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(54) **Title:** PREPARATION OF POLYCLONAL CANCER VACCINE FOR PERSONALIZED IMMUNOTHERAPY

Figure 1

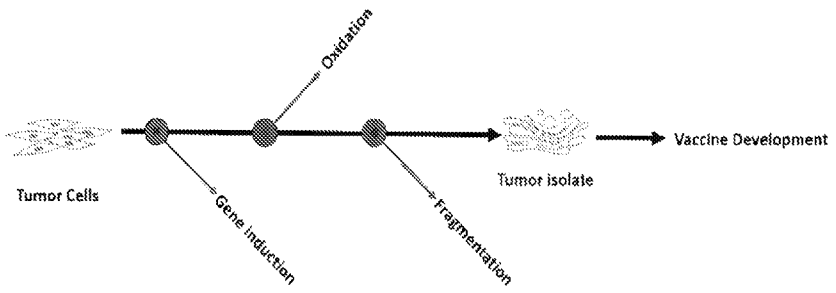

Figure 1. Steps involved in polyclonal Vaccine development

(57) **Abstract:** The present invention relates to immunotherapy of cancer. Specifically, the present invention relates to polyclonal vaccines and their method of preparation. The present invention further relates to a method of preparation of polyclonal vaccines, applicable against all leukemic and solid tumors. The present invention relates to a method of preparation of polyclonal vaccine, wherein the method comprises the following steps: a) purification and classification of tumor cells; b) induction of Gene expression; c) oxidation; and d) fragmentation.

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PREPARATION OF POLYCLONAL CANCER VACCINE FOR PERSONALIZED IMMUNOTHERAPY

FIELD OF THE INVENTION

[0001] The present invention relates to immunotherapy of cancer. Specifically, the present invention relates to polyclonal vaccines and their method of preparation. The present invention further relates to a method of preparation of polyclonal vaccines, applicable against all leukemic and solid tumors.

BACKGROUND OF THE INVENTION

[0002] Background description includes information that may be useful in understanding the present invention. It is not an admission that any of the information provided herein is prior art or relevant to the presently claimed invention, or that any publication specifically or implicitly referenced is prior art.

[0003] Cancer is a stepwise evolution of malignant cells with acquired failures to halt disease progression. The incidence of cancer continues to rise with 14.1 million new cases every year. Several studies in the last decades have identified somatic driver mutations leading to molecular classification of tumor types. Detection of relevant somatic mutations driving the growth and progression of tumors has led to development of small molecules and antibody therapies that has improved clinical outcome. However, a subset of patients with aggressive disease develop resistance to all existing therapies (radiotherapy, chemotherapy antibody therapy and small molecule therapy) leading to fatality. This could be due to the clonal evolution of tumors leading to resistance to therapy by accumulation of novel somatic driver mutations. Due to evolving lifestyle cancers of oral cavity and lungs in males and cervix and breast in females account for over 50% of all cancer deaths in India. Although there has been considerable advances in targeted cancer therapeutics in the last decade but it has not increased the overall survival in patients in India due to exemplary costs involved in treatment and follow up. For the past 2 years, immunotherapy has been in the spotlight with the advent of immune checkpoint inhibitors. This innovative shift in the way in which cancer is treated has transformed care for many patients. In fact, it is estimated that immune checkpoint inhibitors could save 250,000 years of life patients with advanced lung cancer for whom a checkpoint inhibitor could be prescribed.

Immune checkpoint inhibitors are medicines that release the brakes on the body's immune system, unleashing it to fight cancer. When the immune system is functioning normally, immune cells move around the body looking for things that do not belong, like bacteria and viruses. These immune cells search for invaders using "receptors," which can be thought of as antennae or feelers. When receptors find invaders in the body, special immune cells come in to destroy them. These special cells are called cytotoxic T cells. Unfortunately, cancer cells are often able to hide from immune cells, which is why the cancer cells can grow out of control. Immunotherapy is a cancer treatment intended to make the body's immune system able to detect and destroy cancer cells. There are three common approaches in the field of cancer immunotherapy currently being practiced i) use of Immune checkpoint inhibitors ii) CAR-T Therapy and iii) Dendritic Cell Therapy, each of which has several advantages and disadvantages.

[0004] Immune checkpoint inhibitors (ICI) have been a successful approach because it pushes the immune system into high gear to fight cancer. Immune checkpoint inhibitors (ICI) targeting CTLA-4 and the PD-1/PD-L1 axis have shown unprecedented clinical activity in several types of cancer and are rapidly transforming the practice of medical oncology. Whereas cytotoxic chemotherapy and small molecule inhibitors largely act on cancer cells directly, immune checkpoint inhibitors reinvigorate anti-cancer immune responses by disrupting co-inhibitory T-cell signaling. Immune checkpoint inhibitor therapy has been particularly successful in melanoma, on-small cell lung cancer, renal cell carcinoma, bladder cancer, head and neck squamous cell carcinoma, MSI-high colorectal carcinoma, Merkel cell carcinoma, and Hodgkin lymphoma, for which approved treatments now include anti-PD-1 (nivolumab and pembrolizumab), anti-CTLA-4 (ipilimumab), and combination anti-PD-1/CTLA-4 regimens (nivolumab–ipilimumab). The major drawback associated with ICI is the routine development resistance. Some patients do not respond to the treatment and succumb at a rate consistent with the natural history of disease. In addition, late relapses are now emerging with longer follow-up of clinical trial populations, suggesting the emergence of acquired resistance. As robust biomarkers to predict clinical response and/or resistance remain elusive, the mechanisms underlying innate (primary) and acquired (secondary) resistance are largely inferred from pre-clinical studies and correlative clinical data. Improved understanding of molecular and immunologic mechanisms of ICI response (and resistance) may not only identify novel

predictive and/or prognostic biomarkers, but also ultimately guide optimal combination/sequencing of ICI therapy in the clinic.

[0005] Another approach is CAR T-cell therapy, wherein a patient's T cells are removed and taken to a laboratory. The T cells are genetically changed so they will attack cancer cells. These CAR T cells are grown in large numbers and then injected into the patient. One of the remarkable things about this treatment is that it is a "living therapy." CAR T cells typically have to be injected only once, because they go on to multiply in the body. CAR T cells continue fighting the cancer in the patient's body, and their effectiveness may even grow over time. However, the major disadvantages of this approach are i) CAR T cells target a unique antigen on tumor cells, thereby leading to clonal selection of tumor cells. ii) This approach is only available for blood cancers but not for solid tumors. iii) The costs involved in generating such cells might be heavy burden on the patient.

[0006] Dendritic Cell therapy or DC therapy is a immunotherapeutic approach involving dendritic cells aim to capitalize on the ability of the cells to direct cytotoxic T lymphocytes and natural killer cells to become potent antitumor effectors capable of eradicating malignant cell. This is commonly known as Dendritic Cell (DC) therapy. DCs are key mediators of tumor immunity owing to their unique capacity for cross-presenting self-tumor antigens to CD8 + T cells. Briefly, the immature dendritic cells are isolated from patient's peripheral blood and grown under laboratory conditions. In parallel the patients cancer cells are isolated and sequenced to identify and rank neoantigens specific to the tumor. Peptides of the highest-ranking neoantigens are then synthesized and then pulsed with the autologous immature dendritic cells. Then the dendritic cells are allowed to mature, grown in the laboratory in millions, and injected into the patients to activate the CD8 + T cells against tumor. Sipuleucel-T was the first DC-based antitumor vaccine to be approved by the FDA for use against asymptomatic or minimally symptomatic castration-resistant prostate cancer using a single PA2024 antigen. However, the clinical trial failed to show promising results because the tumor cells could evade the anti-immune response due to a weak use of single antigen. The major disadvantages of this approach are i) Dependency of patients' immune cells to generate the vaccine ii) Vaccine only target limited tumor antigens iii) The methods involved are expensive and pose a financial burden in the patients.

[0007] The current immunotherapy approaches extensively relies on targeting tumor cells representing 1-3 tumor antigens. This approach has high risk of developing resistance and leading to failure of the treatment due to considerable heterogeneity in the population. For example, a vaccine developed for individual X against tumor A might not work for individual Y with the same tumor A.

[0008] There is, therefore, a need to develop anti-cancer therapies that can overcome deficiencies associated with the known arts. The present invention tries to overcome the problems by using patient's tumor cells to generate a polyclonal vaccine that will target the tumor against multiple tumor antigens.

OBJECTS OF THE INVENTION

[0009] An object of the present invention is to provide anti-cancer polyclonal vaccine that generally overcomes the deficiencies found in the prior art.

[0010] Another object of the present invention is to provide anti-cancer polyclonal vaccine that will target the tumor against multiple tumor antigens.

[0011] Another object of the present invention is to provide polyclonal vaccine that is prepared using patient's tumor cells, to target the tumor against multiple tumor antigens.

[0012] Another object of the present invention is to provide anti-cancer polyclonal vaccine that leads to recognition of multiple antigens of cancer cells in the body by its own immune cells.

[0013] Another object of the present invention is to provide anti-cancer polyclonal vaccine that leads to effective eradication of tumor cells.

[0014] Another object of the present invention is to provide a method of preparation of anti-cancer polyclonal vaccine.

SUMMARY OF THE INVENTION

[0015] This summary is provided to introduce a selection of concepts in a simplified form that are further described below in Detailed Description section. This summary is not intended to identify key features or essential features of the claimed subject matter, nor is it intended to be used as an aid in determining the scope of the claimed subject matter.

[0016] The present invention relates to immunotherapy of cancer. Specifically, the present invention relates to polyclonal vaccines and their method of preparation.

[0017] In one aspect, the present invention relates to anti-cancer polyclonal vaccine that generally overcomes the deficiencies found in the prior art.

[0018] In another aspect, the present invention relates to anti-cancer polyclonal vaccine that will target the tumor against multiple tumor antigens.

[0019] In yet another aspect, the present invention provides polyclonal vaccine that is prepared using patient's tumor cells, to target the tumor against multiple tumor antigens.

[0020] In another aspect, the present invention relates toanti-cancer polyclonal vaccine that leads to recognition of multiple antigens of cancer cells in the body by its own immune cells.

[0021] In another aspect, the present invention relates to anti-cancer polyclonal vaccine that leads to effective eradication of tumor cells.

[0022] In another aspect, the present invention relates to a method of preparation of polyclonal vaccines, applicable against all leukemic and solid tumors.

[0023] In another aspect, the present invention relates to a method of preparation ofpolyclonal vaccine, wherein the method comprises the following steps:

a) purification and classificationof tumor cells;
b) induction of Gene expression;
c) oxidation; and
d) fragmentation.

[0024] In another aspect, the present invention relates to a method of preparation ofpolyclonal vaccine, wherein the method comprises the following steps:

a) processing a fresh tumor tissueto a pure tumor cell population, classifying the tumor cell and growing it at standard laboratory conditions;
b) treating the pure population of cells with DNA modifying drugs, optionally in combination with xCT inhibitors, for 24 hours in culture;
c) oxidizing the resulting cells with specialized reagents;
d) subjecting the tumor cells to multiple freeze thaw cycles; and
e) diluting the resulting cell isolate in sterile physiological saline, quantifying and storing at -120 ºC in aliquots for vaccine development.

[0025] In another aspect, the present invention relates to a method of preparation ofpolyclonal vaccine, wherein the method comprises the following steps:

a) Transferring a fresh tumor specimen, representative of different metastatic sites from oncology clinic to the laboratory under sterile conditions;

b) Dissociating the tumor tissue to a single cell suspension using enzymatic process;

c) Quantifying the purity of the tumor cell population using tumor markers;

d) Classification of tumor cells based on the expression of the gene xCT (SLC7A11, Gene ID: 23657) as xCT positive and xCT negative cells;

e) Culturing one million purified tumor cells in enriched cell culture medium for 24 hours;

f) Treating the cells with DNA modifying drugs wherein cells are xCT negative cells and treating the cells with xCT inhibitors in combination with DNA modifying drugs wherein cells are xCT positive cells;

g) Exposing the cells to oxidant mixture for additional 12 hours;

h) Washing the cells in sterile solution and freezing the cell pellet in liquid nitrogen for 30 minutes;

i) Thawing the cells at room temperature for additional 30 minutes;

j) Repeating the thawing process for 10 cycles to effectively fragment the tumor cells into smaller proteins and peptides; and

k) Diluting the fragmented tumor cell isolate in physiological saline and freezing at -120 ℃ in multiple aliquots for further vaccine development for personalized immunotherapy.

[0026] In an embodiment, the size of fresh tumor specimen is of 0.1 to 5 cubic mm.

[0027] In a preferred embodiment, the size of fresh tumor specimen is of 1 cubic mm.

[0028] In an embodiment, the tumor tissue is dissociated to a single cell suspension using a commercial tumor cell isolation kit.

[0029] In another embodiment, the purity of the tumor cell population is quantified by previously established methods.

[0030] In another embodiment, the purity of the tumor cell population is quantified by flow cytometry.

[0031] In still another embodiment, the DNA modifying drugs are FDA approved.

[0032] In yet another embodiment, DNA modifying drugs can be selected from but not limited to histone deacetlyase inhibitors, DNA methylation inhibitors or platinum based drugs. The choice and concentrations of these drug combinations will be based on the therapeutic regimen the patient is undergoing in the clinic.

[0033] In yet another embodiment, histone deacetlyase inhibitors can be selected from but not limited to Vorinostat, Romidepsin, Belinostat, Panobinostat, Entinostat and the like.

[0034] In yet another embodiment, DNA methylation inhibitor can be selected from 5 AZA-CdR and the like.

[0035] In yet another embodiment, platinum based drugs can be selected from but not limited to Oxaliplatin, Cisplatin, Carboplatin and the like.

[0036] In another embodiment, the xCT inhibitors can be selected from but not limited to Sulfasalazine Sorafenib, Erastin and the like. xCT inhibitors will be only used in tumor cells having higher xCT expression to sensitize cells for subsequent oxidation step.

[0037] In another embodiment, the cells can be exposed to oxidant mixture like hydrogen peroxide (H2O2) and hypochlorus acid (HOCl).

[0038] In a preferred embodiment, the concentration range for hydrogen peroxide is 10-100 μM and concentration range for Hypochlorus acid is 10-100 μM.

[0039] In another aspect, the present invention relates to polyclonal vaccines prepared by the above method and applicable against all leukemic and solid tumors.

[0040] Other aspects of the invention will be set forth in the description which follows, and in part will be apparent from the description, or may be learnt by the practice of the invention.

BRIEF DESCRIPTION OF DRAWINGS THE INVENTION

[0041] The following drawings form part of the present specification and are included to further illustrate aspects of the present disclosure. The disclosure may be better understood by reference to the drawings in combination with the detailed description of the specific embodiments presented herein.

[0042] Figure 1: Schematic overview of steps involved for Polyclonal vaccine preparation.

[0043] Figure 2: Illustrates the xCT expression in eleven different tumor cell lines with MeWo classified as xCT positive cells.

[0044] Figure 3: (A) Coomassie staining of protein lysates derived from eight treatment conditions. (B) Quantification of coomassie signals normalized to the control indicate sample 4 and 8 have highest protein load.

[0045] Figure 4: (A) Co-culture of mDCs (red) pulsed with sample 1, 4 and 8 with MeWo tumor cells (gray). iDCs used as negative control. (B) Quantification of tumor cells (gray) from indicated time points show sample 8+mDCs inhibit tumor growth.

DETAILED DESCRIPTION

[0046] The following is a detailed description of embodiments of the disclosure. The embodiments are in such detail as to clearly communicate the disclosure. However, the amount of detail offered is not intended to limit the anticipated variations of embodiments; on the contrary, the intention is to cover all modifications, equivalents, and alternatives falling within the spirit and scope of the present disclosure as defined by the appended claims.

[0047] All publications herein are incorporated by reference to the same extent as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference. Where a definition or use of a term in an incorporated reference is inconsistent or contrary to the definition of that term provided herein, the definition of that term provided herein applies and the definition of that term in the reference does not apply.

[0048] Reference throughout this specification to "one embodiment" or "an embodiment" means that a particular feature, structure or characteristic described in connection with the embodiment is included in at least one embodiment. Thus, the appearances of the phrases "in one embodiment" or "in an embodiment" in various places throughout this specification are not necessarily all referring to the same embodiment. Furthermore, the particular features, structures, or characteristics may be combined in any suitable manner in one or more embodiments.

[0049] In some embodiments, the numbers expressing quantities of ingredients, properties such as concentration, reaction conditions, and so forth, used to describe and claim certain embodiments of the invention are to be understood as being modified in some instances by the term "about."Accordingly, in some embodiments, the numerical parameters set forth in the written description and attached claims are approximations that can vary depending upon the desired properties sought to be obtained by a particular embodiment.In some embodiments, the numerical parameters should be construed in light of the number of reported significant digits and by applying ordinary rounding techniques.Notwithstanding that the numerical ranges and parameters setting forth the broad scope of some embodiments of the invention are approximations, the numerical values set forth in the specific examples are reported as precisely

as practicable.The numerical values presented in some embodiments of the invention may contain certain errors necessarily resulting from the standard deviation found in their respective testing measurements.

[0050] As used in the description herein and throughout the claims that follow, the meaning of "a," "an," and "the" includes plural reference unless the context clearly dictates otherwise.Also, as used in the description herein, the meaning of "in" includes "in" and "on" unless the context clearly dictates otherwise.

[0051] Unless the context requires otherwise, throughout the specification which follow, the word "comprise" and variations thereof, such as, "comprises" and "comprising" are to be construed in an open, inclusive sense that is as "including, but not limited to."

[0052] The recitation of ranges of values herein is merely intended to serve as a shorthand method of referring individually to each separate value falling within the range.Unless otherwise indicated herein, each individual value is incorporated into the specification as if it were individually recited herein.All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context.The use of any and all examples, or exemplary language (e.g. "such as") provided with respect to certain embodiments herein is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention otherwise claimed.No language in the specification should be construed as indicating any non-claimed element essential to the practice of the invention.

[0053] Groupings of alternative elements or embodiments of the invention disclosed herein are not to be construed as limitations.Each group member can be referred to and claimed individually or in any combination with other members of the group or other elements found herein.One or more members of a group can be included in, or deleted from, a group for reasons of convenience and/or patentability.When any such inclusion or deletion occurs, the specification is herein deemed to contain the group as modified thus fulfilling the writtendescription of all Markush groups used in the appended claims.

[0054] The description that follows, and the embodiments described therein, is provided by way of illustration of an example, or examples, of particular embodiments of the principles and aspects of the present disclosure. These examples are provided for the purposes of explanation, and not of limitation, of those principles and of the disclosure.

[0055] It should also be appreciated that the present disclosure can be implementedin numerous ways, including as a system, a method or a device. In this specification, these implementations, or any other form that the invention may take, may be referred to as processes. In general, the order of the steps of the disclosed processes may be altered within the scope of the invention.

[0056] The headings and abstract of the invention provided herein are for convenience only and do not interpret the scope or meaning of the embodiments.

[0057] The following discussion provides many example embodiments of the inventive subject matter. Although each embodiment represents a single combination of inventive elements, the inventive subject matter is considered to include all possible combinations of the disclosed elements. Thus if one embodiment comprises elements A, B, and C, and a second embodiment comprises elements B and D, then the inventive subject matter is also considered to include other remaining combinations of A, B, C, or D, even if not explicitly disclosed.

[0058] Various terms as used herein are shown below. To the extent a term used in a claim is not defined below, it should be given the broadest definition persons in the pertinent art have given that term as reflected in printed publications and issued patents at the time of filing..

[0059] The term "vaccine" refers to an immunogenic composition for administration to a mammal (such as human) for eliciting a protective immune response against a particular antigen or antigens. The primary active ingredient of a vaccine is the immunogen(s).

[0060] The term "treating", "treatment" or "treat" refers to abrogating a disorder, reducing the severity of a disorder, or reducing the severity or occurrence frequency of a symptom of a disorder.

[0061] The term "immunotherapy" refers to a biological therapy for cancer treatment that helps person's own immune system fight cancer. The immune system, which is made up of white blood cells and organs and tissues of the lymph system, helps the body fight infections and other diseases.

[0062] The term "polyclonal" refers to a collection of different cell lineages within the body.

[0063] The term "tumor" refers toswelling or morbid enlargement thatresults from an overabundance of cell growth and division. Normally, the cells grow and divide to produce new cells in a controlled and orderly manner. Tumor may not be the same as a cancer, although some can develop into cancers.

[0064] The term "antigen" refers toa toxin or other foreign substance which induces an immune response in the body, especially the production of antibodies.

[0065] The present invention relates to immunotherapy of cancer. Specifically, the present invention relates to polyclonal vaccines and their method of preparation.

[0066] In an embodiment, the present invention relates to anti-cancer polyclonal vaccine that generally overcomes the deficiencies found in the prior art.

[0067] In anotherembodiment, the present invention relates to anti-cancer polyclonal vaccine that will target the tumor against multiple tumor antigens.

[0068] In yet another embodiment, the present invention provides polyclonal vaccine that is prepared using patient's tumor cells, to target the tumor against multiple tumor antigens.

[0069] In another embodiment, the present invention relates toanti-cancer polyclonal vaccine that leads to recognition of multiple antigens of cancer cells in the body by its own immune cells.

[0070] In another embodiment, the present invention relates to anti-cancer polyclonal vaccine that leads to effective eradication of tumor cells.

[0071] In another embodiment, the present invention relates to a method of preparation of polyclonal vaccines, applicable against all leukemic and solid tumors.

[0072] In another embodiment, the present invention relates to a method of preparation ofpolyclonal vaccine, wherein the method comprises the following steps:

a) purification and classification of tumor cells;
b) induction of Gene expression;
c) oxidation; and
d) fragmentation.

[0073] In another embodiment, the present invention relates to a systematic method to generate polyclonal tumor vaccines as shown in **Figure 1.**

[0074] In another embodiment, the present invention relates to a method of preparation ofpolyclonal vaccine, wherein the method comprises the following steps:

a) processing a fresh tumor tissueto a pure tumor cell population, classifying the tumor cell and growing it at standard laboratory conditions;
b) treating the pure population of cells with DNA modifying drugs, optionally in combination with xCT inhibitors, for 24 hours in culture;
c) oxidizing the resulting cells with specialized reagents;

d) subjecting the tumor cells to multiple freeze thaw cycles; and

e) diluting the resulting cell isolate in sterile physiological saline, quantifying and storing at -120 ºC in aliquots for vaccine development.

[0075] In another embodiment, the present invention relates to a method of preparation ofpolyclonal vaccine, wherein the method comprises the following steps:

a) Transferring a fresh tumor specimen, representative of different metastatic sites from oncology clinic to the laboratory under sterile conditions;

b) Dissociating the tumor tissue to a single cell suspension using enzymatic process;

c) Quantifying the purity of the tumor cell population using tumor markers;

d) Classification of tumor cells based on the expression of the gene xCT (SLC7A11, Gene ID: 23657) as xCT positive and xCT negative cells;

e) Culturing one million purified tumor cells in enriched cell culture medium for 24 hours;

f) Treating the cells with DNA modifying drugs wherein cells are xCT negative cells and treating the cells with xCT inhibitors in combination with DNA modifying drugs wherein cells are xCT positive cells;

g) Exposing the cells to oxidant mixture for additional 12 hours;

h) Washing the cells in sterile solution and freezing the cell pellet in liquid nitrogen for 30 minutes;

i) Thawing the cells at room temperature for additional 30 minutes;

j) Repeating the thawing process for 10 cycles to effectively fragment the tumor cells into smaller proteins and peptides; and

k) Diluting the fragmented tumor cell isolate in physiological saline and freezing at -120 ºC in multiple aliquots for further vaccine development for personalized immunotherapy.

[0076] In an embodiment, the size of fresh tumor specimen is of 0.1 to 5 cubic mm.

[0077] In a preferred embodiment, the size of fresh tumor specimen is of 1 cubic mm.

[0078] In an embodiment, the tumor tissue is dissociated to a single cell suspension using a commercial tumor cell isolation kit.

[0079] In another embodiment, the purity of the tumor cell population is quantified by previously established methods.

[0080] In another embodiment, the purity of the tumor cell population is quantified by flow cytometry.

[0081] In yet another embodiment, DNA modifying drugs are the drugs approved by FDA.

[0082] In yet another embodiment, FDA approved DNA modifying drugs can be selected from but not limited to histone deacetlyase inhibitors, DNA methylation inhibitors or platinum based drugs. The choice and concentrations of these drug combinations will be based on the therapeutic regimen the patient is undergoing in the clinic.

[0083] In yet another embodiment, histone deacetlyase inhibitors can be selected from but not limited to Vorinostat, Romidepsin, Belinostat, Panobinostat, Entinostat and the like.

[0084] In yet another embodiment, DNA methylation inhibitor can be 5 AZA-CdR and the like.

[0085] In yet another embodiment, platinum based drugs can be selected from but not limited to Oxaliplatin, Cisplatin, Carboplatin and the like.

[0086] In another embodiment, the xCT inhibitors can be selected from but not limited to Sulfasalazine Sorafenib, Erastin and the like. xCT inhibitors will be only used in tumor cells having higher xCT expression to sensitize cells for subsequent oxidation step.

[0087] In another embodiment, the cells can be exposed to oxidant mixture like hydrogen peroxide (H_2O_2) and hypochlorus acid (HOCl).

[0088] In a preferred embodiment, the concentration range for hydrogen peroxide is 10-100 μM and concentration range for Hypochlorus acid is 10-100 μM.

[0089] Sub toxic concentrations of oxidants are known to enhance gene expression patterns and immunogenic cell death in multiple tumor cells.The induction of gene expression is greatly amplified upon incubating cells with oxidants. The oxidation stepis performed to enhance the antigenic/immunogenic profile of the tumor cells, which has been previously suppressed in the patient's body.

[0090] In another embodiment, repeated freeze thaw cycle is process of isolating and fragmenting proteins and other biological molecules in the most efficient way. The immunogenic proteins derived from the tumor cells will be fragmented into a high-grade antigen mixture that can be used in as vaccine in DC therapy.

[0091] In another embodiment, the present invention relates to polyclonal vaccines prepared by the above method and applicable against all leukemic and solid tumors.

[0092] While the foregoing describes various embodiments of the disclosure, other and further embodiments of the disclosure may be devised without departing from the basic scope thereof. The scope of the invention is determined by the claims that follow. The invention is not limited to the described embodiments, versions or examples, which are included to enable a person having ordinary skill in the art to make and use the invention when combined with information and knowledge available to the person having ordinary skill in the art.

[0093] The present invention is further explained in the form of following examples. However, it is to be understood that the followingexamples are merely illustrative and are not to be taken as limitations upon the scope of the invention.

[0094] **Example 1:Generation of tumor cell lysates**

[0095] Tumor lysates were prepared as described below. These are used as a polyclonal vaccine starting froma pure commercially available population of a melanoma tumor cell line MeWo(ATCC) as a model. Hence, this method does not require steps for isolation and quantification of purity of the tumor cells. However, if the tumor is obtained from a cancer patient from the clinic then pure population of tumor cells will be obtained using commercially available Tumor Cell Isolation kit (MiltenyiBiotec) according to manufacturer's instructions.

[0096] The lysates were prepared using different conditions and the optimal condition for high yield and high antigenicity was identified. The FDA approved drug Voronistat (VOR) was used as DNA modifying agent, hydrogen peroxide (H2O2) as an oxidizing agent, and 5 cycles of freeze thaw (FT) in liquid nitrogen for fragmentation. The cell lysates were generated from eight different conditions to identify the best condition resulting in higher protein yield.

1. Melanoma tumor cell line MeWo was grown in DMEM (Dulbecco's Minimal Essential Media; Sigma Aldrich) media supplemented with 10% FCS (Fetal Calf Serum; Gibco) and 1% penicillin/streptomycin (Gibco) solution.
2. The expression of xCT in MeWo cells was quantified by immunoblotting as follows. 1x106 cells was lysed in RIPA buffer (Radioimmunoprecipitation assay buffer; Thermo Scientific). Protein concentrations was estimated by bradford assay (Carl Roth) and 50 micrograms of protein was run in SDS gel electrophoresis and transferred to PVDF membrane using commercial kit (Thermo Scientific). The PVDF membrane was incubated with primary anti-xCT antibody and anti-beta actin antibody (Cell signaling) followed by HRP conjugated secondary antibody. Chemiluminescence assay (Thermo

Scientific) was used to detect the levels of xCT and beta actin (loading control) protein according to manufacturer's instructions. Figure 2 shows the xCT expression in eleven different tumor cell lines with MeWo classified as xCT positive cells.

3. MeWo cells were then treated with 500 µM of xCT inhibitor sulfasalazine (Santa Cruz).
4. MeWo cells were then divided into eight treatment conditions($1x10^6$ /condition) as follows :
    1. Control
    2. Freeze Thaw (FT)
    3. 100 µM H2O2
    4. 100 µM H2O2 + FT
    5. 10 µM VOR
    6. 10 µM VOR + FT
    7. 100 µM H2O2 +10 µM VOR
    8. 100 µM H2O2 +10 µM VOR + FT
5. Cells from conditions 5, 6 and 8 were incubated with the Voronistat for 24 hours to induce gene expression.
6. Cells were then Isolated from all conditions by using trypsin-EDTA (0.05%) and washed in PBS (Phosphate Buffered Saline) once and placed on ice.
7. The cell pellet from conditions 3, 4, 7 and 8 were then treated with 100 µM of H_2O_2 in a final volume of 100 µl for 30 minutes on ice.
8. After 30 minutes, cells were centrifuged,H_2O_2 was removed and the cell pellets were washed in PBS once. The cell pellets were kept on ice.
9. The cell pellet from conditions 2, 4, 6 and 8 were then subjected to snap freeze in liquid nitrogen for 20 minutes and gradual thaw to room temperature. This process was repeated for 5 cycles.
10. Finally, to the resulting tumor cell lysates from all 8 samples, 500 µl of PBS was added. The protein concentration was estimated by Bradford method. 30microgram of protein from samples were run in SDS gel electrophoresis, stained with coomassie blue, and imaged using a gel trans-illuminator. Representative images (**Figure 3A-B**) show samples 4 and 8 having the highest protein content normalized to the control.
11. The remaining tumor lysates were stored in -120°C for further vaccine development.

[0097] Example 2: Determination of anti-tumor response of the tumor lysates in vitro.

[0098] Samples 4 (100μM H2O2 + FT) and 8 (100μM H2O2 +10μM VOR + FT) were identified in Example 1 as having the highest but similar protein load compared to the control. However, it does not identify which of these samples is the best candidate for polyclonal vaccine. To determine this afunctional assay using immature dendritic cells (iDCs) derived from human monocyte cell line THP1 was performed. The iDCs were pulsed with the sample lysates from 4 and 8 for 24 hours. The iDCs will take the polyclonal antigens from the lysates and process them to become tumor specific mature dendritic cells (mDCs). The mDCs then easily home into the target autologous tumor cell (MeWo) based on sample lysate it has been pulsed leading to antitumor effects.

1. THP1 monocytes purchased from cell repository ATCC, were grown in RPMI 1640 (Roswell Park Memorial Institute 1640; Thermo Scientific) media with 10% FCS (Fetal Calf serum; Gibco) and 1% penicillin/streptomycin (Thermo Scientific).
2. $2x10^6$ THP1 monocytes were incubated with rhIL-4 (Interleukin-4) and rhGMCSF (human Granulocyte Macrophage Colony Stimulating Factor) for five days to induce differentiation to iDCs.
3. After 5 days, the iDCs were isolated and split in to three wells with $1x10^6$ cells/well. 100 μl of the lysates from samples 1, 4 and 8 were mixed to these cells and incubated for another 24 hours.
4. After 24 hours, the media was changed and the cells were grown for another 48 hours for maturation leading to mDCs.
5. The mDCs was then isolated and washed with Cell Trace Red™ dye, which labels cells red for fluorescent microscopy.
6. mDCs (Red) and MeWo tumor cell line (gray) were co-cultured and time-lapse microscopy was initiated to determine whether the mDCs inhibit the growth of the tumor cell line MeWo. iDCs that were not treated with any lysate was used as a control for specificity.
7. Quantification of the results from the 72-hour time-lapse microscopy revealed that the sample 8 had significant effect inhibiting tumor growth when compared to sample 4. The

iDCs that were not treated with any lysate did not inhibit the growth of the tumor is used as the control (**Figure 4 A-B**).

[0099] The above data confirmsthe activation of gene expression, oxidation and fragmentation that corresponds to sample 4.

The foregoing examples are merely illustrative and are not to be taken as limitations upon the scope of the invention. Various changes and modifications to the disclosed embodiments will be apparent to those skilled in the art. Such changes and modifications may be made without departing from the scope of the invention.

ADVANTAGES OF THE PRESENT INVENTION

[00100] The present invention provides anti-cancer polyclonal vaccine that generally overcomes the deficiencies found in the prior art.

[00101] The present invention provides a method of preparation of anti-cancer polyclonal vaccine that generally overcomes the deficiencies found in the prior art.

[00102] The present invention provides anti-cancer polyclonal vaccine that will target the tumor against multiple tumor antigens.

[00103] The present invention provides anti-cancer polyclonal vaccine that leads to recognition of multiple antigens of cancer cells in the body by its own immune cells.

[00104] The present invention provides anti-cancer polyclonal vaccine that leads to effective eradication of tumor cells.

I Claim:

1. A method of preparation of anti-cancer polyclonal vaccine, wherein the method comprises the following steps:
   a) purification and classification of tumor cells;
   b) induction of Gene expression;
   c) oxidation; and
   d) fragmentation.

2. The method as claimed in claim 1, wherein the step of purification of tumor cells comprises processing a fresh tumor tissue to a pure tumor cell population.

3. The method as claimed in claim 1, wherein the step of classification of tumor cells comprises classifying the tumor cell and growing it at standard laboratory conditions.

4. The method as claimed in claim 1, wherein the step of induction of Gene expression comprises treating the pure population of cells with DNA modifying drugs, optionally in combination with xCT inhibitors, for 24 hours in culture.

5. The method as claimed in claim 1, wherein the step of oxidation comprises oxidizing the resulting cells with specialized reagents.

6. The method as claimed in claim 1, wherein the step of fragmentation comprises subjecting the tumor cells to multiple freeze thaw cycles and diluting the resulting cell isolate in sterile physiological saline, quantifying and storing at -120 ℃ in aliquots for vaccine development.

7. A method of preparation of polyclonal vaccine, wherein the method comprises the following steps:
   a) Transferring a fresh tumor specimen, representative of different metastatic sites from oncology clinic to the laboratory under sterile conditions;

b) Dissociating the tumor tissue to a single cell suspension using enzymatic process;
c) Quantifying the purity of the tumor cell population using tumor markers;
d) Classification of tumor cells based on the expression of the gene xCT as xCT positive and xCT negative cells;
e) Culturing one million purified tumor cells in enriched cell culture medium for 24 hours;
f) Treating the cells with DNA modifying drugs wherein cells are xCT negative cells and treating the cells with xCT inhibitors in combination with DNA modifying drugs wherein cells are xCT positive cells;
g) Exposing the cells to oxidant mixture for additional 12 hours;
h) Washing the cells in sterile solution and freezing the cell pellet in liquid nitrogen for 30 minutes;
i) Thawing the cells at room temperature for additional 30 minutes;
j) Repeating the thawing process for 10 cycles to effectively fragment the tumor cells into smaller proteins and peptides; and
k) Diluting the fragmented tumor cell isolate in physiological saline and freezing at -120°C in multiple aliquots for further vaccine development for personalized immunotherapy.

8. The method as claimed in claim 7, wherein the size of fresh tumor specimen is 0.1 to 5 cubic mm, preferably of 1 cubic mm.

9. The method as claimed in claim 7, wherein the tumor tissue is dissociated to a single cell suspension using a commercial tumor cell isolation kit and the purity of the tumor cell population is quantified by flow cytometry.

10. The method as claimed in claim 7, wherein the DNA modifying drugs are FDA approved and are selected from histone deacetlyase inhibitors, DNA methylation inhibitors or platinum based drugs and the xCT inhibitors can be selected from Sulfasalazine Sorafenib or Erastin.

11. The method as claimed in claim 10, wherein histone deacetlyase inhibitors can be selected from Vorinostat, Romidepsin, Belinostat, Panobinostat or Entinostat.

12. The method as claimed in claim 10, wherein DNA methylation inhibitor is 5 AZA-CdR.

13. The method as claimed in claim 10, wherein, platinum based drugs can be selected from Oxaliplatin, Cisplatin or Carboplatin.

14. The method as claimed in claim 7, wherein, the cells can be exposed to oxidant mixture like hydrogen peroxide (H_2O_2) and hypochlorus acid (HOCl), wherein the concentration range for hydrogen peroxide is 10-100 μM and concentration range for Hypochlorus acid is 10-100 μM.

15. An anti-cancer polyclonal vaccine prepared by the method as claimed in claims 1-14, applicable against all leukemic and solid tumors and having the capability to target the tumor against multiple tumor antigens.

Figure 1

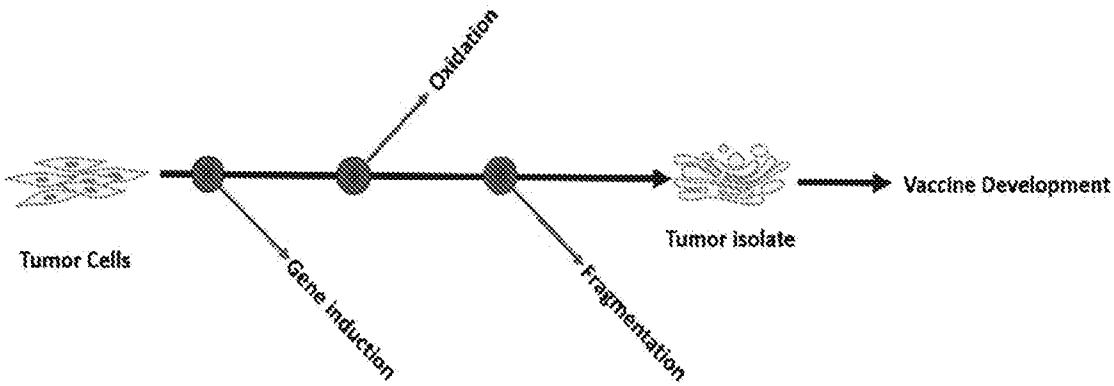


Figure 1. Steps involved in polyclonal Vaccine development

Figure 2

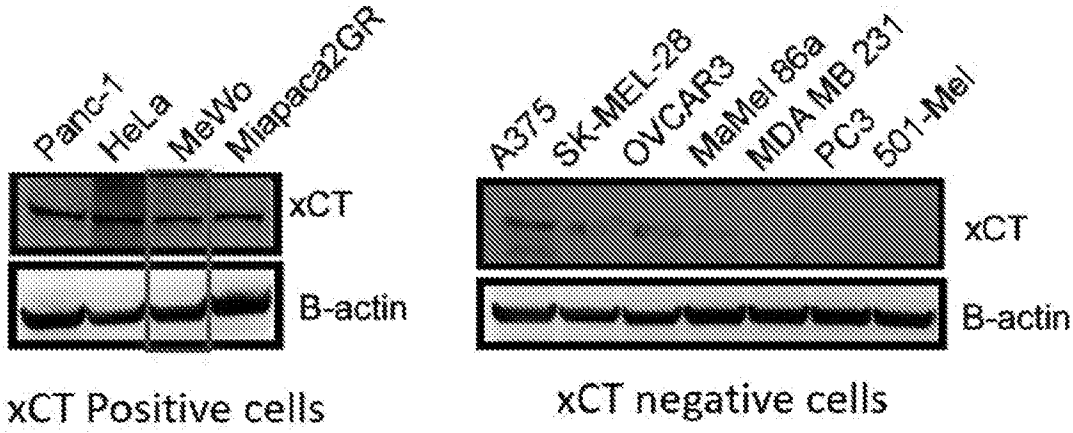


Figure 3

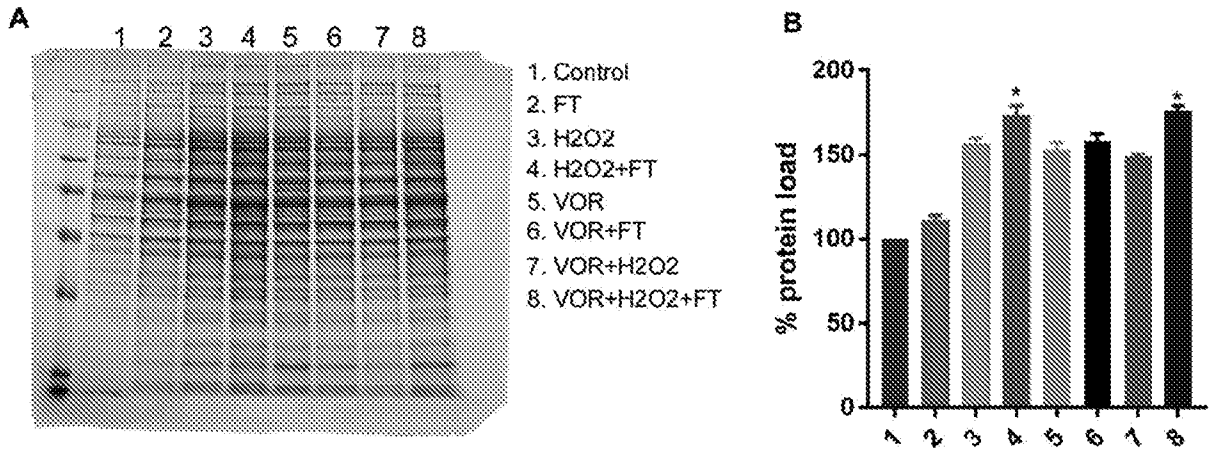
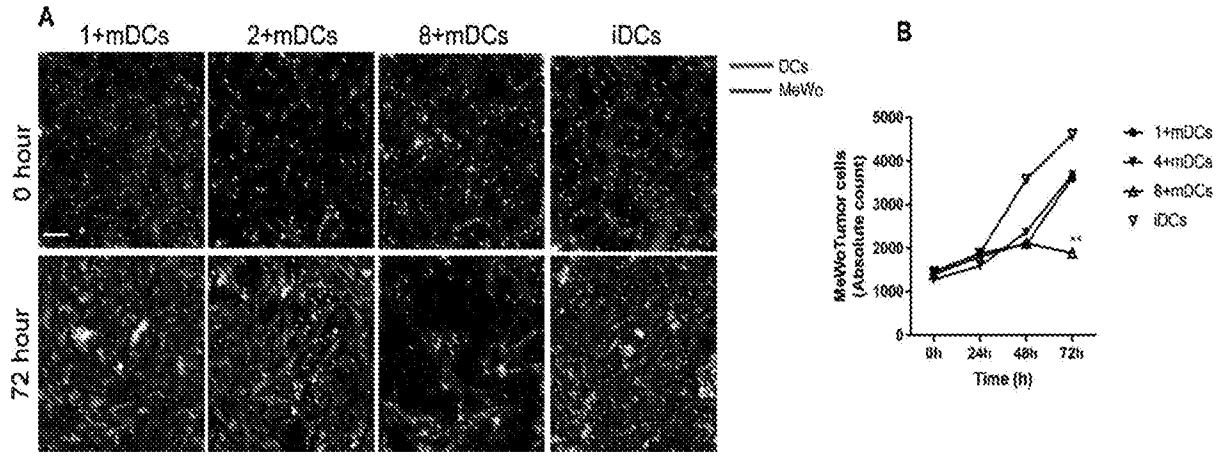


Figure 4



INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB2019/058411

A. CLASSIFICATION OF SUBJECT MATTER
A61K39/00,C07K14/435,C07K14/705 Version=2019.01

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K, C07K

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

TotalPatent One, IPO Internal Database

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO2014102220A1 (AMPHERA B.V) 03 JULY 2014 (03-07-2014) Abstract, claims 12 and 38	15
Y	Whole document	1-14
Y	Chiang et al.: Whole Tumor Antigen Vaccines: Where Are We, Vaccines 2015, 3(2), 344-372. Pages 344-349, Figure 1	1-14
Y	Nava et al.: An Optimized Method for Manufacturing a Clinical Scale Dendritic Cell-Based Vaccine for the Treatment of Glioblastoma; December 20, 2012; Plos One, volume 7, Issue 12, e52301. Figure S2	1-15
Y	Lewerenz et al.: The Cystine/Glutamate Antiporter System xct in Health and Disease: From Molecular Mechanisms to Novel Therapeutic Opportunities, 2013, Antioxidants & Redox Signaling, Vol. 18, No. 5, 522-555. Whole document	7-15

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

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
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Date of the actual completion of the international search	Date of mailing of the international search report
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INTERNATIONAL SEARCH REPORT
Information on patent family members

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Citation	Pub.Date	Family	Pub.Date
WO 2014102220 A1	03-07-2014	EP 2938354 A1	04-11-2015
		CA 2896994 A1	03-07-2014
		KR 20150134314 A	01-12-2015
		US 9962433 B2	08-05-2018
		CN 105050617 A	11-11-2015