Preferably, 6 doses are administered, at intervals of 1 month, and booster vaccinations may be given periodically thereafter. Alternate protocols may be appropriate for individual patients. A suitable dose is an amount of a compound that, when administered as described above, is capable of promoting an anti-tumor immune response that is at least 10-50% above the basal (*i.e.*, untreated) level. Such response can be monitored by measuring the anti-tumor antibodies in a patient or by vaccine-dependent generation of cytolytic effector cells capable of killing the patient's tumor cells *in vitro*. Such vaccines should also be capable of causing an immune response that leads to an improved clinical outcome (*e.g.*, more frequent complete or partial remissions, or longer disease-free and/or overall survival) in vaccinated patients as compared to non-vaccinated patients. In general, for pharmaceutical compositions and vaccines comprising one or more

polypeptides, the amount of each polypeptide present in a dose ranges from about 100 μg to 5 mg. Suitable dose sizes will vary with the size of the patient, but will typically range from about 0.1 mL to about 5 mL.

In general, an appropriate dosage and treatment regimen provides the active compound(s) in an amount sufficient to provide therapeutic and/or prophylactic benefit. Such a response can be monitored by establishing an improved clinical outcome (*e.g.*, more frequent complete or partial remissions, or longer disease-free and/or overall survival) in treated patients as compared to non-treated patients. Increases in preexisting immune responses to WT1 generally correlate with an improved clinical outcome. Such immune responses may generally be evaluated using standard proliferation, cytotoxicity or cytokine assays, which may be performed using samples obtained from a patient before and after treatment.

Within certain embodiments, immunotherapy may be active immunotherapy, in which treatment relies on the *in vivo* stimulation of the endogenous host immune system to react against tumors with the administration of immune response-modifying agents (such as polypeptides and polynucleotides as provided herein).

Within other embodiments, immunotherapy may be passive immunotherapy, in which treatment involves the delivery of agents with established tumor-immune reactivity (such as effector cells or antibodies) that can directly or indirectly mediate antitumor effects and does not necessarily depend on an intact host immune system. Examples of effector cells include T cells as discussed above, T lymphocytes (such as $CD8^+$ cytotoxic T lymphocytes and $CD4^+$ T-helper tumor-infiltrating lymphocytes), killer cells (such as Natural Killer cells and lymphokine-activated killer cells), B cells and antigen-presenting cells (such as dendritic cells and macrophages) expressing a polypeptide provided herein. T cell receptors and antibody receptors specific for the polypeptides recited herein may be cloned, expressed and transferred into other vectors or effector cells for adoptive immunotherapy. The polypeptides provided herein may also be used to generate antibodies or anti-

idiotypic antibodies (as described above and in U.S. Patent No. 4,918,164) for passive immunotherapy.

Monoclonal antibodies may be labeled with any of a variety of labels for desired selective usages in detection, diagnostic assays or therapeutic applications (as described in U.S. Patent Nos. 6,090,365; 6,015,542; 5,843,398; 5,595,721; and 4,708,930, hereby incorporated by reference in their entirety as if each was incorporated individually). In each case, the binding of the labelled monoclonal antibody to the determinant site of the antigen will signal detection or delivery of a particular therapeutic agent to the antigenic determinant on the non-normal cell. A further object of this invention is to provide the specific monoclonal antibody suitably labelled for achieving such desired selective usages thereof.

Effector cells may generally be obtained in sufficient quantities for adoptive immunotherapy by growth *in vitro*, as described herein. Culture conditions for expanding single antigen-specific effector cells to several billion in number with retention of antigen recognition *in vivo* are well known in the art. Such *in vitro* culture conditions typically use intermittent stimulation with antigen, often in the presence of cytokines (such as IL-2) and non-dividing feeder cells. As noted above, immunoreactive polypeptides as provided herein may be used to rapidly expand antigen-specific T cell cultures in order to generate a sufficient number of cells for immunotherapy. In particular, antigen-presenting cells, such as dendritic, macrophage, monocyte, fibroblast and/or B cells, may be pulsed with immunoreactive polypeptides or transfected with one or more polynucleotides using standard techniques well known in the art. For example, antigen-presenting cells can be transfected with a polynucleotide having a promoter appropriate for increasing expression in a recombinant virus or other expression system. Cultured effector cells for use in therapy must be able to grow and distribute widely, and to survive long term *in vivo*. Studies have shown that cultured effector cells can be induced to grow in vivo and to survive long term in substantial numbers by repeated stimulation with antigen

supplemented with IL-2 (*see*, for example, Cheever et al., *Immunological Reviews 157*:177, 1997).

Alternatively, a vector expressing a polypeptide recited herein may be introduced into antigen presenting cells taken from a patient and clonally propagated *ex vivo* for transplant back into the same patient. Transfected cells may be reintroduced into the patient using any means known in the art, preferably in sterile form by intravenous, intracavitary, intraperitoneal or intratumor administration.

Within further aspects, methods for inhibiting the development of a malignant disease associated with WT1 expression involve the administration of autologous T cells that have been activated in response to a WT1 polypeptide or WT1-expressing APC, as described above. Such T cells may be $CD4^+$ and/or $CD8^+$, and may be proliferated as described above. The T cells may be administered to the individual in an amount effective to inhibit the development of a malignant disease. Typically, about 1×10^9 to 1×10^{11} T cells/M^2 are administered intravenously, intracavitary or in the bed of a resected tumor. It will be evident to those skilled in the art that the number of cells and the frequency of administration will be dependent upon the response of the patient.

Within certain embodiments, T cells may be stimulated prior to an autologous bone marrow transplantation. Such stimulation may take place *in vivo* or *in vitro*. For *in vitro* stimulation, bone marrow and/or peripheral blood (or a fraction of bone marrow or peripheral blood) obtained from a patient may be contacted with a WT1 polypeptide, a polynucleotide encoding a WT1 polypeptide and/or an APC that expresses a WT1 polypeptide under conditions and for a time sufficient to permit the stimulation of T cells as described above. Bone marrow, peripheral blood stem cells and/or WT1-specific T cells may then be administered to a patient using standard techniques.

Within related embodiments, T cells of a related or unrelated donor may be stimulated prior to a syngeneic or allogeneic (related or unrelated) bone marrow transplantation. Such stimulation may take place *in*

vivo or *in vitro*. For *in vitro* stimulation, bone marrow and/or peripheral blood (or a fraction of bone marrow or peripheral blood) obtained from a related or unrelated donor may be contacted with a WT1 polypeptide, WT1 polynucleotide and/or APC that expresses a WT1 polypeptide under conditions and for a time sufficient to permit the stimulation of T cells as described above. Bone marrow, peripheral blood stem cells and/or WT1-specific T cells may then be administered to a patient using standard techniques.

Within other embodiments, WT1-specific T cells as described herein may be used to remove cells expressing WT1 from autologous bone marrow, peripheral blood or a fraction of bone marrow or peripheral blood (*e.g.*, $CD34^+$ enriched peripheral blood (PB) prior to administration to a patient). Such methods may be performed by contacting bone marrow or PB with such T cells under conditions and for a time sufficient to permit the reduction of WT1 expressing cells to less than 10%, preferably less than 5% and more preferably less than 1%, of the total number of myeloid or lymphatic cells in the bone marrow or peripheral blood. The extent to which such cells have been removed may be readily determined by standard methods such as, for example, qualitative and quantitative PCR analysis, morphology, immunohistochemistry and FACS analysis. Bone marrow or PB (or a fraction thereof) may then be administered to a patient using standard techniques.

Cancer Detection and Diagnostic Compositions, Methods and Kits

In general, a cancer associated with WT1 expression may be detected in a patient based on the presence of one or more WT1 proteins and/or polynucleotides encoding such proteins in a biological sample (for example, blood, sera, sputum urine and/or tumor biopsies) obtained from the patient. In other words, such WT1 proteins may be used as markers to indicate the presence or absence of a cancer. The binding agents provided herein generally permit detection of the level of antigen that binds to the agent in the biological sample.

Polynucleotide primers and probes may be used to detect the level of mRNA encoding a WT1 protein, which is also indicative of the presence or absence of a cancer. In general, a WT1 sequence should be present at a level that is at least two-fold, preferably three-fold, and more preferably five-fold or higher in tumor tissue than in normal tissue of the same type from which the tumor arose. Expression levels of WT1 in tissue types different from that in which the tumor arose are irrelevant in certain diagnostic embodiments since the presence of tumor cells can be confirmed by observation of predetermined differential expression levels, e.g., 2-fold, 5-fold, etc, in tumor tissue to expression levels in normal tissue of the same type.

Other differential expression patterns can be utilized advantageously for diagnostic purposes. For example, in one aspect of the invention, overexpression of WT1 sequence in tumor tissue and normal tissue of the same type, but not in other normal tissue types, *e.g.* PBMCs, can be exploited diagnostically. In this case, the presence of metastatic tumor cells, for example in a sample taken from the circulation or some other tissue site different from that in which the tumor arose, can be identified and/or confirmed by detecting expression of the tumor sequence in the sample, for example using RT-PCR analysis. In many instances, it will be desired to enrich for tumor cells in the sample of interest, e.g., PBMCs, using cell capture or other like techniques.

There are a variety of assay formats known to those of ordinary skill in the art for using a binding agent to detect WT1 polypeptide markers in a sample. *See, e.g.*, Harlow and Lane, *Antibodies: A Laboratory Manual,* Cold Spring Harbor Laboratory, 1988. In general, the presence or absence of a cancer associated with WT1 in a patient may be determined by (a) contacting a biological sample obtained from a patient with a binding agent; (b) detecting in the sample a level of WT1 polypeptide that binds to the binding agent; and (c) comparing the level of WT1 polypeptide with a predetermined cut-off value.

In a preferred embodiment, the assay involves the use of binding agent immobilized on a solid support to bind to and remove the WT1

polypeptide from the remainder of the sample. The bound WT1 polypeptide may then be detected using a detection reagent that contains a reporter group and specifically binds to the binding agent/WT1 polypeptide complex. Such detection reagents may comprise, for example, a binding agent that specifically binds to a WT1 polypeptide or an antibody or other agent that specifically binds to the binding agent, such as an anti-immunoglobulin, protein G, protein A or a lectin. Alternatively, a competitive assay may be utilized, in which a WT1 polypeptide is labeled with a reporter group and allowed to bind to the immobilized binding agent after incubation of the binding agent with the sample. The extent to which components of the sample inhibit the binding of the labeled WT1 polypeptide to the binding agent is indicative of the reactivity of the sample with the immobilized binding agent. Suitable polypeptides for use within such assays include full length WT1 proteins and polypeptide portions thereof to which the binding agent binds, as described above.

The solid support may be any material known to those of ordinary skill in the art to which the WT1 protein may be attached. For example, the solid support may be a test well in a microtiter plate or a nitrocellulose or other suitable membrane. Alternatively, the support may be a bead or disc, such as glass, fiberglass, latex or a plastic material such as polystyrene or polyvinylchloride. The support may also be a magnetic particle or a fiber optic sensor, such as those disclosed, for example, in U.S. Patent No. 5,359,681. The binding agent may be immobilized on the solid support using a variety of techniques known to those of skill in the art, which are amply described in the patent and scientific literature. In the context of the present invention, the term "immobilization" refers to both noncovalent association, such as adsorption, and covalent attachment (which may be a direct linkage between the agent and functional groups on the support or may be a linkage by way of a cross-linking agent). Immobilization by adsorption to a well in a microtiter plate or to a membrane is preferred. In such cases, adsorption may be achieved by contacting the binding agent, in a suitable buffer, with the solid support for a suitable amount of time. The contact time varies with temperature, but is

typically between about 1 hour and about 1 day. In general, contacting a well of a plastic microtiter plate (such as polystyrene or polyvinylchloride) with an amount of binding agent ranging from about 10 ng to about 10 μg, and preferably about 100 ng to about 1 μg, is sufficient to immobilize an adequate amount of binding agent.

Covalent attachment of binding agent to a solid support may generally be achieved by first reacting the support with a bifunctional reagent that will react with both the support and a functional group, such as a hydroxyl or amino group, on the binding agent. For example, the binding agent may be covalently attached to supports having an appropriate polymer coating using benzoquinone or by condensation of an aldehyde group on the support with an amine and an active hydrogen on the binding partner (*see, e.g.*, Pierce Immunotechnology Catalog and Handbook, 1991, at A12-A13).

In certain embodiments, the assay is a two-antibody sandwich assay. This assay may be performed by first contacting an antibody that has been immobilized on a solid support, commonly the well of a microtiter plate, with the sample, such that WT1 polypeptides within the sample are allowed to bind to the immobilized antibody. Unbound sample is then removed from the immobilized polypeptide-antibody complexes and a detection reagent (preferably a second antibody capable of binding to a different site on the polypeptide) containing a reporter group is added. The amount of detection reagent that remains bound to the solid support is then determined using a method appropriate for the specific reporter group.

More specifically, once the antibody is immobilized on the support as described above, the remaining protein binding sites on the support are typically blocked. Any suitable blocking agent known to those of ordinary skill in the art, such as bovine serum albumin or Tween 20™ (Sigma Chemical Co., St. Louis, MO). The immobilized antibody is then incubated with the sample, and polypeptide is allowed to bind to the antibody. The sample may be diluted with a suitable diluent, such as phosphate-buffered saline (PBS) prior to incubation. In general, an appropriate contact time (*i.e.*, incubation time) is a

period of time that is sufficient to detect the presence of WT1 polypeptide within a sample obtained from an individual with a cancer associated with WT1 least about 95% of that achieved at equilibrium between bound and unbound polypeptide. Those of ordinary skill in the art will recognize that the time necessary to achieve equilibrium may be readily determined by assaying the level of binding that occurs over a period of time. At room temperature, an incubation time of about 30 minutes is generally sufficient.

Unbound sample may then be removed by washing the solid support with an appropriate buffer, such as PBS containing 0.1% Tween 20™. The second antibody, which contains a reporter group, may then be added to the solid support. Preferred reporter groups include those groups recited above.

The detection reagent is then incubated with the immobilized antibody-polypeptide complex for an amount of time sufficient to detect the bound polypeptide. An appropriate amount of time may generally be determined by assaying the level of binding that occurs over a period of time. Unbound detection reagent is then removed and bound detection reagent is detected using the reporter group. The method employed for detecting the reporter group depends upon the nature of the reporter group. For radioactive groups, scintillation counting or autoradiographic methods are generally appropriate. Spectroscopic methods may be used to detect dyes, luminescent groups and fluorescent groups. Biotin may be detected using avidin, coupled to a different reporter group (commonly a radioactive or fluorescent group or an enzyme). Enzyme reporter groups may generally be detected by the addition of substrate (generally for a specific period of time), followed by spectroscopic or other analysis of the reaction products.

To determine the presence or absence of a cancer associated with WT1 expression the signal detected from the reporter group that remains bound to the solid support is generally compared to a signal that corresponds to a predetermined cut-off value. In one preferred embodiment, the cut-off value for the detection of a cancer associated with WT1 is the average mean

signal obtained when the immobilized antibody is incubated with samples from patients without the cancer. In general, a sample generating a signal that is three standard deviations above the predetermined cut-off value is considered positive for the cancer. In an alternate preferred embodiment, the cut-off value is determined using a Receiver Operator Curve, according to the method of Sackett et al., *Clinical Epidemiology: A Basic Science for Clinical Medicine*, Little Brown and Co., 1985, p. 106-7. Briefly, in this embodiment, the cut-off value may be determined from a plot of pairs of true positive rates (*i.e.*, sensitivity) and false positive rates (100%-specificity) that correspond to each possible cut-off value for the diagnostic test result. The cut-off value on the plot that is the closest to the upper left-hand corner (*i.e.*, the value that encloses the largest area) is the most accurate cut-off value, and a sample generating a signal that is higher than the cut-off value determined by this method may be considered positive. Alternatively, the cut-off value may be shifted to the left along the plot, to minimize the false positive rate, or to the right, to minimize the false negative rate. In general, a sample generating a signal that is higher than the cut-off value determined by this method is considered positive for a cancer.

In a related embodiment, the assay is performed in a flow-through or strip test format, wherein the binding agent is immobilized on a membrane, such as nitrocellulose. In the flow-through test, polypeptides within the sample bind to the immobilized binding agent as the sample passes through the membrane. A second, labeled binding agent then binds to the binding agent-polypeptide complex as a solution containing the second binding agent flows through the membrane. The detection of bound second binding agent may then be performed as described above. In the strip test format, one end of the membrane to which binding agent is bound is immersed in a solution containing the sample. The sample migrates along the membrane through a region containing second binding agent and to the area of immobilized binding agent. Concentration of second binding agent at the area of immobilized antibody indicates the presence of a cancer. Typically, the concentration of second binding agent at that site generates a pattern, such as a line, that can be read

visually. The absence of such a pattern indicates a negative result. In general, the amount of binding agent immobilized on the membrane is selected to generate a visually discernible pattern when the biological sample contains a level of polypeptide that would be sufficient to generate a positive signal in the two-antibody sandwich assay, in the format discussed above. Preferred binding agents for use in such assays are antibodies and antigen-binding fragments thereof. Preferably, the amount of antibody immobilized on the membrane ranges from about 25 ng to about 1μg, and more preferably from about 50 ng to about 500 ng. Such tests can typically be performed with a very small amount of biological sample.

Of course, numerous other assay protocols exist that are suitable for use with the WT1 proteins or binding agents of the present invention. The above descriptions are intended to be exemplary only. For example, it will be apparent to those of ordinary skill in the art that the above protocols may be readily modified to use tumor polypeptides to detect antibodies that bind to such polypeptides in a biological sample. The detection of such WT1-specific antibodies may correlate with the presence of a cancer associated with WT1 expression.

A cancer associated with WT1 expression may also, or alternatively, be detected based on the presence of T cells that specifically react with a tumor protein in a biological sample. Within certain methods, a biological sample comprising $CD4^+$ and/or $CD8^+$ T cells isolated from a patient is incubated with a WT1 polypeptide, a polynucleotide encoding such a polypeptide and/or an APC that expresses at least an immunogenic portion of such a polypeptide, and the presence or absence of specific activation of the T cells is detected. Suitable biological samples include, but are not limited to, isolated T cells. For example, T cells may be isolated from a patient by routine techniques (such as by Ficoll/Hypaque density gradient centrifugation of peripheral blood lymphocytes). T cells may be incubated *in vitro* for 2-9 days (typically 4 days) at 37□C with polypeptide (*e.g.*, 5 - 25 □g/ml). It may be desirable to incubate another aliquot of a T cell sample in the absence of WT1

polypeptide to serve as a control. For $CD4^+$ T cells, activation is preferably detected by evaluating proliferation of the T cells. For $CD8^+$ T cells, activation is preferably detected by evaluating cytolytic activity. A level of proliferation that is at least two fold greater and/or a level of cytolytic activity that is at least 20% greater than in disease-free patients indicates the presence of a cancer associated with WT1 expression in the patient.

As noted above, a cancer may also, or alternatively, be detected based on the level of mRNA encoding a WT1 protein in a biological sample. For example, at least two oligonucleotide primers may be employed in a polymerase chain reaction (PCR) based assay to amplify a portion of a WT1 cDNA derived from a biological sample, wherein at least one of the oligonucleotide primers is specific for (*i.e.*, hybridizes to) a polynucleotide encoding the WT1 protein. The amplified cDNA is then separated and detected using techniques well known in the art, such as gel electrophoresis.

Similarly, oligonucleotide probes that specifically hybridize to a polynucleotide encoding a WT1 protein may be used in a hybridization assay to detect the presence of polynucleotide encoding the WT1 protein in a biological sample.

To permit hybridization under assay conditions, oligonucleotide primers and probes should comprise an oligonucleotide sequence that has at least about 60%, preferably at least about 75% and more preferably at least about 90%, identity to a portion of a polynucleotide encoding a WT1 protein of the invention that is at least 10 nucleotides, and preferably at least 20 nucleotides, in length. Preferably, oligonucleotide primers and/or probes hybridize to a polynucleotide encoding a polypeptide described herein under moderately stringent conditions, as defined above. Oligonucleotide primers and/or probes which may be usefully employed in the diagnostic methods described herein preferably are at least 10-40 nucleotides in length. In a preferred embodiment, the oligonucleotide primers comprise at least 10 contiguous nucleotides, more preferably at least 15 contiguous nucleotides, of a DNA molecule having a sequence as disclosed herein. Techniques for both

PCR based assays and hybridization assays are well known in the art (*see*, for example, Mullis et al., *Cold Spring Harbor Symp. Quant. Biol.*, *51*:263, 1987; Erlich ed., *PCR Technology*, Stockton Press, NY, 1989).

One preferred assay employs RT-PCR, in which PCR is applied in conjunction with reverse transcription. Typically, RNA is extracted from a biological sample, such as biopsy tissue, and is reverse transcribed to produce cDNA molecules. PCR amplification using at least one specific primer generates a cDNA molecule, which may be separated and visualized using, for example, gel electrophoresis. Amplification may be performed on biological samples taken from a test patient and from an individual who is not afflicted with a cancer. The amplification reaction may be performed on several dilutions of cDNA spanning two orders of magnitude. A two-fold or greater increase in expression in several dilutions of the test patient sample as compared to the same dilutions of the non-cancerous sample is typically considered positive.

In another aspect of the present invention, cell capture technologies may be used in conjunction, with, for example, real-time PCR to provide a more sensitive tool for detection of metastatic cells expressing WT1 antigens. Detection of WT1-associated cancer cells in biological samples, e.g., bone marrow samples, peripheral blood, and small needle aspiration samples is desirable for diagnosis and prognosis in patients with cancer associated with WT1 expression.

Immunomagnetic beads coated with specific monoclonal antibodies to surface cell markers, or tetrameric antibody complexes, may be used to first enrich or positively select cancer cells in a sample. Various commercially available kits may be used, including Dynabeads® Epithelial Enrich (Dynal Biotech, Oslo, Norway), StemSep™ (StemCell Technologies, Inc., Vancouver, BC), and RosetteSep (StemCell Technologies). A skilled artisan will recognize that other methodologies and kits may also be used to enrich or positively select desired cell populations. Dynabeads® Epithelial Enrich contains magnetic beads coated with mAbs specific for two glycoprotein

membrane antigens expressed on normal and neoplastic epithelial tissues. The coated beads may be added to a sample and the sample then applied to a magnet, thereby capturing the cells bound to the beads. The unwanted cells are washed away and the magnetically isolated cells eluted from the beads and used in further analyses.

RosetteSep can be used to enrich cells directly from a blood sample and consists of a cocktail of tetrameric antibodies that targets a variety of unwanted cells and crosslinks them to glycophorin A on red blood cells (RBC) present in the sample, forming rosettes. When centrifuged over Ficoll, targeted cells pellet along with the free RBC. The combination of antibodies in the depletion cocktail determines which cells will be removed and consequently which cells will be recovered. Antibodies that are available include, but are not limited to: CD2, CD3, CD4, CD5, CD8, CD10, CD11b, CD14, CD15, CD16, CD19, CD20, CD24, CD25, CD29, CD33, CD34, CD36, CD38, CD41, CD45, CD45RA, CD45RO, CD56, CD66B, CD66e, HLA-DR, IgE, and TCR$\alpha\beta$.

In another embodiment, the compositions described herein may be used as markers for the progression of cancer. In this embodiment, assays as described above for the diagnosis of a cancer associated with WT1 expression may be performed over time, and the change in the level of reactive polypeptide(s) or polynucleotide(s) evaluated. For example, the assays may be performed every 24-72 hours for a period of 6 months to 1 year, and thereafter performed as needed. In general, a cancer is progressing in those patients in whom the level of WT1 polypeptide or polynucleotide detected increases over time. In contrast, the cancer is not progressing when the level of reactive polypeptide or polynucleotide either remains constant or decreases with time.

Certain *in vivo* diagnostic assays may be performed directly on a tumor. One such assay involves contacting tumor cells with a binding agent. The bound binding agent may then be detected directly or indirectly via a reporter group. Such binding agents may also be used in histological applications. Alternatively, polynucleotide probes may be used within such applications.

The present invention further provides kits for use within any of the above diagnostic methods. Such kits typically comprise two or more components necessary for performing a diagnostic assay. Components may be compounds, reagents, containers and/or equipment. For example, one container within a kit may contain a monoclonal antibody or fragment thereof that specifically binds to a WT1 protein. Such antibodies or fragments may be provided attached to a support material, as described above. One or more additional containers may enclose elements, such as reagents or buffers, to be used in the assay. Such kits may also, or alternatively, contain a detection reagent as described above that contains a reporter group suitable for direct or indirect detection of antibody binding.

Alternatively, a kit may be designed to detect the level of mRNA encoding a WT1 protein in a biological sample. Such kits generally comprise at least one oligonucleotide probe or primer, as described above, that hybridizes to a polynucleotide encoding a WT1 protein. Such an oligonucleotide may be used, for example, within a PCR or hybridization assay. Additional components that may be present within such kits include a second oligonucleotide and/or a diagnostic reagent or container to facilitate the detection of a polynucleotide encoding a WT1 protein.

The following Examples are offered by way of illustration and not by way of limitation.

EXAMPLES

EXAMPLE 1

IDENTIFICATION OF AN IMMUNE RESPONSE TO WT1 IN PATIENTS WITH HEMATOLOGICAL MALIGNANCIES

This Example illustrates the identification of an existent immune response in patients with a hematological malignancy.

To evaluate the presence of preexisting WT1 specific antibody responses in patients, sera of patients with acute myelogenous leukemia

(AML), acute lymphocytic leukemia (ALL), chronic myelogenous leukemia (CML) and severe aplastic anemia were analyzed using Western blot analysis. Sera were tested for the ability to immunoprecipitate WT1 from the human leukemic cell line K562 (American Type Culture Collection, Manassas, VA). In each case, immunoprecipitates were separated by gel electrophoresis, transferred to membrane and probed with the anti WT1 antibody WT180 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). This Western blot analysis identified potential WT1 specific antibodies in patients with hematological malignancy. A representative Western blot showing the results for a patient with AML is shown in Figure 2. A 52 kD protein in the immunoprecipitate generated using the patient sera was recognized by the WT1 specific antibody. The 52 kD protein migrated at the same size as the positive control.

Additional studies analyzed the sera of patients with AML and CML for the presence of antibodies to full-length and truncated WT1 proteins. CDNA constructs representing the human WT1/full-length (aa 1-449), the N-terminus (aa 1-249) (WT1/N-terminus) and C-terminus (aa 267-449) (WT1/C-terminus) region were subcloned into modified pET28 vectors. The WT1/full-length and WT1/N-terminus proteins were expressed as Ral2 fusion proteins. Ra12 is the C-terminal fragment of a secreted Mycobacterium tuberculosis protein, denoted as MTB32B. (Skeiky et al., *Infect Immun. 67*;3998, 1999). The Ra12-WT1/full-length fusion region was cloned 3' to a histidine-tag in a histidine-tag modified pET28 vector. The WT1/N-terminus region was subcloned into a modified pET28 vector that has a 5' histidine-tag followed by the thioredoxin (TRX)-WT1/N-terminus fusion region followed by a 3' histidine-tag. The WT1/C-terminus coding region was subcloned into a modified pET28 vector without a fusion partner containing only the 5' and 3' histidine-tag, followed by a Thrombin and EK site.

BL21 pLysS *E. coli* (Stratagene, La Jolla, CA) were transformed with the three WT1 expression constructs, grown overnight and induced with isopropyl-β-D-thiogalactoside (IPTG). WT1 proteins were purified as follows: Cells were harvested and lysed by incubation in 10mM Tris, pH 8.0 with

Complete Protease Inhibitor Tablets (Boehringer Mannheim Biochemicals, Indianapolis, IN) at 37°C followed by repeated rounds of sonication. Inclusion bodies were washed twice with 10mM Tris, pH 8.0. Proteins were then purified by metal chelate affinity chromatography over nickel-nitrilotriacetic acid resin (QIAGEN Inc., Valencia, CA; Hochuli et al., *Biologically Active Molecules* :217, 1989) followed by chromatography on a Source Q anion exchange resin (Amersham Pharmacia Biotech, Upsala, Sweden). The identity of the WT1 proteins was confirmed by N-terminal sequencing.

Sera from adult patients with *de nova* AML or CML were studied for the presence of WT1 specific Ab. Recombinant proteins were adsorbed to TC microwell plates (Nunc, Roskilde, Denmark). Plates were washed with PBS/0.5%Tween 20 and blocked with 1% BSA/PBS/0.1%Tween 20. After washing, serum dilutions were added and incubated overnight at 4°C. Plates were washed and Donkey anti-human IgG-HRP secondary antibody was added (Jackson-Immunochem, West Grove, PA) and incubated for 2h at room temperature. Plates were washed, incubated with TMB Peroxidase substrate solution (Kirkegaard and Perry Laboratories, MA), quenched with 1N H_2SO_4, and immediately read (Cyto-Fluor 2350; Millipore, Bedford, MA).

For the serological survey, human sera were tested by ELISA over a range of serial dilutions from 1:50 to 1:20,000. A positive reaction was defined as an OD value of a 1:500 diluted serum that exceeded the mean OD value of sera from normal donors (n=96) by three (WT1/full-length, WT1C-terminus) standard deviations. Due to a higher background in normal donors to the WT1/N-terminus protein a positive reaction to WT1/N-terminus was defined as an OD value of 1:500 diluted serum that exceeded the mean OD value of sera from normal donors by four standard deviations. To verify that the patient Ab response was directed against WT1 and not to the Ra12 or TRX fusion part of the protein or possible *E. coli* contaminant proteins, controls included the Ra12 and TRX protein alone purified in a similar manner. Samples that showed reactivity against the Ra12 and/or TRX proteins were excluded from the analysis.

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To evaluate for the presence of immunity to WT1, Ab to recombinant full-length and truncated WT1 proteins in the sera of normal individuals and patients with leukemia were determined. Antibody reactivity was analyzed by ELISA reactivity to WT1/full-length protein, WT1/N-terminus protein and WT1/C-terminus protein.

Only 2 of 96 normal donors had serum antibodies reactive with WT1/full-length protein (Figure 18). One of those individuals had antibody to WT1/N-terminus protein and one had antibody to WT1/C-terminus protein. In contrast, 16 of 63 patients (25%) with AML had serum antibodies reactive with WT1/full-length protein. By marked contrast, only 2 of 63 patients (3%) had reactivity to WT1/C-terminus protein. Fifteen of 81 patients (19%) with CML had serum antibodies reactive with WT1/full-length protein and 12 of 81 patients (15%) had serum antibodies reactive with WT1/N-terminus. Only 3 of 81 patients (3%) had reactivity to WT1/C-terminus protein. (Figures 16 and 17.)

These data demonstrate that Ab responses to WT1 are detectable in some patients with AML and CML. The greater incidence of antibody in leukemia patients provides strong evidence that immunization to the WT1 protein occurred as a result of patients bearing malignancy that expresses or at some time expressed WT1. Without being limited to a specific theory, it is believed that the observed antibody responses to WT1 most probably result from patients becoming immune to WT1 on their own leukemia cells and provide direct evidence that WT1 can be immunogenic despite being a "self" protein.

The presence of antibody to WT1 strongly implies that concurrent helper T cell responses are also present in the same patients. WT1 is an internal protein. Thus, CTL responses are likely to be the most effective in terms of leukemia therapy and the most toxic arm of immunity. Thus, these data provide evidence that therapeutic vaccines directed against WT1 will be able to elicit an immune response to WT1.

EXAMPLE 3

INDUCTION OF TH AND ANTIBODY RESPONSES IN MICE IMMUNIZED WITH WT1 PEPTIDES

This Example illustrates the ability of immunization with WT1 peptides to elicit an immune response specific for WT1.

Peptides suitable for eliciting Ab and proliferative T cell responses were identified according to the Tsites program (Rothbard and Taylor, *EMBO J.* 7:93-100, 1988; Deavin et al., *Mol. Immunol.* 33:145-155, 1996), which searches for peptide motifs that have the potential to elicit Th responses. Peptides shown in Table I were synthesized and sequenced.

Table I

WT1 Peptides

Peptide	Sequence	Comments
Mouse: p6-22	RDLNALLPAVSSLGGGG (SEQ ID NO:13)	1 mismatch relative to human WT1 sequence
Human: p6-22	RDLNALLPAVPSLGGGG (SEQ ID NO:1)	
Human/mouse: p117-139	PSQASSGQARMFPNAPYLPSCLE (SEQ ID NOs: 2 and 3)	
Mouse: p244-262	GATLKGMAAGSSSSVKWTE (SEQ ID NO:14)	1 mismatch relative to human WT1 sequence
Human: p244-262	GATLKGVAAGSSSSVKWTE (SEQ ID NO:4)	
Human/mouse: p287-301	RIHTHGVFRGIQDVR (SEQ ID NOs: 15 and 16)	
Mouse: p299-313	VRRVSGVAPTLVRS (SEQ ID NO:17)	1 mismatch relative to human WT1 sequence
Human/mouse: p421-435	CQKKFARSDELVRHH (SEQ ID NOs: 19 and 20)	

For immunization, peptides were grouped as follows:

proliferative T cell responses (Figures 5A-5C). The stimulation indices (SI) varied between 8 and 72. Other peptides (P6-22 and P299-313) also were shown to elicit proliferative T cell responses. Immunization with P6-22 resulted in a stimulation index (SI) of 2.3 and immunization with P299-313 resulted in a SI of 3.3. Positive controls included ConA stimulated T cells, as well as T cells stimulated with known antigens, such as CD45 and FBL, and allogeneic T cell lines (DeBruijn et al., *Eur. J. Immunol. 21*:2963-2970, 1991).

Figures 6A and 6B show the proliferative response observed for each of the three peptides within vaccine A (Figure 6A) and vaccine B (Figure 6B). Vaccine A elicited proliferative T cell responses to the immunizing peptides p6-22 and p117-139, with stimulation indices (SI) varying between 3 and 8 (bulk lines). No proliferative response to p244-262 was detected (Figure 6A).

Subsequent *in vitro* stimulations were carried out as single peptide stimulations using only p6-22 and p117-139. Stimulation of the Vaccine A specific T cell line with p117-139 resulted in proliferation to p117-139 with no response to p6-22 (Figure7A). Clones derived from the line were specific for p117-139 (Figure 7B). By contrast, stimulation of the Vaccine A specific T cell line with p6-22 resulted in proliferation to p6-22 with no response to p117-139 (Figure 7C). Clones derived from the line were specific for p6-22 (Figure 7D).

These results show that vaccination with WT1 peptides can elicit antibody responses to WT1 protein and proliferative T cell responses to the immunizing peptides.

EXAMPLE 4

INDUCTION OF CTL RESPONSES IN MICE IMMUNIZED WITH WT1 PEPTIDES

This Example illustrates the ability of WT1 peptides to elicit CTL immunity.

Peptides (9-mers) with motifs appropriate for binding to class I MHC were identified using a BIMAS HLA peptide binding prediction analysis (Parker et al., *J. Immunol. 152*:163, 1994). Peptides identified within such

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
17	427	RSDELVRHH (SEQ ID NO:191)	0.750
18	394	RSDHLKTHT (SEQ ID NO:192)	0.750
19	317	TSEKRPFMC (SEQ ID NO:233)	0.675
20	213	QALLLRTPY (SEQ ID NO:160)	0.500

Table III

Results of BIMAS HLA Peptide Binding Prediction Analysis for

Binding of Human WT1 Peptides to Human HLA A 0201

(HLA-A0201 peptide binding motif anchor residues are L or M at P2; V or L at P9; with an auxiliary anchor being V at P6)

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	126	RMFPNAPYL (SEQ ID NO:185)	313.968
2	187	SLGEQQYSV (SEQ ID NO:214)	285.163
3	10	ALLPAVPSL (SEQ ID NO:34)	181.794
4	242	NLGATLKGV (SEQ ID NO:146)	159.970
5	225	NLYQMTSQL (SEQ ID NO:147)	68.360
6	292	GVFRGIQDV (SEQ ID NO:103)	51.790
7	191	QQYSVPPPV (SEQ ID NO:171)	22.566
8	280	ILCGAQYRI (SEQ ID NO:116)	17.736
9	235	CMTWNQMNL (SEQ ID NO:49)	15.428
10	441	NMTKLQLAL (SEQ ID NO:149)	15.428
11	7	DLNALLPAV (SEQ ID NO:58)	11.998
12	227	YQMTSQLEC (SEQ ID NO:251)	8.573
13	239	NQMNLGATL (SEQ ID NO:151)	8.014
14	309	TLVRSASET (SEQ ID NO:226)	7.452
15	408	KTSEKPFSC (SEQ ID NO:129)	5.743
16	340	LQMHSRKHT (SEQ ID NO:139)	4.752
17	228	QMTSQLECM (SEQ ID NO:169)	4.044
18	93	TVHFSGQFT (SEQ ID NO:235)	3.586
19	37	VLDFAPPGA (SEQ ID NO:241)	3.378
20	86	EQCLSAFTV (SEQ ID NO:69)	3.068

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
2	218	RTPYSSDNL (SEQ ID NO:194)	12.000
3	356	DFKDCERRF (SEQ ID NO:55)	12.000
4	126	RMFPNAPYL (SEQ ID NO:185)	9.600
5	326	AYPGCNKRY (SEQ ID NO:42)	7.500
6	270	GYESDNHT (SEQ ID NO:106)T	7.500
7	239	NQMNLGATL (SEQ ID NO:151)	7.200
8	10	ALLPAVPSL (SEQ ID NO:34)	7.200
9	130	NAPYLPSCL (SEQ ID NO:144)	7.200
10	329	GCNKRYFKL (SEQ ID NO:90)	6.600
11	417	RWPSCQKKF (SEQ ID NO:196)	6.600
12	47	AYGSLGGPA (SEQ ID NO:41)	6.000
13	180	DPMGQQGSL (SEQ ID NO:59)	6.000
14	4	DVRDLNALL (SEQ ID NO:62)	5.760
15	285	QYRIHTHGV (SEQ ID NO:175)	5.000
16	192	QYSVPPPVY (SEQ ID NO:176)	5.000
17	207	DSCTGSQAL (SEQ ID NO:61)	4.800
18	441	NMTKLQLAL (SEQ ID NO:149)	4.800
19	225	NLYQMTSQL (SEQ ID NO:147)	4.000
20	235	CMTWNQMNL (SEQ ID NO:49)	4.000

Table VI

Results of BIMAS HLA Peptide Binding Prediction Analysis for Binding of Human WT1 Peptides to Human HLA A3

(HLA-A3 peptide binding motif anchor residues are L, V, or M at P2; K, Y, or F at P9; auxiliary anchors are F or Y at P3, I, M, F, V, or L at P6; I, L, M, F at P7)

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	436	NMHQRNMTK (SEQ ID NO:148)	40.000
2	240	QMNLGATLK (SEQ ID NO:168)	20.000
3	88	CLSAFTVHF (SEQ ID NO:48)	6.000
4	126	RMFPNAPYL (SEQ ID NO:185)	4.500
5	169	AQFPNHSFK (SEQ ID NO:36)	4.500
6	10	ALLPAVPSL (SEQ ID NO:34)	4.050
7	137	CLESQPAIR (SEQ ID NO:47)	4.000
8	225	NLYQMTSQL (SEQ ID NO:147)	3.000
9	32	AQWAPVLDF (SEQ ID NO:37)	2.700
10	280	ILCGAQYRI (SEQ ID NO:116)	2.700
11	386	KTCQRKFSR (SEQ ID NO:128)	1.800

Table VIII

Results of BIMAS HLA Peptide Binding Prediction Analysis for

Binding of Human WT1 Peptides to Human HLA A 1101

(HLA-A1101 peptide binding motif anchor residues are K at P9; auxiliary anchor residues are V, I, F, Y at P2, M, L, F, Y, I, A, at P3, L, I, Y, V, or F at P7)

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	386	KTCQRKFSR (SEQ ID NO:128)	1.800
2	169	AQFPNHSFK (SEQ ID NO:36)	1.200
3	436	NMHQRNMTK (SEQ ID NO:148)	0.800
4	391	KFSRSDHLK (SEQ ID NO:120)	0.600
5	373	HQRRHTGVK (SEQ ID NO:109)	0.600
6	383	FQCKTCQRK (SEQ ID NO:80)	0.600
7	363	RFSRSDQLK (SEQ ID NO:178)	0.600
8	240	QMNLGATLK (SEQ ID NO:168)	0.400
9	287	RIHTHGVFR (SEQ ID NO:182)	0.240
10	100	FTGTAGACR (SEQ ID NO:84)	0.200
11	324	MCAYPGCNK (SEQ ID NO:142)	0.200
12	251	AAGSSSSVK (SEQ ID NO:28)	0.200
13	415	SCRWPSCQK (SEQ ID NO:201)	0.200
14	118	SQASSGQAR (SEQ ID NO:216)	0.120
15	292	GVFRGIQDV (SEQ ID NO:103)	0.120
16	137	CLESQPAIR (SEQ ID NO:47)	0.080
17	425	FARSDELVR (SEQ ID NO:75)	0.080
18	325	CAYPGCNKR (SEQ ID NO:44)	0.080
19	312	RSASETSEK (SEQ ID NO:190)	0.060
20	65	PPPPHSFIK (SEQ ID NO:156)	0.060

Table IX

Results of BIMAS HLA Peptide Binding Prediction Analysis for

Binding of Human WT1 Peptides to Human HLA A 3101

(HLA-A3101 peptide binding motif anchor residues are R at P9; auxiliary anchors L, V, Y, or F at P2; F, L, Y, W at P3, L, F, V, I, at P6)

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	386	KTCQRKFSR (SEQ ID NO:128)	9.000
2	287	RIHTHGVFR (SEQ ID NO:182)	6.000
3	137	CLESQPAIR (SEQ ID NO:47)	2.000
4	118	SQASSGQAR (SEQ ID NO:216)	2.000

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
15	207	DSCTGSQAL (SEQ ID NO:61)	1.500
16	139	ESQPAIRNQ (SEQ ID NO:72)	1.500
17	299	DVRRVPGVA (SEQ ID NO:63)	1.500
18	419	PSCQKKFAR (SEQ ID NO:159)	1.500
19	272	ESDNHTTPI (SEQ ID NO:71)	1.500
20	4	DVRDLNALL (SEQ ID NO:62)	1.500

Table XI

Results of BIMAS HLA Peptide Binding Prediction Analysis for

Binding of Human WT1 Peptides to Human HLA B14

(HLA-B14 peptide binding motif anchor residues are R at P2, Y at P3, R at P5, L at P9. Three of the 4 anchor residues is sufficient)

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	362	RRFSRSDQL (SEQ ID NO:187)	1000.000
2	332	KRYFKLSHL (SEQ ID NO:127)	300.000
3	423	KKFARSDEL (SEQ ID NO:122)	150.000
4	390	RKFSRSDHL (SEQ ID NO:183)	150.000
5	439	QRNMTKLQL (SEQ ID NO:173)	20.000
6	329	GCNKRYFKL (SEQ ID NO:90)	10.000
7	10	ALLPAVPSL (SEQ ID NO:34)	10.000
8	180	DPMGQQGSL (SEQ ID NO:59)	9.000
9	301	RRVPGVAPT (SEQ ID NO:189)	6.000
10	126	RMFPNAPYL (SEQ ID NO:185)	5.000
11	371	KRHQRRHTG (SEQ ID NO:126)	5.000
12	225	NLYQMTSQL (SEQ ID NO:147)	5.000
13	144	IRNQGYSTV (SEQ ID NO:117)	4.000
14	429	DELVRHHNM (SEQ ID NO:53)	3.000
15	437	MHQRNMTKL (SEQ ID NO:143)	3.000
16	125	ARMFPNAPY (SEQ ID NO:38)	3.000
17	239	NQMNLGATL (SEQ ID NO:151)	3.000
18	286	YRIHTHGVF (SEQ ID NO:252)	3.000
19	174	HSFKHEDPM (SEQ ID NO:110)	3.000
20	372	RHQRRHTGV (SEQ ID NO:181)	3.000

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
5	273	SDNHTTPIL (SEQ ID NO:204)	20.000
6	209	CTGSQALLL (SEQ ID NO:52)	8.000
7	30	GAAQWAPVL (SEQ ID NO:86)	8.000
8	318	SEKRPFMCA (SEQ ID NO:208)	8.000
9	180	DPMGQQGSL (SEQ ID NO:59)	8.000
10	138	LESQPAIRN (SEQ ID NO:132)	5.280
11	239	NQMNLGATL (SEQ ID NO:151)	4.400
12	329	GCNKRYFKL (SEQ ID NO:90)	4.400
13	130	NAPYLPSCL (SEQ ID NO:144)	4.400
14	85	EEQCLSAFT (SEQ ID NO:65)	4.400
15	208	SCTGSQALL (SEQ ID NO:202)	4.000
16	207	DSCTGSQAL (SEQ ID NO:61)	4.000
17	218	RTPYSSDNL (SEQ ID NO:194)	4.000
18	261	TEGQSNHST (SEQ ID NO:221)	4.000
19	18	LGGGGGCAL (SEQ ID NO:134)	4.000
20	221	YSSDNLYQM (SEQ ID NO:253)	2.200

Table XIV

Results of BIMAS HLA Peptide Binding Prediction Analysis for

Binding of Human WT1 Peptides to Human HLA B61

(HLA-B61 peptide binding motif anchor residues are E at P2, V at P9; auxiliary anchors are F, I, L, V, Y, W at P3, I at P6)

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	318	SEKRPFMCA (SEQ ID NO:208)	20.000
2	429	DELVRHHNM (SEQ ID NO:53)	16.000
3	298	QDVRRVPGV (SEQ ID NO:164)	10.000
4	81	AEPHEEQCL (SEQ ID NO:30)	8.000
5	233	LECMTWNQM (SEQ ID NO:131)	8.000
6	6	RDLNALLPA (SEQ ID NO:177)	5.500
7	85	EEQCLSAFT (SEQ ID NO:65)	4.000
8	261	TEGQSNHST (SEQ ID NO:221)	4.000
9	206	TDSCTGSQA (SEQ ID NO:220)	2.500
10	295	RGIQDVRRV (SEQ ID NO:179)	2.200
11	3	SDVRDLNAL (SEQ ID NO:206)	2.000
12	250	VAAGSSSSV (SEQ ID NO:236)	2.000
13	29	SGAAQWAPV (SEQ ID NO:211)	2.000
14	315	SETSEKRPF (SEQ ID NO:209)	1.600

To evaluate for the presence of immunity to WT1, Ab to recombinant full-length and truncated WT1 proteins in the sera of normal individuals and patients with leukemia were determined. Antibody reactivity was analyzed by ELISA reactivity to WT1/full-length protein, WT1/N-terminus protein and WT1/C-terminus protein.

Only 2 of 96 normal donors had serum antibodies reactive with WT1/full-length protein (Figure 18). One of those individuals had antibody to WT1/N-terminus protein and one had antibody to WT1/C-terminus protein. In contrast, 16 of 63 patients (25%) with AML had serum antibodies reactive with WT1/full-length protein. By marked contrast, only 2 of 63 patients (3%) had reactivity to WT1/C-terminus protein. Fifteen of 81 patients (19%) with CML had serum antibodies reactive with WT1/full-length protein and 12 of 81 patients (15%) had serum antibodies reactive with WT1/N-terminus. Only 3 of 81 patients (3%) had reactivity to WT1/C-terminus protein. (Figures 16 and 17.)

These data demonstrate that Ab responses to WT1 are detectable in some patients with AML and CML. The greater incidence of antibody in leukemia patients provides strong evidence that immunization to the WT1 protein occurred as a result of patients bearing malignancy that expresses or at some time expressed WT1. Without being limited to a specific theory, it is believed that the observed antibody responses to WT1 most probably result from patients becoming immune to WT1 on their own leukemia cells and provide direct evidence that WT1 can be immunogenic despite being a "self" protein.

The presence of antibody to WT1 strongly implies that concurrent helper T cell responses are also present in the same patients. WT1 is an internal protein. Thus, CTL responses are likely to be the most effective in terms of leukemia therapy and the most toxic arm of immunity. Thus, these data provide evidence that therapeutic vaccines directed against WT1 will be able to elicit an immune response to WT1.

The majority of the antibodies detected were reactive with epitopes within the N-terminus while only a small subgroup of patients showed a weak antibody response to the C-terminus. This is consistent with observations in the animal model, where immunization with peptides derived from the N-terminus elicited antibody, helper T cell and CTL responses, whereas none of the peptides tested from the C-terminus elicited antibody or T cell responses (Gaiger et al., *Blood 96*:1334, 2000).

EXAMPLE 2

INDUCTION OF ANTIBODIES TO WT1 IN MICE IMMUNIZED WITH CELL LINES EXPRESSING WT1

This Example illustrates the use of cells expressing WT1 to induce a WT1 specific antibody response *in vivo*.

Detection of existent antibodies to WT1 in patients with leukemia strongly implied that it is possible to immunize to WT1 protein to elicit immunity to WT1. To test whether immunity to WT1 can be generated by vaccination, mice were injected with TRAMP-C, a WT1 positive tumor cell line of B6 origin. Briefly, male B6 mice were immunized with 5×10^6 TRAMP-C cells subcutaneously and boosted twice with 5×10^6 cells at three week intervals. Three weeks after the final immunization, sera were obtained and single cell suspensions of spleens were prepared in RPMI 1640 medium (GIBCO) with 25μM β-2-mercaptoethanol, 200 units of penicillin per ml, 10mM L-glutamine, and 10% fetal bovine serum.

Following immunization to TRAMP-C, a WT1 specific antibody response in the immunized animals was detectable. A representative Western blot is shown in Figure 3. These results show that immunization to WT1 protein can elicit an immune response to WT1 protein.

EXAMPLE 3

INDUCTION OF TH AND ANTIBODY RESPONSES IN MICE IMMUNIZED WITH WT1 PEPTIDES

This Example illustrates the ability of immunization with WT1 peptides to elicit an immune response specific for WT1.

Peptides suitable for eliciting Ab and proliferative T cell responses were identified according to the Tsites program (Rothbard and Taylor, *EMBO J.* 7:93-100, 1988; Deavin et al., *Mol. Immunol.* 33:145-155, 1996), which searches for peptide motifs that have the potential to elicit Th responses. Peptides shown in Table I were synthesized and sequenced.

Table I

WT1 Peptides

Peptide	Sequence	Comments
Mouse: p6-22	RDLNALLPAVSSLGGGG (SEQ ID NO:13)	1 mismatch relative to human WT1 sequence
Human: p6-22	RDLNALLPAVPSLGGGG (SEQ ID NO:1)	
Human/mouse: p117-139	PSQASSGQARMFPNAPYLPSCLE (SEQ ID NOs: 2 and 3)	
Mouse: p244-262	GATLKGMAAGSSSSVKWTE (SEQ ID NO:14)	1 mismatch relative to human WT1 sequence
Human: p244-262	GATLKGVAAGSSSSVKWTE (SEQ ID NO:4)	
Human/mouse: p287-301	RIHTHGVFRGIQDVR (SEQ ID NOs: 15 and 16)	
Mouse: p299-313	VRRVSGVAPTLVRS (SEQ ID NO:17)	1 mismatch relative to human WT1 sequence
Human/mouse: p421-435	CQKKFARSDELVRHH (SEQ ID NOs: 19 and 20)	

For immunization, peptides were grouped as follows:

Group A: p6-22 human: 10.9mg in 1ml (10μl = 100μg)
p117-139 human/mouse: 7.6mg in 1ml (14μl = 100μg)
p244-262 human: 4.6.mg in 1ml (22μl = 100μg)

Group B: p287-301 human/mouse: 7.2mg in 1ml (14μl = 100μg)
mouse p299-313: 6.6.mg in 1ml (15μl = 100μg)
p421-435 human/mouse: 3.3mg in 1ml (30μl = 100μg)

Control: (FBL peptide 100μg) + CFA/IFA

Control: (CD45 peptide 100μg) + CFA/IFA

Group A contained peptides present within the amino terminus portion of WT1 (exon 1) and Group B contained peptides present within the carboxy terminus, which contains a four zinc finger region with sequence homology to other DNA-binding proteins. Within group B, p287-301 and p299-313 were derived from exon 7, zinc finger 1, and p421-435 was derived from exon 10, zinc finger IV.

B6 mice were immunized with a group of WT1 peptides or with a control peptide. Peptides were dissolved in 1ml sterile water for injection, and B6 mice were immunized 3 times at time intervals of three weeks. Adjuvants used were CFA/IFA, GM-CSF, and Montinide. The presence of antibodies specific for WT1 was then determined as described in Examples 1 and 2, and proliferative T cell responses were evaluated using a standard thymidine incorporation assay, in which cells were cultured in the presence of antigen and proliferation was evaluated by measuring incorporated radioactivity (Chen et al., *Cancer Res. 54*:1065-1070, 1994). In particular, lymphocytes were cultured in 96-well plates at $2x10^5$ cells per well with $4x10^5$ irradiated (3000 rads) syngeneic spleen cells and the designated peptide.

Immunization of mice with the group of peptides designated as Group A elicited an antibody response to WT1 (Figure 4). No antibodies were detected following immunization to Vaccine B, which is consistent with a lack of helper T cell response from immunization with Vaccine B. P117-139 elicited

proliferative T cell responses (Figures 5A-5C). The stimulation indices (SI) varied between 8 and 72. Other peptides (P6-22 and P299-313) also were shown to elicit proliferative T cell responses. Immunization with P6-22 resulted in a stimulation index (SI) of 2.3 and immunization with P299-313 resulted in a SI of 3.3. Positive controls included ConA stimulated T cells, as well as T cells stimulated with known antigens, such as CD45 and FBL, and allogeneic T cell lines (DeBruijn et al., *Eur. J. Immunol. 21*:2963-2970, 1991).

Figures 6A and 6B show the proliferative response observed for each of the three peptides within vaccine A (Figure 6A) and vaccine B (Figure 6B). Vaccine A elicited proliferative T cell responses to the immunizing peptides p6-22 and p117-139, with stimulation indices (SI) varying between 3 and 8 (bulk lines). No proliferative response to p244-262 was detected (Figure 6A).

Subsequent *in vitro* stimulations were carried out as single peptide stimulations using only p6-22 and p117-139. Stimulation of the Vaccine A specific T cell line with p117-139 resulted in proliferation to p117-139 with no response to p6-22 (Figure7A). Clones derived from the line were specific for p117-139 (Figure 7B). By contrast, stimulation of the Vaccine A specific T cell line with p6-22 resulted in proliferation to p6-22 with no response to p117-139 (Figure 7C). Clones derived from the line were specific for p6-22 (Figure 7D).

These results show that vaccination with WT1 peptides can elicit antibody responses to WT1 protein and proliferative T cell responses to the immunizing peptides.

EXAMPLE 4

INDUCTION OF CTL RESPONSES IN MICE IMMUNIZED WITH WT1 PEPTIDES

This Example illustrates the ability of WT1 peptides to elicit CTL immunity.

Peptides (9-mers) with motifs appropriate for binding to class I MHC were identified using a BIMAS HLA peptide binding prediction analysis (Parker et al., *J. Immunol. 152*:163, 1994). Peptides identified within such

analyses are shown in Tables II - XLIV. In each of these tables, the score reflects the theoretical binding affinity (half-time of dissociation) of the peptide to the MHC molecule indicated. Also indicated in the tables are the defined peptide binding motifs as described by Rammensee, *et al.*, Immunogenetics 41:178-228, 1995, incorporated herein in its entirety. The peptide binding motif for HLA-B14 is described in DiBrino *et al.*, J. Biol. Chem. 269:23426-23434, 1994, also incorporated herein it its entirety. Peptide positions are abreviated as P1, P2, P3, P4, P5, P6, P7, P8, and P9.

Peptides identified using the Tsites program (Rothbard and Taylor, *EMBO J.* 7:93-100, 1988; Deavin et al., *Mol. Immunol.* 33:145-155, 1996), which searches for peptide motifs that have the potential to elicit Th responses are further shown in Figures 8A and 8B, and Table XLV.

Table II

Results of BIMAS HLA Peptide Binding Prediction Analysis for Binding of Human WT1 Peptides to Human HLA A1

(HLA-A1 peptide binding motif anchor residues are D or E at position 3 (P3); Y at P9; with auxiliary anchors being P at P4 and L at P7)

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	137	CLESQPAIR (SEQ ID NO:47)	18.000
2	80	GAEPHEEQC (SEQ ID NO:87)	9.000
3	40	FAPPGASAY (SEQ ID NO:74)	5.000
4	354	QCDFKDCER (SEQ ID NO:162)	5.000
5	2	GSDVRDLNA (SEQ ID NO:101)	3.750
6	152	VTFDGTPSY (SEQ ID NO:244)	2.500
7	260	WTEGQSNHS (SEQ ID NO:247)	2.250
8	409	TSEKPFSCR (SEQ ID NO:232)	1.350
9	73	KQEPSWGGA (SEQ ID NO:125)	1.350
10	386	KTCQRKFSR (SEQ ID NO:128)	1.250
11	37	VLDFAPPGA (SEQ ID NO:241)	1.000
12	325	CAYPGCNKR (SEQ ID NO:44)	1.000
13	232	QLECMTWNQ (SEQ ID NO:167)	0.900
14	272	ESDNHTTPI (SEQ ID NO:71)	0.750
15	366	RSDQLKRHQ (SEQ ID NO:193)	0.750
16	222	SSDNLYQMT (SEQ ID NO:217)	0.750

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
17	427	RSDELVRHH (SEQ ID NO:191)	0.750
18	394	RSDHLKTHT (SEQ ID NO:192)	0.750
19	317	TSEKRPFMC (SEQ ID NO:233)	0.675
20	213	QALLLRTPY (SEQ ID NO:160)	0.500

Table III

Results of BIMAS HLA Peptide Binding Prediction Analysis for

Binding of Human WT1 Peptides to Human HLA A 0201

(HLA-A0201 peptide binding motif anchor residues are L or M at P2; V or L at P9; with an auxiliary anchor being V at P6)

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	126	RMFPNAPYL (SEQ ID NO:185)	313.968
2	187	SLGEQQYSV (SEQ ID NO:214)	285.163
3	10	ALLPAVPSL (SEQ ID NO:34)	181.794
4	242	NLGATLKGV (SEQ ID NO:146)	159.970
5	225	NLYQMTSQL (SEQ ID NO:147)	68.360
6	292	GVFRGIQDV (SEQ ID NO:103)	51.790
7	191	QQYSVPPPV (SEQ ID NO:171)	22.566
8	280	ILCGAQYRI (SEQ ID NO:116)	17.736
9	235	CMTWNQMNL (SEQ ID NO:49)	15.428
10	441	NMTKLQLAL (SEQ ID NO:149)	15.428
11	7	DLNALLPAV (SEQ ID NO:58)	11.998
12	227	YQMTSQLEC (SEQ ID NO:251)	8.573
13	239	NQMNLGATL (SEQ ID NO:151)	8.014
14	309	TLVRSASET (SEQ ID NO:226)	7.452
15	408	KTSEKPFSC (SEQ ID NO:129)	5.743
16	340	LQMHSRKHT (SEQ ID NO:139)	4.752
17	228	QMTSQLECM (SEQ ID NO:169)	4.044
18	93	TVHFSGQFT (SEQ ID NO:235)	3.586
19	37	VLDFAPPGA (SEQ ID NO:241)	3.378
20	86	EQCLSAFTV (SEQ ID NO:69)	3.068

Table IV

Results of BIMAS HLA Peptide Binding Prediction Analysis for

Binding of Human WT1 Peptides to Human HLA A 0205

(HLA-A0205 peptide binding motif anchor residues are L at P9 with auxiliary anchors being V, L, I, M at P2 and I, V, L, A, at P6)

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	10	ALLPAVPSL (SEQ ID NO:34)	42.000
2	292	GVFRGIQDV (SEQ ID NO:103)	24.000
3	126	RMFPNAPYL (SEQ ID NO:185)	21.000
4	225	NLYQMTSQL (SEQ ID NO:147)	21.000
5	239	NQMNLGATL (SEQ ID NO:151)	16.800
6	302	RVPGVAPTL (SEQ ID NO:195)	14.000
7	441	NMTKLQLAL (SEQ ID NO:149)	7.000
8	235	CMTWNQMNL (SEQ ID NO:49)	7.000
9	187	SLGEQQYSV (SEQ ID NO:214)	6.000
10	191	QQYSVPPPV (SEQ ID NO:171)	4.800
11	340	LQMHSRKHT (SEQ ID NO:139)	4.080
12	242	NLGATLKGV (SEQ ID NO:146)	4.000
13	227	YQMTSQLEC (SEQ ID NO:251)	3.600
14	194	SVPPPVYGC (SEQ ID NO:218)	2.000
15	93	TVHFSGQFT (SEQ ID NO:235)	2.000
16	280	ILCGAQYRI (SEQ ID NO:116)	1.700
17	98	GQFTGTAGA (SEQ ID NO:99)	1.200
18	309	TLVRSASET (SEQ ID NO:226)	1.000
19	81	AEPHEEQCL (SEQ ID NO:30)	0.980
20	73	KQEPSWGGA (SEQ ID NO:125)	0.960

Table V

Results of BIMAS HLA Peptide Binding Prediction Analysis for

Binding of Human WT1 Peptides to Human HLA A24

(HLA-A24 peptide binding motif anchor residues are Y at P2; I, L, or F at P9; with auxiliary anchors I or V at P5 and F at P6)

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	302	RVPGVAPTL (SEQ ID NO:195)	16.800

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
2	218	RTPYSSDNL (SEQ ID NO:194)	12.000
3	356	DFKDCERRF (SEQ ID NO:55)	12.000
4	126	RMFPNAPYL (SEQ ID NO:185)	9.600
5	326	AYPGCNKRY (SEQ ID NO:42)	7.500
6	270	GYESDNHT (SEQ ID NO:106)T	7.500
7	239	NQMNLGATL (SEQ ID NO:151)	7.200
8	10	ALLPAVPSL (SEQ ID NO:34)	7.200
9	130	NAPYLPSCL (SEQ ID NO:144)	7.200
10	329	GCNKRYFKL (SEQ ID NO:90)	6.600
11	417	RWPSCQKKF (SEQ ID NO:196)	6.600
12	47	AYGSLGGPA (SEQ ID NO:41)	6.000
13	180	DPMGQQGSL (SEQ ID NO:59)	6.000
14	4	DVRDLNALL (SEQ ID NO:62)	5.760
15	285	QYRIHTHGV (SEQ ID NO:175)	5.000
16	192	QYSVPPPVY (SEQ ID NO:176)	5.000
17	207	DSCTGSQAL (SEQ ID NO:61)	4.800
18	441	NMTKLQLAL (SEQ ID NO:149)	4.800
19	225	NLYQMTSQL (SEQ ID NO:147)	4.000
20	235	CMTWNQMNL (SEQ ID NO:49)	4.000

Table VI

Results of BIMAS HLA Peptide Binding Prediction Analysis for Binding of Human WT1 Peptides to Human HLA A3

(HLA-A3 peptide binding motif anchor residues are L, V, or M at P2; K, Y, or F at P9; auxiliary anchors are F or Y at P3, I, M, F, V, or L at P6; I, L, M, F at P7)

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	436	NMHQRNMTK (SEQ ID NO:148)	40.000
2	240	QMNLGATLK (SEQ ID NO:168)	20.000
3	88	CLSAFTVHF (SEQ ID NO:48)	6.000
4	126	RMFPNAPYL (SEQ ID NO:185)	4.500
5	169	AQFPNHSFK (SEQ ID NO:36)	4.500
6	10	ALLPAVPSL (SEQ ID NO:34)	4.050
7	137	CLESQPAIR (SEQ ID NO:47)	4.000
8	225	NLYQMTSQL (SEQ ID NO:147)	3.000
9	32	AQWAPVLDF (SEQ ID NO:37)	2.700
10	280	ILCGAQYRI (SEQ ID NO:116)	2.700
11	386	KTCQRKFSR (SEQ ID NO:128)	1.800

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
12	235	CMTWNQMNL (SEQ ID NO:49)	1.200
13	441	NMTKLQLAL (SEQ ID NO:149)	1.200
14	152	VTFDGTPSY (SEQ ID NO:244)	1.000
15	187	SLGEQQYSV (SEQ ID NO:214)	0.900
16	383	FQCKTCQRK (SEQ ID NO:80)	0.600
17	292	GVFRGIQDV (SEQ ID NO:103)	0.450
18	194	SVPPPVYGC (SEQ ID NO:218)	0.405
19	287	RIHTHGVFR (SEQ ID NO:182)	0.400
20	263	GQSNHSTGY (SEQ ID NO:100)	0.360

Table VII

Results of BIMAS HLA Peptide Binding Prediction Analysis for Binding of Human WT1 Peptides to Human HLA A68.1

(HLA-A68.1 peptide binding motif anchor residues are V or T at P2; R or K at P9)

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	100	FTGTAGACR (SEQ ID NO:84)	100.000
2	386	KTCQRKFSR (SEQ ID NO:128)	50.000
3	368	DQLKRHQRR (SEQ ID NO:60)	30.000
4	312	RSASETSEK (SEQ ID NO:190)	18.000
5	337	LSHLQMHSR (SEQ ID NO:141)	15.000
6	364	FSRSDQLKR (SEQ ID NO:83)	15.000
7	409	TSEKPFSCR (SEQ ID NO:232)	15.000
8	299	DVRRVPGVA (SEQ ID NO:63)	12.000
9	4	DVRDLNALL (SEQ ID NO:62)	12.000
10	118	SQASSGQAR (SEQ ID NO:216)	10.000
11	343	HSRKHTGEK (SEQ ID NO:111)	9.000
12	169	AQFPNHSFK (SEQ ID NO:36)	9.000
13	292	GVFRGIQDV (SEQ ID NO:103)	8.000
14	325	CAYPGCNKR (SEQ ID NO:44)	7.500
15	425	FARSDELVR (SEQ ID NO:75)	7.500
16	354	QCDFKDCER (SEQ ID NO:162)	7.500
17	324	MCAYPGCNK (SEQ ID NO:142)	6.000
18	251	AAGSSSSVK (SEQ ID NO:28)	6.000
19	379	GVKPFQCKT (SEQ ID NO:104)	6.000
20	137	CLESQPAIR (SEQ ID NO:47)	5.000

Table VIII

Results of BIMAS HLA Peptide Binding Prediction Analysis for

Binding of Human WT1 Peptides to Human HLA A 1101

(HLA-A1101 peptide binding motif anchor residues are K at P9; auxiliary anchor residues are V, I, F, Y at P2, M, L, F, Y, I, A, at P3, L, I, Y, V, or F at P7)

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	386	KTCQRKFSR (SEQ ID NO:128)	1.800
2	169	AQFPNHSFK (SEQ ID NO:36)	1.200
3	436	NMHQRNMTK (SEQ ID NO:148)	0.800
4	391	KFSRSDHLK (SEQ ID NO:120)	0.600
5	373	HQRRHTGVK (SEQ ID NO:109)	0.600
6	383	FQCKTCQRK (SEQ ID NO:80)	0.600
7	363	RFSRSDQLK (SEQ ID NO:178)	0.600
8	240	QMNLGATLK (SEQ ID NO:168)	0.400
9	287	RIHTHGVFR (SEQ ID NO:182)	0.240
10	100	FTGTAGACR (SEQ ID NO:84)	0.200
11	324	MCAYPGCNK (SEQ ID NO:142)	0.200
12	251	AAGSSSSVK (SEQ ID NO:28)	0.200
13	415	SCRWPSCQK (SEQ ID NO:201)	0.200
14	118	SQASSGQAR (SEQ ID NO:216)	0.120
15	292	GVFRGIQDV (SEQ ID NO:103)	0.120
16	137	CLESQPAIR (SEQ ID NO:47)	0.080
17	425	FARSDELVR (SEQ ID NO:75)	0.080
18	325	CAYPGCNKR (SEQ ID NO:44)	0.080
19	312	RSASETSEK (SEQ ID NO:190)	0.060
20	65	PPPPHSFIK (SEQ ID NO:156)	0.060

Table IX

Results of BIMAS HLA Peptide Binding Prediction Analysis for

Binding of Human WT1 Peptides to Human HLA A 3101

(HLA-A3101 peptide binding motif anchor residues are R at P9; auxiliary anchors L, V, Y, or F at P2; F, L, Y, W at P3, L, F, V, I, at P6)

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	386	KTCQRKFSR (SEQ ID NO:128)	9.000
2	287	RIHTHGVFR (SEQ ID NO:182)	6.000
3	137	CLESQPAIR (SEQ ID NO:47)	2.000
4	118	SQASSGQAR (SEQ ID NO:216)	2.000

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
5	368	DQLKRHQRR (SEQ ID NO:60)	1.200
6	100	FTGTAGACR (SEQ ID NO:84)	1.000
7	293	VFRGIQDVR (SEQ ID NO:238)	0.600
8	325	CAYPGCNKR (SEQ ID NO:44)	0.600
9	169	AQFPNHSFK (SEQ ID NO:36)	0.600
10	279	PILCGAQYR (SEQ ID NO:155)	0.400
11	436	NMHQRNMTK (SEQ ID NO:148)	0.400
12	425	FARSDELVR (SEQ ID NO:75)	0.400
13	32	AQWAPVLDF (SEQ ID NO:37)	0.240
14	240	QMNLGATLK (SEQ ID NO:168)	0.200
15	354	QCDFKDCER (SEQ ID NO:162)	0.200
16	373	HQRRHTGVK (SEQ ID NO:109)	0.200
17	383	FQCKTCQRK (SEQ ID NO:80)	0.200
18	313	SASETSEKR (SEQ ID NO:197)	0.200
19	358	KDCERRFSR (SEQ ID NO:118)	0.180
20	391	KFSRSDHLK (SEQ ID NO:120)	0.180

Table X

Results of BIMAS HLA Peptide Binding Prediction Analysis for

Binding of Human WT1 Peptides to Human HLA A 3302

(HLA-A3302 peptide binding motif anchor residues are R at P9; auxiliary anchors A, I, L, F, Y, or V at P2)

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	337	LSHLQMHSR (SEQ ID NO:141)	15.000
2	409	TSEKPFSCR (SEQ ID NO:232)	15.000
3	364	FSRSDQLKR (SEQ ID NO:83)	15.000
4	137	CLESQPAIR (SEQ ID NO:47)	9.000
5	368	DQLKRHQRR (SEQ ID NO:60)	9.000
6	287	RIHTHGVFR (SEQ ID NO:182)	4.500
7	210	TGSQALLLR (SEQ ID NO:223)	3.000
8	425	FARSDELVR (SEQ ID NO:75)	3.000
9	313	SASETSEKR (SEQ ID NO:197)	3.000
10	293	VFRGIQDVR (SEQ ID NO:238)	3.000
11	354	QCDFKDCER (SEQ ID NO:162)	3.000
12	100	FTGTAGACR (SEQ ID NO:84)	3.000
13	118	SQASSGQAR (SEQ ID NO:216)	3.000
14	325	CAYPGCNKR (SEQ ID NO:44)	3.000

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
15	207	DSCTGSQAL (SEQ ID NO:61)	1.500
16	139	ESQPAIRNQ (SEQ ID NO:72)	1.500
17	299	DVRRVPGVA (SEQ ID NO:63)	1.500
18	419	PSCQKKFAR (SEQ ID NO:159)	1.500
19	272	ESDNHTTPI (SEQ ID NO:71)	1.500
20	4	DVRDLNALL (SEQ ID NO:62)	1.500

Table XI

Results of BIMAS HLA Peptide Binding Prediction Analysis for

Binding of Human WT1 Peptides to Human HLA B14

(HLA-B14 peptide binding motif anchor residues are R at P2, Y at P3, R at P5, L at P9. Three of the 4 anchor residues is sufficient)

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	362	RRFSRSDQL (SEQ ID NO:187)	1000.000
2	332	KRYFKLSHL (SEQ ID NO:127)	300.000
3	423	KKFARSDEL (SEQ ID NO:122)	150.000
4	390	RKFSRSDHL (SEQ ID NO:183)	150.000
5	439	QRNMTKLQL (SEQ ID NO:173)	20.000
6	329	GCNKRYFKL (SEQ ID NO:90)	10.000
7	10	ALLPAVPSL (SEQ ID NO:34)	10.000
8	180	DPMGQQGSL (SEQ ID NO:59)	9.000
9	301	RRVPGVAPT (SEQ ID NO:189)	6.000
10	126	RMFPNAPYL (SEQ ID NO:185)	5.000
11	371	KRHQRRHTG (SEQ ID NO:126)	5.000
12	225	NLYQMTSQL (SEQ ID NO:147)	5.000
13	144	IRNQGYSTV (SEQ ID NO:117)	4.000
14	429	DELVRHHNM (SEQ ID NO:53)	3.000
15	437	MHQRNMTKL (SEQ ID NO:143)	3.000
16	125	ARMFPNAPY (SEQ ID NO:38)	3.000
17	239	NQMNLGATL (SEQ ID NO:151)	3.000
18	286	YRIHTHGVF (SEQ ID NO:252)	3.000
19	174	HSFKHEDPM (SEQ ID NO:110)	3.000
20	372	RHQRRHTGV (SEQ ID NO:181)	3.000

Table XII

Results of BIMAS HLA Peptide Binding Prediction Analysis for

Binding of Human WT1 Peptides to Human HLA B40

(HLA-B40 peptide binding motif anchor residues are E at P2, L, W, M, or A at P9; auxiliary anchors are F, I, or V at P3)

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	81	AEPHEEQCL (SEQ ID NO:30)	40.000
2	429	DELVRHHNM (SEQ ID NO:53)	24.000
3	410	SEKPFSCRW (SEQ ID NO:207)	20.000
4	318	SEKRPFMCA (SEQ ID NO:208)	15.000
5	233	LECMTWNQM (SEQ ID NO:131)	12.000
6	3	SDVRDLNAL (SEQ ID NO:206)	10.000
7	349	GEKPYQCDF (SEQ ID NO:91)	8.000
8	6	RDLNALLPA (SEQ ID NO:177)	5.000
9	85	EEQCLSAFT (SEQ ID NO:65)	4.000
10	315	SETSEKRPF (SEQ ID NO:209)	4.000
11	261	TEGQSNHST (SEQ ID NO:221)	4.000
12	23	GCALPVSGA (SEQ ID NO:89)	3.000
13	38	LDFAPPGAS (SEQ ID NO:130)	3.000
14	273	SDNHTTPIL (SEQ ID NO:204)	2.500
15	206	TDSCTGSQA (SEQ ID NO:220)	2.500
16	24	CALPVSGAA (SEQ ID NO:43)	2.000
17	98	GQFTGTAGA (SEQ ID NO:99)	2.000
18	30	GAAQWAPVL (SEQ ID NO:86)	2.000
19	84	HEEQCLSAF (SEQ ID NO:107)	2.000
20	26	LPVSGAAQW (SEQ ID NO:138)	2.000

Table XIII

Results of BIMAS HLA Peptide Binding Prediction Analysis for

Binding of Human WT1 Peptides to Human HLA B60

(HLA-B60 peptide binding motif anchor residues are E at P2, L at P9; auxiliary anchors are I or V at P7)

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	81	AEPHEEQCL (SEQ ID NO:30)	160.000
2	3	SDVRDLNAL (SEQ ID NO:206)	40.000
3	429	DELVRHHNM (SEQ ID NO:53)	40.000
4	233	LECMTWNQM (SEQ ID NO:131)	22.000

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
5	273	SDNHTTPIL (SEQ ID NO:204)	20.000
6	209	CTGSQALLL (SEQ ID NO:52)	8.000
7	30	GAAQWAPVL (SEQ ID NO:86)	8.000
8	318	SEKRPFMCA (SEQ ID NO:208)	8.000
9	180	DPMGQQGSL (SEQ ID NO:59)	8.000
10	138	LESQPAIRN (SEQ ID NO:132)	5.280
11	239	NQMNLGATL (SEQ ID NO:151)	4.400
12	329	GCNKRYFKL (SEQ ID NO:90)	4.400
13	130	NAPYLPSCL (SEQ ID NO:144)	4.400
14	85	EEQCLSAFT (SEQ ID NO:65)	4.400
15	208	SCTGSQALL (SEQ ID NO:202)	4.000
16	207	DSCTGSQAL (SEQ ID NO:61)	4.000
17	218	RTPYSSDNL (SEQ ID NO:194)	4.000
18	261	TEGQSNHST (SEQ ID NO:221)	4.000
19	18	LGGGGGCAL (SEQ ID NO:134)	4.000
20	221	YSSDNLYQM (SEQ ID NO:253)	2.200

Table XIV

Results of BIMAS HLA Peptide Binding Prediction Analysis for

Binding of Human WT1 Peptides to Human HLA B61

(HLA-B61 peptide binding motif anchor residues are E at P2, V at P9; auxiliary anchors are F, I, L, V, Y, W at P3, I at P6)

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	318	SEKRPFMCA (SEQ ID NO:208)	20.000
2	429	DELVRHHNM (SEQ ID NO:53)	16.000
3	298	QDVRRVPGV (SEQ ID NO:164)	10.000
4	81	AEPHEEQCL (SEQ ID NO:30)	8.000
5	233	LECMTWNQM (SEQ ID NO:131)	8.000
6	6	RDLNALLPA (SEQ ID NO:177)	5.500
7	85	EEQCLSAFT (SEQ ID NO:65)	4.000
8	261	TEGQSNHST (SEQ ID NO:221)	4.000
9	206	TDSCTGSQA (SEQ ID NO:220)	2.500
10	295	RGIQDVRRV (SEQ ID NO:179)	2.200
11	3	SDVRDLNAL (SEQ ID NO:206)	2.000
12	250	VAAGSSSSV (SEQ ID NO:236)	2.000
13	29	SGAAQWAPV (SEQ ID NO:211)	2.000
14	315	SETSEKRPF (SEQ ID NO:209)	1.600

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
15	138	LESQPAIRN (SEQ ID NO:132)	1.200
16	244	GATLKGVAA (SEQ ID NO:88)	1.100
17	20	GGGGCALPV (SEQ ID NO:92)	1.100
18	440	RNMTKLQLA (SEQ ID NO:186)	1.100
19	23	GCALPVSGA (SEQ ID NO:89)	1.100
20	191	QQYSVPPPV (SEQ ID NO:171)	1.000

Table XV

Results of BIMAS HLA Peptide Binding Prediction Analysis for

Binding of Human WT1 Peptides to Human HLA B62

(HLA-B62 peptide binding motif anchor residues are Q or L at P2, F or Y at P9; auxiliary anchors are I or V at P5)

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	146	NQGYSTVTF (SEQ ID NO:150)	211.200
2	32	AQWAPVLDF (SEQ ID NO:37)	96.000
3	263	GQSNHSTGY (SEQ ID NO:100)	96.000
4	88	CLSAFTVHF (SEQ ID NO:48)	96.000
5	17	SLGGGGGCA (SEQ ID NO:215)	9.600
6	239	NQMNLGATL (SEQ ID NO:151)	8.800
7	191	QQYSVPPPV (SEQ ID NO:171)	8.000
8	98	GQFTGTAGA (SEQ ID NO:99)	8.000
9	384	QCKTCQRKF (SEQ ID NO:163)	6.000
10	40	FAPPGASAY (SEQ ID NO:74)	4.800
11	227	YQMTSQLEC (SEQ ID NO:251)	4.800
12	187	SLGEQQYSV (SEQ ID NO:214)	4.400
13	86	EQCLSAFTV (SEQ ID NO:69)	4.400
14	152	VTFDGTPSY (SEQ ID NO:244)	4.400
15	101	TGTAGACRY (SEQ ID NO:224)	4.000
16	242	NLGATLKGV (SEQ ID NO:146)	4.000
17	92	FTVHFSGQF (SEQ ID NO:85)	4.000
18	7	DLNALLPAV (SEQ ID NO:58)	4.000
19	123	GQARMFPNA (SEQ ID NO:98)	4.000
20	280	ILCGAQYRI (SEQ ID NO:116)	3.120

Table XVI

Results of BIMAS HLA Peptide Binding Prediction Analysis for

Binding of Human WT1 Peptides to Human HLA B7

(HLA-B7 peptide binding motif anchor residues are P at P2, L or F at P9; auxiliary anchor is R at P2)

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	180	DPMGQQGSL (SEQ ID NO:59)	240.000
2	4	DVRDLNALL (SEQ ID NO:62)	200.000
3	302	RVPGVAPTL (SEQ ID NO:195)	20.000
4	30	GAAQWAPVL (SEQ ID NO:86)	12.000
5	239	NQMNLGATL (SEQ ID NO:151)	12.000
6	130	NAPYLPSCL (SEQ ID NO:144)	12.000
7	10	ALLPAVPSL (SEQ ID NO:34)	12.000
8	299	DVRRVPGVA (SEQ ID NO:63)	5.000
9	208	SCTGSQALL (SEQ ID NO:202)	4.000
10	303	VPGVAPTLV (SEQ ID NO:242)	4.000
11	18	LGGGGGCAL (SEQ ID NO:134)	4.000
12	218	RTPYSSDNL (SEQ ID NO:194)	4.000
13	207	DSCTGSQAL (SEQ ID NO:61)	4.000
14	209	CTGSQALLL (SEQ ID NO:52)	4.000
15	329	GCNKRYFKL (SEQ ID NO:90)	4.000
16	235	CMTWNQMNL (SEQ ID NO:49)	4.000
17	441	NMTKLQLAL (SEQ ID NO:149)	4.000
18	126	RMFPNAPYL (SEQ ID NO:185)	4.000
19	225	NLYQMTSQL (SEQ ID NO:147)	4.000
20	143	AIRNQGYST (SEQ ID NO:33)	3.000

Table XVII

Results of BIMAS HLA Peptide Binding Prediction Analysis for

Binding of Human WT1 Peptides to Human HLA B8

(HLA-B8 peptide binding motif anchor residues are K at P3, K or R at P5, L at P9)

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	329	GCNKRYFKL (SEQ ID NO:90)	16.000
2	4	DVRDLNALL (SEQ ID NO:62)	12.000
3	316	ETSEKRPFM (SEQ ID NO:73)	3.000
4	180	DPMGQQGSL (SEQ ID NO:59)	1.600

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
5	208	SCTGSQALL (SEQ ID NO:202)	0.800
6	130	NAPYLPSCL (SEQ ID NO:144)	0.800
7	244	GATLKGVAA (SEQ ID NO:88)	0.800
8	30	GAAQWAPVL (SEQ ID NO:86)	0.800
9	299	DVRRVPGVA (SEQ ID NO:63)	0.400
10	420	SCQKKFARS (SEQ ID NO:200)	0.400
11	387	TCQRKFSRS (SEQ ID NO:219)	0.400
12	225	NLYQMTSQL (SEQ ID NO:147)	0.400
13	141	QPAIRNQGY (SEQ ID NO:170)	0.400
14	10	ALLPAVPSL (SEQ ID NO:34)	0.400
15	207	DSCTGSQAL (SEQ ID NO:61)	0.400
16	384	QCKTCQRKF (SEQ ID NO:163)	0.400
17	136	SCLESQPAI (SEQ ID NO:198)	0.300
18	347	HTGEKPYQC (SEQ ID NO:112)	0.300
19	401	HTRTHTGKT (SEQ ID NO:114)	0.200
20	332	KRYFKLSHL (SEQ ID NO:127)	0.200

Table XVIII

Results of BIMAS HLA Peptide Binding Prediction Analysis for

Binding of Human WT1 Peptides to Human HLA B 2702

(HLA-B2702 peptide binding motif anchor residues are R at P2; F, Y, I, L, or W at P9)

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	332	KRYFKLSHL (SEQ ID NO:127)	900.000
2	362	RRFSRSDQL (SEQ ID NO:187)	900.000
3	286	YRIHTHGVF (SEQ ID NO:252)	200.000
4	125	ARMFPNAPY (SEQ ID NO:38)	200.000
5	375	RRHTGVKPF (SEQ ID NO:188)	180.000
6	32	AQWAPVLDF (SEQ ID NO:37)	100.000
7	301	RRVPGVAPT (SEQ ID NO:189)	60.000
8	439	QRNMTKLQL (SEQ ID NO:173)	60.000
9	126	RMFPNAPYL (SEQ ID NO:185)	22.500
10	426	ARSDELVRH (SEQ ID NO:39)	20.000
11	146	NQGYSTVTF (SEQ ID NO:150)	20.000
12	144	IRNQGYSTV (SEQ ID NO:117)	20.000
13	389	QRKFSRSDH (SEQ ID NO:172)	20.000
14	263	GQSNHSTGY (SEQ ID NO:100)	20.000

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
15	416	CRWPSCQKK (SEQ ID NO:50)	20.000
16	191	QQYSVPPPV (SEQ ID NO:171)	10.000
17	217	LRTPYSSDN (SEQ ID NO:140)	10.000
18	107	CRYGPFGPP (SEQ ID NO:51)	10.000
19	98	GQFTGTAGA (SEQ ID NO:99)	10.000
20	239	NQMNLGATL (SEQ ID NO:151)	6.000

Table XIX

Results of BIMAS HLA Peptide Binding Prediction Analysis for Binding of Human WT1 Peptides to Human HLA B 2705

(HLA-B2705 peptide binding motif anchor residues are R at P2; L or F at P9; Y, R, H, and K, have also been found at P9 for naturally processed epitopes)

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	332	KRYFKLSHL (SEQ ID NO:127)	30000.000
2	362	RRFSRSDQL (SEQ ID NO:187)	30000.000
3	416	CRWPSCQKK (SEQ ID NO:50)	10000.000
4	439	QRNMTKLQL (SEQ ID NO:173)	2000.000
5	286	YRIHTHGVF (SEQ ID NO:252)	1000.000
6	125	ARMFPNAPY (SEQ ID NO:38)	1000.000
7	294	FRGIQDVRR (SEQ ID NO:81)	1000.000
8	432	VRHHNMHQR (SEQ ID NO:243)	1000.000
9	169	AQFPNHSFK (SEQ ID NO:36)	1000.000
10	375	RRHTGVKPF (SEQ ID NO:188)	900.000
11	126	RMFPNAPYL (SEQ ID NO:185)	750.000
12	144	IRNQGYSTV (SEQ ID NO:117)	600.000
13	301	RRVPGVAPT (SEQ ID NO:189)	600.000
14	32	AQWAPVLDF (SEQ ID NO:37)	500.000
15	191	QQYSVPPPV (SEQ ID NO:171)	300.000
16	373	HQRRHTGVK (SEQ ID NO:109)	200.000
17	426	ARSDELVRH (SEQ ID NO:39)	200.000
18	383	FQCKTCQRK (SEQ ID NO:80)	200.000
19	239	NQMNLGATL (SEQ ID NO:151)	200.000
20	389	QRKFSRSDH (SEQ ID NO:172)	200.000

Table XX

Results of BIMAS HLA Peptide Binding Prediction Analysis for

Binding of Human WT1 Peptides to Human HLA B 3501

(HLA-B3501 peptide binding motif anchor residues are P at P2; Y, F, M, L, or I at P9)

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	278	TPILCGAQY (SEQ ID NO:227)	40.000
2	141	QPAIRNQGY (SEQ ID NO:170)	40.000
3	219	TPYSSDNLY (SEQ ID NO:231)	40.000
4	327	YPGCNKRYF (SEQ ID NO:250)	20.000
5	163	TPSHHAAQF (SEQ ID NO:228)	20.000
6	180	DPMGQQGSL (SEQ ID NO:59)	20.000
7	221	YSSDNLYQM (SEQ ID NO:253)	20.000
8	26	LPVSGAAQW (SEQ ID NO:138)	10.000
9	174	HSFKHEDPM (SEQ ID NO:110)	10.000
10	82	EPHEEQCLS (SEQ ID NO:68)	6.000
11	213	QALLLRTPY (SEQ ID NO:160)	6.000
12	119	QASSGQARM (SEQ ID NO:161)	6.000
13	4	DVRDLNALL (SEQ ID NO:62)	6.000
14	40	FAPPGASAY (SEQ ID NO:74)	6.000
15	120	ASSGQARMF (SEQ ID NO:40)	5.000
16	207	DSCTGSQAL (SEQ ID NO:61)	5.000
17	303	VPGVAPTLV (SEQ ID NO:242)	4.000
18	316	ETSEKRPFM (SEQ ID NO:73)	4.000
19	152	VTFDGTPSY (SEQ ID NO:244)	4.000
20	412	KPFSCRWPS (SEQ ID NO:123)	4.000

Table XXI

Results of BIMAS HLA Peptide Binding Prediction Analysis for

Binding of Human WT1 Peptides to Human HLA B 3701

(HLA-B3701 peptide binding motif anchor residues are F, M, or L at P8; I or L at P9; auxiliary anchors are D, E at P2 and V, I at P5)

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	3	SDVRDLNAL (SEQ ID NO:206)	40.000
2	273	SDNHTTPIL (SEQ ID NO:204)	40.000
3	81	AEPHEEQCL (SEQ ID NO:30)	10.000
4	298	QDVRRVPGV (SEQ ID NO:164)	8.000

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
5	428	SDELVRHHN (SEQ ID NO:203)	6.000
6	85	EEQCLSAFT (SEQ ID NO:65)	5.000
7	208	SCTGSQALL (SEQ ID NO:202)	5.000
8	4	DVRDLNALL (SEQ ID NO:62)	5.000
9	209	CTGSQALLL (SEQ ID NO:52)	5.000
10	38	LDFAPPGAS (SEQ ID NO:130)	4.000
11	223	SDNLYQMTS (SEQ ID NO:205)	4.000
12	179	EDPMGQQGS (SEQ ID NO:64)	4.000
13	206	TDSCTGSQA (SEQ ID NO:220)	4.000
14	6	RDLNALLPA (SEQ ID NO:177)	4.000
15	84	HEEQCLSAF (SEQ ID NO:107)	2.000
16	233	LECMTWNQM (SEQ ID NO:131)	2.000
17	429	DELVRHHNM (SEQ ID NO:53)	2.000
18	315	SETSEKRPF (SEQ ID NO:209)	2.000
19	349	GEKPYQCDF (SEQ ID NO:91)	2.000
20	302	RVPGVAPTL (SEQ ID NO:195)	1.500

Table XXII

Results of BIMAS HLA Peptide Binding Prediction Analysis for Binding of Human WT1 Peptides to Human HLA B 3801

(HLA-B3801 peptide binding motif anchor residues are F or L at P9;Auxiliary anchors are H at P2 and D or E at P3)

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	437	MHQRNMTKL (SEQ ID NO:143)	36.000
2	434	HHNMHQRNM (SEQ ID NO:108)	6.000
3	372	RHQRRHTGV (SEQ ID NO:181)	6.000
4	180	DPMGQQGSL (SEQ ID NO:59)	4.000
5	433	RHHNMHQRN (SEQ ID NO:180)	3.900
6	165	SHHAAQFPN (SEQ ID NO:213)	3.900
7	202	CHTPTDSCT (SEQ ID NO:45)	3.000
8	396	DHLKTHTRT (SEQ ID NO:57)	3.000
9	161	GHTPSHHAA (SEQ ID NO:94)	3.000
10	302	RVPGVAPTL (SEQ ID NO:195)	2.600
11	417	RWPSCQKKF (SEQ ID NO:196)	2.400
12	327	YPGCNKRYF (SEQ ID NO:250)	2.400
13	208	SCTGSQALL (SEQ ID NO:202)	2.000
14	163	TPSHHAAQF (SEQ ID NO:228)	2.000

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
15	120	ASSGQARMF (SEQ ID NO:40)	2.000
16	18	LGGGGGCAL (SEQ ID NO:134)	2.000
17	177	KHEDPMGQQ (SEQ ID NO:121)	1.800
18	83	PHEEQCLSA (SEQ ID NO:154)	1.800
19	10	ALLPAVPSL (SEQ ID NO:34)	1.300
20	225	NLYQMTSQL (SEQ ID NO:147)	1.300

Table XXIII

Results of BIMAS HLA Peptide Binding Prediction Analysis for

Binding of Human WT1 Peptides to Human HLA B 3901

(HLA-B3901 peptide binding motif anchor residues are R or H at P2; L at P9; auxiliary anchors are I, V, or L at P6)

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	437	MHQRNMTKL (SEQ ID NO:143)	135.000
2	332	KRYFKLSHL (SEQ ID NO:127)	45.000
3	434	HHNMHQRNM (SEQ ID NO:108)	30.000
4	362	RRFSRSDQL (SEQ ID NO:187)	30.000
5	372	RHQRRHTGV (SEQ ID NO:181)	30.000
6	10	ALLPAVPSL (SEQ ID NO:34)	9.000
7	439	QRNMTKLQL (SEQ ID NO:173)	7.500
8	390	RKFSRSDHL (SEQ ID NO:183)	6.000
9	396	DHLKTHTRT (SEQ ID NO:57)	6.000
10	239	NQMNLGATL (SEQ ID NO:151)	6.000
11	423	KKFARSDEL (SEQ ID NO:122)	6.000
12	126	RMFPNAPYL (SEQ ID NO:185)	6.000
13	225	NLYQMTSQL (SEQ ID NO:147)	6.000
14	180	DPMGQQGSL (SEQ ID NO:59)	6.000
15	144	IRNQGYSTV (SEQ ID NO:117)	5.000
16	136	SCLESQPAI (SEQ ID NO:198)	4.000
17	292	GVFRGIQDV (SEQ ID NO:103)	3.000
18	302	RVPGVAPTL (SEQ ID NO:195)	3.000
19	208	SCTGSQALL (SEQ ID NO:202)	3.000
20	207	DSCTGSQAL (SEQ ID NO:61)	3.000

Table XXIV

Results of BIMAS HLA Peptide Binding Prediction Analysis for Binding of Human WT1 Peptides to Human HLA B 3902

(HLA-B3902 peptide binding motif anchor residues are K or Q at P2; L at P9; auxiliary anchors are I, L, F, or V at P5)

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	239	NQMNLGATL (SEQ ID NO:151)	24.000
2	390	RKFSRSDHL (SEQ ID NO:183)	20.000
3	423	KKFARSDEL (SEQ ID NO:122)	20.000
4	32	AQWAPVLDF (SEQ ID NO:37)	5.000
5	146	NQGYSTVTF (SEQ ID NO:150)	5.000
6	130	NAPYLPSCL (SEQ ID NO:144)	2.400
7	225	NLYQMTSQL (SEQ ID NO:147)	2.400
8	30	GAAQWAPVL (SEQ ID NO:86)	2.400
9	441	NMTKLQLAL (SEQ ID NO:149)	2.400
10	302	RVPGVAPTL (SEQ ID NO:195)	2.400
11	126	RMFPNAPYL (SEQ ID NO:185)	2.000
12	218	RTPYSSDNL (SEQ ID NO:194)	2.000
13	209	CTGSQALLL (SEQ ID NO:52)	2.000
14	332	KRYFKLSHL (SEQ ID NO:127)	2.000
15	180	DPMGQQGSL (SEQ ID NO:59)	2.000
16	437	MHQRNMTKL (SEQ ID NO:143)	2.000
17	207	DSCTGSQAL (SEQ ID NO:61)	2.000
18	208	SCTGSQALL (SEQ ID NO:202)	2.000
19	329	GCNKRYFKL (SEQ ID NO:90)	2.000
20	10	ALLPAVPSL (SEQ ID NO:34)	2.000

Table XXV

Results of BIMAS HLA Peptide Binding Prediction Analysis for Binding of Human WT1 Peptides to Human HLA B 4403

(HLA-B4403 peptide binding motif anchor residues are E at P2; Y or F at P9)

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	315	SETSEKRPF (SEQ ID NO:209)	80.000
2	349	GEKPYQCDF (SEQ ID NO:91)	80.000
3	84	HEEQCLSAF (SEQ ID NO:107)	60.000
4	410	SEKPFSCRW (SEQ ID NO:207)	48.000
5	429	DELVRHHNM (SEQ ID NO:53)	24.000

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
6	278	TPILCGAQY (SEQ ID NO:227)	15.000
7	141	QPAIRNQGY (SEQ ID NO:170)	9.000
8	40	FAPPGASAY (SEQ ID NO:74)	9.000
9	213	QALLLRTPY (SEQ ID NO:160)	9.000
10	318	SEKRPFMCA (SEQ ID NO:208)	8.000
11	81	AEPHEEQCL (SEQ ID NO:30)	8.000
12	152	VTFDGTPSY (SEQ ID NO:244)	4.500
13	101	TGTAGACRY (SEQ ID NO:224)	4.500
14	120	ASSGQARMF (SEQ ID NO:40)	4.500
15	261	TEGQSNHST (SEQ ID NO:221)	4.000
16	85	EEQCLSAFT (SEQ ID NO:65)	4.000
17	233	LECMTWNQM (SEQ ID NO:131)	4.000
18	104	AGACRYGPF (SEQ ID NO:31)	4.000
19	3	SDVRDLNAL (SEQ ID NO:206)	3.000
20	185	QGSLGEQQY (SEQ ID NO:166)	3.000

Table XXVI

Results of BIMAS HLA Peptide Binding Prediction Analysis for

Binding of Human WT1 Peptides to Human HLA B 5101

(HLA-B5101 peptide binding motif anchor residues are A, P, or G at P2; F or I at P9)

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	303	VPGVAPTLV (SEQ ID NO:242)	314.600
2	180	DPMGQQGSL (SEQ ID NO:59)	242.000
3	250	VAAGSSSSV (SEQ ID NO:236)	157.300
4	130	NAPYLPSCL (SEQ ID NO:144)	50.000
5	30	GAAQWAPVL (SEQ ID NO:86)	50.000
6	20	GGGGCALPV (SEQ ID NO:92)	44.000
7	64	PPPPPHSFI (SEQ ID NO:157)	40.000
8	29	SGAAQWAPV (SEQ ID NO:211)	40.000
9	18	LGGGGGCAL (SEQ ID NO:134)	31.460
10	295	RGIQDVRRV (SEQ ID NO:179)	22.000
11	119	QASSGQARM (SEQ ID NO:161)	18.150
12	418	WPSCQKKFA (SEQ ID NO:246)	12.100
13	82	EPHEEQCLS (SEQ ID NO:68)	12.100
14	110	GPFGPPPPS (SEQ ID NO:96)	11.000
15	272	ESDNHTTPI (SEQ ID NO:71)	8.000

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
16	306	VAPTLVRSA (SEQ ID NO:237)	7.150
17	280	ILCGAQYRI (SEQ ID NO:116)	6.921
18	219	TPYSSDNLY (SEQ ID NO:231)	6.600
19	128	FPNAPYLPS (SEQ ID NO:79)	6.500
20	204	TPTDSCTGS (SEQ ID NO:230)	6.050

Table XXVII

Results of BIMAS HLA Peptide Binding Prediction Analysis for

Binding of Human WT1 Peptides to Human HLA B 5102

(HLA-B5102 peptide binding motif anchor residues are P, A, or G at P2; I or V at P9; auxiliary anchor is Y at P3)

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	295	RGIQDVRRV (SEQ ID NO:179)	290.400
2	303	VPGVAPTLV (SEQ ID NO:242)	200.000
3	180	DPMGQQGSL (SEQ ID NO:59)	133.100
4	250	VAAGSSSSV (SEQ ID NO:236)	110.000
5	30	GAAQWAPVL (SEQ ID NO:86)	55.000
6	130	NAPYLPSCL (SEQ ID NO:144)	50.000
7	20	GGGGCALPV (SEQ ID NO:92)	44.000
8	29	SGAAQWAPV (SEQ ID NO:211)	44.000
9	64	PPPPPHSFI (SEQ ID NO:157)	40.000
10	119	QASSGQARM (SEQ ID NO:161)	36.300
11	110	GPFGPPPPS (SEQ ID NO:96)	27.500
12	412	KPFSCRWPS (SEQ ID NO:123)	25.000
13	18	LGGGGGCAL (SEQ ID NO:134)	24.200
14	24	CALPVSGAA (SEQ ID NO:43)	16.500
15	219	TPYSSDNLY (SEQ ID NO:231)	15.000
16	292	GVFRGIQDV (SEQ ID NO:103)	14.641
17	136	SCLESQPAI (SEQ ID NO:198)	14.520
18	418	WPSCQKKFA (SEQ ID NO:246)	12.100
19	269	TGYESDNHT (SEQ ID NO:225)	11.000
20	351	KPYQCDFKD (SEQ ID NO:124)	11.000

Table XXVIII

Results of BIMAS HLA Peptide Binding Prediction Analysis for

Binding of Human WT1 Peptides to Human HLA B 5201

(HLA-B5201 peptide binding motif anchor residues are I or V at P8; I or V at P9; auxiliary anchors are Q at P2, F, Y, or W at P3, L, I, or V at P5)

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	191	QQYSVPPPV (SEQ ID NO:171)	100.000
2	32	AQWAPVLDF (SEQ ID NO:37)	30.000
3	243	LGATLKGVA (SEQ ID NO:133)	16.500
4	303	VPGVAPTLV (SEQ ID NO:242)	13.500
5	86	EQCLSAFTV (SEQ ID NO:69)	12.000
6	295	RGIQDVRRV (SEQ ID NO:179)	10.000
7	98	GQFTGTAGA (SEQ ID NO:99)	8.250
8	292	GVFRGIQDV (SEQ ID NO:103)	8.250
9	29	SGAAQWAPV (SEQ ID NO:211)	6.000
10	146	NQGYSTVTF (SEQ ID NO:150)	5.500
11	20	GGGGCALPV (SEQ ID NO:92)	5.000
12	239	NQMNLGATL (SEQ ID NO:151)	4.000
13	64	PPPPPHSFI (SEQ ID NO:157)	3.600
14	273	SDNHTTPIL (SEQ ID NO:204)	3.300
15	286	YRIHTHGVF (SEQ ID NO:252)	3.000
16	269	TGYESDNHT (SEQ ID NO:225)	3.000
17	406	TGKTSEKPF (SEQ ID NO:222)	2.750
18	327	YPGCNKRYF (SEQ ID NO:250)	2.750
19	7	DLNALLPAV (SEQ ID NO:58)	2.640
20	104	AGACRYGPF (SEQ ID NO:31)	2.500

Table XXIX

Results of BIMAS HLA Peptide Binding Prediction Analysis for

Binding of Human WT1 Peptides to Human HLA B 5801

(HLA-B5801 peptide binding motif anchor residues are A, S, or T at P2; F or W at P9; auxiliary anchors are P, E, or K at P4, V, I, L, M, of F, at P5)

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	230	TSQLECMTW (SEQ ID NO:234)	96.800
2	92	FTVHFSGQF (SEQ ID NO:85)	60.000
3	120	ASSGQARMF (SEQ ID NO:40)	40.000
4	168	AAQFPNHSF (SEQ ID NO:29)	20.000

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
5	408	KTSEKPFSC (SEQ ID NO:129)	12.000
6	394	RSDHLKTHT (SEQ ID NO:192)	9.900
7	276	HTTPILCGA (SEQ ID NO:115)	7.200
8	218	RTPYSSDNL (SEQ ID NO:194)	6.600
9	152	VTFDGTPSY (SEQ ID NO:244)	6.000
10	40	FAPPGASAY (SEQ ID NO:74)	6.000
11	213	QALLLRTPY (SEQ ID NO:160)	4.500
12	347	HTGEKPYQC (SEQ ID NO:112)	4.400
13	252	AGSSSSVKW (SEQ ID NO:32)	4.400
14	211	GSQALLLRT (SEQ ID NO:102)	4.356
15	174	HSFKHEDPM (SEQ ID NO:110)	4.000
16	317	TSEKRPFMC (SEQ ID NO:233)	4.000
17	26	LPVSGAAQW (SEQ ID NO:138)	4.000
18	289	HTHGVFRGI (SEQ ID NO:113)	3.600
19	222	SSDNLYQMT (SEQ ID NO:217)	3.300
20	96	FSGQFTGTA (SEQ ID NO:82)	3.300

Table XXX

Results of BIMAS HLA Peptide Binding Prediction Analysis for

Binding of Human WT1 Peptides to Human HLA CW0301

(HLA-CW0301 peptide binding motif anchor residues are L, F, M, or I at P9;
auxiliary anchors are V, I, Y, L, or M, at P3, P at P4, F or Y at P6)

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	10	ALLPAVPSL (SEQ ID NO:34)	100.000
2	332	KRYFKLSHL (SEQ ID NO:127)	48.000
3	126	RMFPNAPYL (SEQ ID NO:185)	36.000
4	3	SDVRDLNAL (SEQ ID NO:206)	30.000
5	239	NQMNLGATL (SEQ ID NO:151)	24.000
6	225	NLYQMTSQL (SEQ ID NO:147)	24.000
7	180	DPMGQQGSL (SEQ ID NO:59)	20.000
8	362	RRFSRSDQL (SEQ ID NO:187)	12.000
9	329	GCNKRYFKL (SEQ ID NO:90)	10.000
10	286	YRIHTHGVF (SEQ ID NO:252)	10.000
11	301	RRVPGVAPT (SEQ ID NO:189)	10.000
12	24	CALPVSGAA (SEQ ID NO:43)	10.000
13	136	SCLESQPAI (SEQ ID NO:198)	7.500
14	437	MHQRNMTKL (SEQ ID NO:143)	7.200

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
15	390	RKFSRSDHL (SEQ ID NO:183)	6.000
16	423	KKFARSDEL (SEQ ID NO:122)	6.000
17	92	FTVHFSGQF (SEQ ID NO:85)	5.000
18	429	DELVRHHNM (SEQ ID NO:53)	5.000
19	130	NAPYLPSCL (SEQ ID NO:144)	4.800
20	30	GAAQWAPVL (SEQ ID NO:86)	4.000

Table XXXI

Results of BIMAS HLA Peptide Binding Prediction Analysis for

Binding of Human WT1 Peptides to Human HLA CW0401

(HLA-CW0401 peptide binding motif anchor residues are Y, P, or F at P2; L, F, or M at P9; auxiliary anchors are V, I, or L at P6)

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	356	DFKDCERRF (SEQ ID NO:55)	120.000
2	334	YFKLSHLQM (SEQ ID NO:248)	100.000
3	180	DPMGQQGSL (SEQ ID NO:59)	88.000
4	163	TPSHHAAQF (SEQ ID NO:228)	52.800
5	327	YPGCNKRYF (SEQ ID NO:250)	40.000
6	285	QYRIHTHGV (SEQ ID NO:175)	27.500
7	424	KFARSDELV (SEQ ID NO:119)	25.000
8	326	AYPGCNKRY (SEQ ID NO:42)	25.000
9	192	QYSVPPPVY (SEQ ID NO:176)	25.000
10	417	RWPSCQKKF (SEQ ID NO:196)	22.000
11	278	TPILCGAQY (SEQ ID NO:227)	12.000
12	10	ALLPAVPSL (SEQ ID NO:34)	11.616
13	141	QPAIRNQGY (SEQ ID NO:170)	11.000
14	303	VPGVAPTLV (SEQ ID NO:242)	11.000
15	219	TPYSSDNLY (SEQ ID NO:231)	10.000
16	39	DFAPPGASA (SEQ ID NO:54)	7.920
17	99	QFTGTAGAC (SEQ ID NO:165)	6.000
18	4	DVRDLNALL (SEQ ID NO:62)	5.760
19	70	SFIKQEPSW (SEQ ID NO:210)	5.500
20	63	PPPPPPHSF (SEQ ID NO:158)	5.280

Table XXXII

Results of BIMAS HLA Peptide Binding Prediction Analysis for

Binding of Human WT1 Peptides to Human HLA CW0602

(HLA-CW0602 peptide binding motif anchor residues are L, I, V, or Y at P9; auxiliary anchors are I, L, F, or M at P5, V, I, or L at P6)

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	332	KRYFKLSHL (SEQ ID NO:127)	9.680
2	239	NQMNLGATL (SEQ ID NO:151)	6.600
3	130	NAPYLPSCL (SEQ ID NO:144)	6.600
4	7	DLNALLPAV (SEQ ID NO:58)	6.000
5	441	NMTKLQLAL (SEQ ID NO:149)	6.000
6	225	NLYQMTSQL (SEQ ID NO:147)	6.000
7	4	DVRDLNALL (SEQ ID NO:62)	6.000
8	3	SDVRDLNAL (SEQ ID NO:206)	4.400
9	10	ALLPAVPSL (SEQ ID NO:34)	4.000
10	213	QALLLRTPY (SEQ ID NO:160)	3.300
11	319	EKRPFMCAY (SEQ ID NO:67)	3.000
12	30	GAAQWAPVL (SEQ ID NO:86)	2.200
13	242	NLGATLKGV (SEQ ID NO:146)	2.200
14	292	GVFRGIQDV (SEQ ID NO:103)	2.200
15	207	DSCTGSQAL (SEQ ID NO:61)	2.200
16	362	RRFSRSDQL (SEQ ID NO:187)	2.200
17	439	QRNMTKLQL (SEQ ID NO:173)	2.200
18	295	RGIQDVRRV (SEQ ID NO:179)	2.200
19	423	KKFARSDEL (SEQ ID NO:122)	2.200
20	180	DPMGQQGSL (SEQ ID NO:59)	2.200

Table XXXIII

Results of BIMAS HLA Peptide Binding Prediction Analysis for

Binding of Human WT1 Peptides to Human HLA CW0702

(HLA-CW0702 peptide binding motif anchor residues are Y, F, or L at P9; auxiliary anchors are Y or P at P2, V, Y, I, L, F, or M at P5,V, I, L, or M at P6)

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	319	EKRPFMCAY (SEQ ID NO:67)	26.880
2	326	AYPGCNKRY (SEQ ID NO:42)	24.000
3	40	FAPPGASAY (SEQ ID NO:74)	14.784
4	192	QYSVPPPVY (SEQ ID NO:176)	12.000

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
5	278	TPILCGAQY (SEQ ID NO:227)	12.000
6	219	TPYSSDNLY (SEQ ID NO:231)	12.000
7	213	QALLLRTPY (SEQ ID NO:160)	8.800
8	125	ARMFPNAPY (SEQ ID NO:38)	8.000
9	327	YPGCNKRYF (SEQ ID NO:250)	6.600
10	152	VTFDGTPSY (SEQ ID NO:244)	5.600
11	141	QPAIRNQGY (SEQ ID NO:170)	4.800
12	345	RKHTGEKPY (SEQ ID NO:184)	4.000
13	185	QGSLGEQQY (SEQ ID NO:166)	4.000
14	101	TGTAGACRY (SEQ ID NO:224)	4.000
15	375	RRHTGVKPF (SEQ ID NO:188)	4.000
16	263	GQSNHSTGY (SEQ ID NO:100)	4.000
17	163	TPSHHAAQF (SEQ ID NO:228)	3.000
18	33	QWAPVLDFA (SEQ ID NO:174)	2.688
19	130	NAPYLPSCL (SEQ ID NO:144)	2.640
20	84	HEEQCLSAF (SEQ ID NO:107)	2.400

Table XXXIV

Results of BIMAS HLA Peptide Binding Prediction Analysis for Binding of Human WT1 Peptides to Mouse MHC Class I Db

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	235	CMTWNQMNL (SEQ ID NO:49)	5255.712
2	126	RMFPNAPYL (SEQ ID NO:185)	1990.800
3	221	YSSDNLYQM (SEQ ID NO:253)	930.000
4	228	QMTSQLECM (SEQ ID NO:169)	33.701
5	239	NQMNLGATL (SEQ ID NO:151)	21.470
6	441	NMTKLQLAL (SEQ ID NO:149)	19.908
7	437	MHQRNMTKL (SEQ ID NO:143)	19.837
8	136	SCLESQPAI (SEQ ID NO:198)	11.177
9	174	HSFKHEDPM (SEQ ID NO:110)	10.800
10	302	RVPGVAPTL (SEQ ID NO:195)	10.088
11	130	NAPYLPSCL (SEQ ID NO:144)	8.400
12	10	ALLPAVPSL (SEQ ID NO:34)	5.988
13	208	SCTGSQALL (SEQ ID NO:202)	4.435
14	209	CTGSQALLL (SEQ ID NO:52)	3.548
15	238	WNQMNLGAT (SEQ ID NO:245)	3.300

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
16	218	RTPYSSDNL (SEQ ID NO:194)	3.185
17	24	CALPVSGAA (SEQ ID NO:43)	2.851
18	18	LGGGGGCAL (SEQ ID NO:134)	2.177
19	142	PAIRNQGYS (SEQ ID NO:152)	2.160
20	30	GAAQWAPVL (SEQ ID NO:86)	1.680

Table XXXV

Results of BIMAS HLA Peptide Binding Prediction Analysis for Binding of Human WT1 Peptides to Mouse MHC Class I Dd

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	112	FGPPPPSQA (SEQ ID NO:76)	48.000
2	122	SGQARMFPN (SEQ ID NO:212)	36.000
3	104	AGACRYGPF (SEQ ID NO:31)	30.000
4	218	RTPYSSDNL (SEQ ID NO:194)	28.800
5	130	NAPYLPSCL (SEQ ID NO:144)	20.000
6	302	RVPGVAPTL (SEQ ID NO:195)	20.000
7	18	LGGGGGCAL (SEQ ID NO:134)	20.000
8	81	AEPHEEQCL (SEQ ID NO:30)	10.000
9	29	SGAAQWAPV (SEQ ID NO:211)	7.200
10	423	KKFARSDEL (SEQ ID NO:122)	7.200
11	295	RGIQDVRRV (SEQ ID NO:179)	7.200
12	390	RKFSRSDHL (SEQ ID NO:183)	6.000
13	332	KRYFKLSHL (SEQ ID NO:127)	6.000
14	362	RRFSRSDQL (SEQ ID NO:187)	6.000
15	417	RWPSCQKKF (SEQ ID NO:196)	6.000
16	160	YGHTPSHHA (SEQ ID NO:249)	6.000
17	20	GGGGCALPV (SEQ ID NO:92)	6.000
18	329	GCNKRYFKL (SEQ ID NO:90)	5.000
19	372	RHQRRHTGV (SEQ ID NO:181)	4.500
20	52	GGPAPPPAP (SEQ ID NO:93)	4.000

Table XXXVI

Results of BIMAS HLA Peptide Binding Prediction Analysis for Binding of Human WT1 Peptides to Mouse MHC Class I Kb

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	329	GCNKRYFKL (SEQ ID NO:90)	24.000
2	225	NLYQMTSQL (SEQ ID NO:147)	10.000
3	420	SCQKKFARS (SEQ ID NO:200)	3.960
4	218	RTPYSSDNL (SEQ ID NO:194)	3.630
5	437	MHQRNMTKL (SEQ ID NO:143)	3.600
6	387	TCQRKFSRS (SEQ ID NO:219)	3.600
7	302	RVPGVAPTL (SEQ ID NO:195)	3.300
8	130	NAPYLPSCL (SEQ ID NO:144)	3.000
9	289	HTHGVFRGI (SEQ ID NO:113)	3.000
10	43	PGASAYGSL (SEQ ID NO:153)	2.400
11	155	DGTPSYGHT (SEQ ID NO:56)	2.400
12	273	SDNHTTPIL (SEQ ID NO:204)	2.200
13	126	RMFPNAPYL (SEQ ID NO:185)	2.200
14	128	FPNAPYLPS (SEQ ID NO:79)	2.000
15	3	SDVRDLNAL (SEQ ID NO:206)	1.584
16	207	DSCTGSQAL (SEQ ID NO:61)	1.584
17	332	KRYFKLSHL (SEQ ID NO:127)	1.500
18	18	LGGGGGCAL (SEQ ID NO:134)	1.320
19	233	LECMTWNQM (SEQ ID NO:131)	1.320
20	441	NMTKLQLAL (SEQ ID NO:149)	1.200

Table XXXVII

Results of BIMAS HLA Peptide Binding Prediction Analysis for Binding of Human WT1 Peptides to Mouse MHC Class I Kd

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	285	QYRIHTHGV (SEQ ID NO:175)	600.000
2	424	KFARSDELV (SEQ ID NO:119)	288.000
3	334	YFKLSHLQM (SEQ ID NO:248)	120.000

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
4	136	SCLESQPTI (SEQ ID NO:199)	115.200
5	239	NQMNLGATL (SEQ ID NO:151)	115.200
6	10	ALLPAVSSL (SEQ ID NO:35)	115.200
7	47	AYGSLGGPA (SEQ ID NO:41)	86.400
8	180	DPMGQQGSL (SEQ ID NO:59)	80.000
9	270	GYESDNHTA (SEQ ID NO:105)	72.000
10	326	AYPGCNKRY (SEQ ID NO:42)	60.000
11	192	QYSVPPPVY (SEQ ID NO:176)	60.000
12	272	ESDNHTAPI (SEQ ID NO:70)	57.600
13	289	HTHGVFRGI (SEQ ID NO:113)	57.600
14	126	DVRDLNALL (SEQ ID NO:62)	57.600
15	4	CTGSQALLL (SEQ ID NO:52)	57.600
16	208	SCTGSQALL (SEQ ID NO:202)	48.000
17	441	NMTKLQLAL (SEQ ID NO:149)	48.000
18	207	DSCTGSQAL (SEQ ID NO:61)	48.000
19	130	NAPYLPSCL (SEQ ID NO:144)	48.000
20	235	CMTWNQMNL (SEQ ID NO:49)	48.000

Table XXXVIII

Results of BIMAS HLA Peptide Binding Prediction Analysis for Binding of Human WT1 Peptides to Mouse MHC Class I Kk

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	81	AEPHEEQCL (SEQ ID NO:30)	40.000
2	85	EEQCLSAFT (SEQ ID NO:65)	40.000
3	429	DELVRHHNM (SEQ ID NO:53)	20.000
4	315	SETSEKRPF (SEQ ID NO:209)	20.000
5	261	TEGQSNHST (SEQ ID NO:221)	20.000
6	410	SEKPFSCRW (SEQ ID NO:207)	10.000
7	272	ESDNHTTPI (SEQ ID NO:71)	10.000
8	318	SEKRPFMCA (SEQ ID NO:208)	10.000
9	138	LESQPAIRN (SEQ ID NO:132)	10.000
10	233	LECMTWNQM (SEQ ID NO:131)	10.000
11	298	QDVRRVPGV (SEQ ID NO:164)	10.000
12	84	HEEQCLSAF (SEQ ID NO:107)	10.000
13	349	GEKPYQCDF (SEQ ID NO:91)	10.000
14	289	HTHGVFRGI (SEQ ID NO:113)	10.000

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
15	179	EDPMGQQGS (SEQ ID NO:64)	8.000
16	136	SCLESQPAI (SEQ ID NO:198)	5.000
17	280	ILCGAQYRI (SEQ ID NO:116)	5.000
18	273	SDNHTTPIL (SEQ ID NO:204)	4.000
19	428	SDELVRHHN (SEQ ID NO:203)	4.000
20	3	SDVRDLNAL (SEQ ID NO:206)	4.000

Table XXXIX

Results of BIMAS HLA Peptide Binding Prediction Analysis for Binding of Human WT1 Peptides to Mouse MHC Class I Ld

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	163	TPSHHAAQF (SEQ ID NO:228)	360.000
2	327	YPGCNKRYF (SEQ ID NO:250)	300.000
3	180	DPMGQQGSL (SEQ ID NO:59)	150.000
4	26	LPVSGAAQW (SEQ ID NO:138)	93.600
5	278	TPILCGAQY (SEQ ID NO:227)	72.000
6	141	QPAIRNQGY (SEQ ID NO:170)	60.000
7	219	TPYSSDNLY (SEQ ID NO:231)	60.000
8	303	VPGVAPTLV (SEQ ID NO:242)	60.000
9	120	ASSGQARMF (SEQ ID NO:40)	50.000
10	63	PPPPPPHSF (SEQ ID NO:158)	45.000
11	113	GPPPPSQAS (SEQ ID NO:97)	45.000
12	157	TPSYGHTPS (SEQ ID NO:229)	39.000
13	207	DSCTGSQAL (SEQ ID NO:61)	32.500
14	110	GPFGPPPPS (SEQ ID NO:96)	30.000
15	82	EPHEEQCLS (SEQ ID NO:68)	30.000
16	412	KPFSCRWPS (SEQ ID NO:123)	30.000
17	418	WPSCQKKFA (SEQ ID NO:246)	30.000
18	221	YSSDNLYQM (SEQ ID NO:253)	30.000
19	204	TPTDSCTGS (SEQ ID NO:230)	30.000
20	128	FPNAPYLPS (SEQ ID NO:79)	30.000

Table XL

Results of BIMAS HLA Peptide Binding Prediction Analysis for

Binding of Human WT1 Peptides to Cattle HLA A20

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	350	EKPYQCDFK (SEQ ID NO:66)	1000.00
2	319	EKRPFMCAY (SEQ ID NO:67)	500.000
3	423	KKFARSDEL (SEQ ID NO:122)	500.000
4	345	RKHTGEKPY (SEQ ID NO:184)	500.000
5	390	RKFSRSDHL (SEQ ID NO:183)	500.000
6	137	CLESQPAIR (SEQ ID NO:47)	120.000
7	380	VKPFQCKTC (SEQ ID NO:239)	100.000
8	407	GKTSEKPFS (SEQ ID NO:95)	100.000
9	335	FKLSHLQMH (SEQ ID NO:78)	100.000
10	247	LKGVAAGSS (SEQ ID NO:135)	100.000
11	370	LKRHQRRHT (SEQ ID NO:136)	100.000
12	258	VKWTEGQSN (SEQ ID NO:240)	100.000
13	398	LKTHTRTHT (SEQ ID NO:137)	100.000
14	331	NKRYFKLSH (SEQ ID NO:145)	100.000
15	357	FKDCERRFS (SEQ ID NO:77)	100.000
16	385	CKTCQRKFS (SEQ ID NO:46)	100.000
17	294	FRGIQDVRR (SEQ ID NO:81)	80.000
18	368	DQLKRHQRR (SEQ ID NO:60)	80.000
19	432	VRHHNMHQR (SEQ ID NO:243)	80.000
20	118	SQASSGQAR (SEQ ID NO:216)	80.000

Table XLI

Results of BIMAS HLA Peptide Binding Prediction Analysis for

Binding of Mouse WT1 Peptides to Human HLA A_0201

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	126	RMFPNAPYL (SEQ ID NO:293)	313.968
2	187	SLGEQQYSV (SEQ ID NO:299)	285.163
3	10	ALLPAVSSL (SEQ ID NO:255)	181.794
4	225	NLYQMTSQL (SEQ ID NO:284)	68.360
5	292	GVFRGIQDV (SEQ ID NO:270)	51.790
6	93	TLHFSGQFT (SEQ ID NO:302)	40.986

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
7	191	QQYSVPPPV (SEQ ID NO:290)	22.566
8	280	ILCGAQYRI (SEQ ID NO:274)	17.736
9	441	NMTKLHVAL (SEQ ID NO:285)	15.428
10	235	CMTWNQMNL (SEQ ID NO:258)	15.428
11	7	DLNALLPAV (SEQ ID NO:261)	11.998
12	242	NLGATLKGM (SEQ ID NO:283)	11.426
13	227	YQMTSQLEC (SEQ ID NO:307)	8.573
14	239	NQMNLGATL (SEQ ID NO:286)	8.014
15	309	TLVRSASET (SEQ ID NO:303)	7.452
16	408	KTSEKPFSC (SEQ ID NO:277)	5.743
17	340	LQMHSRKHT (SEQ ID NO:280)	4.752
18	228	QMTSQLECM (SEQ ID NO:289)	4.044
19	37	VLDFAPPGA (SEQ ID NO:304)	3.378
20	302	RVSGVAPTL (SEQ ID NO:295)	1.869

Table XLII

Results of BIMAS HLA Peptide Binding Prediction Analysis for Binding of Mouse WT1 Peptides to Mouse MHC Class I Db

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	221	YSSDNLYQM (SEQ ID NO:308)	312.000
2	126	RMFPNAPYL (SEQ ID NO:293)	260.000
3	235	CMTWNQMNL (SEQ ID NO:258)	260.000
4	437	MHQRNMTKL (SEQ ID NO:281)	200.000
5	238	WNQMNLGAT (SEQ ID NO:305)	12.000
6	130	NAPYLPSCL (SEQ ID NO:282)	8.580
7	3	SDVRDLNAL (SEQ ID NO:298)	7.920
8	136	SCLESQPTI (SEQ ID NO:296)	7.920
9	81	AEPHEEQCL (SEQ ID NO:254)	6.600
10	10	ALLPAVSSL (SEQ ID NO:255)	6.600
11	218	RTPYSSDNL (SEQ ID NO:294)	6.000
12	441	NMTKLHVAL (SEQ ID NO:285)	3.432
13	228	QMTSQLECM (SEQ ID NO:289)	3.120
14	174	HSFKHEDPM (SEQ ID NO:272)	3.120
15	242	NLGATLKGM (SEQ ID NO:283)	2.640
16	261	TEGQSNHGI (SEQ ID NO:301)	2.640
17	225	NLYQMTSQL (SEQ ID NO:284)	2.640

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
18	207	DSCTGSQAL (SEQ ID NO:263)	2.600
19	119	QASSGQARM (SEQ ID NO:288)	2.600
20	18	LGGGGGCGL (SEQ ID NO:279)	2.600

Table XLIII

Results of BIMAS HLA Peptide Binding Prediction Analysis for Binding of Mouse WT1 Peptides to Mouse MHC Class I Kb

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	329	GCNKRYFKL (SEQ ID NO:268)	24.000
2	225	NLYQMTSQL (SEQ ID NO:284)	10.000
3	420	SCQKKFARS (SEQ ID NO:297)	3.960
4	218	RTPYSSDNL (SEQ ID NO:294)	3.630
5	437	MHQRNMTKL (SEQ ID NO:281)	3.600
6	387	TCQRKFSRS (SEQ ID NO:300)	3.600
7	289	HTHGVFRGI (SEQ ID NO:273)	3.000
8	130	NAPYLPSCL (SEQ ID NO:282)	3.000
9	43	PGASAYGSL (SEQ ID NO:287)	2.400
10	155	DGAPSYGHT (SEQ ID NO:260)	2.400
11	126	RMFPNAPYL (SEQ ID NO:293)	2.200
12	128	FPNAPYLPS (SEQ ID NO:267)	2.000
13	207	DSCTGSQAL (SEQ ID NO:263)	1.584
14	3	SDVRDLNAL (SEQ ID NO:298)	1.584
15	332	KRYFKLSHL (SEQ ID NO:276)	1.500
16	233	LECMTWNQM (SEQ ID NO:278)	1.320
17	18	LGGGGGCGL (SEQ ID NO:279)	1.320
18	242	NLGATLKGM (SEQ ID NO:283)	1.200
19	123	GQARMFPN (SEQ ID NO:269)A	1.200
20	441	NMTKLHVAL (SEQ ID NO:285)	1.200

Table XLIV

Results of BIMAS HLA Peptide Binding Prediction Analysis for

Binding of Mouse WT1 Peptides to Mouse MHC Class I Kd

Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	285	QYRIHTHGV (SEQ ID NO:291)	600.000
2	424	KFARSDELV (SEQ ID NO:275)	288.000
3	334	YFKLSHLQM (SEQ ID NO:306)	120.000
4	136	SCLESQPTI (SEQ ID NO:296)	115.200
5	239	NQMNLGATL (SEQ ID NO:286)	115.200
6	10	ALLPAVSSL (SEQ ID NO:255)	115.200
7	47	AYGSLGGPA (SEQ ID NO:256)	86.400
8	180	DPMGQQGSL (SEQ ID NO:262)	80.000
9	270	GYESDNHTA (SEQ ID NO:271)	72.000
10	192	QYSVPPPVY (SEQ ID NO:292)	60.000
11	326	AYPGCNKRY (SEQ ID NO:257)	60.000
12	289	HTHGVFRGI (SEQ ID NO:273)	57.600
13	4	DVRDLNALL (SEQ ID NO:264)	57.600
14	126	RMFPNAPYL (SEQ ID NO:293)	57.600
15	209	CTGSQALLL (SEQ ID NO:259)	48.000
16	86	EQCLSAFTL (SEQ ID NO:265)	48.000
17	302	RVSGVAPTL (SEQ ID NO:295)	48.000
18	218	RTPYSSDNL (SEQ ID NO:294)	48.000
19	272	ESDNHTAPI (SEQ ID NO:266)	48.000
20	225	NLYQMTSQL (SEQ ID NO:284)	48.000

Table XLV

Results of TSites Peptide Binding Prediction Analysis for

Human WT1 Peptides Capable of Eliciting a Helper T cell Response

Peptide	Sequence
p6-23	RDLNALLPAVPSLGGGG (SEQ ID NO:1)
p30-35	GAAQWA (SEQ ID NO:309)
p45-56	ASAYGSLGGPAP (SEQ ID NO:310)
p91-105	AFTVHFSGQFTGTAG (SEQ ID NO:311)
p117-139	PSQASSGQARMFPNAPYLPSCLE (SEQ ID NO:2)
p167-171	HAAQF (SEQ ID NO:312)
p202-233	CHTPTDSCTGSQALLLRTPYSSDNLYQMTSQL (SEQ ID NO:313)
p244-262	GATLKGVAAGSSSSVKWTE (SEQ ID NO:4)

Peptide	Sequence
p287-318	RIHTHGVFRGIQDVRRVPGVAPTLVRSASETS (SEQ ID NO:314)
p333-336	RYFK (SEQ ID NO:315)
p361-374	ERRFSRSDQLKRHQ (SEQ ID NO:316)
p389-410	QRKFSRSDHLKTHTRTHTGKTS (SEQ ID NO:317)
p421-441	CQKKFARSDELVRHHNMHQRN (SEQ ID NO:318)

Certain CTL peptides (shown in Table XLVI) were selected for further study. For each peptide in Table XLVI, scores obtained using BIMAS HLA peptide binding prediction analysis are provided.

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Table XLVI

WT1 Peptide Sequences and HLA Peptide Binding Predictions

Peptide	Sequence	Comments
p329-337	GCNKRYFKL (SEQ ID NOs: 90 and 268)	Score 24,000
p225-233	NLYQMTSQL (SEQ ID NOs: 147 and 284)	binds also to class II and HLA A2, Kd, score 10,000
p235-243	CMTWNQMNL (SEQ ID NOs: 49 and 258)	binds also to HLA A2, score 5,255,712
p126-134	RMFPNAPYL (SEQ ID NOs: 185 and 293)	binds also to Kd, class II and HLA A2, score 1,990,800
p221-229	YSSDNLYQM (SEQ ID NOs: 253 and 308)	binds also to Ld, score 312,000
p228-236	QMTSQLECM (SEQ ID NOs: 169 and 289)	score 3,120
p239-247	NQMNLGATL (SEQ ID NOs: 151 and 286)	binds also to HLA A 0201, Kd, score 8,015
mouse p136-144	SCLESQPTI (SEQ ID NO:296)	binds also to Kd, 1mismatch to human
human p136-144	SCLESQPAI (SEQ ID NO:198)	score 7,920
mouse p10-18	ALLPAVSSL (SEQ ID NO:255)	binds also to Kd, HLA A2, 1 mismatch to human
human p10-18	ALLPAVPSL (SEQ ID NO:34)	score 6,600

Peptide binding to C57Bl/6 murine MHC was confirmed using the leukemia cell line RMA-S, as described by Ljunggren et al., *Nature* 346:476-480, 1990. In brief, RMA-S cells were cultured for 7 hours at 26°C in complete medium supplemented with 1% FCS. A total of 10^6 RMA-S cells were added into each well of a 24-well plate and incubated either alone or with the designated peptide (25ug/ml) for 16 hours at 26°C and additional 3 hours at 37°C in complete medium. Cells were then washed three times and stained with fluorescein isothiocyanate-conjugated anti D^b or anti-K^b antibody (PharMingen, San Diego, CA). Labeled cells were washed twice, resuspended and fixed in 500ul of PBS with 1% paraformaldehyde and analyzed for fluorescence intensity in a flow cytometer (Becton-Dickinson FACSCalibur®). The percentage of increase of D^b or K^b molecules on the surface of the RMA-S cells was measured by increased mean fluorescent intensity of cells incubated with peptide compared with that of cells incubated in medium alone.

Mice were immunized with the peptides capable of binding to murine class I MHC. Following immunization, spleen cells were stimulated *in vitro* and tested for the ability to lyse targets incubated with WT1 peptides. CTL were evaluated with a standard chromium release assay (Chen et al., *Cancer Res. 54*:1065-1070, 1994). 10^6 target cells were incubated at 37°C with 150μCi of sodium ^{51}Cr for 90 minutes, in the presence or absence of specific peptides. Cells were washed three times and resuspended in RPMI with 5% fetal bovine serum. For the assay, 10^4 ^{51}Cr-labeled target cells were incubated with different concentrations of effector cells in a final volume of 200μl in U-bottomed 96-well plates. Supernatants were removed after 4 to 7 hours at 37°C, and the percentage specific lysis was determined by the formula:
% specific lysis = 100 x (experimental release - spontaneous release)/(maximum release-spontaneous release).

The results, presented in Table XLVII, show that some WT1 peptides can bind to class I MHC molecules, which is essential for generating CTL. Moreover, several of the peptides were able to elicit peptide specific CTL (Figures 9A and 9B), as determined using chromium release assays. Following

immunization to CTL peptides p10-18 human, p136-144 human, p136-144 mouse and p235-243, peptide specific CTL lines were generated and clones were established. These results indicate that peptide specific CTL can kill malignant cells expressing WT1.

Table XLVII

Binding of WT1 CTL Peptides to mouse B6 class I antigens

Peptide	Binding Affinity to Mouse MHC Class I
Positive control	91%
negative control	0.5.-1.3%
p235-243	33.6%
p136-144 mouse	27.9%
p136-144 human	52%
p10-18: human	2.2%
p225-233	5.8%
p329-337	1.2%
p126-134	0.9%
p221-229	0.8%
p228-236	1.2%
p239-247	1%

EXAMPLE 5

USE OF A WT1 POLYPEPTIDE TO ELICIT WT1 SPECIFIC CTL IN MICE

This Example illustrates the ability of a representative WT1 polypeptide to elicit CTL immunity capable of killing WT1 positive tumor cell lines.

P117-139, a peptide with motifs appropriate for binding to class I and class II MHC, was identified as described above using TSITES and BIMAS HLA peptide binding prediction analyses. Mice were immunized as described in Example 3. Following immunization, spleen cells were stimulated *in vitro* and tested for the ability to lyse targets incubated with WT1 peptides, as well as WT1 positive and negative tumor cells. CTL were evaluated with a standard chromium release assay. The results, presented in Figures 10A-10D, show that P117 can elicit WT1 specific CTL capable of killing WT1 positive tumor cells, whereas no killing of WT1 negative cells was observed. These results demonstrate that peptide specific CTL in fact kill malignant cells expressing

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WT1 and that vaccine and T cell therapy are effective against malignancies that express WT1.

Similar immunizations were performed using the 9-mer class I MHC binding peptides p136-144, p225-233, p235-243 as well as the 23-mer peptide p117-139. Following immunization, spleen cells were stimulated *in vitro* with each of the 4 peptides and tested for ability to lyse targets incubated with WT1 peptides. CTL were generated specific for p136-144, p235-243 and p117-139, but not for p225-233. CTL data for p235-243 and p117-139 are presented in Figures 11A and 11B. Data for peptides p136-144 and p225-233 are not depicted.

CTL lysis demands that the target WT1 peptides are endogenously processed and presented in association with tumor cell class I MHC molecules. The above WT1 peptide specific CTL were tested for ability to lyse WT1 positive versus negative tumor cell lines. CTL specific for p235-243 lysed targets incubated with the p235-243 peptides, but failed to lyse cell lines that expressed WT1 proteins (Figure 11A). By marked contrast, CTL specific for p117-139 lysed targets incubated with p117-139 peptides and also lysed malignant cells expressing WT1 (Figure 11B). As a negative control, CTL specific for p117-139 did not lyse WT1 negative EL-4 (also referred to herein as E10).

Specificity of WT1 specific lysis was confirmed by cold target inhibition (Figures 12A-12B). Effector cells were plated for various effector: target ratios in 96-well U-bottom plates. A ten-fold excess (compared to hot target) of the indicated peptide-coated target without ^{51}Cr labeling was added. Finally, 10^4 ^{51}Cr-labeled target cells per well were added and the plates incubated at 37°C for 4 hours. The total volume per well was 200µl.

Lysis of TRAMP-C by p117-139 specific CTL was blocked from 58% to 36% by EL-4 incubated with the relevant peptide p117-139, but not with EL-4 incubated with an irrelevant peptide (Figure 12A). Similarly, lysis of BLK-SV40 was blocked from 18% to 0% by EL-4 incubated with the relevant peptide

p117-139 (Figure 12B). Results validate that WT1 peptide specific CTL specifically kill malignant cells by recognition of processed WT1.

Several segments with putative CTL motifs are contained within p117-139. To determine the precise sequence of the CTL epitope all potential 9-mer peptides within p117-139 were synthesized (Table XLVIII). Two of these peptides (p126-134 and p130-138) were shown to bind to H-2^b class I molecules (Table XLVIII). CTL generated by immunization with p117-139 lysed targets incubated with p126-134 and p130-138, but not the other 9-mer peptides within p117-139 (Figure 13A).

The p117-139 specific CTL line was restimulated with either p126-134 or p130-138. Following restimulation with p126-134 or p130-138, both T cell lines demonstrated peptide specific lysis, but only p130-138 specific CTL showed lysis of a WT1 positive tumor cell line (Figures 13B and 13C). Thus, p130-138 appears to be the naturally processed epitope.

Table XLVIII

Binding of WT1 CTL 9mer Peptides within p117-139 to mouse B6 class I antigens

Peptide		Binding Affinity to Mouse MHC Class I
P117-125	PSQASSGQA (SEQ ID NO:221)	2%
P118-126	SQASSGQAR (SEQ ID NO:216)	2%
P119-127	QASSGQARM (SEQ ID Nos: 161 and 288)	2%
P120-128	ASSGQARMF (SEQ ID NO:40	1%
P121-129	SSGQARMFP (SEQ ID NO:222)	1%
P122-130	SGQARMFPN (SEQ ID NO:212)	1%
P123-131	GQARMFPNA (SEQ ID Nos: 98 and 269)	1%
P124-132	QARMFPNAP (SEQ ID NO:223)	1%
P125-133	ARMFPNAPY (SEQ ID NO:38)	1%
P126-134	RMFPNAPYL (SEQ ID NOs: 185 and 293)	79%
P127-135	MFPNAPYLP (SEQ ID NO:224)	2%
P128-136	FPNAPYLPS (SEQ ID NOs: 79 and 267)	1%
P129-137	PNAPYLPSC (SEQ ID NO:225)	1%
P130-138	NAPYLPSCL (SEQ ID NOs: 144 and 282)	79%
P131-139	APYLPSCLE (SEQ ID NO:226)	1%

EXAMPLE 6

IDENTIFICATION OF WT1 SPECIFIC MRNA IN MOUSE TUMOR CELL LINES

This Example illustrates the use of RT-PCR to detect WT1 specific mRNA in cells and cell lines.

Mononuclear cells were isolated by density gradient centrifugation, and were immediately frozen and stored at -80°C until analyzed by RT-PCR for the presence of WT1 specific mRNA. RT-PCR was generally performed as described by Fraizer et al., *Blood 86*:4704-4706, 1995. Total RNA was extracted from 10^7 cells according to standard procedures. RNA pellets were resuspended in 25 μL diethylpyrocarbonate treated water and used directly for reverse transcription. The zinc-finger region (exons 7 to 10) was amplified by PCR as a 330 bp mouse cDNA. Amplification was performed in a thermocycler during one or, when necessary, two sequential rounds of PCR. AmpliTaq DNA Polymerase (Perkin Elmer Cetus, Norwalk, CT), 2.5 mM $MgCl_2$ and 20 pmol of each primer in a total reaction volume of 50μl were used. Twenty μL aliquots of the PCR products were electrophoresed on 2% agarose gels stained with ethidium bromide. The gels were photographed with Polaroid film (Polaroid 667, Polaroid Ltd., Hertfordshire, England). Precautions against cross contamination were taken following the recommendations of Kwok and Higuchi, *Nature 339*:237-238, 1989. Negative controls included the cDNA- and PCR-reagent mixes with water instead of cDNA in each experiment. To avoid false negatives, the presence of intact RNA and adequate cDNA generation was evaluated for each sample by a control PCR using β-actin primers. Samples that did not amplify with these primers were excluded from analysis.

Primers for amplification of WT1 in mouse cell lines were: P115: 1458-1478: 5' CCC AGG CTG CAA TAA GAG ATA 3' (forward primer; SEQ ID NO:21); and P116: 1767-1787: 5' ATG TTG TGA TGG CGG ACC AAT 3' (reverse primer; SEQ ID NO:22) (*see* Inoue et al, *Blood 88*:2267-2278, 1996; Fraizer et al., *Blood 86*:4704-4706, 1995).

Beta Actin primers used in the control reactions were: 5' GTG GGG CGC CCC AGG CAC CA 3' (sense primer; SEQ ID NO:23); and 5' GTC CTT AAT GTC ACG CAC GAT TTC 3' (antisense primer; SEQ ID NO:24)

Primers for use in amplifying human WT1 include: P117: 954-974: 5' GGC ATC TGA GAC CAG TGA GAA 3' (SEQ ID NO:25); and P118: 1434-1414: 5' GAG AGT CAG ACT TGA AAG CAGT 3' (SEQ ID NO:5). For nested RT-PCR, primers may be: P119: 1023-1043: 5' GCT GTC CCA CTT ACA GAT GCA 3' (SEQ ID NO:26); and P120: 1345-1365: 5' TCA AAG CGC CAG CTG GAG TTT 3' (SEQ ID NO:27).

Table XLVIII shows the results of WT1 PCR analysis of mouse tumor cell lines. Within Table IV, (+++) indicates a strong WT1 PCR amplification product in the first step RT PCR, (++) indicates a WT1 amplification product that is detectable by first step WT1 RT PCR, (+) indicates a product that is detectable only in the second step of WT1 RT PCR, and (-) indicates WT1 PCR negative.

Table XLIX

Detection of WT1 mRNA in Mouse Tumor Cell Lines

Cell Line	WT1 mRNA
K562 (human leukemia; ATCC): Positive control; (Lozzio and Lozzio, *Blood 45*:321-334, 1975)	+++
TRAMPC (SV40 transformed prostate, B6); Foster et al., *Cancer Res. 57*:3325-3330, 1997	+++
BLK-SV40 HD2 (SV40-transf. fibroblast, B6; ATCC); *Nature 276*:510-511, 1978	++
CTLL (T-cell, B6; ATCC); Gillis, *Nature 268*:154-156, 1977)	+
FM (FBL-3 subline, leukemia, B6); Glynn and Fefer, *Cancer Res. 28*:434-439, 1968	+
BALB 3T3 (ATCC); Aaroston and Todaro, *J. Cell. Physiol. 72*:141-148, 1968	+
S49.1 (Lymphoma, T-cell like, B/C; ATCC); Horibata and Harris, *Exp. Cell. Res. 60*:61, 1970	+
BNL CL.2 (embryonic liver, B/C; ATCC); *Nature 276*:510-511, 1978	+
MethA (sarcoma, B/C); Old et al., *Ann. NY Acad. Sci. 101*:80-106, 1962	-

Cell Line	WT1 mRNA
P3.6.2.8.1 (myeloma, B/C; ATCC); *Proc. Natl. Acad. Sci. USA 66*:344, 1970	-
P2N (leukemia, DBA/2; ATCC); Melling et al., *J. Immunol. 117*:1267-1274, 1976	-
BCL1 (lymphoma, B/C; ATCC); Slavin and Strober, *Nature 272*:624-626, 1977	-
LSTRA (lymphoma, B/C); Glynn et al., *Cancer Res. 28*:434-439, 1968	-
E10/EL-4 (lymphoma, B6); Glynn et al., *Cancer Res. 28*:434-439, 1968	-

EXAMPLE 7

EXPRESSION IN E. COLI OF WT1 TRX FUSION CONSTRUCT

The truncated open reading frame of WT1 (WT1B) was PCR amplified with the following primers:

Forward Primer starting at amino acid 2

P-37 (SEQ ID NO. 342) 5' ggctccgacgtgcgggacctg 3' Tm 64°C

Reverse Primer creating EcoRI site after stop codon

P-23 (SEQ ID NO. 343) 5' gaattctcaaagcgccagctggagtttggt 3'

Tm 63°C

The PCR was performed under the following conditions:

10μl 10X Pfu buffer

1μl 10mM dNTPs

2μl 10μM each oligo

83μL sterile water

1.5μl Pfu DNA polymerase (Stratagene, La Jolla, CA)

50 ng DNA (pPDM FL WT1)

96°C 2 minutes

96°C 20 seconds 63°C 15 seconds 72°C 3 minutes x 40 cycles

72°C 4 minutes

The PCR product was digested with EcoRI restriction enzyme, gel purified and then cloned into pTrx 2H vector (a modified pET28 vector with a Trx fusion on the N-terminal and two His tags surrounding the Trx fusion. After the Trx fusion there exists protease cleavage sites for thrombin and enterokinase). The pTrx2H construct was digested with StuI and EcoRI restriction enzymes. The correct constructs were confirmed by DNA sequence analysis and then transformed into BL21 (DE3) pLys S and BL21 (DE3) CodonPlus expression host cells.

EXAMPLE 8

EXPRESSION IN E. COLI OF WT1 A HIS TAG FUSION CONSTRUCTS

The N-terminal open reading frame of WT1 (WT1A) was PCR amplified with the following primers:

Forward Primer starting at amino acid 2

P-37 (SEQ ID NO. 344) 5'ggctccgacgtgcgggacctg 3' Tm 64°C

Reverse Primer creating EcoRI site after an artificial stop codon put after amino acid 249.

PDM-335 (SEQ ID NO. 345)

5'gaattctcaaagcgccagctggagtttggt 3' Tm 64°C

The PCR was performed under the following conditions:

10µl 10X Pfu buffer

1µl 10mM dNTPs

2µl 10µM each oligo

83µL sterile water

1.5µl Pfu DNA polymerase (Stratagene, La Jolla, CA)

50 ng DNA (pPDM FL WT1)

96°C 2 minutes

96°C 20 seconds 63°C 15 seconds 72°C 1 minute 20 seconds x

40 cycles

72°C 4 minutes

The PCR product was digested with EcoRI restriction enzyme, gel purified and then cloned into pPDM, a modified pET28 vector with a His tag in frame, which had been digested with Eco72I and EcoRI restriction enzymes. The PCR product was also transformed into pTrx 2H vector. The pTrx2H construct was digested with StuI and EcoRI restriction enzymes. The correct constructs were confirmed by DNA sequence analysis and then transformed into BL21 (DE3) pLys S and BL21 (DE3) CodonPlus expression host cells.

EXAMPLE 9

EXPRESSION IN E. COLI OF WT1 B HIS TAG FUSION CONSTRUCTS

The truncated open reading frame of WT1 (WT1A) was PCR amplified with the following primers:

Forward Primer starting at amino acid 250
PDM-346 (SEQ ID NO. 346) 5′ cacagcacagggtacgagagc 3′
Tm 58°C

Reverse Primer creating EcoRI site after stop codon
P-23 (SEQ ID NO. 347) 5′gaattctcaaagcgccagctggagtttggt 3′
Tm 63°C
The PCR was performed under the following conditions:

10μl 10X Pfu buffer
1μl 10mM dNTPs
2μl 10μM each oligo
83μL sterile water
1.5μl Pfu DNA polymerase (Stratagene, La Jolla, CA)
50 ng DNA (pPDM FL WT1)

96°C 2 minutes
96°C 20 seconds 63°C 15 seconds 72°C 1 minute 30 seconds x
40 cycles
72°C 4 minutes

The PCR product was digested with EcoRI restriction enzyme, gel purified and then cloned into pPDM, a modified pET28 vector with a His tag in frame, which had been digested with Eco72I and EcoRI restriction enzymes. The PCR product was also transformed into pTrx 2H vector. The pTrx 2H construct was digested with StuI and EcoRI restriction enzymes. The correct constructs were confirmed by DNA sequence analysis and then transformed into BL21 (DE3) pLys S and BL21 (DE3) CodonPlus expression host cells.

For Examples 7-9, the following SEQ ID NOs. are disclosed:

SEQ ID NO. 327 is the determined cDNA sequence for Trx_WT1_B

SEQ ID NO. 328 is the determined cDNA sequence for Trx_WT1_A

SEQ ID NO. 329 is the determined cDNA sequence for Trx_WT1

SEQ ID NO. 330 is the determined cDNA sequence for WT1_A

SEQ ID NO. 331 is the determined cDNA sequence for WT1_B

SEQ ID NO. 332 is the predicted amino acid sequence encoded by SEQ ID No. 327

SEQ ID NO. 333 is the predicted amino acid sequence encoded by SEQ ID No. 328

SEQ ID NO. 334 is the predicted amino acid sequence encoded by SEQ ID No. 329

SEQ ID NO. 335 is the predicted amino acid sequence encoded by SEQ ID No. 330

SEQ ID NO. 336 is the predicted amino acid sequence encoded by SEQ ID No. 331

EXAMPLE 10

TRUNCATED FORMS OF WT1 EXPRESSED IN E. COLI

Three reading frames of WT1 were amplified by PCR using the following primers:

For WT1 Tr2:

PDM-441 (SEQ ID NO. 348) 5' cacgaagaacagtgcctgagcgcattcac 3' Tm 63°C

PDM-442 (SEQ ID NO. 349) 5'
ccggcgaattcatcagtataaattgtcactgc 3' TM 62°C

For WT1 Tr3:

PDM-443 (SEQ ID NO. 350) 5'
caggctttgctgctgaggacgccc 3' Tm 64°C
PDM-444 (SEQ ID NO. 351) 5'
cacggagaattcatcactggtatggtttctcacc Tm 64°C

For WT1 Tr4:

PDM-445 (SEQ ID NO. 352) 5'
cacagcaggaagcacactggtgagaaac 3' Tm 63°C
PDM-446 (SEQ ID NO. 353) 5'
ggatatctgcagaattctcaaagcgccagc 3' TM 63°C

The PCR was performed under the following conditions:

10μl 10X Pfu buffer
1μl 10mM dNTPs
2μl 10μM each oligo
83μL sterile water
1.5μl Pfu DNA polymerase (Stratagene, La Jolla, CA)
50 ng DNA (pPDM FL WT1)

96°C 2 minutes
96°C 20 seconds 63°C 15 seconds 72°C 30 seconds x 40 cycles
72°C 4 minutes

The PCR products were digested with EcoRI and cloned into pPDM His (a modified pET28 vector with a His tag in frame on the 5' end) which has been digested with Eco72I and EcoRI. The constructs were confirmed to be correct through sequence analysis and transformed into BL21 pLys S and BL21 CodonPlus cells or BLR pLys S and BLR CodonPlus cells.

EXAMPLE 11

WT1 C (amino acids 76-437) AND WT1 D (amino acids 91-437) EXPRESSION IN E. COLI

The WT1 C reading frame was amplified by PCR using the following primers:

PDM-504 (SEQ ID NO. 354) 5′ cactccttcatcaaacaggaac 3′ Tm 61°C

PDM-446 (SEQ ID NO. 355) 5′ ggatatctgcagaattctcaaagcgccagc 3′ Tm 63°C

The PCR was performed under the following conditions:

10μl 10X Pfu buffer
1μl 10mM dNTPs
2μl 10μM each oligo
83μL sterile water
1.5μl Pfu DNA polymerase (Stratagene, La Jolla, CA)
50 ng DNA (pPDM FL WT1)

96°C 2 minutes
96°C 20 seconds 63°C 15 seconds 72°C 2 minutes x 40 cycles
72°C 4 minutes

The PCR product was digested with EcoRI and cloned into pPDM His which had been digested with Eco72I and EcoRI. The sequence was confirmed through sequence analysis and then transformed into BLR pLys S and BLR which is co-transformed with CodonPlus RP.

EXAMPLE 12

SYNTHETIC PRODUCTION OF WT1 TR-1 BY ANNEALING OVERLAPPING OLIGOS

This example was performed to determine the effect of changing proline codon usage on expression.

The following pairs of oligos were annealed:

1. PDM-505 (SEQ ID NO. 356) 5′
ggttccgacgtgcgggacctgaacgcactgctg 3′
PDM-506 (SEQ ID NO. 357) 5′
ctgccggcagcagtgcgttcaggtcccgcacgtcggaacc 3′

2. PDM-507 (SEQ ID NO. 358) 5′
ccggcagttccatccctgggtggcggtggaggctg 3′
PDM-508 (SEQ ID NO. 359) 5′
cggcagtgcgcagcctccaccgccacccagggatggaa 3′

3. PDM-509 (SEQ ID NO. 360) 5′
cgcactgccggttagcggtgcagcacagtgggctc 3′
PDM-510 (SEQ ID NO. 361) 5′
cagaactggagcccactgtgctgcaccgctaac 3′

4. PDM-511 (SEQ ID NO. 362) 5′
cagttctggacttcgcaccgcctggtgcatccgcatac 3′
PDM-512 (SEQ ID NO. 363) 5′
cagggaaccgtatgcggatgcaccaggcggtgcgaagtc 3′

5. PDM-513 (SEQ ID NO. 364) 5′
ggttccctgggtggtccagcacctccgcccgcaacgcc 3′
PDM-514 (SEQ ID NO. 365) 5′
ggcggtgggggcgttgcgggcggaggtgctggaccacc 3′

6. PDM-515 (SEQ ID NO. 366) 5′
cccaccgcctccaccgcccccgcactccttcatcaaacag 3′
PDM-516 (SEQ ID NO. 367) 5′
ctaggttcctgtttgatgaaggagtgcgggggcggtgga 3′

7. PDM-517 (SEQ ID NO. 368) 5′
gaacctagctggggtggtgcagaaccgcacgaagaaca 3′
PDM-518 (SEQ ID NO. 369) 5′
ctcaggcactgttcttcgtgcggttctgcaccaccccag 3′

8. PDM-519 (SEQ ID NO. 370) 5'
gtgcctgagcgcattctgagaattctgcagat 3'
PDM-520 (SEQ ID NO. 371) 5'
gtgtgatggatatctgcagaattctcagaatgcg 3'

Each oligo pair was separately combined then annealed. The pairs were then ligated together and one µl of ligation mix was used for PCR conditions below:

10µl 10X Pfu buffer
1µl 10mM dNTPs
2µl 10µM each oligo
83µL sterile water
1.5µl Pfu DNA polymerase (Stratagene, La Jolla, CA)

96°C 2 minutes
96°C 20 seconds 63°C 15 seconds 72°C 30 seconds x 40 cycles
72°C 4 minutes

The PCR product was digested with EcoRI and cloned into pPDM His which had been digested with Eco72I and EcoRI. The sequence was confirmed and then transformed into BLR pLys S and BLR which is co-transformed with CodonPlus RP.

For examples 10-12, the following SEQ ID NOs. are disclosed:
SEQ ID NO:337 is the determined cDNA sequence for WT1_Tr1
SEQ ID NO:338 is the determined cDNA sequence for WT1_Tr2
SEQ ID NO:339 is the determined cDNA sequence for WT1_Tr3
SEQ ID NO:340 is the determined cDNA sequence for WT1_Tr4
SEQ ID NO:341 is the determined cDNA sequence for WT1_C
SEQ ID NO:342 is the predicted amino acid sequence encoded by SEQ ID NO:337
SEQ ID NO:343 is the predicted amino acid sequence encoded by SEQ ID NO:338
SEQ ID NO:344 is the predicted amino acid sequence encoded by SEQ ID

NO:339

SEQ ID NO:345 is the predicted amino acid sequence encoded by SEQ ID NO:340

SEQ ID NO:346 is the predicted amino acid sequence encoded by SEQ ID NO:341

The WT1 C sequence represents a polynucleotide having the coding regions of TR2, TR3 and TR4.

The WT1 TR-1 synthetic sequence represents a polynucleotide in which alternative codons for proline were substituted for the native codons, producing a polynucleotide capable of expressing WT1 TR-1 in E. coli.

EXAMPLE 13

EVALUATION OF THE SYSTEMIC HISTOPATHOLOGICAL AND TOXICOLOGICAL EFFECTS OF WT1 IMMUNIZATION IN MICE

The purpose of this example is to analyze the immunogenicity and potential systemic histopathological and toxicological effects of WT1 protein immunization in a multiple dose titration in mice.

The experimental design for immunization of mice with WT1 protein is outlined in Table L.

Table L

Experimental Design of WT1 Immunization in Mice

Histology Group	Corixa Group	Treatment Description	Dose Level	Total No. (Females)
1	0	No treatment	0	4
2	1.1	MPL-SE (adjuvants alone), 6x, 1 week apart	10ug	4
3	1.2	MPL-SE, 3x, 2 weeks apart	10ug	4

4	2.1	Ra12-WT1+ MPL-SE, 6x	25ug	4
5	2.2	Ra12-WT1 + MPL-SE, 3x	25ug	4
6	3.1	Ra12-WT1 + MPL-SE, 6x	100ug	4
7	3.2	Ra12-WT1 + MPL-SE, 3x	100ug	4
8	4.1	Ra12-WT1 + MPL-SE, 6x	1000ug	4
9	4.2	Ra12-WT1 + MPL-SE, 3x	1000ug	4

Vaccination to WT1 protein using MPL-SE as adjuvant, in a multiple dose titration study (doses ranging from 25μg, 100μg to 1000μg WT1 protein) in female C57/B6 mice elicited a strong WT1-specific antibody response (Figure 19) and cellular T-cell responses (Figure 20).

No systemic histopathological or toxicological effects of immunization with WT1 protein was observed. No histological evidence for toxicity was seen in the following tissues: adrenal gland, brain, cecum, colon, duodenum, eye, femur and marrow, gall bladder, heart, ileum, jejunum, kidney, larynx, lacrimal gland, liver, lung, lymph node, muscle, esophagus, ovary, pancreas, parathyroid, salivary gland, sternum and marrow, spleen, stomach, thymus, trachea, thyroid, urinary bladder and uterus.

Special emphasis was put on evaluation of potential hematopoietic toxicity. The myeloid/erythroid ratio in sternum and femur marrow was normal. All evaluable blood cell counts and blood chemistry (BUN, creatinine, bilirubin, albumin, globulin) were within the normal range (Table LI).

Given that existent immunity to WT1 is present in some patients with leukemia and that vaccination to WT1 protein can elicit WT1 specific Ab and cellular T-cell responses in mice without toxicity to normal tissues, these experiments validate WT1 as a tumor/leukemia vaccine.

Table LI

Clinical Chemistry and Hematology Analysis

Table LI: WT1 Dose Titration Study
Clinical Chemistry and Hematology Analysis

Animal #	K/uL WBC	M/uL RBC	g/dl Hg.	% HCT	fL MCV	pg MCH	% MCHC
Normal	5.4-16.0	6.7-12.5	10.2-16.6	32-54	31-62	9.2-20.8	22.0-35.5
Group 1							
1 (0)	5.6	8.41	12.8	43.5	53	15.2	29.4
2 (0)	5.5	9.12	13.4	47.5	53	14.7	28.2
3 (0)	7.5	9.22	13.5	48	54	14.7	28.1
4 (0)	3.9	9.27	13.6	46	52	14.7	29.6
Mean	5.6	9.0	13.3	46.3	53.0	14.8	28.8
STD	1.5	0.4	0.4	2.0	0.8	0.3	0.8
Group 2							
5 (1.5)	6.6	9	13.1	46	54	14.5	28.5
6 (1.6)	5.2	8.58	12.6	44	53	14.7	28.6
7 (1.7)	7.8	9.21	13.6	46	53	14.7	29.6
8 (1.8)	6.3	NA	NA	41	NA	NA	NA
Mean	6.5	8.9	13.1	44.3	53.3	14.6	28.9
STD	1.1	0.3	0.5	2.4	0.6	0.1	0.6
Group 3							
9 (2.5)	8.3	9.16	13.6	50.3	55	14.9	27.1
10 (2.6)	5	8.78	13	44.2	50	14.8	29.3
11 (2.7)	4	8.94	13.2	48.3	54	14.7	27.3
12 (2.8)	8.2	NA	NA	41	NA	NA	NA
Mean	6.4	9.0	13.3	46.0	53.0	14.8	27.9
STD	2.2	0.2	0.3	4.2	2.6	0.1	1.2
Group 4							
13 (3.5)	6.1	8.82	13.1	46	54	14.9	28.5
14 (3.6)	6.1	8.64	12.9	46	54	15	28
15 (3.7)	9.3	8.93	13.2	48	55	14.8	27.5
16 (3.8)	4.8	8.19	12.6	44	55	15.3	28.6
Mean	6.6	8.6	13.0	46.0	54.5	15.0	28.2
STD	1.9	0.3	0.3	1.6	0.6	0.2	0.5
Group 5							
17 (4.5)	3.1	8.48	12.6	46	54	14.9	27.5
18 (4.6)	5.7	9.12	13.7	48	54	15	28.5
19 (4.7)	5.3	8.58	13	44.5	55	15.2	29.2
20 (4.8)	5.3	NA	NA	40	NA	NA	NA
Mean	4.9	8.7	13.1	44.6	54.3	15.0	28.4

Table LI: WT1 Dose Titration Study
Clinical Chemistry and Hematology Analysis

Animal #	K/uL WBC	M/uL RBC	g/dl Hg.	% HCT	fL MCV	pg MCH	% MCHC
Normal	5.4-16.0	6.7-12.5	10.2-16.6	32-54	31-62	9.2-20.8	22.0-35.5
STD	1.2	0.3	0.6	3.4	0.6	0.2	0.9
Group 6							
21 (1.1)	3.5	9.36	13.5	37.6	40	14.4	35.9
22 (1.2)	6.9	8.93	13.6	37.3	42	15.3	36.6
23 (1.3)	3.6	8.3	12.5	35.3	43	15.1	35.5
24 (1.4)	NA	NA	NA	NA	NA	NA	NA
Mean	4.7	8.9	13.2	36.7	41.7	14.9	36.0
STD	1.9	0.5	0.6	1.3	1.5	0.5	0.6
Group 7							
25 (2.1)	4	NA	NA	40	NA	NA	NA
26 (2.2)	7.4	9.12	13.2	38.5	42	14.5	34.3
27 (2.3)	4.5	8.19	12.1	34.5	42	14.8	35.1
28 (2.4)	5.8	8.25	12.3	34.1	41	14.9	36.1
Mean	5.4	8.5	12.5	36.8	41.7	14.7	35.2
STD	1.5	0.5	0.6	2.9	0.6	0.2	0.9
Group 8							
29 (3.1)	5.1	8.53	12.6	34.9	41	14.7	36
30 (3.2)	7.6	8.42	13	36.1	43	15.4	35.9
31 (3.3)	3.4	8.45	12.6	34.9	41	14.9	36.1
32 (3.4)	6.1	8.11	12.3	34.8	43	15.2	35.5
Mean	5.6	8.4	12.6	35.2	42.0	15.1	35.9
STD	1.8	0.2	0.3	0.6	1.2	0.3	0.3
Group 9							
33 (4.1)	NA	NA	NA	NA	NA	NA	NA
34 (4.2)	4.5	8.63	12.8	36.2	42	14.8	35.2
35 (4.3)	3.9	8.85	13	36.6	41	14.7	35.6
36 (4.4)	4.7	8.14	12.3	33.8	42	15.1	36.3
Mean	4.4	8.5	12.7	35.5	41.7	14.9	35.7
STD	0.4	0.4	0.4	1.5	0.6	0.2	0.6

Table LI (cont'd): WT1 Dose Titration Study
Clinical Chemistry and Hematology Analysis

Animal #	yes/no Plt. clump	K/uL Platelets	Abs. Baso	Abs. Eos	Abs. Bands	Abs. Polys	Abs. Lymph	Abs. Mono
Normal	no	150-1500	0.0-0.15	0.0-0.51	0.0-0.32	8.0-42.9	8.0-18.0	0.0-1.5
Group 1			K/uL	K/uL	K/uL	K/uL	K/uL	K/uL
1 (0)	yes	726	0	56	0	336	5208	0
2 (0)	no	860	0	0	0	55	5445	0
3 (0)	no	875	0	375	0	525	6525	75
4 (0)	yes	902	0	0	0	156	3744	0
Mean		840.8	0.0	107.8	0.0	268.0	5230.5	18.8
STD		78.4	0.0	180.1	0.0	207.0	1144.8	37.5
Group 2								
5 (1.5)	no	1193	0	132	0	792	5214	462
6 (1.6)	no	1166	0	52	0	624	4472	52
7 (1.7)	no	1087	0	234	0	1170	6396	0
8 (1.8)	yes	NA	0	126	0	126	5922	126
Mean		1148.7	0.0	136.0	0.0	678.0	5501.0	160.0
STD		55.1	0.0	74.8	0.0	433.1	840.5	207.9
Group 3								
9 (2.5)	no	705	0	166	0	664	7387	83
10 (2.6)	no	1140	0	150	0	500	4350	0
11 (2.7)	no	952	0	120	0	680	3200	0
12 (2.8)	yes	NA	0	164	0	656	7216	164
Mean		932.3	0.0	150.0	0.0	625.0	5538.3	61.8
STD		218.2	0.0	21.2	0.0	83.9	2090.6	78.6
Group 4								
13 (3.5)	no	785	0	488	0	732	4636	244
14 (3.6)	yes	973	0	0	0	488	5307	305
15 (3.7)	yes	939	0	465	0	558	7812	465
16 (3.8)	yes	1622	0	192	0	480	4080	48
Mean		1079.8	0.0	286.3	0.0	564.5	5458.8	265.5
STD		370.6	0.0	233.4	0.0	117.0	1647.1	172.4
Group 5								
17 (4.5)	no	892	0	31	0	620	2449	0
18 (4.6)	yes	966	57	114	0	855	4674	0
19 (4.7)	yes	883	0	53	0	742	4452	53
20 (4.8)	yes	NA	0	106	0	53	5141	0
Mean		913.7	14.3	76.0	0.0	567.5	4179.0	13.3
STD		45.5	28.5	40.4	0.0	356.2	1188.5	26.5

Table LI (cont'd): WT1 Dose Titration Study
Clinical Chemistry and Hematology Analysis

Animal #	yes/no Plt. clump	K/uL Platelets	Abs. Baso	Abs. Eos	Abs. Bands	Abs. Polys	Abs. Lymph	Abs. Mono
Normal	no	150-1500	0.0-0.15	0.0-0.51	0.0-0.32	8.0-42.9	8.0-18.0	0.0-1.5
Group 6								
21 (1.1)	yes	784	0	35	0	385	2870	210
22 (1.2)	yes	806	0	69	0	207	6486	138
23 (1.3)	yes	790	0	180	0	396	2988	36
24 (1.4)	NA	NA	NA	NA	NA	NA	NA	NA
Mean		793.3	0.0	94.7	0.0	329.3	4114.7	128.0
STD		11.4	0.0	75.8	0.0	106.1	2054.5	87.4
Group 7								
25 (2.1)	yes	NA	0	80	0	200	3720	0
26 (2.2)	yes	753	0	0	0	518	6734	148
27 (2.3)	yes	725	0	90	0	225	4140	45
28 (2.4)	yes	792	0	232	0	754	4814	0
Mean		756.7	0.0	100.5	0.0	424.3	4852.0	48.3
STD		33.7	0.0	96.5	0.0	263.0	1333.1	69.8
Group 8								
29 (3.1)	yes	784	0	153	0	561	4233	153
30 (3.2)	yes	512	0	152	0	304	6992	152
31 (3.3)	yes	701	0	0	0	238	3094	68
32 (3.4)	yes	631	0	305	0	305	5368	122
Mean		657.0	0.0	152.5	0.0	352.0	4921.8	123.8
STD		115.1	0.0	124.5	0.0	142.8	1663.3	39.9
Group 9								
33 (4.1)	NA	NA	NA	NA	NA	NA	NA	NA
34 (4.2)	yes	724	0	125	0	540	3780	45
35 (4.3)	yes	758	0	117	0	429	3315	39
36 (4.4)	yes	808	0	47	0	329	4089	235
Mean		763.3	0.0	96.3	0.0	432.7	3728.0	106.3
STD		42.3	0.0	42.9	0.0	105.5	389.6	111.5

Table LI (cont'd): WT1 Dose Titration Study
Clinical Chemistry and Hematology Analysis

Animal #	mg/dl BUN	mg/dl Creatinine	g/dl T. protein	g/dl Albumin	g/dl Globulin	mg/dl T. Bilirubin
Normal	13.9-28.3	0.3-1.0	4.0-8.6	2.5-4.8	1.5-3.8	0.10-0.90
Group 1						
1 (0)	NA	NA	NA	NA	NA	NA
2 (0)	28	0.5	4.9	3.7	1.2	0.3
3 (0)	25	0.5	4.9	3.8	1.1	0.2
4 (0)	27	0.5	4.7	3.7	1	0.2
Mean	26.7	0.5	4.8	3.7	1.1	0.2
STD	1.5	0.0	0.1	0.1	0.1	0.1
Group 2						
5 (1.5)	34	0.5	4.6	3.6	1	0.2
6 (1.6)	31	0.4	4.6	3.3	1.3	0.2
7 (1.7)	34	0.6	4.9	4	0.9	0.3
8 (1.8)	NA	NA	NA	NA	NA	NA
Mean	33.0	0.5	4.7	3.6	1.1	0.2
STD	1.7	0.1	0.2	0.4	0.2	0.1
Group 3						
9 (2.5)	NA	NA	NA	NA	NA	NA
10 (2.6)	33	0.5	4.6	3.6	1	0.3
11 (2.7)	NA	NA	NA	NA	NA	NA
12 (2.8)	31	0.5	4.8	3.7	1.1	0.2
Mean	32.0	0.5	4.7	3.7	1.1	0.3
STD	1.4	0.0	0.1	0.1	0.1	0.1
Group 4						
13 (3.5)	32	0.7	4.6	3.4	1.2	0.2
14 (3.6)	34	0.4	4.8	3.8	1	0.2
15 (3.7)	30	0.4	4.7	3.4	1.3	0.2
16 (3.8)	24	0.3	5.1	3.8	1.3	0.2
Mean	30.0	0.5	4.8	3.6	1.2	0.2
STD	4.3	0.2	0.2	0.2	0.1	0.0
Group 5						
17 (4.5)	22	0.4	4.6	3.3	1.3	0.2
18 (4.6)	31	0.5	4.9	3.7	1.2	0.2
19 (4.7)	23	0.6	4.8	3.6	1.2	0.2
20 (4.8)	28	0.5	4.5	3.4	1.1	0.2
Mean	26.0	0.5	4.7	3.5	1.2	0.2
STD	4.2	0.1	0.2	0.2	0.1	0.0

Table LI (cont'd): WT1 Dose Titration Study
Clinical Chemistry and Hematology Analysis

Animal #	Mg/dl BUN	mg/dl Creatinine	g/dl T. protein	g/dl Albumin	g/dl Globulin	mg/dl T. Bilirubin
Normal	13.9-28.3	0.3-1.0	4.0-8.6	2.5-4.8	1.5-3.8	0.10-0.90
Group 6						
21 (1.1)	28	0.3	5.1	3.4	1.7	0.2
22 (1.2)	36	0.3	5.1	3.8	1.3	0.2
23 (1.3)	32	0.4	4.9	3.5	1.4	0.1
24 (1.4)	NA	NA	NA	NA	NA	NA
Mean	32.0	0.3	5.0	3.6	1.5	0.2
STD	4.0	0.1	0.1	0.2	0.2	0.1
Group 7						
25 (2.1)	32	0.2	5	3.4	1.6	0.2
26 (2.2)	24	0.3	4.2	2.8	1.4	0.1
27 (2.3)	28	0.3	4.8	3.2	1.6	0.2
28 (2.4)	27	0.3	5	3.4	1.6	0.1
Mean	27.8	0.3	4.8	3.2	1.6	0.2
STD	3.3	0.0	0.4	0.3	0.1	0.1
Group 8						
29 (3.1)	32	0.3	4.9	3.3	1.6	0.2
30 (3.2)	NA	NA	NA	NA	NA	NA
31 (3.3)	18	0.3	4.8	3.1	1.7	0.2
32 (3.4)	26	0.2	4.2	2.9	1.3	0
Mean	25.3	0.3	4.6	3.1	1.5	0.1
STD	7.0	0.1	0.4	0.2	0.2	0.1
Group 9						
33 (4.1)	25	0.2	4.1	2.7	1.4	0.3
34 (4.2)	NA	NA	NA	NA	NA	NA
35 (4.3)	23	0.2	4.7	3.1	1.6	0.2
36 (4.4)	29	0.3	4.7	3.2	1.5	0.3
Mean	25.7	0.2	4.5	3.0	1.5	0.3
STD	3.1	0.1	0.3	0.3	0.1	0.1

Abbreviations: WBC: white blood cells; RBC: red blood cells; Hg.: hemoglobin; HCT: hematocrit ; MCV: Mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; Plt.: platelets; Abs.: Absolute; Baso: basophils; Eos: eosinophils; Abs. Bands: immature neutrophils ; Polys: polymorphonuclear cells; Lymph: lymphocytes; Mono: monocytes; BUN: blood urea nitrogen.

EXAMPLE 14

ELICITATION OF HUMAN WT1-SPECIFIC T-CELL RESPONSES BY WHOLE GENE IN VITRO PRIMING

This example demonstrates that WT1 specific T-cell responses can be generated from the blood of normal individuals.

Dendritic cells (DC) were differentiated from monocyte cultures derived from PBMC of normal donors by growth for 4-10 days in RPMI medium containing 10% human serum, 50 ng/ml GMCSF and 30 ng/ml IL-4. Following culture, DC were infected 16 hours with recombinant WT1-expressing vaccinia virus at an M.O.I. of 5, or for 3 days with recombinant WT1-expressing adenovirus at an M.O.I. of 10 (Figures 21 and 22). Vaccinia virus was inactivated by U.V. irradiation. CD8+ T-cells were isolated by positive selection using magnetic beads, and priming cultures were initiated in 96-well plates. Cultures were restimulated every 7-10 days using autologous dendritic cells adeno or vaccinia infected to express WT1. Following 3-6 stimulation cycles, CD8+ lines could be identified that specifically produced interferon-gamma when stimulated with autologous-WT1-expressing dendritic cells or fibroblasts. The WT1-specific activity of these lines could be maintained following additional stimulation cycles. These lines were demonstrated to specifically recognize adeno or vaccinia WT1 infected autologous dendritic cells but not adeno or vaccinia EGFP-infected autologous dendritic cells by Elispot assays (Figure 23).

EXAMPLE 15

FORMULATION OF RA12-WT1 FOR INJECTION: USE OF EXCIPIENTS TO STABILIZE LYOPHILIZED PRODUCT

This example describes the formulation that allows the complete solubilization of lyophilized Ra12-WT1.

The following formulation allowed for the recombinant protein Ra12-WT1 to be dissolved into an aqueous medium after being lyophylized to dryness:

Recombinant Ra12-WT1 concentration: 0.5 – 1.0 mg/ml; Buffer: 10-20 mM Ethanolamine, pH 10.0; 1.0 – 5.0 mM Cysteine; 0.05 % Tween-80 (Polysorbate-80); Sugar: 10% Trehalose (T5251, Sigma, MO) 10% Maltose (M9171, Sigma, MO) 10% Sucrose (S7903, Sigma, MO) 10% Fructose (F2543, Sigma, MO) 10% Glucose (G7528, Sigma, MO).

The lyophilized protein with the sugar excipient was found to dissolve significantly more than without the sugar excipient. Analysis by coomassie stained SDS-PAGE showed no signs of remaining solids in the dissolved material.

EXAMPLE 16

FORMULATION OF A WT1 PROTEIN VACCINE

This example describes the induction of WT1-specific immune responses following immunization with WT1 protein and 2 different adjuvant formulations.

According to this example, WT1 protein in combination with MPL-SE induces a strong Ab and Interferon-γ (IFN-γ) response to WT1. Described in detail below are the methods used to induce WT1 specific immune responses following WT1 protein immunization using MPL-SE or Enhanzyn as adjuvant in C57/B6 mice.

C57BL/6 mice were immunized with 20 μg rRa12-WT1 combined with either MPL-SE or Enhanzyn adjuvants. One group of control mice was immunized with rRa12-WT1 without adjuvant and one group was immunized with saline alone. Three intramuscular (IM) immunizations were given, three weeks apart. Spleens and sera were harvested 2 weeks post-final immunization. Sera were analyzed for antibody responses by ELISA on plates coated with Ra12-WT1 fusion, Ra12 or WT1TRX. Similar levels of IgG2a and IgG1 antibody titers were observed in mice immunized with Ra12-WT1+MPL-SE and Ra12-WT1+Enhanzyn. Mice immunized with rRa12-WT1 without adjuvant showed lower levels of IgG2a antibodies.

CD4 responses were assessed by measuring Interferon-γ production following stimulation of splenocytes *in vitro* with rRa12-WT1, rRa12 or with WT1 peptides p6, p117 and p287. Both adjuvants improved the CD4 responses over mice immunized with rRA12-WT1 alone. Additionally, the results indicate that rRA12-WT1+MPL-SE induced a stronger CD4 response than did rRA12-WT1+Enhanzyn. IFN-γ OD readings ranged from 1.4-1.6 in the mice immunized with rRA12-WT1+MPL-SE as compared to 1-1.2 in the mice immunized with rRA12-WT1+Enhanzyn. Peptide responses were only observed against p117, and then only in mice immunized with rRa12-WT1+MPL-SE. Strong IFN-γ responses to the positive control, ConA, were observed in all mice. Only responses to ConA were observed in the negative control mice immunized with saline indicating that the responses were specific to rRA12-WT1.

EXAMPLE 17

CONSTRUCTION OF A RANDOMLY MUTATED WT1 LIBRARY

The nucleic acid sequence of human WT1 was randomly mutated using a polymerase chain reaction method in the presence of 8-oxo dGTP and dPTP (journal of Molecular Biology 1996; 255:589-603). The complete unspliced human WT1 gene is disclosed in SEQ ID NO:380 and the corresponding protein sequence is set forth in SEQ ID NO:404. A splice variant of WT1 was used as a template for the PCR reactions and is disclosed in SEQ ID NOs:381 (DNA) and 408 (protein). Conditions were selected so that the frequency of nucleic acid alterations led to a targeted change in the amino acid sequence, usually 5-30% of the PCR product. The mutated PCR product was then amplified in the absence of the nucleotide analogues using the four normal dNTPs. This PCR product was subcloned into mammalian expression vectors and viral vectors for immunization. This library, therefore, contains a mixed population of randomly mutated WT1 clones. Several clones were selected and sequenced. The mutated WT1 variant DNA sequences are disclosed in SEQ ID NOs:377-379 and the predicted amino acid sequences of

the variants are set forth in SEQ ID NOs:405-407. These altered sequences, and others from the library, can be used as immunogens to induce stronger T cell responses against WT1 protein in cancer cells.

EXAMPLE 18

CONSTRUCTION OF WT1-LAMP FUSIONS

A tripartite fusion was constructed using the polymerase chain reaction and synthetic oligonucleotides containing the desired junctions of human lysosomal associated membrane protein-1 (LAMP-1) and a splice variant of the human WT1 sequence. The splice variant of WT1 and the LAMP-1 sequence used for these fusions are disclosed in SEQ ID NOs:381 and 383. Specifically, the signal peptide of LAMP-1 (base pairs 1-87 of LAMP) was fused to the 5-prime end of the human WT1 open reading frame (1,290 base pairs in length), then the transmembrane and cytoplasmic domain of LAMP-1 (base pairs 1161 to 1281 of LAMP) was fused to the 3-prime end of the WT1 sequence. The sequence of the resulting WT1-LAMP construct is set forth in SEQ ID NO:382 (DNA) and SEQ ID NO:409 (protein). The construct was designed so that when it is expressed in eukaryotic cells, the signal peptide directs the protein to the endoplasmic reticulum (ER) where the localization signals in the transmembrane and cytoplasmic domain of LAMP-1 direct transport of the fusion protein to the lysosomal location where peptides are loaded on to Class II MHC molecules.

EXAMPLE 19

CONSTRUCTION OF WT1-UBIQUITIN FUSIONS FOR ENHANCED MHC CLASS I PRESENTATION

The human ubiquitin open reading frame (SEQ ID NO:384) was mutated such that the nucleotides encoding the last amino acid encode an alanine instead of a glycine. This mutated open reading frame was cloned in frame just upstream of the first codon of a splice variant of human WT1 (SEQ ID NOs:381 and 408, DNA and protein, respectively). The G->A mutation

prevents co-translational cleavage of the nacent protein by the proteases that normally process poly-ubiquitin during translation. The DNA and predicted amino acid sequence for the resulting contruct are set forth in SEQ ID NOs:385 and 410, respectively. The resulting protein demonstrated decreased cellular cytotoxicity when it was expressed in human cells. Whereas it was not possible to generate stable lines expressing native WT1, cell lines expressing the fusion protein were readily obtained. The resulting protein is predicted to be targeted to the proteosome by virtue of the added ubiquitin molecule. This should result in more efficient recognition of the protein by WT1 specific CD8+ T cells.

EXAMPLE 20

CONSTRUCTION OF AN ADENOVIRUS VECTOR EXPRESSING HUMAN WT1

A splice variant of human WT1 (SEQ ID NO:381) was cloned into an E1 and E3 deleted adenovirus serotype 5 vector. The expression of the WT1 gene is controlled by the CMV promoter mediating high levels of WT1 protein expression. Infection of human cells with this reagent leads to a high level of expression of the WT1 protein. The antigenic nature of the adenoviral proteins introduced into the host cell during and produced at low levels subsequent to infection can act to increase immune surveillance and immune recognition of WT1 as an immunological target. This vector can be also used to generate immune responses against the WT1 protein when innoculated into human subjects. If these subjects are positive for WT1 expressing tumor cells the immune response could have a theraputic or curative effect on the course of the disease.

EXAMPLE 21

CONSTRUCTION OF A VACCINIA VIRUS VECTOR EXPRESSING HUMAN WT1

A splice variant of the full length human WT1 gene (SEQ ID NO:381) was cloned into the thymidine kinase locus of the Western Reserve strain of the vaccinia virus using the pSC11 shuttle vector. The WT1 gene is under the control of a hybrid vaccinia virus promoter that mediates gene

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expression throughout the course of vaccinia virus infection. This reagent can be used to express the WT1 protein in human cells in vivo or in vitro. WT1 is a self protein that is overexpressed on some human tumor cells. Thus, immunological responses to WT1 delivered as a protein are unlikely to lead to Major Histocompatibility Class I (MHC class I)-mediated recognition of WT1. However, expression of the protein in the intracellular compartment by the vaccinia virus vector will allow high level MHC class I presentation and recognition of the WT1 protein by CD8+ T cells. Expression of the WT1 protein by the vaccinia virus vector will also lead to presentation of WT1 peptides in the context of MHC class II and thus to recognition by CD4+ T cells.

The uses of this invention include its use as a cancer vaccine. Immunization of human subjects bearing WT1 positive tumors could lead to a theraputic or curative response. The expression of WT1 within the cell will lead to recognition of the protein by both CD4 and CD8 positive T cells.

EXAMPLE 22

GENERATION OF WT1-SPECIFIC CD8+ T-CELL CLONES USING WHOLE GENE PRIMING

Dendritic cells (DC) were differentiated from monocyte cultures derived from PBMC of normal donors by growth for 4-6 days in RPMI medium containing 10% human serum, 50 ng/ml GM-CSF and 30 ng/ml IL-4. Following culture, DC were infected 16 hours with recombinant WT1-expressing vaccinia virus (described in Example 21) at a multiplicity of infection (MOI) of 5 or for 3 days with recombinat WT1-expressing adenovirus at an MOI of 10. Vaccinia virus was inactivated by U.V. irradiation. CD8+ T-cells were isolated by negative depletion using magnetic beads, and priming cultures were initiated in 96-well plates. Cultures were restimulated every 7-10 days using autologous dendritic cells infected with adeno or vaccinia virus engineered to express WT1. Following 4-5 stimulation cycles, CD8+ T-cell lines could be identified that specifically produced interferon-gamma when stimulated with autologous-WT1 expressing dendritic cells or fibroblasts. These lines were cloned and

demonstrated to specifically recognize WT1 transduced autologous fibroblasts but not EGFP transduced fibroblasts by Elispot assays.

The Wilms' tumor (WT1) gene participates in leukemogenesis and is overexpressed in most human leukemias as well as in several solid tumors. Previous studies in humans have demonstrated the presence of WT1 specific antibody (Ab) responses in 16/63 (25%) of AML and in 15/81 (19%) of CML patients studied. Previous studies in mice have shown that WT1 peptide based vaccines elicit WT1 specific Ab, Th and CTL responses. The use of peptides as vaccines in humans is limited by their HLA restriction and the tendency to elicit peptide specific responses and only in a minority of patients tumor specific CTL. The advantages of whole gene immunization are that several helper and CTL epitopes can be included in a single vaccine, thus not restricting the vaccine to specific HLA types. The data disclosed herein demonstrate the induction of WT1 specific immune responses using whole gene in vitro priming. and that WT1 specific CD8+ T-cell clones can be generated. Given that existent immunity to WT1 is present in some patients with leukemia and that murine and human WT1 are 96% identical at the amino acid level and vaccination to WT1 protein, DNA or peptides can elicit WT1 specific Ab, and cellular T-cell responses in mice without toxicity to normal tissues in mice, these human in vitro priming experiments provide further validation of WT1 as a tumor/leukemia vaccine. Furthermore, the ability to generate WT1 specific CD8+ T-cell clones may lead to the treatment of malignancies associated with WT1 overexpression using genetically engineered T-cells.

EXAMPLE 23

RECOMBINANT CONSTRUCTS FOR CLINICAL MANUFACTURING OF WT1

Five constructs were made as described in detail below, for the production of clinical grade WT1.

Design of Ra12/WT-E (SEQ ID NOs:388 (cDNA) and 391 (protein)) and WT-1 E (SEQ ID NOs:386 (cDNA) and 395 (protein)) with No His tag:

The WT-1 E reading frame was PCR amplified with the following primers for the non-His non fusion construct:

PDM-780 (SEQ ID NO:396) 5' gacgaaagcatatgcactccttcatcaaac 3' Tm 66°C

PDM-779 (SEQ ID NO:397) 5' cgcgtgaattcatcactgaatgcctctgaag 3' Tm 63°C

The following PCR cycling conditions were used: 1μl 10X Pfu buffer, 1μl 10mM dNTPs, 2μl 1μM each oligo, 83μl sterile water 1.5μl Pfu DNA polymerase (Stratagene, La Jolla, CA), 50 ηg DNA (pPDMRa12 WT-1 No His). The reaction was denatured initially at 96°C for 2 minutes, followed by 40 cylces of 96°C for 20 seconds, 62°C for 15 seconds, and 72°C for 1 minute and 40 seconds. This was followed by a final extension of 72°C for 4 minutes. The PCR product was digested with NdeI and EcoRI and cloned into pPDM His (a modified pET28 vector) that had been digested with NdeI and EcoRI. The construct was confirmed through sequence analysis and then transformed into BLR (DE3) pLys S and HMS 174 (DE3) pLys S cells. This construct – pPDM WT-1 E was then digested with NcoI and XbaI and used as the vector backbone for the NcoI and XbaI insert from pPDM Ra12 WT-1 F (see below). The construct was confirmed through sequence analysis and then tranformed into BLR (DE3) pLys S and HMS 174 (DE3) pLys S cells. Protein expression was confirmed by Coomassie stained SDS-PAGE and N-terminal protein sequence analysis.

Design of Ra12-WT-1-F (a.a. 1-281) with No His tag (SEQ ID NOs:389 (cDNA) and 393 (protein)):

The Ra12 WT-1 reading frame was PCR amplified with the following primers:

PDM-777 (SEQ ID NO:398) 5' cgataagcatatgacggccgcgtccgataac 3' Tm 66°C

PDM-779 (SEQ ID NO:399) 5' cgcgtgaattcatcactgaatgcctctgaag 3' Tm 63°C

The following PCR cycling conditions were used: 1μl 10X Pfu buffer, 1μl 10mM dNTPs, 2μl 1μM each oligo, 83μl sterile water 1.5μl Pfu DNA polymerase (Stratagene, La Jolla, CA), 50 ηg DNA (pPDMRa12 WT-1 No His). The reaction was denatured initially at 96°C for 2 minutes, followed by 40 cylces of 96°C for 20 seconds, 58°C for 15 seconds, and 72°C for 3 minutes. This was followed by a final extension of 72°C for 4 minutes. The PCR product was digested with NdeI and cloned into pPDM His that had been digested with NdeI and Eco72I. The sequence was confirmed through sequence analysis and then transformed into BLR (DE3) pLys S and HMS 174 (DE3) pLysS cells. Protein expression was confirmed by Coomassie stained SDS-PAGE and N-terminal protein sequence analysis.

Design of Ra12-WT-1 with No His tag (SEQ ID NOs:390 (cDNA) and 392 (protein)):

The Ra12 WT-1 reading frame was PCR amplified with the following primers:

PDM-777 (SEQ ID NO:400) 5' cgataagcatatgacggccgcgtccgataac 3' Tm 66°C

PDM-778 (SEQ ID NO:401) 5' gtctgcagcggccgctcaaagcgccagc 3' Tm 7°C

The following PCR cycling conditions were used: 1μl 10X Pfu buffer, 1μl 10mM dNTPs, 2μl 1μM each oligo, 83μl sterile water 1.5μl Pfu DNA polymerase (Stratagene, La Jolla, CA), 50 ηg DNA (pPDMRa12 WT-1 No His). The reaction was denatured initially at 96°C for 2 minutes, followed by 40 cylces of 96°C for 20 seconds, 68°C for 15 seconds, and 72°C for 2 minutes and 30 seconds. This was followed by a final extension of 72°C for 4 minutes. The PCR product was digested with NotI and NdeI and cloned into pPDM His that had been digested with NdeI and NotI. The sequence was confirmed through sequence anaysis and then transformed into BLR (DE3) pLys S and HMS 174 (DE3) pLysS cells. Protein expression was confirmed by Coomassie stained SDS-PAGE and N-terminal protein sequence analysis.

Design of WT-1 C (a.a. 69-430) in *E. coli* without His tag (SEQ ID NOs:387 (cDNA) and 394 (protein)):

The WT-1 C reading frame was PCR amplified with the following primers:

PDM-780 (SEQ ID NO:402) 5' gacgaaagcatatgcactccttcatcaaac 3' Tm ~6°C

PDM-778 (SEQ ID NO:403) 5' gtctgcagcggccgctcaaagcgccagc 3' Tm ~7°C

The following PCR cycling conditions were used: 1μl 10X Pfu buffer, 1μl 10mM dNTPs, 2μl 1μM each oligo, 83μl sterile water 1.5μl Pfu DNA polymerase (Stratagene, La Jolla, CA), 50 ηg DNA (pPDMRa12 WT-1 No His). The reaction was denatured initially at 96°C for 2 minutes, followed by 40 cylces of 96°C for 20 seconds, 62°C for 15 seconds, and 72°C for 2 minutes. This was followed by a final extension of 72°C for 4 minutes. The PCR product was digested with NdeI and cloned into pPDM His that had been digested with NdeI and Eco72I. The sequence was confirmed through sequence analysis and then transformed into BLR (DE3) pLys S and HMS 174 (DE3) pLys S cells. Protein expression was confirmed by Coomassie stained SDS-PAGE and N-terminal protein sequence analysis.

EXAMPLE 24

GENERATION OF WT1-SPECIFIC $CD8^+$ T CELL CLONES USING WHOLE GENE PRIMING AND IDENTIFICATION OF AN HLA-A2-RESTRICTED WT1 EPITOPE

In this example, Adeno and Vaccinia virus delivery vehicles were used to generate WT1-specific T cell lines. A T cell clone from the line was shown to be specific for WT1 and further, the epitope recognized by this clone was identified.

Dendritic cells (DC) were differentiated from monocyte cultures derived from PBMC of normal donors by growth for 4-6 days in RPMI medium containing 10% human serum, 50 ng/ml GM-CSF and 30 ng/ml IL-4. Following culture, DC were infected 16 hours with recombinant WT1-expressing vaccinia

virus at a multiplicity of infection (MOI) of 5 or for 2-3 days with recombinant WT1-expressing adeno virus at an MOI of 3-10. Vaccinia virus was inactivated by U.V. irradiation. CD8+ T-cells were isolated by negative depletion using antibodies to CD4, CD14, CD16, CD19 and CD56+ cells, followed by magnetic beads specific for the Fc portion of these Abs.

Priming cultures were initiated in 96-well plates. Cultures were restimulated every 7-14 days using autologous dendritic cells infected with adeno or vaccinia virus engineered to express WT1. Following 4-5 stimulation cycles, CD8+ T cell lines could be identified that specifically produced interferon-γ (IFN-γ) when stimulated with autologous-WT1 expressing dendritic cells or fibroblasts. These lines were cloned and demonstrated to specifically recognize WT1 transduced autologous fibroblasts but not control transduced fibroblasts by Elispot assays.

To further analyze HLA restriction of these WT1 specific CD8+ T-cell clones, fibroblasts derived from an additional donor (D475), sharing only the HLA-A2 allele with the donor (D349) from which the T-cell clone was established, were transduced with WT1. ELISPOT analysis demonstrated recognition of these D475 target cells by the T-cell clone. To further demonstrate HLA A2 restriction and demonstrate that this epitope is expressed by tumor cells "naturally" overxpressing WT1 (as part of their malignant transformation), the leukemia cell line K562 was tested. K562 was transduced with the HLA A2 molecule, and HLA-A2 negative K562 cells were used as controls for nonspecific IFN-γ release. ELISPOT analysis demonstrated that the T cells recognized the A2 positive K562 cell line, but not the A2 negative K562 cells. Further proof of specificity and HLA-A2 restriction of the recognition was documented by HLA-A2 antibody blocking experiments.

To further define the WT1 epitope, 4 truncated WT1 retroviral constructs were generated. Donor 475 fibroblasts were then transduced with these constructs. ELISPOT assays demonstrated recognition of D475 fibroblasts transduced with the WT1 Tr1 construct (aa2-aa92), thus demonstrating that the WT1 epitope is localized within the first 91 N-terminal

amino acids of the WT1 protein. To fine map the epitope, 15mer peptides of the WT1 protein, overlapping by 11 amino acids, were synthesized. The WT1 specific T-cell clone recognized two overlapping 15mer peptides, peptide 9 (QWAPVLDFAPPGASA) (SEQ ID NO: 412) and peptide 10 (VLDFAPPGASAYGSL) (SEQ ID NO: 413). To further characterize the minimal epitope recognized, shared 9mer and 10mer peptides of the 15mers (5 total) were used to analyse the specificity of the clone. The clone specifically recognized the 9mer, VLDFAPPGA (SEQ ID NO:241), and the 10mer, VLDFAPPGAS (SEQ ID NO:411).

EXAMPLE 25

CLONING AND SEQUENCING OF TCR ALPHA AND BETA CHAINS DERIVED FROM A CD8 T CELL SPECIFIC FOR WT1

T cell receptor (TCR) alpha and beta chains from CD8+ T cell clones specific for WT1 are cloned. Sequence analysis is carried to demonstrate the family origin of the the alpha and beta chains of the TCR. Additionally, unique diversity and joining segments (contributing to the specificity of the response) are identified.

Total mRNA from 2 x 10^6 cells from a WT1 specific CD8+ T cell clone is isolated using Trizol reagent and cDNA is synthesized using Ready-to-go kits (Pharmacia). To determine Vα and Vβ sequences in a clone, a panel of Vα and Vβ subtype specific primers are synthesized (based on primer sequences generated by Clontech, Palo Alto, CA) and used in RT-PCR reactions with cDNA generated from each clone. The RT-PCR reactions demonstrate which Vβ and Vα sequence is expressed by each clone.

To clone the full-length TCR alpha and beta chains from a clone, primers are designed that span the initiator and terminator-coding TCR nucleotides. Standard 35 cycle RT-PCR reactions are established using cDNA synthesized from the CTL clone and the above primers using the proofreading thermostable polymerase PWO (Roche, Basel, Switzerland). The resultant specific bands (~850 bp for alpha and ~950 for beta) are ligated into the PCR

blunt vector (Invitrogen, Carlsbad, CA) and transformed into *E.coli*. *E.coli* transformed with plasmids containing full-length alpha and beta chains are identified, and large scale preparations of the corresponding plasmids are generated. Plasmids containing full-length TCR alpha and beta chains are then sequenced using standard methods. The diversity-joining (DJ) region that contributes to the specificity of the TCR is thus determined.

EXAMPLE 26

WT1 SPECIFIC CD8+ T-CELL CLONE LYSES WT1-EXPRESSING LEUKEMIC BLASTS

The CD8+ T cell clone intially disclosed in Example 24 that recognizes peptide sequence VLDFAPPGA (human WT1 residues 37-45; SEQ ID NO:241) was further tested for the ability to kill (lyse) WT1 expressing leukemia target cells in an HLA A2 restricted fashion. K562 target cells transduced with the HLA A2 molecule, GFP, A2Kb, or untransduced, were used in a standard 4.5 hour ^{51}Chromium release assay with effector to target cell (E:T) ratios of 25:1 and 5:1. At an E:T ratio of 25:1, the CD8+ T-cell clone lysed the K562/A2 and K562/A2Kb cells (40% and 49% specific lysis, respectively) while the control GFP transduced and the K562 cells were not lysed. At an E:T of 5:1, specific lysis of the K562/A2 and K562/A2Kb cells was 21% and 24%, respectively. Thus, this CD8+ T cell clone recognizes and lyses leukemic cells expressing WT1 in an HLA-A2-restricted fashion. The ability to generate WT1 specific CD8+ T-cell clones has utility in the treatment of malignancies associated with WT1 overexpression using genetically engineered T-cells.

EXAMPLE 27

CONSTRUCTION OF HLA-A2-PEPTIDE-MHC TETRAMERIC COMPLEXES

This example describes the cloning and expression of soluble HLA-A2 in insect cells, and the purification and assembly of HLA-A2 into fluorescent, multivalent peptide-MHC tetramer complexes for the detection and isolation of antigen-specific CD8 T cells.

This system is similar to that developed and described by Altman, et al. (Altman, J., et al., Science, 1996 274(5284):94-6) in that soluble HLA-A2 was singly biotinylated at a birA recognition sequence and was subsequently assembled into multimers on a phycoerythrin-conjugated streptavidin scaffolding. The materials described herein differ in that the HLA-A2 was expressed in a glycosylated, soluble form from insect cells and the heterodimer was purified using an anti-human class I MHC antibody affinity column.

The HLA-A2 heavy chain gene, appended with the birA biotinylation sequence, and the human beta-2-microglobulin gene were cloned into the baculovirus expression vector pFASTBAC-dual. Upon infection of insect cells the genes were concomitantly transcribed from divergent promoters and fully assembled, glycosylated soluble HLA-A2 heterodimer was secreted into the growth medium. The infected insect cells were cultured in cell factories for 4 days at 21°C before the supernatants were harvested. HLA-A2 production was monitored by a capture ELISA employing the W6/32 and biotinylated B9.12.1 antibodies. HLA-A2 was purified from the culture supernatant to >90% purity in one step by affinity chromatography using 2 anti-human class I MHC monoclonal antibodies linked to Sepharose beads. The antibodies used were PA2.1 and W6/32. Purified HLA-A2 was singly biotinylated on the birA recognition sequence on the C-terminus of the heavy chain using the commercially available birA enzyme. The efficiency of biotinylation was evaluated essentially as described (Crawford et al (1998) Immunity June ;8(6):675-82.), and the material was further purified by size exclusion chromatography (SEC). Phycoerythrin-conjugated streptavidin was saturated with bio-HLA-A2 and the mulivalent staining reagent was purified from free HLA-A2 by SEC. HLA-A2 tetramer was incubated for 48 hours at room temperature with a 10-fold molar excess of Her-2/neu E75 peptide or Influenza matrix MI peptide before the specific T cell clones were stained at 4°C for 30 minutes in the presence of peptide loaded tetramer and anti-CD8 antibody. Results indicated that the tetramers incubated in the presence of molar excess of the M1 58-66 M1 influenza peptide specifically stained an

influenza-specific T cell clone and the tetramers incubated with an excess of the Her-2/neu E75 peptide specifically stained the Her-2/new specific T cell clone.

EXAMPLE 28

DETECTION OF WT1 SPECIFIC T-CELLS USING WT1 MHC-PEPTIDE TETRAMERS

HLA-A2 tetramers described in Example 27 were incubated with a molar excess of the WT1 p37-45 peptide (VLDFAPPGA) (human WT1 residues 37-45; SEQ ID NO:241) previously shown in Example 24 to be restricted by HLA-A2. This tetramer was used to stain the WT1-specific CD8+ T cell clone described in Example 24. This clone was shown to specifically recognize the p37-45 epitope. When the tetramers were incubated with an excess of p37-45 peptide, they specifically stained the CD8+ T cell clone while those tetramers incubated with an excess of irrelevant HLA-A2 peptides (Her2/neu, WT1p38-46, WT1p39-47), the tetramers did not stain the CD8+ T cell clone. Thus, the WT1p37-45-specific CD8+ T cell clone is specifically recognized by the HLA-A2-p37-45 peptide MHC tetramer.

A WT1-specific T cell line generated as described in Example 24 was then stained with the HLA-A2-p37-45, irrelevant Her2/neu or WT1p37-46 tetramers. The HLA-A2-p37-45 tetramers stained 1% of the total population of this WT1-specific T cell line and 7% of the gated CD8+ population while the control HLA-A2-p37-46 tetramer stained at the same background levels as the control HLA-A2-Her2/neu tetramers.

These results indicate that MHC-peptide tetramers are a highly sensitive and specific tool for detecting WT1 specific immune responses. The peptide-MHC tetramers can be used for early detection of WT1 associated malignancies, monitoring WT1-specific responses,, and for monitoring minimal residual disease. Detection of WT1 specific T-cells by tetramer staining is also a useful tool to identify groups within a patient population suffering from a WT1 asssociated disease at a higher risk for relapse or disease progression.

EXAMPLE 29

GENERATION OF A WT1-SPECIFIC CD8$^+$ T CELL LINE FROM AN HLA-A24-POSITIVE DONOR USING WHOLE GENE PRIMING

In this example, Adeno and Vaccinia virus delivery vehicles were used to generate WT1-specific T cell lines from an HLA-A24 positive donor. This T cell line was shown to be MHC class I restricted. These experiments further confirm the immunogenicity of the WT1 protein and support its use as a target for vaccine and/or other immunotherapeutic approaches.

Dendritic cells (DC) were differentiated from monocyte cultures derived from PBMC of a normal HLA-A24-positive donor by growth for 4-6 days in RPMI medium containing 10% human serum, 50 ng/ml GM-CSF and 30 ng/ml IL-4. Following culture, DC were infected 16 hours with recombinant WT1-expressing vaccinia virus at a multiplicity of infection (MOI) of 5 or for 2-3 days with recombinant WT1-expressing adeno virus at an MOI of 3-10. Vaccinia virus was inactivated by U.V. irradiation. CD8+ T-cells were isolated by negative depletion using antibodies to CD4, CD14, CD16, CD19 and CD56+ cells, followed by magnetic beads specific for the Fc portion of these Abs.

Priming cultures were initiated in 96-well plates. Cultures were restimulated every 7-14 days using autologous dendritic cells infected with adeno or vaccinia virus engineered to express WT1. Following 4-5 stimulation cycles, CD8+ T cell lines could be identified that specifically produced interferon-γ (IFN-γ) when stimulated with autologous-WT1 expressing dendritic cells or fibroblasts. These lines were cloned and shown by Elispot assays to specifically recognize WT1 transduced autologous fibroblasts but not control transduced fibroblasts in an MHC class I-restricted manner.

These experiments show that the WT1 protein can be used to generate a T cell response and thus, further confirm the immunogenicity of the WT1 antigen and support its use as a target for vaccine and other immunotherapeutic approaches.

EXAMPLE 30

IDENTIFICATION OF HLA-A2 HIGH AFFINITY WT1 EPITOPES

This experiment describes the *in silico* identification of WT1 epitopes predicted to bind to HLA-A2 with higher affinity than naturally processed epitopes. The epitopes identified herein have utility in vaccine and/or immunotherapeutic strategies for the treatment of cancers associated with WT1 expression.

Peptide analogs of the naturally processed HLA A2 restricted WT1 epitope p37-45 (VLDFAPPGA; human WT1 residues 37-45; SEQ ID NO:241; previously shown in Example 24 to be restricted by HLA-A2) with motifs for binding to HLA-A2.1 with higher affinity than the naturally processed peptide were constructed as described in further detail below.

A peptide motif searching program based on algorithms developed by Rammensee, et al (Hans-Georg Rammensee, Jutta Bachmann, Niels Nikolaus Emmerich, Oskar Alexander Bachor, Stefan Stevanovic: SYFPEITHI: database for MHC ligands and peptide motifs. Immunogenetics (1999) 50: 213-219) and by Parker, et al (Parker, K. C., M. A. Bednarek, and J. E. Coligan. 1994. Scheme for ranking potential HLA-A2 binding peptides based on independent binding of individual peptide side-chains. J. Immunol. 152:163.) was used to identify analogs of the WT1 p37-45 peptide epitope that are predicted to bind to HLA-A2 with higher affinity than the natural p37-45 peptide. The peptides shown in Table LII have predicted peptide binding scores equal to or greater than the naturally processed p37-45 peptide. The binding score is derived from a predicted half-time of dissociation to the HLA-A2 class I molecule. The analysis is based on coefficient tables deduced from the published literature by Dr. Kenneth Parker kparker@atlas.niaid.nih.gov, NIAID, NIH.

TableLII

p37-45 Peptide Analogs

Position Modified	Sequence	Theoretical Binding Score	SEQ ID NO:
Wild Type	**VLDFAPPGA**	**3.378**	241
P1	**I**LDFAPPGA	3.378	414
P1	**L**LDFAPPGA	3.378	415
P1	**F**LDFAPPGA	9.141	416
P1	**K**LDFAPPGA	6.955	417
P1	**M**LDFAPPGA	3.378	418
P1	**Y**LDFAPPGA	9.141	419
P2	V**M**DFAPPGA	2.44	420
P4	VLDE**A**PPGA	13.85	421
P4	VLDK**A**PPGA	3.378	422
P6	VLDFA**V**PGA	7.77	423
P8	VLDFAPP**K**A	3.378	424
P9	VLDFAPPG**V**	47.3	425
P9	VLDFAPPG**L**	14.53	426
P1 and P4	**F**LDE**A**PPGA	37.48	427
P1 and P4	**K**LDE**A**PPGA	28.52	428
P1 and P4	**Y**LDE**A**PPGA	37.48	429
P1 and P4	**F**LDK**A**PPGA	9.141	430
P1 and P4	**K**LDK**A**PPGA	6.955	431
P1 and P4	**Y**LDK**A**PPGA	9.141	432
P1 and P9	**F**LDFAPPG**V**	128	433
P1 and P9	**K**LDFAPPG**V**	97.37	434
P1 and P9	**Y**LDFAPPG**V**	128	435
P1 and P9	**F**LDFAPPG**L**	39.31	436
P1 and P9	**K**LDFAPPG**L**	29.91	437

Position Modified	Sequence	Theoretical Binding Score	SEQ ID NO:
P1 and P9	YLDFAPPGL	39.31	438
P1, P4 and P9	FLDEAPPGV	524.7	439
P1, P4 and P9	KLDEAPPGV	399.2	440
P1, P4 and P9	YLDEAPPGV	524.7	441
P1, P4 and P9	FLDEAPPGL	161.2	442
P1, P4 and P9	KLDEAPPGL	122.6	443
P1, P4 and P9	YLDEAPPGL	161.2	444

In a separate analysis, computer modeling was used to identify peptide epitope analogs of the p37-45 WT1 epitope. The coordinates of the HLA-A2 native structure were downloaded from the Brookhaven protein database (pdb I.D.: 3HLA) (L. L. Walsh, "Annotated PDB File Listing", Protein Science 1:5, Diskette Appendix (1992). This file was used as a template for manipulations with the SwissModel (Peitsch MC (1996) ProMod and Swiss-Model: Internet-based tools for automated comparative protein modeling. *Biochem. Soc. Trans.* 24:274-279.) program available through the Expasy web site (Appel R.D., Bairoch A., Hochstrasser D.F.A new generation of information retrieval tools for biologists: the example of the ExPASy WWW server.Trends Biochem. Sci. 19:258-260(1994). The peptide bound to the protein was mutated manually to yield the bound WT p37-45 peptide. The new structure was submitted for three rounds of energy minimization with the GROMOS96 implementation of the Swiss-PdbViewer; two energy minimizations were performed on the whole structure, followed by one round with unfavorable residues selected. A final evaluation showed an overall favorable energy state for the model. Ramachandran plotting indicated that only one non-glycinyl residue is far in disallowed regions. Peptides identified using the modeling method described herein are set forth in Table LIII below.

Table LIII

p37-45 Peptide Analogs Identified by Computer Modeling

Position Modified	Sequence	SEQ ID NO:
Wild Type	**VLDFAPPGA**	241
P6	VLDFAGPGA	445
P6	VLDFATPGA	446
P6 and P9	VLDFATPGV	447
P6 and P9	VLDFATPGL	448
P6 and P9	VLDFATPGS	449
P6 and P9	VLDFATPGA	450

Several peptides identified using the two methods described above were then tested for the ability to be recognized by the p37-45 specific CTL clone (see Example 24). ELISPOT analysis showed that peptides p37-1 (SEQ ID NO:426) and model-1 (SEQ ID NO:445) were recognized by the p37-45 CTL clone. These results suggest that these 2 peptide analogs are predicted to bind to HLA-A2 with higher affinity than the naturally processed epitope and still be recognized by a native T cell receptor.

Thus, this experiment describes the *in silico* identification of WT1 epitopes predicted to bind to HLA-A2 with higher affinity than naturally processed epitopes. Two of the epitopes identified were tested and shown to be recognized by a CTL clone generated with the native WT1 p37-45 epitope. The epitopes identified herein have utility in vaccine and/or immunotherapeutic strategies for the treatment of cancers associated with WT1 expression.

EXAMPLE 31

THE *IN VIVO* IMMUNOGENECITY OF THE WT1 ANTIGEN

This example describes three *in vivo* immunogenicity studies to evaluate vaccination strategies with WT1 in mice. The three strategies

comprised: 1) a naked DNA vaccine prime and boost; 2) an attenuated adenovirus prime followed by an attenuated alphavirus boost; or 3) a naked DNA prime followed by an adenovirus boost. The full-length cDNA of the splice variant of WT1 used in these studies is set forth in SEQ ID NO:381. The results described herein provide support for the use of WT1 DNA/DNA, DNA/adenovirus or adenovirus/alphavirus prime/boost regimens as vaccine strategies for treating cancers associated with WT1 expression.

In the first study, C57/Bl6 mice were immunized 3 times at 2 week intervals with 100 μg of naked DNA encoding for WT1. Mice were sacrificed 2-3 weeks after the final immunization and CTL were evaluated by standard Chromium release assay. This first study showed that WT1 DNA immunization elicits WT1-specific cytotoxic T cell responses in these mice with a 25:1 E:T ratio showing 40% lysis.

In the second study, HLA-A2/Kb transgenic mice were immunized once with 5 X 10^8 PFU of attenuated adenovirus encoding WT1 (as described in Example 20) followed 4 weeks later by one boost with 5 X 10^6 PFU of alphavirus (AlphaVax) encoding WT1. Mice were sacrificed 2-3 weeks after the final immunization and CTL were evaluated by standard Chromium release assay. The results showed that WT1-specific CTL in HLA-A2/Kb transgenic mice specifically lysed dendritic cells (DC) transduced with WT1-expressing viral construct as well as DC pulsed with WT1 peptides. Thus, this immunization strategy also effectively elicits WT1-specific CTL *in vivo*.

In the third study, C57/Bl6 and HLA-A2/Kb transgenic mice were immunized twice with 100 μg of naked WT1 DNA 2 weeks apart followed 3 weeks later by a boost with 7 X 10^8 PFU adenovirus encoding WT1. Mice were sacrificed 2-3 weeks after the final immunization and CTL were evaluated by IFN-γ ELISPOT assay. The results showed that the WT1 DNA and adenovirus prime-boost generates a WT1-specific CD8 T cell response in HLA-A2/Kb transgenic mice. About 42% of CD8 positive cells stained positive for IFN-γ following a 7 day stimulation with DCs transduced with WT-1. The results from the C57/BL6 mice showed that this immunization strategy generates CD8

responses detectable in fresh splenocytes. Splenocytes were stimulated for 6 hours with pools of 10 15-mer peptides overlapping by 11 amino acids that span the entire WT1 protein. Only cells stimulated with the p121-171 showed IFN-γ staining. About 1.1% of those CD8 T cells stimulated with the p121-171 peptide pool stained positive for IFN-γ. This peptide contains the p117-139 peptide (SEQ ID NO:2) shown in Example 3 to elicit **CTL**, T helper cell and antibody responses in mice.

In summary, these results show that the three immunization strategies tested herein generate T cell responses *in vivo*. Thus, these studies further confirm the immunogenicity of the WT1 protein and provide support for the use of WT1 DNA/DNA, DNA/adenovirus or adenovirus/alphavirus prime/boost regimens as vaccine strategies for treating cancers associated with WT1 expression.

EXAMPLE 32

REDUCTION IN WT1+ TUMOR GROWTH IN HLA-A2/Kb TRANSGENIC MICE IMMUNIZED WITH WT1 PROTEIN

This example describes the reduction of WT1+ tumors in transgenic mice immunized with a WT1 vaccine. These results further validate WT1 as a vaccine target and provide support for the use of WT1 in vaccine strategies for treating cancers associated with WT1 expression.

The murine dendritic cell (DC) line DC2.4. was stably transduced with a WT1-LAMP construct (see Example 18, cDNA and protein sequences set forth in SEQ ID NO:382 and 409, respectively). Mice were then inoculated either subcutaneously (s.c.) or intraperitoneally (i.p.) with 2 X 10^6 cells. This resulted in tumor growth in 80-100% of the mice. The tumors established in mice *in vivo* retained their WT1 expression. Thus, this model provides a system in which to validate the efficacy of WT1 vaccine strategies.

Three groups of A2/Kb mice were then immunized 3 times, 2 weeks apart as follows:

Group 1: saline alone s.c.(control, n=10 mice)

Group 2: MPL-SE 10 μg alone s.c. (control, n=10 mice)

Group 3: Ra12/WT1 protein 100 μg + 10 μg MPL-SE s.c. (n=9 mice)

Two to three weeks after the last WT1 immunization, mice were inoculated with 2 X 10^6 A2/Kb DC2.4 tumor cells overexpressing WT1. After tumor challenge mice were monitored and tumor size measured twice per week up to 4 weeks after tumor challenge.

The results showed that the percentage of mice with tumor growth in the group that received the WT1 protein vaccine was reduced from about 100% (saline control) or 90% (MPL-SE adjuvant control) to 45% (WT1 protein immunized group). Further, the average tumor volume was reduced in this group from an average tumor size of 1233 cmm (saline control) or 753cmm (MPL-SE adjuvant control) observed in the control group to 226 cmm in the WT1 protein immunized group. Histopathological analyses showed that tumor margins in vaccinated animals were mixed with host immunological reactions including histiocytes, eosinophils, lymphocytes, mast cells and plasmacytes. Taken together, the results demonstrate that WT1 protein immunization protects against or delays the growth of WT1-positive tumors in the animals immunized with WT1. Thus, these results support the use of WT1 protein as a vaccine for malignancies associated with WT1 expression.

EXAMPLE 33

IDENTIFICATION OF A NATURALLY PROCESSED WT1 CYTOTOXIC T CELL EPITOPE

This example describes the identification of a naturally processed epitope of the WT1 protein recognized by cytotoxic T cells. This experiment further confirms the immunogenicity of the WT1 protein and provides support for its use as a target for vaccine and/or other immunotherapeutic approaches. Additionally, this experiment identifies epitopes of the WT1 protein that may be used in these applications.

HLA-A2/Kb transgenic mice were immunized twice with 100 ug of naked WT1 DNA 2 weeks apart followed 3 weeks later by a boost with 10^7 PFU

adenovirus encoding WT1. Mice were sacrificed 2-3 weeks after the final immunization and CTL were evaluated by standard chromium release assay. As observed in previous experiments, immunization with WT1 DNA followed by adenoviral boost elicited a WT1-specific CTL response in HLA-A2 transgenic mice. In order to identify which epitopes were recognized by the T cells, CTL lines were generated and cloned by limiting dilution using standard protocols. A positive clone was then tested using as target cells DC2.4 A2/Kb cells pulsed with peptides corresponding to the top 20 predicted HLA-A2 restricted CTL epitopes. The results showed that the WT1 p10-18 9mer peptide (amino acids: ALLPAVPSL, set forth in SEQ ID NO:451) was recognized by this CTL clone. This epitope was previously predicted to be an epitope, as described in Table XLVI, SEQ ID NO:34. In an additional experiment, CTL responses to the p10 peptide were observed in 4 of 5 WT1 immunized animals tested. Thus, this experiment demonstrates that the predicted p10-18 WT1 epitope is naturally processed and recognized by CTLs. Moreover, this experiment confirms the immunogenicity of the WT1 protein and further defines a naturally processed HLA-A2-restricted CTL epitope that can be used in vaccine and immunotherpeutic strategies for the treatment of malignancies associated with WT1 overexpression.

EXAMPLE 34

WT1 Expression Constructs Using Twin Arginine Translocator (TAT) Signal Peptide

This example describes the construction of WT1-TAT vectors and expression of WT1-TAT from these vectors. These constructs have utility in the expression of WT1-TAT molecules for the use in vaccination strategies.

WT-1-F (a.a. 2-281 of the WT1 protein; cDNA and amino acid sequence of 2-281 of WT1 are set forth in SEQ ID NOs:460 and 461, respectively) and full-length WT-1 were constructed as pTAT fusions with no His tag as described below. The cDNA and amino acid sequences of the

resulting fusions are set forth in SEQ ID NOs:452 and 453 and SEQ ID NOs:454 and 455, respectively.

The WT-1-F open reading frame was PCR amplified with the following primers:

PDM-439 (SEQ ID NO:456):
5' GGCTCCGACGTGCGGGACCTGAAC 3' Tm 66°C
PDM-779 (SEQ ID NO:457):
5' CGCGTGAATTCATCACTGAATGCCTCTGAAG 3' Tm 63°C

The WT-1 full-length open reading frame was amplified with the following primers:

p37 (SEQ ID NO:458): 5' GGCTCCGACGTGCGGGACCTG 3'
p23 (SEQ ID NO:459):
5' GAATTCTCAAAGCGCCAGCTGGAGTTTGGT 3'

The PCR conditions were as follows: 1μl 10X Pfu buffer, 1μl 10mM dNTPs 2μl 1μM each oligo 83μl sterile water 1.5μl Pfu DNA polymerase (Stratagene, La Jolla, CA) 50 ng DNA (pPDM FL WT-1). The reaction was denatured at 96°C for 2 minutes followed by 40 cycles of 96°C for 20 seconds, 64°C for 15 seconds, and 72°C for 2 minutes, 30 seconds and a single, final extension of 4 minutes at 72°C.

The PCR products were digested with EcoRI and cloned into pTAT (a modified pET28 vector with a Twin Arginine Translocation (TAT) signal peptide from the TorA signal peptide in *E. coli* on the N-terminus; see J. Mol. Microbiol. (2000) 2(2): 179-189; Journal of Bacteriology, Jan 2001 p604-610 Vol 183, No 2; Journal of Biochemistry Vol 276, March 16 2001 pp 8159-8164) at the Eco72I and EcoRI sites. The sequences were confirmed through sequence analysis and then transformed into BLR (DE3) pLys S and HMS 174 (DE3) pLysS cells. Expression of the WT1-TAT proteins was confirmed by Western analysis.

EXAMPLE 35

THE N-TERMINUS OF WT1 IS THE DOMINANT *IN VIVO* IMMUNOGENIC PORTION OF THE PROTEIN

In this Example, mice were immunized with different protein constructs of WT-1, (F truncation (2-281) and full length (2-430) as described in Example 34)) formulated with MPL-SE adjuvant. Improved CD4 responses were elicited by the truncated constructs relative to the full length protein. Thus, this example demonstrates that the N-terminal portion of the WT1 protein spanning from amino acid 2 to 281 is the dominant immunogenic portion of the WT1 protein *in vivo*.

Groups of four C57BL/6 mice were immunized subcutaneously with 20 μg WT-1 proteins: WT-1-F or WT-1 full length (FL), with Ra12, HIS or TAT fusions. Immunizations were performed at weeks 0, 3 and 9, and spleens were harvested at week 11. Splenocytes were then stimulated *in vitro* for 6 hours with medium alone, with a 15-mer peptide "p32" (ARMFPNAPYLPSCLE, amino acids 125-139 of WT-1; found within the p117-139 peptide set forth in SEQ ID NO:2), with the DC2.4-WT-1/LAMP cell line, or with rRa12. CD4 cells were then stained for intracellular interferon-gamma and quantified by FACS analysis. A portion of these splenocytes were then stimulated *in vitro* for 8 days, after which CD4+ IFN+ cells were enumerated. After the 6 hour stimulation with p32, 0.33% of CD4-positive cells were positive for intracellular IFN-gamma staining in mice immunized with the truncated N-terminal construct rWT1-F-TAT. By contrast, only 0.10% of CD4-positive cells stained positive for intracellular IFN-gamma in mice immunized with rWT1-FL-TAT. After the 8 day stimulation, mice immunized with the rWT1-F-TAT construct showed IFN-gamma staining in 10.72% of the CD4+ cells. By contrast, 0.24% of CD4-positive cells from mice immunized with the full-length WT1-TAT construct stained positive for intracellular IFN-gamma. These data indicate that improved CD4 responses were elicited by the truncated rWT1-TAT construct relative to the full-length rWT1-TAT construct.

In a second assay splenocytes were stimulated *in vitro* with the 23-mer peptide, p117-139 (SEQ ID NO:2; PSQASSGQARMFPNAPYLPSCLE, containing a known CD4 epitope and encompassing "p32"), for 3 days, after which supernatants were assayed for secreted IFN-gamma by ELISA. There was no detectable IFN-gamma secretion from splenocytes from mice immunized with the full-length WT1 constructs. By contrast, an average of 2477 pg/ml IFN-gamma was detected from splenocytes from mice immunized with rWT1-F without a HIS tag. An average of 4658 pg/ml IFN-gamma was detected from splenocytes from mice immunized with rWT1-F-TAT. These data further support the observation that improved CD4 responses were elicited by the truncated N-terminal WT1 constructs relative to the full length protein.

The WT1 protein is a transcription factor which is composed of two functional domains: a proline-glutamine rich domain at the N-terminus, and a zinc finger domain composed of four zinc fingers at the C-terminus with homology to the EGR1/Sp1 family of transcription factors. WT1 is a self-protein. The C-terminus is homologous to other self-proteins and is thus less immunogenic, *i.e.* the subject of a greater degree of immunological tolerance. Of note, the 4 zinc-finger domains within the C-terminus have homology to EGR family members. The results described in this example indicate that tolerance will vary between different portions of a protein, possibly depending on sequence homologies and functional domains.

In summary, the data described in this example support the notion that the most efficient WT1 vaccine will comprise the WT1 N-terminus, either as a recombinant protein or gene-based construct.

EXAMPLE 36

BACULOVIRUS EXPRESSION CONSTRUCTS FOR EXPRESSION OF THE N-TERMINAL FRAGMENT OF WT1 (WT-1-F: AMINO ACIDS 1-281) AND LARGE SCALE PRODUCTION OF PROTEIN USING INSECT CELLS

The cDNA for the N-terminal fragment of WT-1, together with a Kozak consensus sequence, were obtained by PCR using the WT1-F plasmid

as a template (WT-1-F: amino acids 2-281 of the WT1 protein cloned downstream of a start methionine; cDNA and amino acid sequence of 2-281 of WT1 are set forth in SEQ ID NOs:460 and 461, respectively. The pTAT fusion construct used as template in the experiments described herein is described in Example 34. The cDNA of this construct is set forth in SEQ ID NO:452). The following primers were used for amplification:

WT1F1 (SEQ ID NO:466):

5' CGGCTCTAGAGCCGCCACCATGGGCTCCGACGTGCG

WT1RV4 (SEQ ID NO:467):

5' CGGCTCTAGACTACTGAATGCCTCTGAAGACACCGTG

The cDNA for the same ORF plus a C-terminal 10 residue His Tag was obtained by PCR similarly as above except using WT1RV3 (SEQ ID NO:468) as reverse primer (5' CGGCTCTAGACTAATGGTGATGGTGATGATGATGGTGATGATGCTGAATG CCTCTGAAGACACCGTG).

The purified PCR products were cloned into the Xba I site of the donor plasmid, pFastBac1. The recombinant donor plasmid pFBWT1-F (cDNA and amino acid sequences set forth in SEQ ID NOs:463 and 465, respectively) and pFBWT1-FH (with the 10X His Tag; cDNA and amino acid sequence set forth in SEQ ID NOs:462 and 464, respectively) were transformed into *E. coli* strain DH10Bac (Invitrogen, Carlsbad, CA) to make recombinant bacmids in *E. coli* through site-specific transposition. The recombinant bacmids were confirmed by PCR analysis and then transfected into Sf-9 insect cells to make recombinant baculoviruses BVWT1-F and BVWT1-FH. The recombinant viruses were amplified to high titer viral stock in Sf-9 cells.

The High Five insect cell line was used to optimize conditions for the protein expression and for the large-scale production of the recombinant proteins. To optimize the conditions for protein expression, High 5 insect cell monolayers were infected with the recombinant baculoviruses BVWT1-F and BVWT1-FH at different multiplicities of infection (MOI) and harvested the

transduced cells at different periods of time. The identities of the proteins were confirmed by Western blot analysis with a rabbit anti-WT1 polyclonal antibody [#942-32 (799L)]. Both WT1-F and WT1-FH recombinant proteins were well expressed at either 48 hours or 54 hours post-infection when High 5 cells were infected by the recombinant viruses at MOI 1.0 or 2.0.

The amplification of the recombinant baculoviruses and the expression of the recombinant WT1-F and WT1-FH proteins was then further optimized. Both BVWT1-F and BVWT1-FH were amplified in the Sf9 insect cell line in ESF921 medium containing 2% fatal calf serum and 0.5X PSF. The amplification was carried out for 4 days at MOI 0,05 at 28°C. The High Five insect cell line was used to optimize conditions for the protein expression. High 5 insect cells were infected by the recombinant baculoviruses at MOIs of 0.5, 1 or 2 for 30, 48, 54, or 72 hours before harvesting. The identity of the recombinant protein was confirmed by Western blot analysis with a specific rabbit polyclonal antibody against WT1 (Antibody #942-32), and by capillary LC-ESI-tandem mass spectrometry. These experiments indicate that optimal protein expression for large scale production of WT1-F and WT1-FH occurs at 43 hours post-infection when High 5 cells were infected by the recombinant viruses at an MOI 0.5-1.0.

In summary, the above WT1 baculovirus can be used for large-scale protein production of the N-terminal portion of WT1 for use in a variety of vaccine strategies for the treatment of malignancies associated with WT1 expression.

EXAMPLE 37

INDUCTION OF *IN VIVO* CD4 AND CD8 T CELL RESPONSES IN MICE USING RECOMBINANT TRICOM VACCINIA AND FOWL POX VECTORS

This example describes *in vivo* immunogenicity studies to evaluate vaccination strategies with WT1 in mice. The purpose of these experiments was to test the ability to rV-WT1/TRICOM and rF-WT1/TRICOM to

induce immunity, in particular T cell immunity, to WT1. The results described herein provide support for the use of TRICOM vaccinia and fowlpox vectors expressing WT1 and containing a triad of costimulatory molecules (B7-1, ICAM-1 and LFA-3) in vaccine strategies for treating cancers associated with WT1 expression.

In the first study, C57Bl/6 mice (12 mice per group) were immunized two or three times with 14 days between the primary, secondary and tertiary immunizations as shown below in Table 1. Mice were harvested at 21 days following the secondary and tertiary immunizations. CD8 and CD4 T cell responses were assayed by IFN-γ intracellular cytokine staining of WT1-peptide activated spleen cells as described in further detail below. CD4 T cell responses were additionally assayed by IFN-γ release from rWT1 protein stimulated spleen cells. Serum IgG_1 and IgG_{2b} antibody responses were assayed by ELISA. T cell responses were evaluated using pooled splenocyte cultures (4 mice/group/time point). Antibody titers were determined for individual mice (4 mice/group/time point).

Table 1: Vaccination Strategy

Groups:
A. Non-immune
B. rWT1 + SE-Vehicle
C. rWT1 + MPL-SE
D. 1°: WT1-DNA, 2°: WT1-DNA, 3°: WT1-Adeno
E. rF-WT1/TRICOM
F. 1°: rV-WT1/TRICOM, 2°: rF-WT1/TRICOM, 3°: rF-WT1/TRICOM
Dose:
rWT1: 50 ug
MPL-SE: 10 ug
WT1-DNA: 100 ug
WT1-Adeno: $5X10^5$ pfu
rF-WT1/TRICOM: $1X10^8$ pfu

rV-WT1/TRICOM: 1X10^8 pfu
Route:
Subcutaneous (200 ul) immunization for protein/adjuvant and vectors.
Intramuscular immunization (50 ul) for WT1-DNA.

The full-length cDNA of the splice variant of WT1 used in these studies is set forth in SEQ ID NO:381. The WT1-adenovirus used herein is as described in Example 20. The rF-WT1/TRICOM recombinant fowlpox and the rV-WT1/TRICOM recombinant vaccinia vectors both expressing WT1 and containing a triad of costimulatory molecules (B7-1, ICAM-1 and LFA-3) were generated by Therion Biologics (Cambridge, MA, USA).

To evaluate T cell responses, splenocytes were stimulated *in vitro* with WT1 peptide "p32" (ARMFPNAPYLPSCLE, amino acids 125-139 of WT-1; found within the p117-139 peptide set forth in SEQ ID NO:2) known to contain a CTL and a helper T cell epitope. Intracellular cytokine staining for IFN-γ of p125-139 activated splenocytes at 21 days following the secondary immunization showed a significant percentage WT1 responsive $CD4^+$ and $CD8^+$ T cells in mice immunized with rV-WT1/TRICOM (prime) and rF-WT1/TRICOM (boost) whereas no other groups showed significant WT1 specific T cell immune responses. Note that the DNA group was primed and boosted with DNA only. The same group subsequently had an rWT1-Adv tertiary immunization.

Following tertiary immunization, responses to pools of overlapping 15-mer peptides were evaluated rather than responses to the single peptide #32. WT1 peptide $CD8^+$ and $CD4^+$ T-cells specific for peptide pool #32-36 were found to respond at levels similar to the responses following secondary immunization in mice vaccinated with rV-WT1/TRICOM (prime) and rF-WT1/TRICOM (boost X2). In addition, $CD4^+$ and $CD8^+$ T-cells from these mice were found to respond, albeit at a much lower level than peptide #32, to a second WT1 epitope contained within two overlapping 15-mer peptides #57-58 (Peptide 57: DNLYQMTSQLECMTWN (amino acids 224-239); Peptide 58:

MTSQLECMTWNQMNL (amino acids 229-243); overlap corresponds to amino acid residues 224-243 of WT1).

Antibody responses were evaluated using a standard ELISA. Low levels of serum IgG_{2b} antibodies to WT1 were measurable in all 8 mice 21 days post secondary immunization (titer of 1:1350) and 21 days post tertiary immunization (titer of 1:3325) in mice immunized with rWT1 + MPL-SE. By contrast, in all other groups serum IgG_{2b} antibodies titers were <1:100 (Table 2).

Table 2: Antibody Responses in WT1-Immunized Mice

Group	Serum Titers*			
	Secondary		Tertiary	
	IgG_1	IgG_{2b}	IgG_1	IgG_{2b}
Non-immune	ND	ND	ND	ND
WT1 + Vehicle-SE	<75	<100	475	<50
WT1 + MPL-SE	<50	1350	338	3325
WT1-DNA, WT1-DNA, WT1-Adeno	ND	ND	ND	ND
rF-WT1/TRICOM	ND	ND	<50	<50
rV-WT/TRICOM, rFWT1/TRICOM	ND	<50	<50	ND

*Serum titers are the average of 4 mice per group. Titers written as <50, <75, or <100 were also averages, but in all instances less than 4 mice had detectable titers. ND indicates antibody was not detected in any of the mice.

In summary, immunization of C57Bl/6 mice with rV-WT1/TRICOM (prime) followed by rF-WT1/TRICOM (boost) or by WT1-DNA (prime) followed by WT1-Adeno (boost) elicited both CD8 and CD4 T cell responses against WT1. The T cell responses to rV-WT1/TRICOM followed by rF-WT1/TRICOM versus WT1-DNA followed by WT1-Adeno were equivalent within the power of the experimental design. Without being bound by theory, a major advantage of

rF-WT1/TRICOM is that multiple boosts can continue to increase the level of immunity. Thus, these studies further confirm the immunogenicity of the WT1 protein and provide support for the use of WT1 DNA/adenovirus or rv-WT1/TRICOM/rF-WT1/TRICOM immunization regimens in vaccine strategies for treating cancers associated with WT1 expression.

EXAMPLE 38

CONSTRUCTION OF THE STUMPY-WT1-F VECTOR FOR EXPRESSION OF WT1-F IN *E. COLI*

This example describes the construction of an expression vector containing a truncated twin arginine translocator (TAT) signal peptide fused to the WT1-F reading frame (2-281 N-terminal portion of the WT1 protein). This vector can be used to produce a single species truncated TAT-WT1-F protein for use in immunization strategies for the treatment of malignancies associated with expression of WT1.

As described previously in Example 34, the TAT signal sequence was used to make various WT1 vectors. When these TAT vectors were used in expression, multiple forms of the protein were observed. N-terminal sequencing of these forms showed that each of the three separate proteins being expressed were truncations of the TAT peptide. These cleavages were occurring at each of the twin arginine sites. Thus, a truncated TAT vector was constructed to shorten the TAT signal peptide from 39 amino acids to 12 amino acids to avoid generation of these cleavage products during expression. The TAT "Stumpy" vector was generated by maintaining the first 12 residues of the TAT signal peptide up to the first twin arginine. This vector was constructed as follows:

The following pairs of oligonucleotides were combined and then annealed.

Pair 1:

Stumpy 1 (SEQ ID NO:486): 5' tatgaacaataacgatctgtttcaggc 3'

Stumpy 3 (SEQ ID NO:487): 5' CTGAAACAGATCGTTATTGTTCA 3'

Pair 2:

Stumpy 4 (SEQ ID NO:488): 5' aattcttggtcattcacgtggcggctcgc 3'

Stumpy 2 (SEQ ID NO:489): 5' gagccgccacgtgaatgaccaag 3'

The pairs were then ligated together with pPDM, a modified pET28 vector, that had been digested with NdeI and EcoRI. The resulting plasmid, pStumpy, is set forth in SEQ ID NO:471. The amino acid sequence of the truncated TAT protein is set forth in SEQ ID NO:506. The full-length TAT polynucleotide is set forth in SEQ ID NO:503 and encodes the amino acid sequence of the TAT polypeptide set forth in SEQ ID NO:504.

The pStumpy vector was then used to construct the Stumpy-WT1-F vector to express the N-terminal portion of WT1 without cleavage products. The Stumpy-WT1-F vector was constructed as follows:

The coding region of WT1-F was PCR amplified with the following primer set:

PDM-1005 (SEQ ID NO:484):

5' GGCTCCGACGTTCGGGACCTGAACGCACTG 3'

PDM-1004 (SEQ ID NO:485):

5' CTGCAGAATTCATCACTGAATGCCTCTGAAG 3'

The amplification reaction contained 10 ul 10X Pfu buffer, 1 ul 10mM dNTPs, 2 ul each 10 uM primer, 83 ul sterile water, and 1.5 ul Pfu DNA polymerase (Stratagene, La Jolla, CA). The reaction was first denatured for 2 minutes at 96°C followed by 40 cycles of 96°C for 20 seconds, 63°C for 15 seconds, and 72°C for 30 seconds. The reaction was then extended for a final extension of 72°C for 4 minutes.

The PCR product was then digested with EcoRI and cloned into the pStumpy vector that had been digested with Eco72I and EcoRI. The

construct was confirmed through sequence analysis and then transformed into BLR (DE3) pLys S cells. The resulting truncated TAT WT1-F fusion protein expressed as a single species. The polynucleotide sequence of the coding region of pStumpy WT1-F is set forth in SEQ ID NO:469 which encodes the amino acid sequence set forth in SEQ ID NO:470.

EXAMPLE 39

CONSTRUCTION OF WT1-F GMP, WT1-G, AND WT1-DELTA G VECTORS FOR EXPRESSION IN *E. COLI*

This example describes the construction of WT1-F, WT1-G, and WT1-Delta G vectors optimized for protein production in *E. coli*.

The TAT WT-1 F DNA sequence was codon optimized and synthetically engineered by Blue Heron Biotechnology (Bothel, WA) to be optimized for expression in *E. coli*. This codon optimized cDNA sequence of WT1-F is set forth in SEQ ID NOs:477 and encodes the amino acid sequence set forth in SEQ ID NO:479. The plasmid containing this sequence was then digested with NdeI and EcoRI and sub-cloned into a modified pET28 vector that had been digested with NdeI and EcoRI for expression in *E. coli*. This construct was confirmed to be correct by DNA sequence analysis and then transformed into BLR (DE3) pLys S cells for expression. This construct is a purely synthetic traceable source of TAT WT1-F template DNA.

The codon optimized sequence was then used as template for PCR to delete a proline rich region of fourteen amino acids from the N-terminal region (deletion of amino acids 54-67 of WT-1 F sequence set forth in SEQ ID NO:461; corresponding to amino acids 55-68 of full-length WT1), creating the WT-1 G cDNA sequence set forth in SEQ ID NO:473 which encodes the amino acid sequence set forth in SEQ ID NO:478. This was done by first generating the pTAT-WT-1 G construct as follows:

The 5' coding region of WT-1 G was PCR amplified using the following primer set:

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PDM-1007 (SEQ ID NO:490) 5' caagcgctcatgagcccgaagtggcgagccc 3' Tm 76°C

PDM-1006 (SEQ ID NO:491) 5' cataaatttgcccgggcccgcccagagagccg 3' Tm 76°C

The 3' coding region of the WT-1 G region was PCR amplified using the following primer set:

PDM-1008 (SEQ ID NO:492) 5' cattcattcatcaaacaggagcc 3' Tm 61°C

PDM-1009 (SEQ ID NO:493) 5' ccattaagaattcatcactgaatacc 3' Tm 60°C

The PCR reaction for amplification of WT1-G contained 10 ul 10X Pfu buffer, 1 ul 10 mM dNTPs, 2 ul each 10 uM primer, 83 ul sterile water, 1.5 ul Pfu DNA polymerase (Stratagene, La Jolla, CA), and 50 ng DNA template (pTAT WT1-F codon optimized as set forth in SEQ ID NO: 477). The reaction was first denatured for 2 minutes at 96°C followed by 40 cycles of 96°C for 20 seconds, 63°C for 15 seconds, and 72°C for 30 seconds. The reaction was then extended for a final extension of 72°C for 4 minutes.

The first PCR product was then digested with XbaI and cloned into pPDM (a modified pET28 vector) that had been digested with XbaI and Eco72I. The resulting construct (pTAT WT1-GA) was then digested with SrfI and EcoRI. The second PCR construct was digested with EcoRI and cloned into the pTAT WT1-GA plasmid construct. At the same time a three-way ligation was carried out by digesting the first PCR construct with XbaI and SrfI and digesting the second PCR construct with EcoRI and cloning into pPDM that had been digested with XbaI and EcoRI. The resulting pTAT-WT1-G construct was confirmed to be correct through sequence analysis and then transformed into an expression host. The cDNA of pTAT-WT1-G is set forth in SEQ ID NO:475 and encodes the amino acid sequence of SEQ ID NO:482.

Due to incomplete SrfI digestion during the second method described above, an unexpected form of WT-1 G (WT-1 Delta G was created that deleted 14 amino acids (amino acids 55-68 of WT-1 F) and added three

additional amino acids (WT-1 Delta G polynucleotide sequence set forth in SEQ ID NO:505 which encodes the polypeptide sequence set forth in SEQ ID NO:502). This TAT WT-1 Delta G construct resulted in an unexpected 5-fold over expression as compared to the TAT WT-1 F constructs. The cDNA of the pTAT-WT1 Delta G construct is set forth in SEQ ID NO:476 and encodes the amino acid sequence set forth in SEQ ID NO:481.

Following generation of the pTAT-WT-1 G, pStumpy-WT1 G and WT1-G with and without a His tag were constructed using the pTAT-WT1 G as a template in the PCR reactions described below.

The coding region of the WT1-G region was PCR amplified using the following primer sets:

PCR 1:

PDM-1010 (SEQ ID NO:494) 5' ggttcggatgtacgcgatctgaacg 3' Tm 68°C

PDM-1011 (SEQ ID NO:495) 5' caaagaattcatcactgaataccgcg 3' Tm 63°C

PCR 2:

PDM-1023 (SEQ ID NO:496) 5' gcttttggcatatgggttcggatgtacgcgatc 3' Tm 71°C

PDM-1011 (SEQ ID NO:497) 5' caaagaattcatcactgaataccgcg 3' Tm 63°C

The PCR reaction and amplification conditions were as described above using the pTAT-WT1 G set forth in SEQ ID NO:475 as template. The first PCR product was digested with EcoRI and cloned into pPDM (a modified pET28 vector with a his tag in-frame) and pSTUMPY (a modified pET28 vector with a 12 amino acid truncated TAT leader peptide in frame set forth in SEQ ID NO:471) that were digested with EcoRI and Eco72I. The second PCR product was digested with NdeI and EcoRI and cloned into a modified pET28 vector

(pPDM) that had been digested with NdeI and EcoRI. The constructs were confirmed to be correct through sequence analysis and then transformed into an expression host. The resulting constructs are as follows: The polynucleotide of WT1-G with His tag is set forth in SEQ ID NO:472 and encodes the amino acid sequence set forth in SEQ ID NO:480. The polynucleotide of WT1-G without His tag is set forth in SEQ ID NO:473 which encodes the amino acid sequence set forth in SEQ ID NO:478. The pStumpy-WT1-G polynucleotide sequence is set forth in SEQ ID NO:474 and encodes the amino acid sequence set forth in SEQ ID NO:483.

In a related experiment, the pStumpy WT-1 F GMP (codon optimized) vector was constructed as follows:

The coding region of the WT-1 F region was amplified using the following primers

PDM-1010 (SEQ ID NO:500) 5' ggttcggatgtacgcgatctgaacg 3' Tm 68oC

PDM-1011 (SEQ ID NO:501) 5' caaagaattcatcactgaataccgcg 3' Tm 63oC

The PCR reaction and amplification conditions were as described above using pTAT WT-1F GMP as template. The PCR product was digested with EcoRI and cloned into the pStumpy vector described in Example 38 that was digested with EcoRI and Eco72I. The construct was confirmed to be correct through sequence analysis and then transformed into an expression host. The polynucleotide sequence of pStumpy WT-1 F GMP is set forth in SEQ ID NO:498 which encodes the amino acid sequence of SEQ ID NO:499.

In summary, the expression vectors described herein can be used for optimal expression and production of WT1, truncated WT1, and WT1 fusion proteins for use in vaccine strategies for the treatment of malignancies associated with expression of WT1.

From the foregoing it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

1. An isolated polypeptide comprising an immunogenic portion of a Wilms' tumor antigen (WT1), selected from the group consisting of SEQ ID NOs:414-450, 478, and 502, or a variant thereof that differs in one or more substitutions, deletions, additions and/or insertions such that the ability of the variant to react with WT1-specific antisera and/or T-cell lines or clones is not substantially diminished.

2. The isolated polypeptide according to claim 1 wherein the immunogenic portion has been modified such that the ability of the immunogenic portion to bind to an MHC molecule is increased relative to that of the immunogenic portion.

3. The isolated polypeptide according to claim 2 wherein the immunogenic portion has been modified such that the ability of the immunogenic portion to bind to HLA-A2 is increased relative to that of the unmodified immunogenic portion.

4. An isolated polypeptide comprising a Wilms' tumor antigen having a deletion of a proline rich region.

5. An isolated polypeptide according to claim 4 wherein said proline rich region is from about amino acid positions 54 to 68 of the Wilms' tumor antigen.

6. The isolated polypeptide of claim 5 wherein said polypeptide comprises the amino acid sequence set forth in any one of SEQ ID NO:478 and 502.

7. A fusion protein comprising at least one polypeptide according to any one of claims 1, 2 or 4.

8. The fusion protein of claim 7 wherein said fusion partner is selected from the group consisting of Ra12, protein D, LYTA, a HIS tag, a targeting signal capable of directing a polypeptide to the endosomal/lysosomal compartment, twin arginine translocator , and truncated twin arginine translocator.

9. The fusion protein of claim 8 wherein the fusion protein comprises a twin arginine translocator signal peptide.

10. The fusion protein of claim 8 wherein the fusion partner comprises the amino acid sequence of any one of SEQ ID NOs:504 and 506.

11. The fusion protein of claim 10 wherein the fusion protein comprises the amino acid sequence of any one of SEQ ID NOs:~~453~~, 454, 455, 470, 479-483, and 499.

12. An isolated polynucleotide encoding the fusion proteins of any one of claims 7, 9, and 11.

13. The isolated polynucleotide of claim 12 wherein the polynucleotide has been codon optimized for expression in *E. coli.*

14. The isolated polynucleotide of claim 12 wherein the polynucleotide comprises a sequence of any one of SEQ ID NOs:452, 454, 469, 471, and 472-477 and 503, 505.

15. A composition comprising a polypeptide of claim 1, 4, 6, or 11 in combination with a pharmaceutically acceptable carrier or excipient.

16. A vaccine comprising a polypeptide of claim 1, 4, 6, or 11 in combination with a non-specific immune response enhancer.

17. The vaccine according to claim 15 wherein the non-specific immune response enhancer preferentially enhances a T cell response in a patient.

18. The vaccine according to claim 15, wherein the immune response enhancer is selected from the group consisting of 3d-MPL, MPL, RC-529, AGP's, Montanide ISA50, Seppic Montanide ISA 720, a cytokine, a microsphere, dimethyl dioctadecyl ammoniumbromide (DDA) based adjuvants, AS-1, AS-2, Ribi Adjuvant system based adjuvant, QS21, saponin based adjuvants, Syntex adjuvant in its microfluidized form, MV, ddMV, immune stimulating complex (iscom) based adjuvants, and inactivated toxins.

19. An expression vector comprising a polynucleotide of claim 12 operably linked to an expression control sequence.

20. A host cell transformed or transfected with an expression vector according to claim 19.

21. An isolated polypeptide comprising an immunogenic portion of the Wilms' tumor antigen wherein said polypeptide has been modified such that the ability of the polypeptide to bind HLA-A2 is increased and wherein the immunogenicity of the polypeptide is increased relative to that of SEQ ID NO:241.

22. The isolated polypeptide of claim 21 wherein said polypeptide comprises an amino acid sequence selected from the group consisting of any one of SEQ ID NOs:414-450.

23. The isolated polypeptide of claim 21, wherein said modification comprises one or more substitutions at any one of positions 1 through 9 of SEQ ID NO:241.

24. The isolated polypeptide according to claim 23 wherein said modification comprises a substitution at position 6 (P6) of SEQ ID NO:241.

25. The isolated polypeptide of claim 24 wherein said polypeptide comprises the amino acid sequence set forth in SEQ ID NO:445

26. The isolated polypeptide according to claim 23 wherein said modification comprises a substitution at position 9 (P9) of SEQ ID NO:241.

27. The isolated polypeptide according to claim 26 wherein said polypeptide comprises an amino acid sequence set forth in SEQ ID NO:426.

28. A method for inducing an immune response in an animal, comprising:

(a) administering to said animal a first composition comprising a first viral vector wherein said first viral vector comprises at least an immunogenic portion of a WT1 polynucleotide operably linked to an expression control sequence;

(b) administering to said animal a second composition comprising a second viral vector wherein said second viral vector comprises at least a second immunogenic portion of a WT1 polynucleotide operably linked to an expression control sequence; and

thereby inducing an immune response in the animal.

29. The method of claim 28 wherein said first and said second viral vectors are selected from the group consisting of vaccinia vectors, fowlpox vectors, and adenoviral vectors.

30. The method of claim 29 wherein said first viral vector comprises a recombinant vaccinia vector.

31. The method of claim 28 wherein said first viral vector comprises rV-WT1/TRICOM.

32. The method of claim 28 wherein said second viral vector comprises a recombinant fowlpox vector.

33. The method of claim 28 wherein said second viral vector comprises rF-WT1/TRICOM.

34. The method of claim 28 wherein said first and said second viral vectors further comprise at least one polynucleotide encoding a costimulatory molecule.

35. The method of claim 28 wherein said costimulatory molecule is selected from the group consisting of B7-1, ICAM-1, and LFA-3.

36. The method of claim 28 wherein said first composition is administered at a first timepoint and said second composition is administered subsequently at a second and a third timepoint.

37. A method for inducing an immune response in a patient comprising administering to the patient a composition comprising a first component comprising an immunogenic portion of a Wilms' tumor antigen selected from SEQ ID NOs: 414-450, 453, 455, 470, 478, 479-483, 499, and 502 and a second component selected from any one or both of a physiologically acceptable carrier or immunostimulant.

38. A method for inducing an immune response in a patient comprising administering to the patient a composition comprising a first component comprising a polynucleotide encoding a polypeptide comprising an amino acid sequence set forth in any one of 414-450, 453, 455, 470, 478, 479-

483, 499, and 502 and a second component comprising a polypeptide comprising an amino acid sequence set forth in any one of 414-450, 453, 455, 470, 478, 479-483, 499, and 502.

39. An isolated polypeptide according to any one of claims 1-6 and 21, wherein the polypeptide is not a wild type WT1 polypeptide.

40. An isolated polypeptide according to any one of claims 1-6 and 21, wherein the polypeptide comprises fewer than 100 amino acid residues.

41. An isolated polypeptide according to any one of claims 1-6 and 21, wherein the polypeptide comprises fewer than 50 amino acid residues.

42. An isolated polypeptide according to any one of claims 1-6 and 21, wherein the polypeptide comprises no more than 23 amino acid residues of a native WT1 polypeptide sequence.

43. An isolated polypeptide according to any one of claims 1-6 and 21, wherein the polypeptide comprises no more than 16 amino acid residues of a native WT1 polypeptide sequence.

44. A method according to claim 28, wherein said immunogenic portions of a WT1 polynucleotide are selected from SEQ ID NOs: 414-450, 470, 478 and 502.

SEQUENCE LISTING

<110> Corixa Corporation
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McNeill, Patricia D.
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Carter, Darrick

<120> COMPOSITIONS AND METHODS FOR WT1
SPECIFIC IMMUNOTHERAPY

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His Thr His Gly Val Phe Arg Gly Ile
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<211> 32
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<210> 317
<211> 22
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1 5 10 15
His Thr Gly Lys Thr Ser
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<211> 21
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1 5 10 15
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<213> Homo sapien

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20 25 30
Gln Trp Ala Pro Val Leu Asp Phe Ala Pro Pro Gly Ala Ser Ala Tyr
35 40 45

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Gly Ser Leu Gly Gly Pro Ala Pro Pro Pro Ala Pro Pro Pro Pro Pro
    50                  55                  60
Pro Pro Pro Pro His Ser Phe Ile Lys Gln Glu Pro Ser Trp Gly Gly
65                  70                  75                  80
Ala Glu Pro His Glu Glu Gln Cys Leu Ser Ala Phe Thr Val His Phe
                85                  90                  95
Ser Gly Gln Phe Thr Gly Thr Ala Gly Ala Cys Arg Tyr Gly Pro Phe
            100                 105                 110
Gly Pro Pro Pro Pro Ser Gln Ala Ser Ser Gly Gln Ala Arg Met Phe
        115                 120                 125
Pro Asn Ala Pro Tyr Leu Pro Ser Cys Leu Glu Ser Gln Pro Ala Ile
    130                 135                 140
Arg Asn Gln Gly Tyr Ser Thr Val Thr Phe Asp Gly Thr Pro Ser Tyr
145                 150                 155                 160
Gly His Thr Pro Ser His His Ala Ala Gln Phe Pro Asn His Ser Phe
                165                 170                 175
Lys His Glu Asp Pro Met Gly Gln Gln Gly Ser Leu Gly Glu Gln Gln
            180                 185                 190
Tyr Ser Val Pro Pro Pro Val Tyr Gly Cys His Thr Pro Thr Asp Ser
        195                 200                 205
Cys Thr Gly Ser Gln Ala Leu Leu Leu Arg Thr Pro Tyr Ser Ser Asp
    210                 215                 220
Asn Leu Tyr Gln Met Thr Ser Gln Leu Glu Cys Met Thr Trp Asn Gln
225                 230                 235                 240
Met Asn Leu Gly Ala Thr Leu Lys Gly Val Ala Ala Gly Ser Ser Ser
                245                 250                 255
Ser Val Lys Trp Thr Glu Gly Gln Ser Asn His Ser Thr Gly Tyr Glu
            260                 265                 270
Ser Asp Asn His Thr Thr Pro Ile Leu Cys Gly Ala Gln Tyr Arg Ile
        275                 280                 285
His Thr His Gly Val Phe Arg Gly Ile Gln Asp Val Arg Arg Val Pro
    290                 295                 300
Gly Val Ala Pro Thr Leu Val Arg Ser Ala Ser Glu Thr Ser Glu Lys
305                 310                 315                 320
Arg Pro Phe Met Cys Ala Tyr Pro Gly Cys Asn Lys Arg Tyr Phe Lys
                325                 330                 335
Leu Ser His Leu Gln Met His Ser Arg Lys His Thr Gly Glu Lys Pro
            340                 345                 350
Tyr Gln Cys Asp Phe Lys Asp Cys Glu Arg Arg Phe Ser Arg Ser Asp
        355                 360                 365
Gln Leu Lys Arg His Gln Arg Arg His Thr Gly Val Lys Pro Phe Gln
    370                 375                 380
Cys Lys Thr Cys Gln Arg Lys Phe Ser Arg Ser Asp His Leu Lys Thr
385                 390                 395                 400
His Thr Arg Thr His Thr Gly Lys Thr Ser Glu Lys Pro Phe Ser Cys
                405                 410                 415
Arg Trp Pro Ser Cys Gln Lys Lys Phe Ala Arg Ser Asp Glu Leu Val
            420                 425                 430
Arg His His Asn Met His Gln Arg Asn Met Thr Lys Leu Gln Leu Ala
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Leu
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<213> Mus musculus

<400> 320

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Gln Trp Ala Pro Val Leu Asp Phe Ala Pro Pro Gly Ala Ser Ala Tyr
        35                  40                  45
Gly Ser Leu Gly Gly Pro Ala Pro Pro Pro Ala Pro Pro Pro Pro Pro
    50                  55                  60
Pro Pro Pro Pro His Ser Phe Ile Lys Gln Glu Pro Ser Trp Gly Gly
65                  70                  75                  80
Ala Glu Pro His Glu Glu Gln Cys Leu Ser Ala Phe Thr Leu His Phe
                85                  90                  95
Ser Gly Gln Phe Thr Gly Thr Ala Gly Ala Cys Arg Tyr Gly Pro Phe
            100                 105                 110
Gly Pro Pro Pro Pro Ser Gln Ala Ser Ser Gly Gln Ala Arg Met Phe
        115                 120                 125
Pro Asn Ala Pro Tyr Leu Pro Ser Cys Leu Glu Ser Gln Pro Thr Ile
    130                 135                 140
Arg Asn Gln Gly Tyr Ser Thr Val Thr Phe Asp Gly Ala Pro Ser Tyr
145                 150                 155                 160
Gly His Thr Pro Ser His His Ala Ala Gln Phe Pro Asn His Ser Phe
                165                 170                 175
Lys His Glu Asp Pro Met Gly Gln Gln Gly Ser Leu Gly Glu Gln Gln
            180                 185                 190
Tyr Ser Val Pro Pro Pro Val Tyr Gly Cys His Thr Pro Thr Asp Ser
        195                 200                 205
Cys Thr Gly Ser Gln Ala Leu Leu Leu Arg Thr Pro Tyr Ser Ser Asp
    210                 215                 220
Asn Leu Tyr Gln Met Thr Ser Gln Leu Glu Cys Met Thr Trp Asn Gln
225                 230                 235                 240
Met Asn Leu Gly Ala Thr Leu Lys Gly Met Ala Ala Gly Ser Ser Ser
                245                 250                 255
Ser Val Lys Trp Thr Glu Gly Gln Ser Asn His Gly Ile Gly Tyr Glu
            260                 265                 270
Ser Asp Asn His Thr Ala Pro Ile Leu Cys Gly Ala Gln Tyr Arg Ile
        275                 280                 285
His Thr His Gly Val Phe Arg Gly Ile Gln Asp Val Arg Arg Val Ser
    290                 295                 300
Gly Val Ala Pro Thr Leu Val Arg Ser Ala Ser Glu Thr Ser Glu Lys
305                 310                 315                 320
Arg Pro Phe Met Cys Ala Tyr Pro Gly Cys Asn Lys Arg Tyr Phe Lys
                325                 330                 335
Leu Ser His Leu Gln Met His Ser Arg Lys His Thr Gly Glu Lys Pro
            340                 345                 350
Tyr Gln Cys Asp Phe Lys Asp Cys Glu Arg Arg Phe Ser Arg Ser Asp
        355                 360                 365
Gln Leu Lys Arg His Gln Arg Arg His Thr Gly Val Lys Pro Phe Gln
    370                 375                 380
Cys Lys Thr Cys Gln Arg Lys Phe Ser Arg Ser Asp His Leu Lys Thr
385                 390                 395                 400
His Thr Arg Thr His Thr Gly Lys Thr Ser Glu Lys Pro Phe Ser Cys
                405                 410                 415
Arg Trp His Ser Cys Gln Lys Lys Phe Ala Arg Ser Asp Glu Leu Val
            420                 425                 430
Arg His His Asn Met His Gln Arg Asn Met Thr Lys Leu His Val Ala
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Leu
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1 5

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aaactgaccg ttgcaaaact gaacatcgat caaaaccctg gcactgcgcc gaaatatggc 240
atccgtggta tcccgactct gctgctgttc aaaaacggtg aagtggcggc aaccaaagtg 300
ggtgcactgt ctaaaggtca gttgaaagag ttcctcgacg ctaacctggc cggttctggt 360
tctggccata tgcagcatca ccaccatcac cacgtgtcta tcgaaggtcg tgctagctct 420
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caatacagaa tacacacgca cggtgtcttc agaggcattc aggatgtgcg acgtgtgcct 600
ggagtagccc cgactcttgt acggtcggca tctgagacca gtgagaaacg ccccttcatg 660
tgtgcttacc caggctgcaa taagagatat tttaagctgt cccacttaca gatgcacagc 720
aggaagcaca ctggtgagaa accataccag tgtgacttca aggactgtga acgaaggttt 780
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tgtaaaactt gtcagcgaaa gttctcccgg tccgaccacc tgaagaccca caccaggact 900
catacaggtg aaaagccctt cagctgtcgg tggccaagtt gtcagaaaaa gtttgcccgg 960
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<210> 328
<211> 1233
<212> DNA
<213> Homo sapiens

<400> 328
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tgcggtccgt gcaaaatgat cgccccgatt ctggatgaaa tcgctgacga atatcagggc 180
aaactgaccg ttgcaaaact gaacatcgat caaaaccctg gcactgcgcc gaaatatggc 240
atccgtggta tcccgactct gctgctgttc aaaaacggtg aagtggcggc aaccaaagtg 300
ggtgcactgt ctaaaggtca gttgaaagag ttcctcgacg ctaacctggc cggttctggt 360
tctggccata tgcagcatca ccaccatcac cacgtgtcta tcgaaggtcg tgctagctct 420
ggtggcagcg gtctggttcc gcgtggtagc tctggttcgg gggacgacga cgacaaatct 480
agtaggggct ccgacgttcg tgacctgaac gcactgctgc cggcagttcc gtccctgggt 540
ggtggtggtg gttgcgcact gccggttagc ggtgcagcac agtgggctcc ggttctggac 600
ttcgcaccgc cgggtgcatc cgcatacggt tccctgggtg gtccggcacc gccgccggca 660
ccgccgccgc cgccgccgcc gccgccgcac tccttcatca aacaggaacc gagctggggt 720
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tacggtcaca cgccctcgca ccatgcggcg cagttcccca accactcatt caagcatgag 1020
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cagatgaact taggagccac cttaaagggc tga 1233

<210> 329
<211> 1776
<212> DNA
<213> Homo sapiens

<400> 329

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tgcggtccgt gcaaaatgat cgccccgatt ctggatgaaa tcgctgacga atatcagggc 180
aaactgaccg ttgcaaaact gaacatcgat caaaaccctg gcactgcgcc gaaatatggc 240
atccgtggta tcccgactct gctgctgttc aaaaacggtg aagtggcggc aaccaaagtg 300
ggtgcactgt ctaaaggtca gttgaaagag ttcctcgacg ctaacctggc cggttctggt 360
tctggccata tgcagcatca ccaccatcac cacgtgtcta tcgaaggtcg tgctagctct 420
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gacttcgcac cgccgggtgc atccgcatac ggttccctgg gtggtccggc accgccgccg 660
gcaccgccgc cgccgccgcc gccgccgccg cactccttca tcaaacagga accgagctgg 720
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cagttcactg gcacagccgg agcctgtcgc tacgggccct tcggtcctcc tccgcccagc 840
caggcgtcat ccggccaggc caggatgttt cctaacgcgc cctacctgcc cagctgcctc 900
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cagagaaaca tgaccaaact ccagctggcg ctttga 1776

<210> 330
<211> 771
<212> DNA
<213> Homo sapiens

<400> 330
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gcagttccgt ccctgggtgg tggtggtggt tgcgcactgc cggttagcgg tgcagcacag 120
tgggctccgg ttctggactt cgcaccgccg ggtgcatccg catacggttc cctgggtggt 180
ccggcaccgc cgccggcacc gccgccgccg ccgccgccgc cgccgcactc cttcatcaaa 240
caggaaccga gctggggtgg tgcagaaccg cacgaagaac agtgcctgag cgcattcacc 300
gttcacttct ccggccagtt cactggcaca gccggagcct gtcgctacgg gcccttcggt 360
cctcctccgc ccagccaggc gtcatccggc caggccagga tgtttcctaa cgcgccctac 420
ctgcccagct gcctcgagag ccagcccgct attcgcaatc agggttacag cacggtcacc 480
ttcgacggga cgcccagcta cggtcacacg ccctcgcacc atgcggcgca gttccccaac 540
cactcattca agcatgagga tcccatgggc cagcagggct cgctgggtga gcagcagtac 600
tcggtgccgc ccccggtcta tggctgccac acccccaccg acagctgcac cggcagccag 660
gctttgctgc tgaggacgcc ctacagcagt gacaatttat accaaatgac atcccagctt 720
gaatgcatga cctggaatca gatgaactta ggagccacct taaagggctg a 771

<210> 331
<211> 567
<212> DNA
<213> Homo sapiens

<400> 331
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gatgtgcgac gtgtgcctgg agtagccccg actcttgtac ggtcggcatc tgagaccagt 180
gagaaacgcc ccttcatgtg tgcttaccca ggctgcaata agagatattt taagctgtcc 240
cacttacaga tgcacagcag gaagcacact ggtgagaaac cataccagtg tgacttcaag 300
gactgtgaac gaaggttttt tcgttcagac cagctcaaaa gacaccaaag gagacataca 360
ggtgtgaaac cattccagtg taaaacttgt cagcgaaagt tctcccggtc cgaccacctg 420
aagacccaca ccaggactca tacaggtgaa aagcccttca gctgtcggtg gccaagttgt 480
cagaaaaagt ttgcccggtc agatgaatta gtccgccatc acaacatgca tcagagaaac 540
atgaccaaac tccagctggc gctttga                                     567
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<210> 332
<211> 342
<212> PRT
<213> Homo sapiens

<400> 332

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Met Gln His His His His His His Met Ser Asp Lys Ile Ile His Leu
                  5                  10                  15
Thr Asp Asp Ser Phe Asp Thr Asp Val Leu Lys Ala Asp Gly Ala Ile
             20                  25                  30
Leu Val Asp Phe Trp Ala Glu Trp Cys Gly Pro Cys Lys Met Ile Ala
         35                  40                  45
Pro Ile Leu Asp Glu Ile Ala Asp Glu Tyr Gln Gly Lys Leu Thr Val
     50                  55                  60
Ala Lys Leu Asn Ile Asp Gln Asn Pro Gly Thr Ala Pro Lys Tyr Gly
 65                  70                  75                  80
Ile Arg Gly Ile Pro Thr Leu Leu Leu Phe Lys Asn Gly Glu Val Ala
                 85                  90                  95
Ala Thr Lys Val Gly Ala Leu Ser Lys Gly Gln Leu Lys Glu Phe Leu
            100                 105                 110
Asp Ala Asn Leu Ala Gly Ser Gly Ser Gly His Met Gln His His His
        115                 120                 125
His His His Val Ser Ile Glu Gly Arg Ala Ser Ser Gly Gly Ser Gly
    130                 135                 140
Leu Val Pro Arg Gly Ser Ser Gly Ser Gly Asp Asp Asp Asp Lys Ser
145                 150                 155                 160
Ser Arg His Ser Thr Gly Tyr Glu Ser Asp Asn His Thr Thr Pro Ile
                165                 170                 175
Leu Cys Gly Ala Gln Tyr Arg Ile His Thr His Gly Val Phe Arg Gly
            180                 185                 190
Ile Gln Asp Val Arg Arg Val Pro Gly Val Ala Pro Thr Leu Val Arg
        195                 200                 205
Ser Ala Ser Glu Thr Ser Glu Lys Arg Pro Phe Met Cys Ala Tyr Pro
    210                 215                 220
Gly Cys Asn Lys Arg Tyr Phe Lys Leu Ser His Leu Gln Met His Ser
225                 230                 235                 240
Arg Lys His Thr Gly Glu Lys Pro Tyr Gln Cys Asp Phe Lys Asp Cys
                245                 250                 255
Glu Arg Arg Phe Phe Arg Ser Asp Gln Leu Lys Arg His Gln Arg Arg
            260                 265                 270
His Thr Gly Val Lys Pro Phe Gln Cys Lys Thr Cys Gln Arg Lys Phe
        275                 280                 285
Ser Arg Ser Asp His Leu Lys Thr His Thr Arg Thr His Thr Gly Glu
    290                 295                 300
Lys Pro Phe Ser Cys Arg Trp Pro Ser Cys Gln Lys Lys Phe Ala Arg
```

305 310 315 320
Ser Asp Glu Leu Val Arg His His Asn Met His Gln Arg Asn Met Thr
325 330 335
Lys Leu Gln Leu Ala Leu
340

<210> 333
<211> 410
<212> PRT
<213> Homo sapiens

<400> 333
Met Gln His His His His His His Met Ser Asp Lys Ile Ile His Leu
5 10 15
Thr Asp Asp Ser Phe Asp Thr Asp Val Leu Lys Ala Asp Gly Ala Ile
20 25 30
Leu Val Asp Phe Trp Ala Glu Trp Cys Gly Pro Cys Lys Met Ile Ala
35 40 45
Pro Ile Leu Asp Glu Ile Ala Asp Glu Tyr Gln Gly Lys Leu Thr Val
50 55 60
Ala Lys Leu Asn Ile Asp Gln Asn Pro Gly Thr Ala Pro Lys Tyr Gly
65 70 75 80
Ile Arg Gly Ile Pro Thr Leu Leu Leu Phe Lys Asn Gly Glu Val Ala
85 90 95
Ala Thr Lys Val Gly Ala Leu Ser Lys Gly Gln Leu Lys Glu Phe Leu
100 105 110
Asp Ala Asn Leu Ala Gly Ser Gly Ser Gly His Met Gln His His His
115 120 125
His His His Val Ser Ile Glu Gly Arg Ala Ser Ser Gly Gly Ser Gly
130 135 140
Leu Val Pro Arg Gly Ser Ser Gly Ser Gly Asp Asp Asp Asp Lys Ser
145 150 155 160
Ser Arg Gly Ser Asp Val Arg Asp Leu Asn Ala Leu Leu Pro Ala Val
165 170 175
Pro Ser Leu Gly Gly Gly Gly Gly Cys Ala Leu Pro Val Ser Gly Ala
180 185 190
Ala Gln Trp Ala Pro Val Leu Asp Phe Ala Pro Pro Gly Ala Ser Ala
195 200 205
Tyr Gly Ser Leu Gly Gly Pro Ala Pro Pro Pro Ala Pro Pro Pro Pro
210 215 220
Pro Pro Pro Pro Pro His Ser Phe Ile Lys Gln Glu Pro Ser Trp Gly
225 230 235 240
Gly Ala Glu Pro His Glu Glu Gln Cys Leu Ser Ala Phe Thr Val His
245 250 255
Phe Ser Gly Gln Phe Thr Gly Thr Ala Gly Ala Cys Arg Tyr Gly Pro
260 265 270
Phe Gly Pro Pro Pro Pro Ser Gln Ala Ser Ser Gly Gln Ala Arg Met
275 280 285
Phe Pro Asn Ala Pro Tyr Leu Pro Ser Cys Leu Glu Ser Gln Pro Ala
290 295 300
Ile Arg Asn Gln Gly Tyr Ser Thr Val Thr Phe Asp Gly Thr Pro Ser
305 310 315 320
Tyr Gly His Thr Pro Ser His His Ala Ala Gln Phe Pro Asn His Ser
325 330 335
Phe Lys His Glu Asp Pro Met Gly Gln Gln Gly Ser Leu Gly Glu Gln
340 345 350

```
Gln Tyr Ser Val Pro Pro Pro Val Tyr Gly Cys His Thr Pro Thr Asp
        355                 360                 365
Ser Cys Thr Gly Ser Gln Ala Leu Leu Leu Arg Thr Pro Tyr Ser Ser
    370                 375                 380
Asp Asn Leu Tyr Gln Met Thr Ser Gln Leu Glu Cys Met Thr Trp Asn
385                 390                 395                 400
Gln Met Asn Leu Gly Ala Thr Leu Lys Gly
                405                 410
```

<210> 334
<211> 591
<212> PRT
<213> Homo sapiens

<400> 334

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Met Gln His His His His His His Met Ser Asp Lys Ile Ile His Leu
                    5                  10                  15
Thr Asp Asp Ser Phe Asp Thr Asp Val Leu Lys Ala Asp Gly Ala Ile
            20                  25                  30
Leu Val Asp Phe Trp Ala Glu Trp Cys Gly Pro Cys Lys Met Ile Ala
        35                  40                  45
Pro Ile Leu Asp Glu Ile Ala Asp Glu Tyr Gln Gly Lys Leu Thr Val
    50                  55                  60
Ala Lys Leu Asn Ile Asp Gln Asn Pro Gly Thr Ala Pro Lys Tyr Gly
65                  70                  75                  80
Ile Arg Gly Ile Pro Thr Leu Leu Leu Phe Lys Asn Gly Glu Val Ala
                85                  90                  95
Ala Thr Lys Val Gly Ala Leu Ser Lys Gly Gln Leu Lys Glu Phe Leu
            100                 105                 110
Asp Ala Asn Leu Ala Gly Ser Gly Ser Gly His Met Gln His His His
        115                 120                 125
His His His Val Ser Ile Glu Gly Arg Ala Ser Ser Gly Gly Ser Gly
    130                 135                 140
Leu Val Pro Arg Gly Ser Ser Gly Ser Gly Asp Asp Asp Asp Lys Ser
145                 150                 155                 160
Ser Arg Met Gly Ser Asp Val Arg Asp Leu Asn Ala Leu Leu Pro Ala
                165                 170                 175
Val Pro Ser Leu Gly Gly Gly Gly Gly Cys Ala Leu Pro Val Ser Gly
            180                 185                 190
Ala Ala Gln Trp Ala Pro Val Leu Asp Phe Ala Pro Pro Gly Ala Ser
        195                 200                 205
Ala Tyr Gly Ser Leu Gly Gly Pro Ala Pro Pro Pro Ala Pro Pro Pro
    210                 215                 220
Pro Pro Pro Pro Pro Pro His Ser Phe Ile Lys Gln Glu Pro Ser Trp
225                 230                 235                 240
Gly Gly Ala Glu Pro His Glu Glu Gln Cys Leu Ser Ala Phe Thr Val
                245                 250                 255
His Phe Ser Gly Gln Phe Thr Gly Thr Ala Gly Ala Cys Arg Tyr Gly
            260                 265                 270
Pro Phe Gly Pro Pro Pro Pro Ser Gln Ala Ser Ser Gly Gln Ala Arg
        275                 280                 285
Met Phe Pro Asn Ala Pro Tyr Leu Pro Ser Cys Leu Glu Ser Gln Pro
    290                 295                 300
Ala Ile Arg Asn Gln Gly Tyr Ser Thr Val Thr Phe Asp Gly Thr Pro
305                 310                 315                 320
Ser Tyr Gly His Thr Pro Ser His His Ala Ala Gln Phe Pro Asn His
                325                 330                 335
```

```
Ser Phe Lys His Glu Asp Pro Met Gly Gln Gln Gly Ser Leu Gly Glu
            340                 345                 350
Gln Gln Tyr Ser Val Pro Pro Pro Val Tyr Gly Cys His Thr Pro Thr
        355                 360                 365
Asp Ser Cys Thr Gly Ser Gln Ala Leu Leu Leu Arg Thr Pro Tyr Ser
    370                 375                 380
Ser Asp Asn Leu Tyr Gln Met Thr Ser Gln Leu Glu Cys Met Thr Trp
385                 390                 395                 400
Asn Gln Met Asn Leu Gly Ala Thr Leu Lys Gly His Ser Thr Gly Tyr
                405                 410                 415
Glu Ser Asp Asn His Thr Thr Pro Ile Leu Cys Gly Ala Gln Tyr Arg
            420                 425                 430
Ile His Thr His Gly Val Phe Arg Gly Ile Gln Asp Val Arg Arg Val
        435                 440                 445
Pro Gly Val Ala Pro Thr Leu Val Arg Ser Ala Ser Glu Thr Ser Glu
    450                 455                 460
Lys Arg Pro Phe Met Cys Ala Tyr Pro Gly Cys Asn Lys Arg Tyr Phe
465                 470                 475                 480
Lys Leu Ser His Leu Gln Met His Ser Arg Lys His Thr Gly Glu Lys
                485                 490                 495
Pro Tyr Gln Cys Asp Phe Lys Asp Cys Glu Arg Arg Phe Phe Arg Ser
            500                 505                 510
Asp Gln Leu Lys Arg His Gln Arg Arg His Thr Gly Val Lys Pro Phe
        515                 520                 525
Gln Cys Lys Thr Cys Gln Arg Lys Phe Ser Arg Ser Asp His Leu Lys
    530                 535                 540
Thr His Thr Arg Thr His Thr Gly Glu Lys Pro Phe Ser Cys Arg Trp
545                 550                 555                 560
Pro Ser Cys Gln Lys Lys Phe Ala Arg Ser Asp Glu Leu Val Arg His
                565                 570                 575
His Asn Met His Gln Arg Asn Met Thr Lys Leu Gln Leu Ala Leu
            580                 585                 590
```

<210> 335
<211> 256
<212> PRT
<213> Homo sapiens

<400> 335

```
Met Gln His His His His His His Gly Ser Asp Val Arg Asp Leu Asn
                5                  10                  15
Ala Leu Leu Pro Ala Val Pro Ser Leu Gly Gly Gly Gly Gly Cys Ala
            20                  25                  30
Leu Pro Val Ser Gly Ala Ala Gln Trp Ala Pro Val Leu Asp Phe Ala
        35                  40                  45
Pro Pro Gly Ala Ser Ala Tyr Gly Ser Leu Gly Gly Pro Ala Pro Pro
    50                  55                  60
Pro Ala Pro Pro Pro Pro Pro Pro Pro Pro Pro His Ser Phe Ile Lys
65                  70                  75                  80
Gln Glu Pro Ser Trp Gly Gly Ala Glu Pro His Glu Glu Gln Cys Leu
                85                  90                  95
Ser Ala Phe Thr Val His Phe Ser Gly Gln Phe Thr Gly Thr Ala Gly
            100                 105                 110
Ala Cys Arg Tyr Gly Pro Phe Gly Pro Pro Pro Pro Ser Gln Ala Ser
        115                 120                 125
Ser Gly Gln Ala Arg Met Phe Pro Asn Ala Pro Tyr Leu Pro Ser Cys
```

```
    130                 135                 140
Leu Glu Ser Gln Pro Ala Ile Arg Asn Gln Gly Tyr Ser Thr Val Thr
145                 150                 155                 160
Phe Asp Gly Thr Pro Ser Tyr Gly His Thr Pro Ser His His Ala Ala
                165                 170                 175
Gln Phe Pro Asn His Ser Phe Lys His Glu Asp Pro Met Gly Gln Gln
            180                 185                 190
Gly Ser Leu Gly Glu Gln Gln Tyr Ser Val Pro Pro Pro Val Tyr Gly
        195                 200                 205
Cys His Thr Pro Thr Asp Ser Cys Thr Gly Ser Gln Ala Leu Leu Leu
    210                 215                 220
Arg Thr Pro Tyr Ser Ser Asp Asn Leu Tyr Gln Met Thr Ser Gln Leu
225                 230                 235                 240
Glu Cys Met Thr Trp Asn Gln Met Asn Leu Gly Ala Thr Leu Lys Gly
                245                 250                 255
```

<210> 336
<211> 188
<212> PRT
<213> Homo sapiens

<400> 336

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Met Gln His His His His His His His Ser Thr Gly Tyr Glu Ser Asp
                5                  10                  15
Asn His Thr Thr Pro Ile Leu Cys Gly Ala Gln Tyr Arg Ile His Thr
            20                  25                  30
His Gly Val Phe Arg Gly Ile Gln Asp Val Arg Arg Val Pro Gly Val
        35                  40                  45
Ala Pro Thr Leu Val Arg Ser Ala Ser Glu Thr Ser Glu Lys Arg Pro
    50                  55                  60
Phe Met Cys Ala Tyr Pro Gly Cys Asn Lys Arg Tyr Phe Lys Leu Ser
65                  70                  75                  80
His Leu Gln Met His Ser Arg Lys His Thr Gly Glu Lys Pro Tyr Gln
                85                  90                  95
Cys Asp Phe Lys Asp Cys Glu Arg Arg Phe Phe Arg Ser Asp Gln Leu
            100                 105                 110
Lys Arg His Gln Arg Arg His Thr Gly Val Lys Pro Phe Gln Cys Lys
        115                 120                 125
Thr Cys Gln Arg Lys Phe Ser Arg Ser Asp His Leu Lys Thr His Thr
    130                 135                 140
Arg Thr His Thr Gly Glu Lys Pro Phe Ser Cys Arg Trp Pro Ser Cys
145                 150                 155                 160
Gln Lys Lys Phe Ala Arg Ser Asp Glu Leu Val Arg His His Asn Met
                165                 170                 175
His Gln Arg Asn Met Thr Lys Leu Gln Leu Ala Leu
            180                 185
```

<210> 337
<211> 324
<212> DNA
<213> Homo sapiens

<400> 337

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gcagttccat ccctgggtgg cggtggaggc tgcgcactgc cggttagcgg tgcagcacag 120
tgggctccag ttctggactt cgcaccgcct ggtgcatccg catacggttc cctgggtggt 180
ccagcacctc cgcccgcaac gcccccaccg cctccaccgc ccccgcactc cttcatcaaa 240
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caggaaccta gctggggtgg tgcagaaccg cacgaagaac agtgcctgag cgcattctga 300
gaattctgca gatatccatc acac 324

<210> 338
<211> 462
<212> DNA
<213> Homo sapiens

<400> 338
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ttctccggcc agttcactgg cacagccgga gcctgtcgct acgggccctt cggtcctcct 120
ccgcccagcc aggcgtcatc cggccaggcc aggatgtttc ctaacgcgcc ctacctgccc 180
agctgcctcg agagccagcc cgctattcgc aatcagggtt acagcacggt caccttcgac 240
gggacgccca gctacggtca cacgccctcg caccatgcgg cgcagttccc caaccactca 300
ttcaagcatg aggatcccat gggccagcag ggctcgctgg gtgagcagca gtactcggtg 360
ccgcccccgg tctatggctg ccacacccca accgacagct gcaccggcag ccaggctttg 420
ctgctgagga cgccctacag cagtgacaat ttatactgat ga 462

<210> 339
<211> 405
<212> DNA
<213> Homo sapiens

<400> 339
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aatttatacc aaatgacatc ccagcttgaa tgcatgacct ggaatcagat gaacttagga 120
gccaccttaa agggccacag cacagggtac gagagcgata accacacaac gcccatcctc 180
tgcggagccc aatacagaat acacacgcac ggtgtcttca gaggcattca ggatgtgcga 240
cgtgtgcctg gagtagcccc gactcttgta cggtcggcat ctgagaccag tgagaaacgc 300
cccttcatgt gtgcttaccc aggctgcaat aagagatatt ttaagctgtc ccacttacag 360
atgcacagca ggaagcacac tggtgagaaa ccataccagt gatga 405

<210> 340
<211> 339
<212> DNA
<213> Homo sapiens

<400> 340
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tgtgacttca aggactgtga acgaaggttt tttcgttcag accagctcaa aagacaccaa 120
aggagacata caggtgtgaa accattccag tgtaaaactt gtcagcgaaa gttctcccgg 180
tccgaccacc tgaagaccca caccaggact catacaggtg aaaagccctt cagctgtcgg 240
tggccaagtt gtcagaaaaa gtttgcccgg tcagatgaat tagtccgcca tcacaacatg 300
catcagagaa acatgaccaa actccagctg gcgctttga 339

<210> 341
<211> 1110
<212> DNA
<213> Homo sapiens

<400> 341
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gcagaaccgc acgaagaaca gtgcctgagc gcattcaccg ttcacttctc cggccagttc 120
actggcacag ccggagcctg tcgctacggg cccttcggtc ctcctccgcc cagccaggcg 180
tcatccggcc aggccaggat gtttcctaac gcgccctacc tgcccagctg cctcgagagc 240
cagcccgcta ttcgcaatca gggttacagc acggtcacct tcgacgggac gcccagctac 300

ggtcacacgc cctcgcacca tgcggcgcag ttccccaacc actcattcaa gcatgaggat 360
cccatgggcc agcagggctc gctgggtgag cagcagtact cggtgccgcc cccggtctat 420
ggctgccaca cccccaccga cagctgcacc ggcagccagg ctttgctgct gaggacgccc 480
tacagcagtg acaatttata ccaaatgaca tcccagcttg aatgcatgac ctggaatcag 540
atgaacttag gagccacctt aaagggccac agcacagggt acgagagcga taaccacaca 600
acgcccatcc tctgcggagc ccaatacaga atacacacgc acggtgtctt cagaggcatt 660
caggatgtgc gacgtgtgcc tggagtagcc ccgactcttg tacggtcggc atctgagacc 720
agtgagaaac gccccttcat gtgtgcttac ccaggctgca ataagagata ttttaagctg 780
tcccacttac agatgcacag caggaagcac actggtgaga aaccatacca gtgtgacttc 840
aaggactgtg aacgaaggtt ttttcgttca gaccagctca aaagacacca aaggagacat 900
acaggtgtga aaccattcca gtgtaaaact tgtcagcgaa agttctcccg gtccgaccac 960
ctgaagaccc acaccaggac tcatacaggt gaaaagccct tcagctgtcg gtggccaagt 1020
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aacatgacca aactccagct ggcgctttga 1110

<210> 342
<211> 99
<212> PRT
<213> Homo sapiens

<400> 342
Met Gln His His His His His His Gly Ser Asp Val Arg Asp Leu Asn
5 10 15
Ala Leu Leu Pro Ala Val Pro Ser Leu Gly Gly Gly Gly Gly Cys Ala
20 25 30
Leu Pro Val Ser Gly Ala Ala Gln Trp Ala Pro Val Leu Asp Phe Ala
35 40 45
Pro Pro Gly Ala Ser Ala Tyr Gly Ser Leu Gly Gly Pro Ala Pro Pro
50 55 60
Pro Ala Pro Pro Pro Pro Pro Pro Pro Pro Pro His Ser Phe Ile Lys
65 70 75 80
Gln Glu Pro Ser Trp Gly Gly Ala Glu Pro His Glu Glu Gln Cys Leu
85 90 95
Ser Ala Phe

<210> 343
<211> 152
<212> PRT
<213> Homo sapiens

<400> 343
Met Gln His His His His His His His Glu Glu Gln Cys Leu Ser Ala
5 10 15
Phe Thr Val His Phe Ser Gly Gln Phe Thr Gly Thr Ala Gly Ala Cys
20 25 30
Arg Tyr Gly Pro Phe Gly Pro Pro Pro Pro Ser Gln Ala Ser Ser Gly
35 40 45
Gln Ala Arg Met Phe Pro Asn Ala Pro Tyr Leu Pro Ser Cys Leu Glu
50 55 60
Ser Gln Pro Ala Ile Arg Asn Gln Gly Tyr Ser Thr Val Thr Phe Asp
65 70 75 80
Gly Thr Pro Ser Tyr Gly His Thr Pro Ser His His Ala Ala Gln Phe
85 90 95
Pro Asn His Ser Phe Lys His Glu Asp Pro Met Gly Gln Gln Gly Ser
100 105 110
Leu Gly Glu Gln Gln Tyr Ser Val Pro Pro Pro Val Tyr Gly Cys His

```
        115                 120                 125
Thr Pro Thr Asp Ser Cys Thr Gly Ser Gln Ala Leu Leu Leu Arg Thr
    130                 135                 140
Pro Tyr Ser Ser Asp Asn Leu Tyr
145                 150
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<210> 344
<211> 133
<212> PRT
<213> Homo sapiens

<400> 344

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Met Gln His His His His His His Gln Ala Leu Leu Leu Arg Thr Pro
                  5                  10                  15
Tyr Ser Ser Asp Asn Leu Tyr Gln Met Thr Ser Gln Leu Glu Cys Met
             20                  25                  30
Thr Trp Asn Gln Met Asn Leu Gly Ala Thr Leu Lys Gly His Ser Thr
         35                  40                  45
Gly Tyr Glu Ser Asp Asn His Thr Thr Pro Ile Leu Cys Gly Ala Gln
     50                  55                  60
Tyr Arg Ile His Thr His Gly Val Phe Arg Gly Ile Gln Asp Val Arg
 65                  70                  75                  80
Arg Val Pro Gly Val Ala Pro Thr Leu Val Arg Ser Ala Ser Glu Thr
                 85                  90                  95
Ser Glu Lys Arg Pro Phe Met Cys Ala Tyr Pro Gly Cys Asn Lys Arg
            100                 105                 110
Tyr Phe Lys Leu Ser His Leu Gln Met His Ser Arg Lys His Thr Gly
        115                 120                 125
Glu Lys Pro Tyr Gln
    130
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<210> 345
<211> 112
<212> PRT
<213> Homo sapiens

<400> 345

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Met Gln His His His His His His His Ser Arg Lys His Thr Gly Glu
                  5                  10                  15
Lys Pro Tyr Gln Cys Asp Phe Lys Asp Cys Glu Arg Arg Phe Phe Arg
             20                  25                  30
Ser Asp Gln Leu Lys Arg His Gln Arg Arg His Thr Gly Val Lys Pro
         35                  40                  45
Phe Gln Cys Lys Thr Cys Gln Arg Lys Phe Ser Arg Ser Asp His Leu
     50                  55                  60
Lys Thr His Thr Arg Thr His Thr Gly Glu Lys Pro Phe Ser Cys Arg
 65                  70                  75                  80
Trp Pro Ser Cys Gln Lys Lys Phe Ala Arg Ser Asp Glu Leu Val Arg
                 85                  90                  95
His His Asn Met His Gln Arg Asn Met Thr Lys Leu Gln Leu Ala Leu
            100                 105                 110
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<210> 346
<211> 369
<212> PRT

<213> Homo sapiens

<400> 346

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Met Gln His His His His His His His Ser Phe Ile Lys Gln Glu Pro
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Ser Trp Gly Gly Ala Glu Pro His Glu Glu Gln Cys Leu Ser Ala Phe
             20                  25                  30
Thr Val His Phe Ser Gly Gln Phe Thr Gly Thr Ala Gly Ala Cys Arg
         35                  40                  45
Tyr Gly Pro Phe Gly Pro Pro Pro Pro Ser Gln Ala Ser Ser Gly Gln
     50                  55                  60
Ala Arg Met Phe Pro Asn Ala Pro Tyr Leu Pro Ser Cys Leu Glu Ser
 65                  70                  75                  80
Gln Pro Ala Ile Arg Asn Gln Gly Tyr Ser Thr Val Thr Phe Asp Gly
                 85                  90                  95
Thr Pro Ser Tyr Gly His Thr Pro Ser His His Ala Ala Gln Phe Pro
            100                 105                 110
Asn His Ser Phe Lys His Glu Asp Pro Met Gly Gln Gln Gly Ser Leu
        115                 120                 125
Gly Glu Gln Gln Tyr Ser Val Pro Pro Pro Val Tyr Gly Cys His Thr
    130                 135                 140
Pro Thr Asp Ser Cys Thr Gly Ser Gln Ala Leu Leu Leu Arg Thr Pro
145                 150                 155                 160
Tyr Ser Ser Asp Asn Leu Tyr Gln Met Thr Ser Gln Leu Glu Cys Met
                165                 170                 175
Thr Trp Asn Gln Met Asn Leu Gly Ala Thr Leu Lys Gly His Ser Thr
            180                 185                 190
Gly Tyr Glu Ser Asp Asn His Thr Thr Pro Ile Leu Cys Gly Ala Gln
        195                 200                 205
Tyr Arg Ile His Thr His Gly Val Phe Arg Gly Ile Gln Asp Val Arg
    210                 215                 220
Arg Val Pro Gly Val Ala Pro Thr Leu Val Arg Ser Ala Ser Glu Thr
225                 230                 235                 240
Ser Glu Lys Arg Pro Phe Met Cys Ala Tyr Pro Gly Cys Asn Lys Arg
                245                 250                 255
Tyr Phe Lys Leu Ser His Leu Gln Met His Ser Arg Lys His Thr Gly
            260                 265                 270
Glu Lys Pro Tyr Gln Cys Asp Phe Lys Asp Cys Glu Arg Arg Phe Phe
        275                 280                 285
Arg Ser Asp Gln Leu Lys Arg His Gln Arg Arg His Thr Gly Val Lys
    290                 295                 300
Pro Phe Gln Cys Lys Thr Cys Gln Arg Lys Phe Ser Arg Ser Asp His
305                 310                 315                 320
Leu Lys Thr His Thr Arg Thr His Thr Gly Glu Lys Pro Phe Ser Cys
                325                 330                 335
Arg Trp Pro Ser Cys Gln Lys Lys Phe Ala Arg Ser Asp Glu Leu Val
            340                 345                 350
Arg His His Asn Met His Gln Arg Asn Met Thr Lys Leu Gln Leu Ala
        355                 360                 365
Leu
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<210> 347
<211> 21
<212> DNA
<213> Artificial Sequence

<220>

<223> Primer

<400> 347
ggctccgacg tgcgggacct g 21

<210> 348
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer

<400> 348
gaattctcaa agcgccagct ggagtttggt 30

<210> 349
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer

<400> 349
ggctccgacg tgcgggacct g 21

<210> 350
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer

<400> 350
gaattctcaa agcgccagct ggagtttggt 30

<210> 351
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer

<400> 351
cacagcacag ggtacgagag c 21

<210> 352
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer

<400> 352

gaattctcaa agcgccagct ggagtttggt 30

<210> 353
<211> 29
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer

<400> 353
cacgaagaac agtgcctgag cgcattcac 29

<210> 354
<211> 32
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer

<400> 354
ccggcgaatt catcagtata aattgtcact gc 32

<210> 355
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer

<400> 355
caggctttgc tgctgaggac gccc 24

<210> 356
<211> 34
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer

<400> 356
cacggagaat tcatcactgg tatggtttct cacc 34

<210> 357
<211> 28
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer

<400> 357
cacagcagga agcacactgg tgagaaac 28

<210> 358

<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer

<400> 358
ggatatctgc agaattctca aagcgccagc 30

<210> 359
<211> 22
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer

<400> 359
cactccttca tcaaacagga ac 22

<210> 360
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer

<400> 360
ggatatctgc agaattctca aagcgccagc 30

<210> 361
<211> 33
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer

<400> 361
ggttccgacg tgcgggacct gaacgcactg ctg 33

<210> 362
<211> 40
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer

<400> 362
ctgccggcag cagtgcgttc aggtcccgca cgtcggaacc 40

<210> 363
<211> 35
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer

<400> 363
ccggcagttc catccctggg tggcggtgga ggctg 35

<210> 364
<211> 38
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer

<400> 364
cggcagtgcg cagcctccac cgccacccag ggatggaa 38

<210> 365
<211> 35
<212> DNA
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<220>
<223> Primer

<400> 365
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<210> 366
<211> 33
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer

<400> 366
cagaactgga gcccactgtg ctgcaccgct aac 33

<210> 367
<211> 38
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer

<400> 367
cagttctgga cttcgcaccg cctggtgcat ccgcatac 38

<210> 368
<211> 39
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer

<400> 368
cagggaaccg tatgcggatg caccaggcgg tgcgaagtc 39

<210> 369
<211> 38
<212> DNA
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<220>
<223> Primer

<400> 369
ggttccctgg gtggtccagc acctccgccc gcaacgcc 38

<210> 370
<211> 38
<212> DNA
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<220>
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<400> 370
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<210> 371
<211> 40
<212> DNA
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<220>
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<400> 371
cccaccgcct ccaccgcccc cgcactcctt catcaaacag 40

<210> 372
<211> 39
<212> DNA
<213> Artificial Sequence

<220>
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<400> 372
ctaggttcct gtttgatgaa ggagtgcggg ggcggtgga 39

<210> 373
<211> 38
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer

<400> 373
gaacctagct ggggtggtgc agaaccgcac gaagaaca 38

<210> 374
<211> 39
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer

<400> 374
ctcaggcact gttcttcgtg cggttctgca ccaccccag 39

<210> 375
<211> 32
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer

<400> 375
gtgcctgagc gcattctgag aattctgcag at 32

<210> 376
<211> 34
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer

<400> 376
gtgtgatgga tatctgcaga attctcagaa tgcg 34

<210> 377
<211> 1292
<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<222> 253,256,517,518,520,521,522,743,753,754,
758
<223> n = A,T,C or G

<400> 377
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ggtggtggtt gcgcactgcc ggttagcggt gcagcacagt gggctccggt tctggacttc 120
gcaccgccgg gtgcatccgc acacggtccc ctgggtggtc cggcgccgcc gtcggcaccg 180
ccgccgccgc cgccgccgcc gccgcactcc ttcatcaaac agggaccgag ctggggtggc 240
gcggaactgc ackaakaaca gtacctgagc gcgttcaccg ttcactcctc cggtcaggtt 300
cactggcacg gccggggcct gtcgctacgg gcccctcggc ccccctccgc ccagccaggc 360
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ccagcccgct acccgcaatc ggggctacag cacggtcacc ttcgacgggg cgtccggcta 480
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tcccatgggc cagcagggtc cgctgggtga gcagcagtgc tcggcgccgc ccccggcctg 600
tggccgccac acccccgccg acagctgcgc cggcagccag gctttgctgc tgagggcgcc 660

ctgtagcagc gacggtttat accaagtgac gtcccagctt gagtgcatgg cctggagtca 720
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gggcatgacc gaactccagc tggcgctttg aa 1292

<210> 378
<211> 1291
<212> DNA
<213> Homo sapiens

<400> 378
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gtaccgccgg gtgcgcctgt atgcggttcc ctgggtggcc cggcaccgcc gccagcgccg 180
ccgccgctgc cgccgccgcc gtcgcactcc ttcaccaaac aggaaccgag ttggggtggt 240
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actggcacag ccggagcctg tcgctacggg cccttcggtc ctcctccgcc cagccaggcg 360
tcatccggcc aggccaggat gtttcctaac gcgccctacc tgcccagctg cctcgagagc 420
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<210> 379
<211> 1281
<212> DNA
<213> Homo sapiens

<400> 379
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gcaccgccgg gcgcatccgc atacgattcc ctgggtggcc cggcaccgcc gccggcgccg 180
ccgccgccgc cgccgccgcc gccgcactcc tgcggcgaac aggggccgag ctggggtggt 240
gcagaaccgc gcgaggggca atgcctgagt gcgcccgccg tccgcttctc cggccggttc 300
accggcacag tcggagcctg tcgctatggg cccctcggtc ctcctccgcc cagccaggcg 360
ccatccggcc agaccaggat gttgcccagc gcgccctatc tgtcgagttg cctcaggagc 420
cggtccgcta tccgtagtca gggtcgcagc acggcacctt cagcggggcg cccagctatg 480
gcacccaccc tcgcaccacc ggcgcagtcc cactactccc aacatggggt cctacatggg 540

ccagcagggc tcgctgggtg agcagcagta ctcggtgccg cccccggtct atggctgcca 600
caccccacc gacagctgca ccggcagcca ggctttgctg ctgaggacgc cctacagcag 660
tgacaattta taccaaatga catcccagct tgaatgcatg acctggaatc agatgaactt 720
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ggttcgccca gtcagacgaa ttagtccgtc atcacaacat gtatcagcga aacatgacta 1260
aactccagct ggcgctttga a 1281

<210> 380
<211> 3020
<212> DNA
<213> Homo sapiens

<400> 380
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cagcccaggc gcccgggccc cgccgtctcc tcgccgcgat cctggacttc ctcttgctgc 180
aggacccggc ttccacgtgt gtcccggagc cggcgtctca gcacacgctc cgctccgggc 240
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ccgtcccctc cctgggtggc ggcggcggct gtgccctgcc tgtgagcggc gcggcgcagt 480
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agtgtgactt caaggactgt gaacgaaggt tttctcgttc agaccagctc aaaagacacc 1500
aaaggagaca tacaggtgtg aaaccattcc agtgtaaaac ttgtcagcga aagttctccc 1560
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cccattgcca tttggtctgg attttctact gtaagaagag ccatagctga tcatgtcccc 2040
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ttctatcatg gaatatttat aggccagggc atgtgtatgt gtctgctaat gtaaactttg 2160
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aacactggca cactgctctt aagaaactat gaagatctga gatttttttg tgtatgtttt 2460
tgactctttt gagtggtaat catatgtgtc tttatagatg tacatacctc cttgcacaaa 2520
tggaggggaa ttcattttca tcactgggac tgtccttagt gtataaaaac catgctggta 2580
tatggcttca agttgtaaaa atgaaagtga ctttaaaaga aaatagggga tggtccagga 2640
tctccactga taagactgtt tttaagtaac ttaaggacct ttgggtctac aagtatatgt 2700
gaaaaaaatg agacttactg ggtgaggaaa tccattgttt aaagatggtc gtgtgtgtgt 2760
gtgtgtgtgt gtgtgtgttg tgttgtgttt tgtttttttaa gggagggaat ttattattta 2820
ccgttgcttg aaattactgt gtaaatatat gtctgataat gatttgctct ttgacaacta 2880
aaattaggac tgtataagta ctagatgcat cactgggtgt tgatcttaca agatattgat 2940
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aaggaaaaaa aaaaaaaaaa 3020

<210> 381
<211> 1291
<212> DNA
<213> Homo sapiens

<400> 381
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<211> 1491
<212> DNA
<213> Homo sapiens

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<211> 1251
<212> DNA
<213> Homo sapiens

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<210> 384
<211> 228
<212> DNA
<213> Homo sapiens

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cagaggttga tctttgccgg aaaacagctg gaagatggtc gtaccctgtc tgactacaac 180
atccagaaag agtccacctt gcacctggta ctccgtctca gaggtggg 228

<210> 385
<211> 1515
<212> DNA
<213> Homo sapiens

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<210> 386
<211> 648
<212> DNA
<213> Homo sapiens

<400> 386
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aagggccaca gcacagggta cgagagcgat aaccacacaa cgcccatcct ctgcggagcc 600
caatacagaa tacacacgca cggtgtcttc agaggcattc agtgatga 648

<210> 387
<211> 1089

<212> DNA
<213> Homo sapiens

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<210> 388
<211> 1035
<212> DNA
<213> Homo sapiens

<400> 388
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<211> 1263
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<210> 390
<211> 1707
<212> DNA
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<210> 391
<211> 344

<212> PRT
<213> Homo sapiens

<400> 391
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Phe Ala Ile Pro Ile Gly Gln Ala Met Ala Ile Ala Gly Gln Ile Lys
20 25 30

Leu Pro Thr Val His Ile Gly Pro Thr Ala Phe Leu Gly Leu Gly Val
35 40 45

Val Asp Asn Asn Gly Asn Gly Ala Arg Val Gln Arg Val Val Gly Ser
50 55 60

Ala Pro Ala Ala Ser Leu Gly Ile Ser Thr Gly Asp Val Ile Thr Ala
65 70 75 80

Val Asp Gly Ala Pro Ile Asn Ser Ala Thr Ala Met Ala Asp Ala Leu
85 90 95

Asn Gly His His Pro Gly Asp Val Ile Ser Val Thr Trp Gln Thr Lys
100 105 110

Ser Gly Gly Thr Arg Thr Gly Asn Val Thr Leu Ala Glu Gly Pro Pro
115 120 125

Ala Glu Phe His Ser Phe Ile Lys Gln Glu Pro Ser Trp Gly Gly Ala
130 135 140

Glu Pro His Glu Glu Gln Cys Leu Ser Ala Phe Thr Val His Phe Ser
145 150 155 160

Gly Gln Phe Thr Gly Thr Ala Gly Ala Cys Arg Tyr Gly Pro Phe Gly
165 170 175

Pro Pro Pro Pro Ser Gln Ala Ser Ser Gly Gln Ala Arg Met Phe Pro
180 185 190

Asn Ala Pro Tyr Leu Pro Ser Cys Leu Glu Ser Gln Pro Ala Ile Arg
195 200 205

Asn Gln Gly Tyr Ser Thr Val Thr Phe Asp Gly Thr Pro Ser Tyr Gly
210 215 220

His Thr Pro Ser His His Ala Ala Gln Phe Pro Asn His Ser Phe Lys
225 230 235 240

His Glu Asp Pro Met Gly Gln Gln Gly Ser Leu Gly Glu Gln Gln Tyr
245 250 255

Ser Val Pro Pro Pro Val Tyr Gly Cys His Thr Pro Thr Asp Ser Cys
260 265 270

Thr Gly Ser Gln Ala Leu Leu Leu Arg Thr Pro Tyr Ser Ser Asp Asn
275 280 285

Leu Tyr Gln Met Thr Ser Gln Leu Glu Cys Met Thr Trp Asn Gln Met
290 295 300

Asn Leu Gly Ala Thr Leu Lys Gly His Ser Thr Gly Tyr Glu Ser Asp
305 310 315 320

Asn His Thr Thr Pro Ile Leu Cys Gly Ala Gln Tyr Arg Ile His Thr
325 330 335

His Gly Val Phe Arg Gly Ile Gln
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<210> 392
<211> 568
<212> PRT
<213> Homo sapiens

<400> 392
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Phe Ala Ile Pro Ile Gly Gln Ala Met Ala Ile Ala Gly Gln Ile Lys
20 25 30

Leu Pro Thr Val His Ile Gly Pro Thr Ala Phe Leu Gly Leu Gly Val
35 40 45

Val Asp Asn Asn Gly Asn Gly Ala Arg Val Gln Arg Val Val Gly Ser
50 55 60

Ala Pro Ala Ala Ser Leu Gly Ile Ser Thr Gly Asp Val Ile Thr Ala
65 70 75 80

Val Asp Gly Ala Pro Ile Asn Ser Ala Thr Ala Met Ala Asp Ala Leu
85 90 95

Asn Gly His His Pro Gly Asp Val Ile Ser Val Thr Trp Gln Thr Lys
100 105 110

Ser Gly Gly Thr Arg Thr Gly Asn Val Thr Leu Ala Glu Gly Pro Pro
115 120 125

Ala Glu Phe Pro Leu Val Pro Arg Gly Ser Pro Met Gly Ser Asp Val
130 135 140

Arg Asp Leu Asn Ala Leu Leu Pro Ala Val Pro Ser Leu Gly Gly Gly
145 150 155 160

Gly Gly Cys Ala Leu Pro Val Ser Gly Ala Ala Gln Trp Ala Pro Val
165 170 175

Leu Asp Phe Ala Pro Pro Gly Ala Ser Ala Tyr Gly Ser Leu Gly Gly
180 185 190

Pro Ala Pro Pro Pro Ala Pro Pro Pro Pro Pro Pro Pro Pro Pro His
195 200 205

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Ser Phe Ile Lys Gln Glu Pro Ser Trp Gly Gly Ala Glu Pro His Glu
    210                 215                 220

Glu Gln Cys Leu Ser Ala Phe Thr Val His Phe Ser Gly Gln Phe Thr
225                 230                 235                 240

Gly Thr Ala Gly Ala Cys Arg Tyr Gly Pro Phe Gly Pro Pro Pro Pro
                245                 250                 255

Ser Gln Ala Ser Ser Gly Gln Ala Arg Met Phe Pro Asn Ala Pro Tyr
            260                 265                 270

Leu Pro Ser Cys Leu Glu Ser Gln Pro Ala Ile Arg Asn Gln Gly Tyr
        275                 280                 285

Ser Thr Val Thr Phe Asp Gly Thr Pro Ser Tyr Gly His Thr Pro Ser
    290                 295                 300

His His Ala Ala Gln Phe Pro Asn His Ser Phe Lys His Glu Asp Pro
305                 310                 315                 320

Met Gly Gln Gln Gly Ser Leu Gly Glu Gln Gln Tyr Ser Val Pro Pro
                325                 330                 335

Pro Val Tyr Gly Cys His Thr Pro Thr Asp Ser Cys Thr Gly Ser Gln
            340                 345                 350

Ala Leu Leu Leu Arg Thr Pro Tyr Ser Ser Asp Asn Leu Tyr Gln Met
        355                 360                 365

Thr Ser Gln Leu Glu Cys Met Thr Trp Asn Gln Met Asn Leu Gly Ala
    370                 375                 380

Thr Leu Lys Gly His Ser Thr Gly Tyr Glu Ser Asp Asn His Thr Thr
385                 390                 395                 400

Pro Ile Leu Cys Gly Ala Gln Tyr Arg Ile His Thr His Gly Val Phe
                405                 410                 415

Arg Gly Ile Gln Asp Val Arg Arg Val Pro Gly Val Ala Pro Thr Leu
            420                 425                 430

Val Arg Ser Ala Ser Glu Thr Ser Glu Lys Arg Pro Phe Met Cys Ala
        435                 440                 445

Tyr Pro Gly Cys Asn Lys Arg Tyr Phe Lys Leu Ser His Leu Gln Met
    450                 455                 460

His Ser Arg Lys His Thr Gly Glu Lys Pro Tyr Gln Cys Asp Phe Lys
465                 470                 475                 480

Asp Cys Glu Arg Arg Phe Phe Arg Ser Asp Gln Leu Lys Arg His Gln
                485                 490                 495

Arg Arg His Thr Gly Val Lys Pro Phe Gln Cys Lys Thr Cys Gln Arg
            500                 505                 510

Lys Phe Ser Arg Ser Asp His Leu Lys Thr His Thr Arg Thr His Thr
```

515 520 525

Gly Glu Lys Pro Phe Ser Cys Arg Trp Pro Ser Cys Gln Lys Lys Phe
530 535 540

Ala Arg Ser Asp Glu Leu Val Arg His His Asn Met His Gln Arg Asn
545 550 555 560

Met Thr Lys Leu Gln Leu Ala Leu
565

<210> 393
<211> 420
<212> PRT
<213> Homo sapiens

<400> 393
Met Thr Ala Ala Ser Asp Asn Phe Gln Leu Ser Gln Gly Gly Gln Gly
5 10 15

Phe Ala Ile Pro Ile Gly Gln Ala Met Ala Ile Ala Gly Gln Ile Lys
20 25 30

Leu Pro Thr Val His Ile Gly Pro Thr Ala Phe Leu Gly Leu Gly Val
35 40 45

Val Asp Asn Asn Gly Asn Gly Ala Arg Val Gln Arg Val Val Gly Ser
50 55 60

Ala Pro Ala Ala Ser Leu Gly Ile Ser Thr Gly Asp Val Ile Thr Ala
65 70 75 80

Val Asp Gly Ala Pro Ile Asn Ser Ala Thr Ala Met Ala Asp Ala Leu
85 90 95

Asn Gly His His Pro Gly Asp Val Ile Ser Val Thr Trp Gln Thr Lys
100 105 110

Ser Gly Gly Thr Arg Thr Gly Asn Val Thr Leu Ala Glu Gly Pro Pro
115 120 125

Ala Glu Phe Pro Leu Val Pro Arg Gly Ser Pro Met Gly Ser Asp Val
130 135 140

Arg Asp Leu Asn Ala Leu Leu Pro Ala Val Pro Ser Leu Gly Gly Gly
145 150 155 160

Gly Gly Cys Ala Leu Pro Val Ser Gly Ala Ala Gln Trp Ala Pro Val
165 170 175

Leu Asp Phe Ala Pro Pro Gly Ala Ser Ala Tyr Gly Ser Leu Gly Gly
180 185 190

Pro Ala Pro Pro Pro Pro Ala Pro Pro Pro Pro Pro Pro Pro Pro His
195 200 205

Ser Phe Ile Lys Gln Glu Pro Ser Trp Gly Gly Ala Glu Pro His Glu

210 215 220

Glu Gln Cys Leu Ser Ala Phe Thr Val His Phe Ser Gly Gln Phe Thr
225 230 235 240

Gly Thr Ala Gly Ala Cys Arg Tyr Gly Pro Phe Gly Pro Pro Pro Pro
245 250 255

Ser Gln Ala Ser Ser Gly Gln Ala Arg Met Phe Pro Asn Ala Pro Tyr
260 265 270

Leu Pro Ser Cys Leu Glu Ser Gln Pro Ala Ile Arg Asn Gln Gly Tyr
275 280 285

Ser Thr Val Thr Phe Asp Gly Thr Pro Ser Tyr Gly His Thr Pro Ser
290 295 300

His His Ala Ala Gln Phe Pro Asn His Ser Phe Lys His Glu Asp Pro
305 310 315 320

Met Gly Gln Gln Gly Ser Leu Gly Glu Gln Gln Tyr Ser Val Pro Pro
325 330 335

Pro Val Tyr Gly Cys His Thr Pro Thr Asp Ser Cys Thr Gly Ser Gln
340 345 350

Ala Leu Leu Leu Arg Thr Pro Tyr Ser Ser Asp Asn Leu Tyr Gln Met
355 360 365

Thr Ser Gln Leu Glu Cys Met Thr Trp Asn Gln Met Asn Leu Gly Ala
370 375 380

Thr Leu Lys Gly His Ser Thr Gly Tyr Glu Ser Asp Asn His Thr Thr
385 390 395 400

Pro Ile Leu Cys Gly Ala Gln Tyr Arg Ile His Thr His Gly Val Phe
405 410 415

Arg Gly Ile Gln
420

<210> 394
<211> 362
<212> PRT
<213> Homo sapiens

<400> 394
Met His Ser Phe Ile Lys Gln Glu Pro Ser Trp Gly Gly Ala Glu Pro
5 10 15

His Glu Glu Gln Cys Leu Ser Ala Phe Thr Val His Phe Ser Gly Gln
20 25 30

Phe Thr Gly Thr Ala Gly Ala Cys Arg Tyr Gly Pro Phe Gly Pro Pro
35 40 45

Pro Pro Ser Gln Ala Ser Ser Gly Gln Ala Arg Met Phe Pro Asn Ala

```
     50                  55                  60

Pro Tyr Leu Pro Ser Cys Leu Glu Ser Gln Pro Ala Ile Arg Asn Gln
 65                  70                  75                  80

Gly Tyr Ser Thr Val Thr Phe Asp Gly Thr Pro Ser Tyr Gly His Thr
                85                  90                  95

Pro Ser His His Ala Ala Gln Phe Pro Asn His Ser Phe Lys His Glu
            100                 105                 110

Asp Pro Met Gly Gln Gln Gly Ser Leu Gly Glu Gln Gln Tyr Ser Val
        115                 120                 125

Pro Pro Pro Val Tyr Gly Cys His Thr Pro Thr Asp Ser Cys Thr Gly
    130                 135                 140

Ser Gln Ala Leu Leu Leu Arg Thr Pro Tyr Ser Ser Asp Asn Leu Tyr
145                 150                 155                 160

Gln Met Thr Ser Gln Leu Glu Cys Met Thr Trp Asn Gln Met Asn Leu
                165                 170                 175

Gly Ala Thr Leu Lys Gly His Ser Thr Gly Tyr Glu Ser Asp Asn His
            180                 185                 190

Thr Thr Pro Ile Leu Cys Gly Ala Gln Tyr Arg Ile His Thr His Gly
        195                 200                 205

Val Phe Arg Gly Ile Gln Asp Val Arg Arg Val Pro Gly Val Ala Pro
    210                 215                 220

Thr Leu Val Arg Ser Ala Ser Glu Thr Ser Glu Lys Arg Pro Phe Met
225                 230                 235                 240

Cys Ala Tyr Pro Gly Cys Asn Lys Arg Tyr Phe Lys Leu Ser His Leu
                245                 250                 255

Gln Met His Ser Arg Lys His Thr Gly Glu Lys Pro Tyr Gln Cys Asp
            260                 265                 270

Phe Lys Asp Cys Glu Arg Arg Phe Phe Arg Ser Asp Gln Leu Lys Arg
        275                 280                 285

His Gln Arg Arg His Thr Gly Val Lys Pro Phe Gln Cys Lys Thr Cys
    290                 295                 300

Gln Arg Lys Phe Ser Arg Ser Asp His Leu Lys Thr His Thr Arg Thr
305                 310                 315                 320

His Thr Gly Glu Lys Pro Phe Ser Cys Arg Trp Pro Ser Cys Gln Lys
                325                 330                 335

Lys Phe Ala Arg Ser Asp Glu Leu Val Arg His His Asn Met His Gln
            340                 345                 350

Arg Asn Met Thr Lys Leu Gln Leu Ala Leu
        355                 360
```

<210> 395
<211> 214
<212> PRT
<213> Homo sapiens

<400> 395
Met His Ser Phe Ile Lys Gln Glu Pro Ser Trp Gly Gly Ala Glu Pro
5 10 15

His Glu Glu Gln Cys Leu Ser Ala Phe Thr Val His Phe Ser Gly Gln
20 25 30

Phe Thr Gly Thr Ala Gly Ala Cys Arg Tyr Gly Pro Phe Gly Pro Pro
35 40 45

Pro Pro Ser Gln Ala Ser Ser Gly Gln Ala Arg Met Phe Pro Asn Ala
50 55 60

Pro Tyr Leu Pro Ser Cys Leu Glu Ser Gln Pro Ala Ile Arg Asn Gln
65 70 75 80

Gly Tyr Ser Thr Val Thr Phe Asp Gly Thr Pro Ser Tyr Gly His Thr
85 90 95

Pro Ser His His Ala Ala Gln Phe Pro Asn His Ser Phe Lys His Glu
100 105 110

Asp Pro Met Gly Gln Gln Gly Ser Leu Gly Glu Gln Gln Tyr Ser Val
115 120 125

Pro Pro Pro Val Tyr Gly Cys His Thr Pro Thr Asp Ser Cys Thr Gly
130 135 140

Ser Gln Ala Leu Leu Leu Arg Thr Pro Tyr Ser Ser Asp Asn Leu Tyr
145 150 155 160

Gln Met Thr Ser Gln Leu Glu Cys Met Thr Trp Asn Gln Met Asn Leu
165 170 175

Gly Ala Thr Leu Lys Gly His Ser Thr Gly Tyr Glu Ser Asp Asn His
180 185 190

Thr Thr Pro Ile Leu Cys Gly Ala Gln Tyr Arg Ile His Thr His Gly
195 200 205

Val Phe Arg Gly Ile Gln
210

<210> 396
<211> 30
<212> DNA
<213> Artificial Sequence

<220>

<223> PCR primer

<400> 396
gacgaaagca tatgcactcc ttcatcaaac 30

<210> 397
<211> 31
<212> DNA
<213> Artificial Sequence

<220>
<223> PCR primer

<400> 397
cgcgtgaatt catcactgaa tgcctctgaa g 31

<210> 398
<211> 31
<212> DNA
<213> Artificial Sequence

<220>
<223> PCR primer

<400> 398
cgataagcat atgacggccg cgtccgataa c 31

<210> 399
<211> 31
<212> DNA
<213> Artificial Sequence

<220>
<223> PCR primer

<400> 399
cgcgtgaatt catcactgaa tgcctctgaa g 31

<210> 400
<211> 31
<212> DNA
<213> Artificial Sequence

<220>
<223> PCR primer

<400> 400
cgataagcat atgacggccg cgtccgataa c 31

<210> 401
<211> 28
<212> DNA
<213> Artificial Sequence

<220>
<223> PCR primer

<400> 401

gtctgcagcg gccgctcaaa gcgccagc 28

<210> 402
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> PCR primer

<400> 402
gacgaaagca tatgcactcc ttcatcaaac 30

<210> 403
<211> 28
<212> DNA
<213> Artificial Sequence

<220>
<223> PCR primer

<400> 403
gtctgcagcg gccgctcaaa gcgccagc 28

<210> 404
<211> 449
<212> PRT
<213> Homo sapiens

<400> 404

```
Met Gly Ser Asp Val Arg Asp Leu Asn Ala Leu Leu Pro Ala Val Pro
 1               5                  10                  15
Ser Leu Gly Gly Gly Gly Gly Cys Ala Leu Pro Val Ser Gly Ala Ala
            20                  25                  30
Gln Trp Ala Pro Val Leu Asp Phe Ala Pro Pro Gly Ala Ser Ala Tyr
        35                  40                  45
Gly Ser Leu Gly Gly Pro Ala Pro Pro Pro Ala Pro Pro Pro Pro Pro
    50                  55                  60
Pro Pro Pro Pro His Ser Phe Ile Lys Gln Glu Pro Ser Trp Gly Gly
65                  70                  75                  80
Ala Glu Pro His Glu Glu Gln Cys Leu Ser Ala Phe Thr Val His Phe
                85                  90                  95
Ser Gly Gln Phe Thr Gly Thr Ala Gly Ala Cys Arg Tyr Gly Pro Phe
            100                 105                 110
Gly Pro Pro Pro Pro Ser Gln Ala Ser Ser Gly Gln Ala Arg Met Phe
        115                 120                 125
Pro Asn Ala Pro Tyr Leu Pro Ser Cys Leu Glu Ser Gln Pro Ala Ile
    130                 135                 140
Arg Asn Gln Gly Tyr Ser Thr Val Thr Phe Asp Gly Thr Pro Ser Tyr
145                 150                 155                 160
Gly His Thr Pro Ser His His Ala Ala Gln Phe Pro Asn His Ser Phe
                165                 170                 175
Lys His Glu Asp Pro Met Gly Gln Gln Gly Ser Leu Gly Glu Gln Gln
            180                 185                 190
Tyr Ser Val Pro Pro Pro Val Tyr Gly Cys His Thr Pro Thr Asp Ser
        195                 200                 205
Cys Thr Gly Ser Gln Ala Leu Leu Leu Arg Thr Pro Tyr Ser Ser Asp
```

```
    210                 215                 220
Asn Leu Tyr Gln Met Thr Ser Gln Leu Glu Cys Met Thr Trp Asn Gln
225                 230                 235                 240
Met Asn Leu Gly Ala Thr Leu Lys Gly Val Ala Ala Gly Ser Ser Ser
                245                 250                 255
Ser Val Lys Trp Thr Glu Gly Gln Ser Asn His Ser Thr Gly Tyr Glu
            260                 265                 270
Ser Asp Asn His Thr Thr Pro Ile Leu Cys Gly Ala Gln Tyr Arg Ile
        275                 280                 285
His Thr His Gly Val Phe Arg Gly Ile Gln Asp Val Arg Arg Val Pro
    290                 295                 300
Gly Val Ala Pro Thr Leu Val Arg Ser Ala Ser Glu Thr Ser Glu Lys
305                 310                 315                 320
Arg Pro Phe Met Cys Ala Tyr Pro Gly Cys Asn Lys Arg Tyr Phe Lys
                325                 330                 335
Leu Ser His Leu Gln Met His Ser Arg Lys His Thr Gly Glu Lys Pro
            340                 345                 350
Tyr Gln Cys Asp Phe Lys Asp Cys Glu Arg Arg Phe Ser Arg Ser Asp
        355                 360                 365
Gln Leu Lys Arg His Gln Arg Arg His Thr Gly Val Lys Pro Phe Gln
    370                 375                 380
Cys Lys Thr Cys Gln Arg Lys Phe Ser Arg Ser Asp His Leu Lys Thr
385                 390                 395                 400
His Thr Arg Thr His Thr Gly Lys Thr Ser Glu Lys Pro Phe Ser Cys
                405                 410                 415
Arg Trp Pro Ser Cys Gln Lys Lys Phe Ala Arg Ser Asp Glu Leu Val
            420                 425                 430
Arg His His Asn Met His Gln Arg Asn Met Thr Lys Leu Gln Leu Ala
        435                 440                 445
Leu
```

<210> 405
<211> 428
<212> PRT
<213> Homo sapiens

<400> 405

```
Met Gly Ser Asp Val Arg Asp Leu Asn Ala Leu Leu Pro Ala Val Pro
 1               5                  10                  15
Ser Pro Gly Gly Gly Gly Gly Cys Ala Leu Pro Val Ser Gly Ala Thr
            20                  25                  30
Gln Trp Ala Pro Val Leu Asp Phe Val Pro Pro Gly Ala Pro Val Cys
        35                  40                  45
Gly Ser Leu Gly Gly Pro Ala Pro Pro Pro Ala Pro Pro Pro Leu Pro
    50                  55                  60
Pro Pro Pro Ser His Ser Phe Thr Lys Gln Glu Pro Ser Trp Gly Gly
65                  70                  75                  80
Thr Glu Pro His Ala Gly Gln Gly Arg Ser Ala Leu Val Ala His Ser
                85                  90                  95
Ser Gly Gln Phe Thr Gly Thr Ala Gly Ala Cys Arg Tyr Gly Pro Phe
            100                 105                 110
Gly Pro Pro Pro Pro Ser Gln Ala Ser Ser Gly Gln Ala Arg Met Phe
        115                 120                 125
Pro Asn Ala Pro Tyr Leu Pro Ser Cys Leu Glu Ser Gln Pro Ala Ile
    130                 135                 140
Arg Asn Gln Gly Tyr Ser Thr Val Thr Phe Asp Gly Thr Pro Ser Tyr
```

```
145                 150                 155                 160
Gly His Thr Pro Ser His His Ala Ala Gln Phe Pro Asn His Ser Ser
                165                 170                 175
Lys His Glu Asp Pro Met Gly Gln Gln Gly Ser Pro Gly Glu Gln Gln
            180                 185                 190
Tyr Ser Ala Pro Pro Pro Val Cys Gly Cys Arg Thr Pro Thr Gly Ser
        195                 200                 205
Cys Thr Gly Ser Gln Ala Leu Leu Leu Arg Ala Pro Tyr Ser Gly Gly
    210                 215                 220
Asp Leu His Gln Thr Thr Ser Gln Leu Gly His Met Ala Trp Asn Gln
225                 230                 235                 240
Thr Asn Leu Gly Ala Thr Leu Lys Gly His Gly Thr Gly Tyr Glu Ser
                245                 250                 255
Asp Asp His Thr Thr Pro Ile Leu Cys Gly Thr Gln Tyr Arg Ile Arg
            260                 265                 270
Ala Arg Gly Val Leu Arg Gly Thr Gln Asp Val Arg Cys Val Pro Gly
        275                 280                 285
Val Ala Pro Thr Leu Val Arg Ser Ala Ser Glu Thr Ser Glu Lys Arg
    290                 295                 300
Pro Leu Met Cys Ala Tyr Pro Gly Cys Asn Lys Arg His Phe Lys Pro
305                 310                 315                 320
Ser Arg Leu Arg Val Arg Gly Arg Glu Arg Thr Gly Glu Lys Pro Tyr
                325                 330                 335
Gln Arg Asp Phe Lys Asp Arg Gly Arg Gly Leu Leu Arg Pro Asp Gln
            340                 345                 350
Leu Lys Arg His Gln Arg Gly His Thr Gly Val Lys Pro Leu Gln Cys
        355                 360                 365
Glu Ala Arg Arg Arg Pro Pro Arg Pro Gly His Leu Lys Val His Thr
    370                 375                 380
Arg Thr His Thr Gly Gly Glu Pro Phe Ser Cys Arg Trp Pro Ser Cys
385                 390                 395                 400
Gln Glu Lys Ser Ala Arg Pro Asp Glu Ser Ala Arg Arg His Asn Met
                405                 410                 415
His Gln Arg Asn Met Thr Lys Leu Gln Leu Ala Leu
            420                 425
```

<210> 406
<211> 414
<212> PRT
<213> Homo sapiens

<220>
<221> VARIANT
<222> 85, 86, 172, 173, 242, 245, 246, 247
<223> Xaa = Any Amino Acid

<400> 406

```
Met Gly Ser Asp Val Arg Asp Leu Ser Ala Leu Leu Pro Ala Val Pro
 1               5                  10                  15
Ser Leu Gly Asp Gly Gly Gly Cys Ala Leu Pro Val Ser Gly Ala Ala
            20                  25                  30
Gln Trp Ala Pro Val Leu Asp Phe Ala Pro Pro Gly Ala Ser Ala His
        35                  40                  45
Gly Pro Leu Gly Gly Pro Ala Pro Pro Ser Ala Pro Pro Pro Pro Pro
    50                  55                  60
Pro Pro Pro Pro His Ser Phe Ile Lys Gln Gly Pro Ser Trp Gly Gly
65                  70                  75                  80
```

```
Ala Glu Leu His Xaa Xaa Gln Tyr Leu Ser Ala Phe Thr Val His Ser
                85                  90                  95
Ser Gly Gln Val His Trp His Gly Arg Gly Leu Ser Leu Arg Ala Pro
            100                 105                 110
Arg Pro Pro Ser Ala Gln Pro Gly Val Ile Arg Pro Gly Gln Asp Val
        115                 120                 125
Ser Arg Ala Leu Pro Ala Gln Pro Pro Arg Glu Pro Ala Arg Tyr Pro
    130                 135                 140
Gln Ser Gly Leu Gln His Gly His Leu Arg Arg Gly Val Arg Leu Arg
145                 150                 155                 160
Ser His Ala Leu Ala Pro Cys Gly Ala Val Leu Xaa Xaa Thr Arg Ala
                165                 170                 175
Gly Ser His Gly Pro Ala Gly Ser Ala Gly Ala Ala Val Leu Gly Ala
            180                 185                 190
Ala Pro Gly Leu Trp Pro Pro His Pro Arg Arg Gln Leu Arg Arg Gln
        195                 200                 205
Pro Gly Phe Ala Ala Glu Gly Ala Leu Gln Arg Arg Phe Ile Pro Ser
    210                 215                 220
Asp Val Pro Ala Val His Gly Leu Glu Ser Asp Glu Pro Arg Gly Arg
225                 230                 235                 240
Leu Xaa Gly Pro Xaa Xaa Xaa Val Arg Glu Arg Ser His Asn Ala Arg
                245                 250                 255
Pro Leu Arg Ser Pro Ile Gln Asn Thr His Ala Arg Cys Leu Gln Gly
            260                 265                 270
Arg Ser Gly Cys Ala Pro Cys Ala Trp Ser Ser Pro Asp Ser Cys Thr
        275                 280                 285
Val Gly Ile Gly Gln Gly Thr Pro Pro His Val Cys Leu Pro Arg Leu
    290                 295                 300
Gln Glu Val Ser Glu Ala Ala Pro Leu Thr Asp Ala Arg Glu Ala Arg
305                 310                 315                 320
Trp Glu Thr Ile Pro Val Leu Gln Gly Leu Trp Thr Glu Val Phe Leu
                325                 330                 335
Leu Arg Pro Ala Gln Lys Thr Pro Gly Glu Ala Tyr Arg Cys Glu Ala
            340                 345                 350
Ile Pro Ala Asp Leu Ser Ala Arg Val Leu Pro Ala Gln Pro Pro Glu
        355                 360                 365
Asp Pro Arg Gln Asp Ser Cys Arg Lys Ala Pro Gln Leu Ser Val Val
    370                 375                 380
Arg Leu Ser Glu Lys Ala Cys Pro Val Lys Val Gly Pro Pro Ser Arg
385                 390                 395                 400
His Ala Ser Glu Gly His Asp Arg Thr Pro Ala Gly Ala Leu
                405                 410
```

<210> 407
<211> 417
<212> PRT
<213> Homo sapiens

<400> 407

```
Met Gly Ser Asp Val Arg Asp Leu Ser Ala Leu Leu Pro Thr Ala Pro
 1               5                  10                  15
Ser Leu Gly Gly Gly Gly Asp Cys Thr Leu Pro Val Ser Gly Thr Ala
            20                  25                  30
Gln Trp Ala Pro Val Pro Ala Ser Ala Pro Pro Gly Ala Ser Ala Tyr
        35                  40                  45
Asp Ser Leu Gly Gly Pro Ala Pro Pro Pro Ala Pro Pro Pro Pro Pro
    50                  55                  60
```

```
Pro Pro Pro Pro His Ser Cys Gly Glu Gln Gly Pro Ser Trp Gly Gly
65                  70                  75                  80
Ala Glu Pro Arg Glu Gly Gln Cys Leu Ser Ala Pro Ala Val Arg Phe
                85                  90                  95
Ser Gly Arg Phe Thr Gly Thr Val Gly Ala Cys Arg Tyr Gly Pro Leu
            100                 105                 110
Gly Pro Pro Pro Pro Ser Gln Ala Pro Ser Gly Gln Thr Arg Met Leu
        115                 120                 125
Pro Ser Ala Pro Tyr Leu Ser Ser Cys Leu Arg Ser Arg Ser Ala Ile
    130                 135                 140
Arg Ser Gln Gly Arg Ser Thr Ala Pro Ser Ala Gly Arg Pro Ala Met
145                 150                 155                 160
Ala Pro Thr Leu Ala Pro Pro Ala Gln Ser His Tyr Ser Gln His Gly
                165                 170                 175
Val Leu His Gly Pro Ala Gly Leu Ala Gly Ala Ala Val Leu Gly Ala
            180                 185                 190
Ala Pro Gly Leu Trp Leu Pro His Pro His Arg Gln Leu His Arg Gln
        195                 200                 205
Pro Gly Phe Ala Ala Glu Asp Ala Leu Gln Gln Gln Phe Ile Pro Asn
    210                 215                 220
Asp Ile Pro Ala Met His Asp Leu Glu Ser Asp Glu Leu Arg Ser His
225                 230                 235                 240
Leu Lys Gly Pro Gln His Arg Val Arg Glu Arg Pro His Asn Ala His
                245                 250                 255
Pro Leu Arg Ser Pro Ile Gln Asn Thr His Ala Arg Cys Leu Gln Arg
            260                 265                 270
His Ser Gly Cys Ala Thr Cys Ala Trp Ser Ser Pro Asp Ser Cys Thr
        275                 280                 285
Val Ala Pro Glu Thr Ser Glu Asn Ala Pro Trp Cys Val Leu Pro Gly
    290                 295                 300
Leu Gln Gly Val Phe Ala Val Pro Leu Thr Gly Ala Gln Gln Glu Ala
305                 310                 315                 320
His Trp Asp Ala Thr Pro Val Arg Leu Gln Gly Pro Trp Thr Arg Ala
                325                 330                 335
Ser Pro Phe Gly Thr Ser Pro Arg Asp Thr Lys Gly Asp Ile Gln Val
            340                 345                 350
Arg Asn His Ser Ser Val Arg Leu Val Ser Glu Gly Ser Pro Gly Pro
        355                 360                 365
Thr Thr Gly Pro Thr Pro Gly Pro Thr Arg Val Gly Ser Pro Ser Ala
    370                 375                 380
Ala Gly Gly Gln Ala Ala Arg Glu Gly Ser Pro Ser Gln Thr Asn Ser
385                 390                 395                 400
Val Ile Thr Thr Cys Ile Ser Glu Thr Leu Asn Ser Ser Trp Arg Phe
                405                 410                 415
Glu
```

<210> 408
<211> 429
<212> PRT
<213> Homo sapiens

<400> 408

```
Met Gly Ser Asp Val Arg Asp Leu Asn Ala Leu Leu Pro Ala Val Pro
 1               5                  10                  15
Ser Leu Gly Gly Gly Gly Gly Cys Ala Leu Pro Val Ser Gly Ala Ala
            20                  25                  30
```

```
Gln Trp Ala Pro Val Leu Asp Phe Ala Pro Pro Gly Ala Ser Ala Tyr
        35                  40                  45
Gly Ser Leu Gly Gly Pro Ala Pro Pro Pro Ala Pro Pro Pro Pro Pro
    50                  55                  60
Pro Pro Pro Pro His Ser Phe Ile Lys Gln Glu Pro Ser Trp Gly Gly
65                  70                  75                  80
Ala Glu Pro His Glu Glu Gln Cys Leu Ser Ala Phe Thr Val His Phe
                85                  90                  95
Ser Gly Gln Phe Thr Gly Thr Ala Gly Ala Cys Arg Tyr Gly Pro Phe
            100                 105                 110
Gly Pro Pro Pro Pro Ser Gln Ala Ser Ser Gly Gln Ala Arg Met Phe
        115                 120                 125
Pro Asn Ala Pro Tyr Leu Pro Ser Cys Leu Glu Ser Gln Pro Ala Ile
    130                 135                 140
Arg Asn Gln Gly Tyr Ser Thr Val Thr Phe Asp Gly Thr Pro Ser Tyr
145                 150                 155                 160
Gly His Thr Pro Ser His His Ala Ala Gln Phe Pro Asn His Ser Phe
                165                 170                 175
Lys His Glu Asp Pro Met Gly Gln Gln Gly Ser Leu Gly Glu Gln Gln
            180                 185                 190
Tyr Ser Val Pro Pro Pro Val Tyr Gly Cys His Thr Pro Thr Asp Ser
        195                 200                 205
Cys Thr Gly Ser Gln Ala Leu Leu Leu Arg Thr Pro Tyr Ser Ser Asp
    210                 215                 220
Asn Leu Tyr Gln Met Thr Ser Gln Leu Glu Cys Met Thr Trp Asn Gln
225                 230                 235                 240
Met Asn Leu Gly Ala Thr Leu Lys Gly His Ser Thr Gly Tyr Glu Ser
                245                 250                 255
Asp Asn His Thr Thr Pro Ile Leu Cys Gly Ala Gln Tyr Arg Ile His
            260                 265                 270
Thr His Gly Val Phe Arg Gly Ile Gln Asp Val Arg Arg Val Pro Gly
        275                 280                 285
Val Ala Pro Thr Leu Val Arg Ser Ala Ser Glu Thr Ser Glu Lys Arg
    290                 295                 300
Pro Phe Met Cys Ala Tyr Pro Gly Cys Asn Lys Arg Tyr Phe Lys Leu
305                 310                 315                 320
Ser His Leu Gln Met His Ser Arg Lys His Thr Gly Glu Lys Pro Tyr
                325                 330                 335
Gln Cys Asp Phe Lys Asp Cys Glu Arg Arg Phe Phe Arg Ser Asp Gln
            340                 345                 350
Leu Lys Arg His Gln Arg Arg His Thr Gly Val Lys Pro Phe Gln Cys
        355                 360                 365
Lys Thr Cys Gln Arg Lys Phe Ser Arg Ser Asp His Leu Lys Thr His
    370                 375                 380
Thr Arg Thr His Thr Gly Glu Lys Pro Phe Ser Cys Arg Trp Pro Ser
385                 390                 395                 400
Cys Gln Lys Lys Phe Ala Arg Ser Asp Glu Leu Val Arg His His Asn
                405                 410                 415
Met His Gln Arg Asn Met Thr Lys Leu Gln Leu Ala Leu
            420                 425
```

<210> 409
<211> 495
<212> PRT
<213> Homo sapiens

<400> 409

```
Met Ala Ala Pro Gly Ala Arg Arg Ser Leu Leu Leu Leu Leu Leu Ala
 1               5                  10                  15
Gly Leu Ala His Gly Ala Ser Ala Leu Phe Glu Asp Leu Met Gly Ser
            20                  25                  30
Asp Val Arg Asp Leu Asn Ala Leu Leu Pro Ala Val Pro Ser Leu Gly
        35                  40                  45
Gly Gly Gly Gly Cys Ala Leu Pro Val Ser Gly Ala Ala Gln Trp Ala
    50                  55                  60
Pro Val Leu Asp Phe Ala Pro Pro Gly Ala Ser Ala Tyr Gly Ser Leu
65                  70                  75                  80
Gly Gly Pro Ala Pro Pro Pro Ala Pro Pro Pro Pro Pro Pro Pro His
                85                  90                  95
Ser Phe Ile Lys Gln Glu Pro Ser Trp Gly Gly Ala Glu Pro His Glu
            100                 105                 110
Glu Gln Cys Leu Ser Ala Phe Thr Val His Phe Ser Gly Gln Phe Thr
        115                 120                 125
Gly Thr Ala Gly Ala Cys Arg Tyr Gly Pro Phe Gly Pro Pro Pro Pro
    130                 135                 140
Ser Gln Ala Ser Ser Gly Gln Ala Arg Met Phe Pro Asn Ala Pro Tyr
145                 150                 155                 160
Leu Pro Ser Cys Leu Glu Ser Gln Pro Ala Ile Arg Asn Gln Gly Tyr
                165                 170                 175
Ser Thr Val Thr Phe Asp Gly Thr Pro Ser Tyr Gly His Thr Pro Ser
            180                 185                 190
His His Ala Ala Gln Phe Pro Asn His Ser Phe Lys His Glu Asp Pro
        195                 200                 205
Met Gly Gln Gln Gly Ser Leu Gly Glu Gln Gln Tyr Ser Val Pro Pro
    210                 215                 220
Pro Val Tyr Gly Cys His Thr Pro Thr Asp Ser Cys Thr Gly Ser Gln
225                 230                 235                 240
Ala Leu Leu Leu Arg Thr Pro Tyr Ser Ser Asp Asn Leu Tyr Gln Met
                245                 250                 255
Thr Ser Gln Leu Glu Cys Met Thr Trp Asn Gln Met Asn Leu Gly Ala
            260                 265                 270
Thr Leu Lys Gly His Ser Thr Gly Tyr Glu Ser Asp Asn His Thr Thr
        275                 280                 285
Pro Ile Leu Cys Gly Ala Gln Tyr Arg Ile His Thr His Gly Val Phe
    290                 295                 300
Arg Gly Ile Gln Asp Val Arg Arg Val Pro Gly Val Ala Pro Thr Leu
305                 310                 315                 320
Val Arg Ser Ala Ser Glu Thr Ser Glu Lys Arg Pro Phe Met Cys Ala
                325                 330                 335
Tyr Pro Gly Cys Asn Lys Arg Tyr Phe Lys Leu Ser His Leu Gln Met
            340                 345                 350
His Ser Arg Lys His Thr Gly Glu Lys Pro Tyr Gln Cys Asp Phe Lys
        355                 360                 365
Asp Cys Glu Arg Arg Phe Phe Arg Ser Asp Gln Leu Lys Arg His Gln
    370                 375                 380
Arg Arg His Thr Gly Val Lys Pro Phe Gln Cys Lys Thr Cys Gln Arg
385                 390                 395                 400
Lys Phe Ser Arg Ser Asp His Leu Lys Thr His Thr Arg Thr His Thr
                405                 410                 415
Gly Glu Lys Pro Phe Ser Cys Arg Trp Pro Ser Cys Gln Lys Lys Phe
            420                 425                 430
Ala Arg Ser Asp Glu Leu Val Arg His His Asn Met His Gln Arg Asn
        435                 440                 445
Met Thr Lys Leu Gln Leu Ala Leu Leu Asn Asn Met Leu Ile Pro Ile
    450                 455                 460
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Ala Val Gly Gly Ala Leu Ala Gly Leu Val Leu Ile Val Leu Ile Ala
465                 470                 475                 480
Tyr Leu Ile Gly Arg Lys Arg Ser His Ala Gly Tyr Gln Thr Ile
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<213> Homo sapiens

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 1               5                  10                  15
Val Glu Pro Ser Asp Thr Ile Glu Asn Val Lys Ala Lys Ile Gln Asp
            20                  25                  30
Lys Glu Gly Ile Pro Pro Asp Gln Gln Arg Leu Ile Phe Ala Gly Lys
        35                  40                  45
Gln Leu Glu Asp Gly Arg Thr Leu Ser Asp Tyr Asn Ile Gln Lys Glu
    50                  55                  60
Ser Thr Leu His Leu Val Leu Arg Leu Arg Gly Ala Met Gly Ser Asp
65                  70                  75                  80
Val Arg Asp Leu Asn Ala Leu Leu Pro Ala Val Pro Ser Leu Gly Gly
                85                  90                  95
Gly Gly Gly Cys Ala Leu Pro Val Ser Gly Ala Ala Gln Trp Ala Pro
            100                 105                 110
Val Leu Asp Phe Ala Pro Pro Gly Ala Ser Ala Tyr Gly Ser Leu Gly
        115                 120                 125
Gly Pro Ala Pro Pro Pro Ala Pro Pro Pro Pro Pro Pro Pro Pro His
    130                 135                 140
Ser Phe Ile Lys Gln Glu Pro Ser Trp Gly Gly Ala Glu Pro His Glu
145                 150                 155                 160
Glu Gln Cys Leu Ser Ala Phe Thr Val His Phe Ser Gly Gln Phe Thr
                165                 170                 175
Gly Thr Ala Gly Ala Cys Arg Tyr Gly Pro Phe Gly Pro Pro Pro Pro
            180                 185                 190
Ser Gln Ala Ser Ser Gly Gln Ala Arg Met Phe Pro Asn Ala Pro Tyr
        195                 200                 205
Leu Pro Ser Cys Leu Glu Ser Gln Pro Ala Ile Arg Asn Gln Gly Tyr
    210                 215                 220
Ser Thr Val Thr Phe Asp Gly Thr Pro Ser Tyr Gly His Thr Pro Ser
225                 230                 235                 240
His His Ala Ala Gln Phe Pro Asn His Ser Phe Lys His Glu Asp Pro
                245                 250                 255
Met Gly Gln Gln Gly Ser Leu Gly Glu Gln Gln Tyr Ser Val Pro Pro
            260                 265                 270
Pro Val Tyr Gly Cys His Thr Pro Thr Asp Ser Cys Thr Gly Ser Gln
        275                 280                 285
Ala Leu Leu Leu Arg Thr Pro Tyr Ser Ser Asp Asn Leu Tyr Gln Met
    290                 295                 300
Thr Ser Gln Leu Glu Cys Met Thr Trp Asn Gln Met Asn Leu Gly Ala
305                 310                 315                 320
Thr Leu Lys Gly His Ser Thr Gly Tyr Glu Ser Asp Asn His Thr Thr
                325                 330                 335
Pro Ile Leu Cys Gly Ala Gln Tyr Arg Ile His Thr His Gly Val Phe
            340                 345                 350
Arg Gly Ile Gln Asp Val Arg Arg Val Pro Gly Val Ala Pro Thr Leu
        355                 360                 365
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Val Arg Ser Ala Ser Glu Thr Ser Glu Lys Arg Pro Phe Met Cys Ala
    370                 375                 380
Tyr Pro Gly Cys Asn Lys Arg Tyr Phe Lys Leu Ser His Leu Gln Met
385                 390                 395                 400
His Ser Arg Lys His Thr Gly Glu Lys Pro Tyr Gln Cys Asp Phe Lys
                405                 410                 415
Asp Cys Glu Arg Arg Phe Phe Arg Ser Asp Gln Leu Lys Arg His Gln
            420                 425                 430
Arg Arg His Thr Gly Val Lys Pro Phe Gln Cys Lys Thr Cys Gln Arg
        435                 440                 445
Lys Phe Ser Arg Ser Asp His Leu Lys Thr His Thr Arg Thr His Thr
    450                 455                 460
Gly Glu Lys Pro Phe Ser Cys Arg Trp Pro Ser Cys Gln Lys Lys Phe
465                 470                 475                 480
Ala Arg Ser Asp Glu Leu Val Arg His His Asn Met His Gln Arg Asn
                485                 490                 495
Met Thr Lys Leu Gln Leu Ala Leu
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<211> 15
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<210> 413
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<220>
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Ile Leu Asp Phe Ala Pro Pro Gly Ala
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<220>
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1 5

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Phe Leu Asp Phe Ala Pro Pro Gly Ala
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Lys Leu Asp Phe Ala Pro Pro Gly Ala
1 5

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Met Leu Asp Phe Ala Pro Pro Gly Ala
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<210> 419

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<210> 420
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<210> 421
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1 5

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1 5

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Val Leu Asp Phe Ala Pro Pro Lys Ala
1 5

<210> 425
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<210> 430
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Phe Leu Asp Lys Ala Pro Pro Gly Ala
1 5

<210> 431
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<400> 431
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<210> 432
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Tyr Leu Asp Lys Ala Pro Pro Gly Ala
1 5

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1 5

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<220>
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<400> 434
Lys Leu Asp Phe Ala Pro Pro Gly Val
1 5

<210> 435
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<220>
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<400> 435
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1 5

<210> 436
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1 5

<210> 440
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<210> 441
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Tyr Leu Asp Glu Ala Pro Pro Gly Val
1 5

<210> 442
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<220>
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<400> 442
Phe Leu Asp Glu Ala Pro Pro Gly Leu
1 5

<210> 443
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<212> PRT
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<220>
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<400> 443
Lys Leu Asp Glu Ala Pro Pro Gly Leu
1 5

<210> 444
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<212> PRT
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<220>
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<400> 444
Tyr Leu Asp Glu Ala Pro Pro Gly Leu
1 5

<210> 445
<211> 9
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<400> 445
Val Leu Asp Phe Ala Gly Pro Gly Ala
1 5

<210> 446
<211> 9
<212> PRT
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<220>
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<400> 446
Val Leu Asp Phe Ala Thr Pro Gly Ala
1 5

<210> 447
<211> 9
<212> PRT
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<220>
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<400> 447
Val Leu Asp Phe Ala Thr Pro Gly Val
1 5

<210> 448
<211> 9
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<400> 448
Val Leu Asp Phe Ala Thr Pro Gly Leu
1 5

<210> 449
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<400> 449
Val Leu Asp Phe Ala Thr Pro Gly Ser
1 5

<210> 450
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<400> 450
Val Leu Asp Phe Ala Thr Pro Gly Ala
1 5

<210> 451
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<400> 451
Ala Leu Leu Pro Ala Val Pro Ser Leu
1 5

<210> 452
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cacggctccg acgttcggga cctgaacgca ctgctgccgg cagttccgtc cctgggtggt 180
ggtggtggtt gcgcactgcc ggttagcggt gcagcacagt gggctccggt tctggacttc 240
gcaccgccgg gtgcatccgc atacggttcc ctgggtggtc cggcaccgcc gccggcaccg 300
ccgccgccgc cgccgccgcc gccgcactcc ttcatcaaac aggaaccgag ctggggtggt 360
gcagaaccgc acgaagaaca gtgcctgagc gcattcaccg ttcacttctc cggccagttc 420
actggcacag ccggagcctg tcgctacggg cccttcggtc ctcctccgcc cagccaggcg 480
tcatccggcc aggccaggat gtttcctaac gcgccctacc tgcccagctg cctcgagagc 540
cagcccgcta ttcgcaatca gggttacagc acggtcacct tcgacgggac gcccagctac 600
ggtcacacgc cctcgcacca tgcggcgcag ttccccaacc actcattcaa gcatgaggat 660
cccatgggcc agcagggctc gctgggtgag cagcagtact cggtgccgcc cccggtctat 720
ggctgccaca cccccaccga cagctgcacc ggcagccagg ctttgctgct gaggacgccc 780
tacagcagtg acaatttata ccaaatgaca tcccagcttg aatgcatgac ctggaatcag 840
atgaacttag gagccacctt aaagggccac agcacagggt acgagagcga taaccacaca 900
acgcccatcc tctgcggagc ccaatacaga atacacacgc acggtgtctt cagaggcatt 960
cagtgatga 969

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<211> 1410
<212> DNA
<213> Homo sapiens

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ctgaccgttg ctggtatgct gggtccgtct ctgctgaccc cgcgtcgtgc taccgctgct 120
cacggctccg acgtgcggga cctgaacgca ctgctgccgg cagttccgtc cctgggtggt 180
ggtggtggtt gcgcactgcc ggttagcggt gcagcacagt gggctccggt tctggacttc 240
gcaccgccgg gtgcatccgc atacggttcc ctgggtggtc cggcaccgcc gccggcaccg 300
ccgccgccgc cgccgccgcc gccgcactcc ttcatcaaac aggaaccgag ctggggtggt 360
gcagaaccgc acgaagaaca gtgcctgagc gcattcaccg ttcacttctc cggccagttc 420
actggcacag ccggagcctg tcgctacggg cccttcggtc ctcctccgcc cagccaggcg 480
tcatccggcc aggccaggat gtttcctaac gcgccctacc tgcccagctg cctcgagagc 540
cagcccgcta ttcgcaatca gggttacagc acggtcacct tcgacgggac gcccagctac 600

ggtcacacgc cctcgcacca tgcggcgcag ttccccaacc actcattcaa gcatgaggat 660
cccatgggcc agcagggctc gctgggtgag cagcagtact cggtgccgcc cccggtctat 720
ggctgccaca cccccaccga cagctgcacc ggcagccagg ctttgctgct gaggacgccc 780
tacagcagtg acaatttata ccaaatgaca tcccagcttg aatgcatgac ctggaatcag 840
atgaacttag gagccacctt aaagggccac agcacagggt acgagagcga taaccacaca 900
acgcccatcc tctgcggagc ccaatacaga atacacacgc acggtgtctt cagaggcatt 960
caggatgtgc gacgtgtgcc tggagtagcc ccgactcttg tacggtcggc atctgagacc 1020
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tcccacttac agatgcacag caggaagcac actggtgaga aaccatacca gtgtgacttc 1140
aaggactgtg aacgaaggtt ttttcgttca gaccagctca aaagacacca aaggagacat 1200
acaggtgtga aaccattcca gtgtaaaact tgtcagcgaa agttctcccg gtccgaccac 1260
ctgaagaccc acaccaggac tcatacaggt gaaaagccct tcagctgtcg gtggccaagt 1320
tgtcagaaaa agtttgcccg gtcagatgaa ttagtccgcc atcacaacat gcatcagaga 1380
aacatgacca aactccagct ggcgctttga 1410

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<211> 469
<212> PRT
<213> Homo sapiens

<400> 454
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Gln Leu Gly Gly Leu Thr Val Ala Gly Met Leu Gly Pro Ser Leu Leu
20 25 30

Thr Pro Arg Arg Ala Thr Ala Ala His Gly Ser Asp Val Arg Asp Leu
35 40 45

Asn Ala Leu Leu Pro Ala Val Pro Ser Leu Gly Gly Gly Gly Gly Cys
50 55 60

Ala Leu Pro Val Ser Gly Ala Ala Gln Trp Ala Pro Val Leu Asp Phe
65 70 75 80

Ala Pro Pro Gly Ala Ser Ala Tyr Gly Ser Leu Gly Gly Pro Ala Pro
85 90 95

Pro Pro Ala Pro Pro Pro Pro Pro Pro Pro Pro Pro His Ser Phe Ile
100 105 110

Lys Gln Glu Pro Ser Trp Gly Gly Ala Glu Pro His Glu Glu Gln Cys
115 120 125

Leu Ser Ala Phe Thr Val His Phe Ser Gly Gln Phe Thr Gly Thr Ala
130 135 140

Gly Ala Cys Arg Tyr Gly Pro Phe Gly Pro Pro Pro Pro Ser Gln Ala
145 150 155 160

Ser Ser Gly Gln Ala Arg Met Phe Pro Asn Ala Pro Tyr Leu Pro Ser
165 170 175

Cys Leu Glu Ser Gln Pro Ala Ile Arg Asn Gln Gly Tyr Ser Thr Val
180 185 190

Thr Phe Asp Gly Thr Pro Ser Tyr Gly His Thr Pro Ser His His Ala

195 200 205

Ala Gln Phe Pro Asn His Ser Phe Lys His Glu Asp Pro Met Gly Gln
210 215 220

Gln Gly Ser Leu Gly Glu Gln Gln Tyr Ser Val Pro Pro Pro Val Tyr
225 230 235 240

Gly Cys His Thr Pro Thr Asp Ser Cys Thr Gly Ser Gln Ala Leu Leu
245 250 255

Leu Arg Thr Pro Tyr Ser Ser Asp Asn Leu Tyr Gln Met Thr Ser Gln
260 265 270

Leu Glu Cys Met Thr Trp Asn Gln Met Asn Leu Gly Ala Thr Leu Lys
275 280 285

Gly His Ser Thr Gly Tyr Glu Ser Asp Asn His Thr Thr Pro Ile Leu
290 295 300

Cys Gly Ala Gln Tyr Arg Ile His Thr His Gly Val Phe Arg Gly Ile
305 310 315 320

Gln Asp Val Arg Arg Val Pro Gly Val Ala Pro Thr Leu Val Arg Ser
325 330 335

Ala Ser Glu Thr Ser Glu Lys Arg Pro Phe Met Cys Ala Tyr Pro Gly
340 345 350

Cys Asn Lys Arg Tyr Phe Lys Leu Ser His Leu Gln Met His Ser Arg
355 360 365

Lys His Thr Gly Glu Lys Pro Tyr Gln Cys Asp Phe Lys Asp Cys Glu
370 375 380

Arg Arg Phe Phe Arg Ser Asp Gln Leu Lys Arg His Gln Arg Arg His
385 390 395 400

Thr Gly Val Lys Pro Phe Gln Cys Lys Thr Cys Gln Arg Lys Phe Ser
405 410 415

Arg Ser Asp His Leu Lys Thr His Thr Arg Thr His Thr Gly Glu Lys
420 425 430

Pro Phe Ser Cys Arg Trp Pro Ser Cys Gln Lys Lys Phe Ala Arg Ser
435 440 445

Asp Glu Leu Val Arg His His Asn Met His Gln Arg Asn Met Thr Lys
450 455 460

Leu Gln Leu Ala Leu
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<210> 455
<211> 321
<212> PRT
<213> Homo sapiens

<400> 455
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5 10 15

Gln Leu Gly Gly Leu Thr Val Ala Gly Met Leu Gly Pro Ser Leu Leu
20 25 30

Thr Pro Arg Arg Ala Thr Ala Ala His Gly Ser Asp Val Arg Asp Leu
35 40 45

Asn Ala Leu Leu Pro Ala Val Pro Ser Leu Gly Gly Gly Gly Gly Cys
50 55 60

Ala Leu Pro Val Ser Gly Ala Ala Gln Trp Ala Pro Val Leu Asp Phe
65 70 75 80

Ala Pro Pro Gly Ala Ser Ala Tyr Gly Ser Leu Gly Gly Pro Ala Pro
85 90 95

Pro Pro Ala Pro Pro Pro Pro Pro Pro Pro Pro Pro His Ser Phe Ile
100 105 110

Lys Gln Glu Pro Ser Trp Gly Gly Ala Glu Pro His Glu Glu Gln Cys
115 120 125

Leu Ser Ala Phe Thr Val His Phe Ser Gly Gln Phe Thr Gly Thr Ala
130 135 140

Gly Ala Cys Arg Tyr Gly Pro Phe Gly Pro Pro Pro Pro Ser Gln Ala
145 150 155 160

Ser Ser Gly Gln Ala Arg Met Phe Pro Asn Ala Pro Tyr Leu Pro Ser
165 170 175

Cys Leu Glu Ser Gln Pro Ala Ile Arg Asn Gln Gly Tyr Ser Thr Val
180 185 190

Thr Phe Asp Gly Thr Pro Ser Tyr Gly His Thr Pro Ser His His Ala
195 200 205

Ala Gln Phe Pro Asn His Ser Phe Lys His Glu Asp Pro Met Gly Gln
210 215 220

Gln Gly Ser Leu Gly Glu Gln Gln Tyr Ser Val Pro Pro Pro Val Tyr
225 230 235 240

Gly Cys His Thr Pro Thr Asp Ser Cys Thr Gly Ser Gln Ala Leu Leu
245 250 255

Leu Arg Thr Pro Tyr Ser Ser Asp Asn Leu Tyr Gln Met Thr Ser Gln
260 265 270

Leu Glu Cys Met Thr Trp Asn Gln Met Asn Leu Gly Ala Thr Leu Lys
275 280 285

Gly His Ser Thr Gly Tyr Glu Ser Asp Asn His Thr Thr Pro Ile Leu
290 295 300

Cys Gly Ala Gln Tyr Arg Ile His Thr His Gly Val Phe Arg Gly Ile
305 310 315 320

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<220>
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<210> 457
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<210> 458
<211> 21
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<220>
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<400> 458
ggctccgacg tgcgggacct g 21

<210> 459
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<210> 460
<211> 843
<212> DNA
<213> Homo sapiens

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ccgccgggtg catccgcata cggttccctg ggtggtccgg caccgccgcc ggcaccgccg 180
ccgccgccgc cgccgccgcc gcactccttc atcaaacagg aaccgagctg gggtggtgca 240
gaaccgcacg aagaacagtg cctgagcgca ttcaccgttc acttctccgg ccagttcact 300
ggcacagccg gagcctgtcg ctacgggccc ttcggtcctc ctccgcccag ccaggcgtca 360
tccggccagg ccaggatgtt tcctaacgcg ccctacctgc ccagctgcct cgagagccag 420
cccgctattc gcaatcaggg ttacagcacg gtcaccttcg acgggacgcc cagctacggt 480
cacacgccct cgcaccatgc ggcgcagttc cccaaccact cattcaagca tgaggatccc 540
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tgccacaccc ccaccgacag ctgcaccggc agccaggctt tgctgctgag gacgccctac 660
agcagtgaca atttatacca aatgacatcc cagcttgaat gcatgacctg gaatcagatg 720
aacttaggag ccaccttaaa gggccacagc acagggtacg agagcgataa ccacacaacg 780
cccatcctct gcggagccca atacagaata cacacgcacg gtgtcttcag aggcattcag 840
tga 843

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<211> 280
<212> PRT
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Leu Gly Gly Gly Gly Gly Cys Ala Leu Pro Val Ser Gly Ala Ala Gln
20 25 30
Trp Ala Pro Val Leu Asp Phe Ala Pro Pro Gly Ala Ser Ala Tyr Gly
35 40 45
Ser Leu Gly Gly Pro Ala Pro Pro Pro Ala Pro Pro Pro Pro Pro Pro
50 55 60
Pro Pro Pro His Ser Phe Ile Lys Gln Glu Pro Ser Trp Gly Gly Ala
65 70 75 80
Glu Pro His Glu Glu Gln Cys Leu Ser Ala Phe Thr Val His Phe Ser
85 90 95
Gly Gln Phe Thr Gly Thr Ala Gly Ala Cys Arg Tyr Gly Pro Phe Gly
100 105 110
Pro Pro Pro Pro Ser Gln Ala Ser Ser Gly Gln Ala Arg Met Phe Pro
115 120 125
Asn Ala Pro Tyr Leu Pro Ser Cys Leu Glu Ser Gln Pro Ala Ile Arg
130 135 140
Asn Gln Gly Tyr Ser Thr Val Thr Phe Asp Gly Thr Pro Ser Tyr Gly
145 150 155 160
His Thr Pro Ser His His Ala Ala Gln Phe Pro Asn His Ser Phe Lys
165 170 175
His Glu Asp Pro Met Gly Gln Gln Gly Ser Leu Gly Glu Gln Gln Tyr
180 185 190
Ser Val Pro Pro Pro Val Tyr Gly Cys His Thr Pro Thr Asp Ser Cys
195 200 205
Thr Gly Ser Gln Ala Leu Leu Leu Arg Thr Pro Tyr Ser Ser Asp Asn
210 215 220
Leu Tyr Gln Met Thr Ser Gln Leu Glu Cys Met Thr Trp Asn Gln Met
225 230 235 240
Asn Leu Gly Ala Thr Leu Lys Gly His Ser Thr Gly Tyr Glu Ser Asp
245 250 255
Asn His Thr Thr Pro Ile Leu Cys Gly Ala Gln Tyr Arg Ile His Thr
260 265 270
His Gly Val Phe Arg Gly Ile Gln

275 280

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<211> 876
<212> DNA
<213> Homo sapiens

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gcaccgccgg gtgcatccgc atacggttcc ctgggtggtc cggcaccgcc gccggcaccg 180
ccgccgccgc cgccgccgcc gccgcactcc ttcatcaaac aggaaccgag ctggggtggt 240
gcagaaccgc acgaagaaca gtgcctgagc gcattcaccg ttcacttctc cggccagttc 300
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<212> PRT
<213> Homo sapiens

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Gly Ser Leu Gly Gly Pro Ala Pro Pro Pro Ala Pro Pro Pro Pro Pro
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Pro Pro Pro Pro His Ser Phe Ile Lys Gln Glu Pro Ser Trp Gly Gly
 65                  70                  75                  80

Ala Glu Pro His Glu Glu Gln Cys Leu Ser Ala Phe Thr Val His Phe
                 85                  90                  95

Ser Gly Gln Phe Thr Gly Thr Ala Gly Ala Cys Arg Tyr Gly Pro Phe
            100                 105                 110

Gly Pro Pro Pro Pro Ser Gln Ala Ser Ser Gly Gln Ala Arg Met Phe
        115                 120                 125

Pro Asn Ala Pro Tyr Leu Pro Ser Cys Leu Glu Ser Gln Pro Ala Ile
    130                 135                 140

Arg Asn Gln Gly Tyr Ser Thr Val Thr Phe Asp Gly Thr Pro Ser Tyr
145                 150                 155                 160

Gly His Thr Pro Ser His His Ala Ala Gln Phe Pro Asn His Ser Phe
                165                 170                 175

Lys His Glu Asp Pro Met Gly Gln Gln Gly Ser Leu Gly Glu Gln Gln
            180                 185                 190

Tyr Ser Val Pro Pro Pro Val Tyr Gly Cys His Thr Pro Thr Asp Ser
        195                 200                 205

Cys Thr Gly Ser Gln Ala Leu Leu Leu Arg Thr Pro Tyr Ser Ser Asp
    210                 215                 220

Asn Leu Tyr Gln Met Thr Ser Gln Leu Glu Cys Met Thr Trp Asn Gln
225                 230                 235                 240

Met Asn Leu Gly Ala Thr Leu Lys Gly His Ser Thr Gly Tyr Glu Ser
                245                 250                 255

Asp Asn His Thr Thr Pro Ile Leu Cys Gly Ala Gln Tyr Arg Ile His
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His His His
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<212> PRT
<213> Homo sapiens

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20 25 30

Gln Trp Ala Pro Val Leu Asp Phe Ala Pro Pro Gly Ala Ser Ala Tyr
35 40 45

Gly Ser Leu Gly Gly Pro Ala Pro Pro Pro Ala Pro Pro Pro Pro Pro
50 55 60

Pro Pro Pro Pro His Ser Phe Ile Lys Gln Glu Pro Ser Trp Gly Gly
65 70 75 80

Ala Glu Pro His Glu Glu Gln Cys Leu Ser Ala Phe Thr Val His Phe
85 90 95

Ser Gly Gln Phe Thr Gly Thr Ala Gly Ala Cys Arg Tyr Gly Pro Phe
100 105 110

Gly Pro Pro Pro Pro Ser Gln Ala Ser Ser Gly Gln Ala Arg Met Phe
115 120 125

Pro Asn Ala Pro Tyr Leu Pro Ser Cys Leu Glu Ser Gln Pro Ala Ile
130 135 140

Arg Asn Gln Gly Tyr Ser Thr Val Thr Phe Asp Gly Thr Pro Ser Tyr
145 150 155 160

Gly His Thr Pro Ser His His Ala Ala Gln Phe Pro Asn His Ser Phe
165 170 175

Lys His Glu Asp Pro Met Gly Gln Gln Gly Ser Leu Gly Glu Gln Gln
180 185 190

Tyr Ser Val Pro Pro Pro Val Tyr Gly Cys His Thr Pro Thr Asp Ser
195 200 205

Cys Thr Gly Ser Gln Ala Leu Leu Leu Arg Thr Pro Tyr Ser Ser Asp
210 215 220

Asn Leu Tyr Gln Met Thr Ser Gln Leu Glu Cys Met Thr Trp Asn Gln
225 230 235 240

Met Asn Leu Gly Ala Thr Leu Lys Gly His Ser Thr Gly Tyr Glu Ser
245 250 255

Asp Asn His Thr Thr Pro Ile Leu Cys Gly Ala Gln Tyr Arg Ile His
260 265 270

Thr His Gly Val Phe Arg Gly Ile Gln
275 280

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<212> DNA
<213> Artificial Sequence

<220>
<223> PCR primer

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<223> PCR primer

<400> 467
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<212> DNA
<213> Artificial Sequence

<220>
<223> PCR primer

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caccgtg 67

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<211> 882
<212> DNA
<213> Homo sapiens

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<210> 470
<211> 292
<212> PRT

<213> Homo sapiens

<400> 470

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Gly Gly Cys Ala Leu Pro Val Ser Gly Ala Ala Gln Trp Ala Pro Val
        35                  40                  45

Leu Asp Phe Ala Pro Pro Gly Ala Ser Ala Tyr Gly Ser Leu Gly Gly
    50                  55                  60

Pro Ala Pro Pro Pro Ala Pro Pro Pro Pro Pro Pro Pro Pro Pro His
65                  70                  75                  80

Ser Phe Ile Lys Gln Glu Pro Ser Trp Gly Gly Ala Glu Pro His Glu
                85                  90                  95

Glu Gln Cys Leu Ser Ala Phe Thr Val His Phe Ser Gly Gln Phe Thr
            100                 105                 110

Gly Thr Ala Gly Ala Cys Arg Tyr Gly Pro Phe Gly Pro Pro Pro Pro
        115                 120                 125

Ser Gln Ala Ser Ser Gly Gln Ala Arg Met Phe Pro Asn Ala Pro Tyr
    130                 135                 140

Leu Pro Ser Cys Leu Glu Ser Gln Pro Ala Ile Arg Asn Gln Gly Tyr
145                 150                 155                 160

Ser Thr Val Thr Phe Asp Gly Thr Pro Ser Tyr Gly His Thr Pro Ser
                165                 170                 175

His His Ala Ala Gln Phe Pro Asn His Ser Phe Lys His Glu Asp Pro
            180                 185                 190

Met Gly Gln Gln Gly Ser Leu Gly Glu Gln Gln Tyr Ser Val Pro Pro
        195                 200                 205

Pro Val Tyr Gly Cys His Thr Pro Thr Asp Ser Cys Thr Gly Ser Gln
    210                 215                 220

Ala Leu Leu Leu Arg Thr Pro Tyr Ser Ser Asp Asn Leu Tyr Gln Met
225                 230                 235                 240

Thr Ser Gln Leu Glu Cys Met Thr Trp Asn Gln Met Asn Leu Gly Ala
                245                 250                 255

Thr Leu Lys Gly His Ser Thr Gly Tyr Glu Ser Asp Asn His Thr Thr
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Pro Ile Leu Cys Gly Ala Gln Tyr Arg Ile His Thr His Gly Val Phe
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<212> DNA
<213> Homo sapiens

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<213> Homo sapiens

<400> 475
atgaacaata acgatctctt ccaagcttcc cgtcgtcgtt tcctggcgca gctcggcggc 60
cttaccgtgg ccggtatgct gggtccgtcg cttttaacac cgcgccgcgc aaccgccgct 120

catggttcgg atgtacgcga tctgaacgcg cttcttccgg cggtcccaag cttgggcggg 180
ggaggtggat gcgccctgcc cgtgagcggc gccgcgcaat gggccccggt ccttgatttt 240
gctccgccgg gtgcgagcgc atacggctct ctgggcgggc cccattcatt catcaaacag 300
gagcccagct ggggtggcgc cgaacctcat gaagaacaat gtctgtctgc ttttaccgta 360
catttttcag gccagtttac cggaactgcc ggtgcatgtc gctatggccc attcggtccg 420
ccgccaccgt cacaagcctc ttcgggacaa gcccgcatgt ttccgaatgc accctacctg 480
ccgtcttgtc tggaaagcca acccgcgatc cgtaaccaag gctacagcac cgtcacgttt 540
gatggtaccc cgagctatgg tcatacgccg agtcatcatg cagcacagtt cccgaaccac 600
tcgttcaaac acgaagatcc tatgggccag cagggtagtc tgggcgaaca acagtatagc 660
gtcccaccac cagtttatgg ttgccatacc ccaacggatt cctgtactgg tagccaagcg 720
ctcctgctcc gcacccccta ttcttctgat aatctctatc agatgaccag ccaactggaa 780
tgtatgacgt ggaaccaaat gaacctgggc gcaaccctga aaggccacag taccggctat 840
gagtcagata accacacaac acccattttg tgcggcgctc aatatcgtat tcatacccat 900
ggcgtttttc gcggtattca gtgatga 927

<210> 476
<211> 936
<212> DNA
<213> Homo sapiens

<400> 476
atgaacaata acgatctctt ccaagcttcc cgtcgtcgtt tcctggcgca gctcggcggc 60
cttaccgtgg ccggtatgct gggtccgtcg cttttaacac cgcgccgcgc aaccgccgct 120
catggttcgg atgtacgcga tctgaacgcg cttcttccgg cggtcccaag cttgggcggg 180
ggaggtggat gcgccctgcc cgtgagcggc gccgcgcaat gggccccggt ccttgatttt 240
gctccgccgg gtgcgagcgc atacggctct ctgggcgggc ccgggcaaat tcattcattc 300
atcaaacagg agcccagctg gggtggcgcc gaacctcatg aagaacaatg tctgtctgct 360
tttaccgtac atttttcagg ccagtttacc ggaactgccg gtgcatgtcg ctatggccca 420
ttcggtccgc cgccaccgtc acaagcctct tcgggacaag cccgcatgtt tccgaatgca 480
ccctacctgc cgtcttgtct ggaaagccaa cccgcgatcc gtaaccaagg ctacagcacc 540
gtcacgtttg atggtacccc gagctatggt catacgccga gtcatcatgc agcacagttc 600
ccgaaccact cgttcaaaca cgaagatcct atgggccagc agggtagtct gggcgaacaa 660
cagtatagcg tcccaccacc agtttatggt tgccataccc caacggattc ctgtactggt 720
agccaagcgc tcctgctccg cacccccctat tcttctgata atctctatca gatgaccagc 780
caactggaat gtatgacgtg gaaccaaatg aacctgggcg caaccctgaa aggccacagt 840
accggctatg agtcagataa ccacacaaca cccattttgt gcggcgctca atatcgtatt 900
catacccatg gcgtttttcg cggtattcag tgatga 936

<210> 477
<211> 969
<212> DNA
<213> Homo sapiens

<400> 477
atgaacaata acgatctctt ccaagcttcc cgtcgtcgtt tcctggcgca gctcggcggc 60
cttaccgtgg ccggtatgct gggtccgtcg cttttaacac cgcgccgcgc aaccgccgct 120
catggttcgg atgtacgcga tctgaacgcg cttcttccgg cggtcccaag cttgggcggg 180
ggaggtggat gcgccctgcc cgtgagcggc gccgcgcaat gggccccggt ccttgatttt 240
gctccgccgg gtgcgagcgc atacggctct ctgggcggtc cggcacctcc tccagcaccg 300
cctccaccgc caccgccgcc gccgcattca ttcatcaaac aggagcccag ctggggtggc 360
gccgaacctc atgaagaaca atgtctgtct gcttttaccg tacatttttc aggccagttt 420
accggaactg ccggtgcatg tcgctatggc ccattcggtc cgccgccacc gtcacaagcc 480
tcttcgggac aagcccgcat gtttccgaat gcaccctacc tgccgtcttg tctggaaagc 540
caacccgcga tccgtaacca aggctacagc accgtcacgt ttgatggtac cccgagctat 600
ggtcatacgc cgagtcatca tgcagcacag ttccccgaacc actcgttcaa acacgaagat 660
cctatgggcc agcagggtag tctgggcgaa caacagtata gcgtcccacc accagtttat 720
ggttgccata ccccaacgga ttcctgtact ggtagccaag cgctcctgct ccgcacccccc 780
tattcttctg ataatctcta tcagatgacc agccaactgg aatgtatgac gtggaaccaa 840

atgaacctgg gcgcaaccct gaaaggccac agtaccggct atgagtcaga taaccacaca 900
acacccattt tgtgcggcgc tcaatatcgt attcataccc atggcgtttt tcgcggtatt 960
cagtgatga 969

<210> 478
<211> 267
<212> PRT
<213> Homo sapiens

<400> 478
Met Gly Ser Asp Val Arg Asp Leu Asn Ala Leu Leu Pro Ala Val Pro
5 10 15

Ser Leu Gly Gly Gly Gly Gly Cys Ala Leu Pro Val Ser Gly Ala Ala
20 25 30

Gln Trp Ala Pro Val Leu Asp Phe Ala Pro Pro Gly Ala Ser Ala Tyr
35 40 45

Gly Ser Leu Gly Gly Pro His Ser Phe Ile Lys Gln Glu Pro Ser Trp
50 55 60

Gly Gly Ala Glu Pro His Glu Glu Gln Cys Leu Ser Ala Phe Thr Val
65 70 75 80

His Phe Ser Gly Gln Phe Thr Gly Thr Ala Gly Ala Cys Arg Tyr Gly
85 90 95

Pro Phe Gly Pro Pro Pro Pro Ser Gln Ala Ser Ser Gly Gln Ala Arg
100 105 110

Met Phe Pro Asn Ala Pro Tyr Leu Pro Ser Cys Leu Glu Ser Gln Pro
115 120 125

Ala Ile Arg Asn Gln Gly Tyr Ser Thr Val Thr Phe Asp Gly Thr Pro
130 135 140

Ser Tyr Gly His Thr Pro Ser His His Ala Ala Gln Phe Pro Asn His
145 150 155 160

Ser Phe Lys His Glu Asp Pro Met Gly Gln Gln Gly Ser Leu Gly Glu
165 170 175

Gln Gln Tyr Ser Val Pro Pro Pro Val Tyr Gly Cys His Thr Pro Thr
180 185 190

Asp Ser Cys Thr Gly Ser Gln Ala Leu Leu Leu Arg Thr Pro Tyr Ser
195 200 205

Ser Asp Asn Leu Tyr Gln Met Thr Ser Gln Leu Glu Cys Met Thr Trp
210 215 220

Asn Gln Met Asn Leu Gly Ala Thr Leu Lys Gly His Ser Thr Gly Tyr
225 230 235 240

Glu Ser Asp Asn His Thr Thr Pro Ile Leu Cys Gly Ala Gln Tyr Arg
245 250 255

Ile His Thr His Gly Val Phe Arg Gly Ile Gln
260 265

<210> 479
<211> 321
<212> PRT
<213> Homo sapiens

<400> 479
Met Asn Asn Asn Asp Leu Phe Gln Ala Ser Arg Arg Arg Phe Leu Ala
5 10 15

Gln Leu Gly Gly Leu Thr Val Ala Gly Met Leu Gly Pro Ser Leu Leu
20 25 30

Thr Pro Arg Arg Ala Thr Ala Ala His Gly Ser Asp Val Arg Asp Leu
35 40 45

Asn Ala Leu Leu Pro Ala Val Pro Ser Leu Gly Gly Gly Gly Gly Cys
50 55 60

Ala Leu Pro Val Ser Gly Ala Ala Gln Trp Ala Pro Val Leu Asp Phe
65 70 75 80

Ala Pro Pro Gly Ala Ser Ala Tyr Gly Ser Leu Gly Gly Pro Ala Pro
85 90 95

Pro Pro Ala Pro Pro Pro Pro Pro Pro Pro Pro Pro His Ser Phe Ile
100 105 110

Lys Gln Glu Pro Ser Trp Gly Gly Ala Glu Pro His Glu Glu Gln Cys
115 120 125

Leu Ser Ala Phe Thr Val His Phe Ser Gly Gln Phe Thr Gly Thr Ala
130 135 140

Gly Ala Cys Arg Tyr Gly Pro Phe Gly Pro Pro Pro Pro Ser Gln Ala
145 150 155 160

Ser Ser Gly Gln Ala Arg Met Phe Pro Asn Ala Pro Tyr Leu Pro Ser
165 170 175

Cys Leu Glu Ser Gln Pro Ala Ile Arg Asn Gln Gly Tyr Ser Thr Val
180 185 190

Thr Phe Asp Gly Thr Pro Ser Tyr Gly His Thr Pro Ser His His Ala
195 200 205

Ala Gln Phe Pro Asn His Ser Phe Lys His Glu Asp Pro Met Gly Gln
210 215 220

Gln Gly Ser Leu Gly Glu Gln Gln Tyr Ser Val Pro Pro Pro Val Tyr
225 230 235 240

Gly Cys His Thr Pro Thr Asp Ser Cys Thr Gly Ser Gln Ala Leu Leu
245 250 255

Leu Arg Thr Pro Tyr Ser Ser Asp Asn Leu Tyr Gln Met Thr Ser Gln
260 265 270

Leu Glu Cys Met Thr Trp Asn Gln Met Asn Leu Gly Ala Thr Leu Lys
275 280 285

Gly His Ser Thr Gly Tyr Glu Ser Asp Asn His Thr Thr Pro Ile Leu
290 295 300

Cys Gly Ala Gln Tyr Arg Ile His Thr His Gly Val Phe Arg Gly Ile
305 310 315 320

Gln

<210> 480
<211> 274
<212> PRT
<213> Homo sapiens

<400> 480
Met Gln His His His His His His Gly Ser Asp Val Arg Asp Leu Asn
5 10 15

Ala Leu Leu Pro Ala Val Pro Ser Leu Gly Gly Gly Gly Gly Cys Ala
20 25 30

Leu Pro Val Ser Gly Ala Ala Gln Trp Ala Pro Val Leu Asp Phe Ala
35 40 45

Pro Pro Gly Ala Ser Ala Tyr Gly Ser Leu Gly Gly Pro His Ser Phe
50 55 60

Ile Lys Gln Glu Pro Ser Trp Gly Gly Ala Glu Pro His Glu Glu Gln
65 70 75 80

Cys Leu Ser Ala Phe Thr Val His Phe Ser Gly Gln Phe Thr Gly Thr
85 90 95

Ala Gly Ala Cys Arg Tyr Gly Pro Phe Gly Pro Pro Pro Pro Ser Gln
100 105 110

Ala Ser Ser Gly Gln Ala Arg Met Phe Pro Asn Ala Pro Tyr Leu Pro
115 120 125

Ser Cys Leu Glu Ser Gln Pro Ala Ile Arg Asn Gln Gly Tyr Ser Thr
130 135 140

Val Thr Phe Asp Gly Thr Pro Ser Tyr Gly His Thr Pro Ser His His
145 150 155 160

Ala Ala Gln Phe Pro Asn His Ser Phe Lys His Glu Asp Pro Met Gly
165 170 175

Gln Gln Gly Ser Leu Gly Glu Gln Gln Tyr Ser Val Pro Pro Pro Val
180 185 190

```
Tyr Gly Cys His Thr Pro Thr Asp Ser Cys Thr Gly Ser Gln Ala Leu
        195                 200                 205

Leu Leu Arg Thr Pro Tyr Ser Ser Asp Asn Leu Tyr Gln Met Thr Ser
    210                 215                 220

Gln Leu Glu Cys Met Thr Trp Asn Gln Met Asn Leu Gly Ala Thr Leu
225                 230                 235                 240

Lys Gly His Ser Thr Gly Tyr Glu Ser Asp Asn His Thr Thr Pro Ile
                245                 250                 255

Leu Cys Gly Ala Gln Tyr Arg Ile His Thr His Gly Val Phe Arg Gly
            260                 265                 270

Ile Gln
```

<210> 481
<211> 310
<212> PRT
<213> Homo sapiens

<400> 481

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Met Asn Asn Asn Asp Leu Phe Gln Ala Ser Arg Arg Arg Phe Leu Ala
                5                   10                  15

Gln Leu Gly Gly Leu Thr Val Ala Gly Met Leu Gly Pro Ser Leu Leu
            20                  25                  30

Thr Pro Arg Arg Ala Thr Ala Ala His Gly Ser Asp Val Arg Asp Leu
        35                  40                  45

Asn Ala Leu Leu Pro Ala Val Pro Ser Leu Gly Gly Gly Gly Gly Cys
    50                  55                  60

Ala Leu Pro Val Ser Gly Ala Ala Gln Trp Ala Pro Val Leu Asp Phe
65                  70                  75                  80

Ala Pro Pro Gly Ala Ser Ala Tyr Gly Ser Leu Gly Gly Pro Gly Gln
                85                  90                  95

Ile His Ser Phe Ile Lys Gln Glu Pro Ser Trp Gly Gly Ala Glu Pro
            100                 105                 110

His Glu Glu Gln Cys Leu Ser Ala Phe Thr Val His Phe Ser Gly Gln
        115                 120                 125

Phe Thr Gly Thr Ala Gly Ala Cys Arg Tyr Gly Pro Phe Gly Pro Pro
    130                 135                 140

Pro Pro Ser Gln Ala Ser Ser Gly Gln Ala Arg Met Phe Pro Asn Ala
145                 150                 155                 160

Pro Tyr Leu Pro Ser Cys Leu Glu Ser Gln Pro Ala Ile Arg Asn Gln
                165                 170                 175
```

```
Gly Tyr Ser Thr Val Thr Phe Asp Gly Thr Pro Ser Tyr Gly His Thr
            180                 185                 190

Pro Ser His His Ala Ala Gln Phe Pro Asn His Ser Phe Lys His Glu
        195                 200                 205

Asp Pro Met Gly Gln Gln Gly Ser Leu Gly Glu Gln Gln Tyr Ser Val
    210                 215                 220

Pro Pro Pro Val Tyr Gly Cys His Thr Pro Thr Asp Ser Cys Thr Gly
225                 230                 235                 240

Ser Gln Ala Leu Leu Leu Arg Thr Pro Tyr Ser Ser Asp Asn Leu Tyr
                245                 250                 255

Gln Met Thr Ser Gln Leu Glu Cys Met Thr Trp Asn Gln Met Asn Leu
            260                 265                 270

Gly Ala Thr Leu Lys Gly His Ser Thr Gly Tyr Glu Ser Asp Asn His
        275                 280                 285

Thr Thr Pro Ile Leu Cys Gly Ala Gln Tyr Arg Ile His Thr His Gly
    290                 295                 300

Val Phe Arg Gly Ile Gln
305                 310


<210> 482
<211> 307
<212> PRT
<213> Homo sapiens

<400> 482
Met Asn Asn Asn Asp Leu Phe Gln Ala Ser Arg Arg Arg Phe Leu Ala
                5                   10                  15

Gln Leu Gly Gly Leu Thr Val Ala Gly Met Leu Gly Pro Ser Leu Leu
            20                  25                  30

Thr Pro Arg Arg Ala Thr Ala Ala His Gly Ser Asp Val Arg Asp Leu
        35                  40                  45

Asn Ala Leu Leu Pro Ala Val Pro Ser Leu Gly Gly Gly Gly Gly Cys
    50                  55                  60

Ala Leu Pro Val Ser Gly Ala Ala Gln Trp Ala Pro Val Leu Asp Phe
65                  70                  75                  80

Ala Pro Pro Gly Ala Ser Ala Tyr Gly Ser Leu Gly Gly Pro His Ser
                85                  90                  95

Phe Ile Lys Gln Glu Pro Ser Trp Gly Gly Ala Glu Pro His Glu Glu
            100                 105                 110

Gln Cys Leu Ser Ala Phe Thr Val His Phe Ser Gly Gln Phe Thr Gly
        115                 120                 125
```

```
Thr Ala Gly Ala Cys Arg Tyr Gly Pro Phe Gly Pro Pro Pro Pro Ser
    130                 135                 140

Gln Ala Ser Ser Gly Gln Ala Arg Met Phe Pro Asn Ala Pro Tyr Leu
145                 150                 155                 160

Pro Ser Cys Leu Glu Ser Gln Pro Ala Ile Arg Asn Gln Gly Tyr Ser
                165                 170                 175

Thr Val Thr Phe Asp Gly Thr Pro Ser Tyr Gly His Thr Pro Ser His
            180                 185                 190

His Ala Ala Gln Phe Pro Asn His Ser Phe Lys His Glu Asp Pro Met
        195                 200                 205

Gly Gln Gln Gly Ser Leu Gly Glu Gln Gln Tyr Ser Val Pro Pro Pro
    210                 215                 220

Val Tyr Gly Cys His Thr Pro Thr Asp Ser Cys Thr Gly Ser Gln Ala
225                 230                 235                 240

Leu Leu Leu Arg Thr Pro Tyr Ser Ser Asp Asn Leu Tyr Gln Met Thr
                245                 250                 255

Ser Gln Leu Glu Cys Met Thr Trp Asn Gln Met Asn Leu Gly Ala Thr
            260                 265                 270

Leu Lys Gly His Ser Thr Gly Tyr Glu Ser Asp Asn His Thr Thr Pro
        275                 280                 285

Ile Leu Cys Gly Ala Gln Tyr Arg Ile His Thr His Gly Val Phe Arg
    290                 295                 300

Gly Ile Gln
305
```

<210> 483
<211> 278
<212> PRT
<213> Homo sapiens

<400> 483

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Met Asn Asn Asn Asp Leu Phe Gln Ala Ser Arg His Gly Ser Asp Val
                5                   10                  15

Arg Asp Leu Asn Ala Leu Leu Pro Ala Val Pro Ser Leu Gly Gly Gly
            20                  25                  30

Gly Gly Cys Ala Leu Pro Val Ser Gly Ala Ala Gln Trp Ala Pro Val
        35                  40                  45

Leu Asp Phe Ala Pro Pro Gly Ala Ser Ala Tyr Gly Ser Leu Gly Gly
    50                  55                  60

Pro His Ser Phe Ile Lys Gln Glu Pro Ser Trp Gly Gly Ala Glu Pro
65                  70                  75                  80
```

```
His Glu Glu Gln Cys Leu Ser Ala Phe Thr Val His Phe Ser Gly Gln
                85                  90                  95

Phe Thr Gly Thr Ala Gly Ala Cys Arg Tyr Gly Pro Phe Gly Pro Pro
            100                 105                 110

Pro Pro Ser Gln Ala Ser Ser Gly Gln Ala Arg Met Phe Pro Asn Ala
        115                 120                 125

Pro Tyr Leu Pro Ser Cys Leu Glu Ser Gln Pro Ala Ile Arg Asn Gln
    130                 135                 140

Gly Tyr Ser Thr Val Thr Phe Asp Gly Thr Pro Ser Tyr Gly His Thr
145                 150                 155                 160

Pro Ser His His Ala Ala Gln Phe Pro Asn His Ser Phe Lys His Glu
                165                 170                 175

Asp Pro Met Gly Gln Gln Gly Ser Leu Gly Glu Gln Gln Tyr Ser Val
            180                 185                 190

Pro Pro Pro Val Tyr Gly Cys His Thr Pro Thr Asp Ser Cys Thr Gly
        195                 200                 205

Ser Gln Ala Leu Leu Leu Arg Thr Pro Tyr Ser Ser Asp Asn Leu Tyr
    210                 215                 220

Gln Met Thr Ser Gln Leu Glu Cys Met Thr Trp Asn Gln Met Asn Leu
225                 230                 235                 240

Gly Ala Thr Leu Lys Gly His Ser Thr Gly Tyr Glu Ser Asp Asn His
                245                 250                 255

Thr Thr Pro Ile Leu Cys Gly Ala Gln Tyr Arg Ile His Thr His Gly
            260                 265                 270

Val Phe Arg Gly Ile Gln
        275
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<210> 484
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> PCR primer

<400> 484
ggctccgacg ttcgggacct gaacgcactg 30

<210> 485
<211> 31
<212> DNA
<213> Artificial Sequence

<220>

<223> PCR primer

<400> 485
ctgcagaatt catcactgaa tgcctctgaa g 31

<210> 486
<211> 27
<212> DNA
<213> Artificial Sequence

<220>
<223> Oligonucleotide used in construction of the TAT "Stumpy" vector.

<400> 486
tatgaacaat aacgatctgt ttcaggc 27

<210> 487
<211> 23
<212> DNA
<213> Artificial Sequence

<220>
<223> Oligonucleotide used in construction of the TAT "Stumpy" vector.

<400> 487
ctgaaacaga tcgttattgt tca 23

<210> 488
<211> 29
<212> DNA
<213> Artificial Sequence

<220>
<223> Oligonucleotide used in construction of the TAT "Stumpy" vector.

<400> 488
aattcttggt cattcacgtg gcggctcgc 29

<210> 489
<211> 23
<212> DNA
<213> Artificial Sequence

<220>
<223> Oligonucleotide used in construction of the TAT "Stumpy" vector.

<400> 489
gagccgccac gtgaatgacc aag 23

<210> 490
<211> 31
<212> DNA
<213> Artificial Sequence

35

<210> 505
<211> 816
<212> DNA
<213> Homo sapiens

<400> 505
atgggttcgg atgtacgcga tctgaacgcg cttcttccgg cggtcccaag cttgggcggg 60
ggaggtggat gcgccctgcc cgtgagcggc gccgcgcaat gggccccggt ccttgatttt 120
gctccgccgg gtgcgagcgc atacggctct ctgggcgggc ccgggcaaat tcattcattc 180
atcaaacagg agcccagctg gggtggcgcc gaacctcatg aagaacaatg tctgtctgct 240
tttaccgtac atttttcagg ccagtttacc ggaactgccg gtgcatgtcg ctatggccca 300
ttcggtccgc cgccaccgtc acaagcctct tcgggacaag cccgcatgtt tccgaatgca 360
ccctacctgc cgtcttgtct ggaaagccaa cccgcgatcc gtaaccaagg ctacagcacc 420
gtcacgtttg atggtacccc gagctatggt catacgccga gtcatcatgc agcacagttc 480
ccgaaccact cgttcaaaca cgaagatcct atgggccagc agggtagtct gggcgaacaa 540
cagtatagcg tcccaccacc agtttatggt tgccataccc caacggattc ctgtactggt 600
agccaagcgc tcctgctccg cacccccctat tcttctgata atctctatca gatgaccagc 660
caactggaat gtatgacgtg gaaccaaatg aacctgggcg caaccctgaa aggccacagt 720
accggctatg agtcagataa ccacacaaca cccattttgt gcggcgctca atatcgtatt 780
catacccatg gcgtttttcg cggtattcag tgatga 816

<210> 506
<211> 12
<212> PRT
<213> Homo sapiens

<400> 506
Met Asn Asn Asn Asp Leu Phe Gln Ala Ser Arg His
1 5 10