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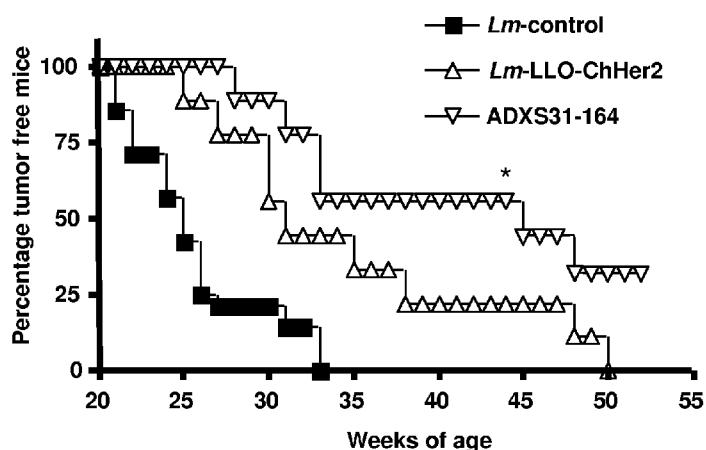


Figure 3

(57) Abstract: This invention provides compositions and methods for treating and vaccinating against an Her2/neu antigen-expressing tumor and inducing an immune response against dominant and several sub-dominant epitopes of the antigen.

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**COMPOSITIONS AND METHODS FOR PREVENTION OF ESCAPE MUTATION
IN THE TREATMENT OF HER2/NEU OVER-EXPRESSING TUMORS**

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority of United States Provisional Application Serial Number 61/260,277, filed 11-Nov-2009. This application is hereby incorporated in its entirety by reference herein.

FIELD OF INVENTION

[0002] This invention provides compositions and methods for treating and vaccinating against an Her2/neu antigen-expressing tumor and inducing an immune response against dominant and several sub-dominant epitopes of the antigen.

BACKGROUND OF THE INVENTION

[0003] Breast cancer is the second most deadly form of cancer in women in the US resulting in over 40,000 deaths annually. Her2/neu overexpression can be detected in 25-30% of all breast cancers and is associated with aggressive disease, hormone resistance and a poor prognosis. Her2/neu oncogene is a potential target for immunotherapy as it is overexpressed in tumors but has limited presence in other tissues, except for the heart.

[0004] Her-2/neu (referred to henceforth as “Her-2”) is a 185 kDa glycoprotein that is a member of the epidermal growth factor receptor (EGFR) family of tyrosine kinases, and consists of an extracellular domain, a transmembrane domain, and an intracellular domain which is known to be involved in cellular signaling (Bargmann CI et al, *Nature* 319: 226, 1986; King CR et al, *Science* 229: 974, 1985). It is overexpressed in 25 to 40% of all breast cancers and is also overexpressed in many cancers of the ovaries, lung, pancreas, and gastrointestinal tract. The overexpression of Her-2 is associated with uncontrolled cell growth and signaling, both of which contribute to the development of tumors. Patients with cancers that overexpress Her-2 exhibit tolerance even with detectable humoral, CD8⁺ T cell, and CD4⁺ T cell responses directed against Her-2.

[0005] *Listeria monocytogenes* is an intracellular pathogen that primarily infects antigen presenting cells and has adapted for life in the cytoplasm of these cells. Host cells, such as macrophages, actively phagocytose *L. monocytogenes* and the majority of the bacteria are degraded in the phagolysosome. Some of the bacteria escape into the host cytosol by

perforating the phagosomal membrane through the action of a hemolysin, listeriolysin O (LLO). Once in the cytosol, *L. monocytogenes* can polymerize the host actin and pass directly from cell to cell further evading the host immune system and resulting in a negligible antibody response to *L. monocytogenes*.

5 [0006] The construction and development of a number of *Listeria monocytogenes* (*Lm*) based vaccines expressing small fragments of human Her2/neu protein from the extra and intra-cellular domains of the protein have been reported. The Her2/neu is too big to fit in *Lm* which necessitated the generation of Her2/neu fragments. Having found activity in each fragment independently the present invention incorporates all of the active sites from each of the
10 independent fragments. Thus, a vaccine based upon a chimeric protein made by fusing of two of the extracellular and one intracellular fragments of the protein which included most of the known MHC class I epitopes of the Her2/neu receptor (*Lm*-LLO-ChHer2) has also been generated. All of these vaccines were shown to be immunogenic and efficacious in regressing pre-established tumors in FVB/N mice and delay the onset of spontaneous mammary tumors in
15 Her2/neu-expressing transgenic animals. The encouraging results from these preliminary experiments suggested that a recombinant *Listeria*-Her2/neu vaccine could be generated which could break the tolerance toward the Her2/neu self-antigen. However, the *Listeria*-Her2/neu vaccines developed thus far have been based on an attenuated *Listeria* platform which used the antibiotic marker (*cat*), for *in vitro* selection of the recombinant bacteria in the presence of
20 chloramphenicol. For clinical use, not only high attenuation is important, but also the absence of resistance to antibiotics.

[0007] Tumor evasion of the host immune response via escape mutations has been well documented and remains a major obstacle in tumor therapy. Thus, there is a need for developing a vaccine that has high therapeutic efficacy and that does not result in escape mutations. The present invention meets this need by providing a recombinant *Listeria*-Her2/neu vaccine (ADXS31-164) that was generated using the *LmddA* vaccine vector which has a well-defined attenuation mechanism and is devoid of antibiotic selection markers. The use of this chimeric antigen does not result in escape mutations indicating that tumors do not mutate away from a therapeutic efficacious response to treatment with this novel antigen.

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SUMMARY OF THE INVENTION

[0008] In one embodiment, the invention provided herein relates to an immunogenic composition comprising a fusion polypeptide, wherein said fusion polypeptide comprises a Her2/neu

chimeric antigen fused to an additional polypeptide, and wherein administering the fusion protein to a subject having an Her2/neu-expressing tumor prevents escape mutations within said tumor.

[0009] In another embodiment, the invention provided herein relates to a recombinant *Listeria* vaccine strain comprising a nucleic acid molecule, wherein and in another embodiment, the nucleic acid molecule comprises a first open reading frame encoding a polypeptide, wherein said polypeptide comprises a Her2/neu chimeric antigen, wherein the nucleic acid molecule further comprises a second open reading frame encoding a metabolic enzyme, and wherein the metabolic enzyme complements an endogenous gene that is lacking in the chromosome of the recombinant *Listeria* strain.

BRIEF DESCRIPTION OF THE FIGURES

[00010] **Figure 1. Construction of ADXS31-164.** (A) Plasmid map of pAdv164, which harbors *bacillus subtilis dal* gene under the control of constitutive *Listeria* p60 promoter for complementation of the chromosomal *dal-dal* deletion in *LmddA* strain. It also contains the fusion of truncated LLO₍₁₋₄₄₁₎ to the chimeric human Her2/neu gene, which was constructed by the direct fusion of 3 fragments the Her2/neu: EC1 (aa 40-170), EC2 (aa 359-518) and ICI (aa 679-808). (B) Expression and secretion of tLLO-ChHer2 was detected in *Lm*-LLO-ChHer2 (*Lm*-LLO-138) and *LmddA*-LLO-ChHer2 (ADXS31-164) by western blot analysis of the TCA precipitated cell culture supernatants blotted with anti-LLO antibody. A differential band of ~104 KD corresponds to tLLO-ChHer2. The endogenous LLO is detected as a 58 KD band. *Listeria* control lacked ChHer2 expression.

[00011] **Figure 2. Immunogenic properties of ADXS31-164** (A) Cytotoxic T cell responses elicited by Her2/neu *Listeria*-based vaccines in splenocytes from immunized mice were tested using NT-2 cells as stimulators and 3T3/neu cells as targets. Lm-control was based on the *LmddA* background that was identical in all ways but expressed an irrelevant antigen (HPV16-E7). (B) IFN- γ secreted by the splenocytes from immunized FVB/N mice into the cell culture medium, measured by ELISA, after 24 hours of *in vitro* stimulation with mitomycin C treated NT-2 cells. (C) IFN- γ secretion by splenocytes from HLA-A2 transgenic mice immunized with the chimeric vaccine, in response to *in vitro* incubation with peptides from different regions of the protein. A recombinant ChHer2 protein was used as positive control and an irrelevant peptide or no peptide groups constituted the negative controls as listed in the figure legend. IFN- γ secretion was detected by an ELISA assay using cell culture supernatants

harvested after 72 hours of co-incubation. Each data point was an average of triplicate data +/- standard error. * P value < 0.001.

[00012] **Figure 3. Tumor Prevention Studies for *Listeria*-ChHer2/neu Vaccines** Her2/neu transgenic mice were injected six times with each recombinant *Listeria*-ChHer2 or a control *Listeria* vaccine. Immunizations started at 6 weeks of age and continued every three weeks until week 21. Appearance of tumors was monitored on a weekly basis and expressed as percentage of tumor free mice. *p<0.05, N = 9 per group.

[00013] **Figure 4. Effect of immunization with ADXS31-164 on the % of Tregs in Spleens.** FVB/N mice were inoculated s.c. with 1×10^6 NT-2 cells and immunized three times with each vaccine at one week intervals. Spleens were harvested 7 days after the second immunization. After isolation of the immune cells, they were stained for detection of Tregs by anti CD3, CD4, CD25 and FoxP3 antibodies. dot-plots of the Tregs from a representative experiment showing the frequency of CD25⁺/FoxP3⁺ T cells, expressed as percentages of the total CD3⁺ or CD3⁺CD4⁺ T cells across the different treatment groups.

[00014] **Figure 5. Effect of immunization with ADXS31-164 on the % of tumor infiltrating Tregs in NT-2 tumors.** FVB/N mice were inoculated s.c. with 1×10^6 NT-2 cells and immunized three times with each vaccine at one week intervals. Tumors were harvested 7 days after the second immunization. After isolation of the immune cells, they were stained for detection of Tregs by anti CD3, CD4, CD25 and FoxP3 antibodies. (A). dot-plots of the Tregs from a representative experiment. (B). Frequency of CD25⁺/FoxP3⁺ T cells, expressed as percentages of the total CD3⁺ or CD3⁺CD4⁺ T cells (left panel) and intratumoral CD8/Tregs ratio (right panel) across the different treatment groups. Data is shown as mean \pm SEM obtained from 2 independent experiments.

[00015] **Figure 6. Vaccination with ADXS31-164 can delay the growth of a breast cancer cell line in the brain.** Balb/c mice were immunized thrice with ADXS31-164 or a control *Listeria* vaccine. EMT6-Luc cells (5,000) were injected intracranially in anesthetized mice. (A) *Ex vivo* imaging of the mice was performed on the indicated days using a Xenogen X-100 CCD camera. (B) Pixel intensity was graphed as number of photons per second per cm² of surface area; this is shown as average radiance. (C) Expression of Her2/neu by EMT6-Luc cells, 4T1-Luc and NT-2 cell lines was detected by Western blots, using an anti-Her2/neu antibody. J774.A2 cells, a murine macrophage like cell line was used as a negative control.

DETAILED DESCRIPTION OF THE INVENTION

[00016] In one embodiment, provided herein are methods and compositions for preventing, treating and vaccinating against a Her2/neu antigen-expressing tumor and inducing an immune response against sub-dominant epitopes of the Her2/neu antigen, while preventing an escape mutation of the tumor.

[00017] In another embodiment, provided herein is an immunogenic composition comprising a fusion polypeptide, wherein said fusion polypeptide comprises a Her2/neu chimeric antigen fused to an additional polypeptide, and wherein administering the fusion protein to a subject having an Her2/neu-expressing tumor prevents escape mutations within said tumor. In another 10 embodiment, provided herein is a recombinant *Listeria* vaccine strain comprising the immunogenic composition.

[00018] In one embodiment, provided herein is a recombinant *Listeria* vaccine strain comprising a nucleic acid molecule, wherein the nucleic acid molecule comprises a first open reading frame encoding a polypeptide, wherein the polypeptide comprises a Her2/neu chimeric antigen, wherein the nucleic acid molecule further comprises a second open reading frame encoding a metabolic enzyme, and wherein the metabolic enzyme complements an endogenous gene that is lacking in the chromosome of the recombinant *Listeria* strain. In another embodiment, the recombinant *Listeria* vaccine strain further comprises a third open reading frame encoding a metabolic enzyme, and wherein the metabolic enzyme complements 15 an endogenous gene that is lacking in the chromosome of the recombinant *Listeria* strain. In another embodiment, the recombinant *Listeria* vaccine strain further comprises a third open reading frame encoding a metabolic enzyme, and wherein the metabolic enzyme complements an endogenous gene that is lacking in the chromosome of the recombinant *Listeria* strain. 20

[00019] In another embodiment, provided herein is a recombinant *Listeria* vaccine strain comprising a nucleic acid molecule, wherein the nucleic acid molecule comprises a first open reading frame encoding a polypeptide, wherein the polypeptide comprises a Her2/neu chimeric antigen, wherein the nucleic acid molecule further comprises a second and a third 25 open reading frame each encoding a metabolic enzyme, and wherein the metabolic enzyme complements an endogenous gene that is lacking in the chromosome of said recombinant *Listeria* strain. In one embodiment, the nucleic acid molecule is integrated into the *Listeria* genome. In another embodiment, the nucleic acid molecule is in a plasmid in the recombinant *Listeria* vaccine strain. In yet another embodiment, the plasmid is stably maintained in the 30 recombinant *Listeria* vaccine strain in the absence of antibiotic selection. In another embodiment, the plasmid does not confer antibiotic resistance upon the recombinant *Listeria*. In another embodiment, the recombinant *Listeria* strain is attenuated. In another embodiment,

the recombinant *Listeria* is an attenuated auxotrophic strain. In another embodiment, the high metabolic burden that the expression of a foreign antigen exerts on a bacterium such as one of the present invention is also an important mechanism of attenuation.

[00020] In one embodiment the attenuated strain is *Lmdda*. In another embodiment, this strain 5 exerts a strong adjuvant effect which is an inherent property of *Listeria*-based vaccines. One manifestation of this adjuvant effect is the 5-fold decrease in the number of the intratumoral Tregs caused by either the irrelevant *Listeria* or the ADXS-31-164 vaccines (see Figure 5 herein). In another embodiment, the *LmddA* vector expressing an irrelevant antigen (HPV16 E7) is also associated with a significant decrease in the frequency of Tregs in the tumors, 10 likely as a consequence of innate immunity responses.

[00021] In one embodiment, the attenuated auxotrophic *Listeria* vaccine strain is the ADXS- 15 31-164 strain. ADXS-31-164 is based on a *Listeria* vaccine vector which is attenuated due to the deletion of virulence gene *actA* and retains the plasmid for Her2/neu expression *in vivo* and *in vitro* by complementation of *dal* gene. In one embodiment, ADXS31-164 expresses and secretes the chimeric Her2/neu protein fused to the first 441 amino acids of listeriolysin O (LLO). In another embodiment, ADXS31-164 exerts strong and antigen specific anti-tumor 20 responses with ability to break tolerance toward HER2/neu in transgenic animals (see Examples). In another embodiment, the ADXS31-164 strain is highly attenuated and has a better safety profile than previous *Listeria* vaccine generation, as it is more rapidly cleared from the spleens of the immunized mice. In another embodiment, the ADXS31-164 results in 25 a longer delay of tumor onset in transgenic animals than *Lm*-LLO-ChHer2, the antibiotic resistant and more virulent version of this vaccine (see Figure 3). In another embodiment, ADXS31-164 strain is highly immunogenic, able to break tolerance toward the HER2/neu self-antigen and prevent tumor formation in Her2/neu transgenic animals. In another 30 embodiment, ADXS31-164 causes a significant decrease in intra-tumoral T regulatory cells (Tregs). In another embodiment, the lower frequency of Tregs in tumors treated with *LmddA* vaccines resulted in an increased intratumoral CD8/Tregs ratio, suggesting that a more favorable tumor microenvironment can be obtained after immunization with *LmddA* vaccines. In another embodiment, the use of this chimeric antigen does not result in escape mutations indicating that tumors do not mutate away from a therapeutic efficacious response to treatment 35 with this novel antigen (see example 6). In another embodiment, peripheral immunization with ADXS31-164 delays the growth of a metastatic breast cancer cell line in the brain (see Example 7).

[00022] In one embodiment, the *Lm*-LLO-ChHer2 strain is Lm-LLO-138.

[00023] In one embodiment, recombinant attenuated, antibiotic-free *Listeria*-expressing chimeric antigens are useful for preventing, and treating a cancer or solid tumors, as exemplified herein. In another embodiment, recombinant *Listeria* expressing a chimeric

5 Her2/neu are useful as a therapeutic vaccine for the treatment of Her2/neu overexpressing solid tumors. In another embodiment, the Her2/neu chimeric antigen provided herein is useful for treating Her2/neu-expressing tumors and preventing escape mutations of the same. In another embodiment, the term “escape mutation” refers to a tumor mutating away from a therapeutic efficacious response to treatment.

10 [00024] In one embodiment, provided herein is a nucleic acid molecule comprising a first open reading frame encoding the immunogenic composition, wherein the nucleic molecule resides within the recombinant *Listeria* vaccine strain. In another embodiment, the nucleic acid molecule provided herein is used to transform the *Listeria* in order to arrive at a recombinant

15 *Listeria*. In another embodiment, the nucleic acid provided herein lacks a virulence gene. In another embodiment, the nucleic acid molecule integrated into the *Listeria* genome carries a non-functional virulence gene. In another embodiment, the virulence gene is mutated in the recombinant *Listeria*. In yet another embodiment, the nucleic acid molecule is used to

inactivate the endogenous gene present in the *Listeria* genome. In yet another embodiment, the virulence gene is an ActA gene. In another embodiment, the virulence gene is a PrfA gene.

20 As will be understood by a skilled artisan, the virulence gene can be any gene known in the art to be associated with virulence in the recombinant *Listeria*.

[00025] In one embodiment, the metabolic gene, the virulence gene, etc. is lacking in a chromosome of the *Listeria* strain. In another embodiment, the metabolic gene, virulence gene, etc. is lacking in the chromosome and in any episomal genetic element of the *Listeria*

25 strain. In another embodiment, the metabolic gene, virulence gene, etc. is lacking in the genome of the virulence strain. In one embodiment, the virulence gene is mutated in the chromosome. In another embodiment, the virulence gene is deleted from the chromosome. Each possibility represents a separate embodiment of the present invention.

[00026] In one embodiment, the metabolic gene, the virulence gene, etc. is lacking in a chromosome of the *Listeria* strain. In another embodiment, the metabolic gene, virulence gene, etc. is lacking in the chromosome and in any episomal genetic element of the *Listeria* strain. In another embodiment, the metabolic gene, virulence gene, etc. is lacking in the

genome of the virulence strain. In one embodiment, the virulence gene is mutated in the chromosome. In another embodiment, the virulence gene is deleted from the chromosome. Each possibility represents a separate embodiment of the present invention.

[00027] In another embodiment, the nucleic acids and plasmids provided herein do not confer 5 antibiotic resistance upon the recombinant *Listeria*.

[00028] “Nucleic acid molecule” refers, in another embodiment, to a plasmid. In another embodiment, the term refers to an integration vector. In another embodiment, the term refers to a plasmid comprising an integration vector. In another embodiment, the integration vector is a site-specific integration vector. In another embodiment, a nucleic acid molecule of 10 methods and compositions of the present invention are composed of any type of nucleotide known in the art. Each possibility represents a separate embodiment of the present invention.

[00029] “Metabolic enzyme” refers, in another embodiment, to an enzyme involved in synthesis of a nutrient required by the host bacteria. In another embodiment, the term refers to an enzyme required for synthesis of a nutrient required by the host bacteria. In another embodiment, the term refers to an enzyme involved in synthesis of a nutrient utilized by the host bacteria. In another embodiment, the term refers to an enzyme involved in synthesis of a nutrient required for sustained growth of the host bacteria. In another embodiment, the enzyme is required for synthesis of the nutrient. Each possibility represents a separate embodiment of the present invention.

[00030] “Stably maintained” refers, in another embodiment, to maintenance of a nucleic acid molecule or plasmid in the absence of selection (e.g. antibiotic selection) for 10 generations, without detectable loss. In another embodiment, the period is 15 generations. In another embodiment, the period is 20 generations. In another embodiment, the period is 25 generations. In another embodiment, the period is 30 generations. In another embodiment, the period is 40 generations. In another embodiment, the period is 50 generations. In another embodiment, the period is 60 generations. In another embodiment, the period is 80 generations. In another embodiment, the period is 100 generations. In another embodiment, the period is 150 generations. In another embodiment, the period is 200 generations. In another embodiment, the period is 300 generations. In another embodiment, the period is 500 generations. In another embodiment, the period is more than generations. In another embodiment, the nucleic acid molecule or plasmid is maintained stably *in vitro* (e.g. in culture). In another embodiment, the nucleic acid molecule or plasmid is maintained stably *in*

vivo. In another embodiment, the nucleic acid molecule or plasmid is maintained stably both *in vitro* and *in vivo*. Each possibility represents a separate embodiment of the present invention.

[00031] In one embodiment, the present invention provides a recombinant *Listeria* strain expressing the antigen. The present invention also provides recombinant peptides comprising a listeriolysin (LLO) protein fragment fused to a Her-2 chimeric protein or fragment thereof, vaccines and immunogenic compositions comprising same, and methods of inducing an anti-Her-2 immune response and treating and vaccinating against a Her-2-expressing tumor, comprising the same.

[00032] In another embodiment, a recombinant *Listeria* strain of the present invention has been passaged through an animal host. In another embodiment, the passaging maximizes efficacy of the strain as a vaccine vector. In another embodiment, the passaging stabilizes the immunogenicity of the *Listeria* strain. In another embodiment, the passaging stabilizes the virulence of the *Listeria* strain. In another embodiment, the passaging increases the immunogenicity of the *Listeria* strain. In another embodiment, the passaging increases the virulence of the *Listeria* strain. In another embodiment, the passaging removes unstable sub-strains of the *Listeria* strain. In another embodiment, the passaging reduces the prevalence of unstable sub-strains of the *Listeria* strain. In another embodiment, the *Listeria* strain contains a genomic insertion of the gene encoding the antigen-containing recombinant peptide. In another embodiment, the *Listeria* strain carries a plasmid comprising the gene encoding the antigen-containing recombinant peptide. In another embodiment, the passaging is performed by any other method known in the art.

[00033] In one embodiment, the polypeptide provided herein is a fusion protein comprising an additional polypeptide selected from the group consisting of: a) non-hemolytic LLO protein or N-terminal fragment, b) a PEST sequence, or c) an ActA fragment, and further wherein said additional polypeptide is fused to said Her2/neu chimeric antigen. In another embodiment, the additional polypeptide is functional. In another embodiment, a fragment of the additional polypeptide is immunogenic. In another embodiment, the additional polypeptide is immunogenic.

[00034] In another embodiment, the polypeptide provided herein is a fusion protein comprising a non-hemolytic LLO protein or N-terminal fragment fused to the Her2/neu chimeric antigen. In another embodiment, a fusion protein of methods and compositions of

the present invention comprises an ActA sequence from a *Listeria* organism. ActA proteins and fragments thereof augment antigen presentation and immunity in a similar fashion to LLO.

[00035] In another embodiment of methods and compositions of the present invention, the 5 fusion protein comprises the Her2/neu antigen and an additional polypeptide. In one embodiment, the additional polypeptide is a non-hemolytic LLO protein or fragment thereof (Examples herein). In another embodiment, the additional polypeptide is a PEST sequence. In another embodiment, the additional polypeptide is an ActA protein or a fragment thereof. ActA proteins and fragments thereof augment antigen presentation and immunity in a similar 10 fashion to LLO.

[00036] The additional polypeptide of methods and compositions of the present invention is, in another embodiment, a listeriolysin (LLO) peptide. In another embodiment, the additional polypeptide is an ActA peptide. In another embodiment, the additional polypeptide is a PEST-like sequence peptide. In another embodiment, the additional polypeptide is any other 15 peptide capable of enhancing the immunogenicity of an antigen peptide. Each possibility represents a separate embodiment of the present invention.

[00037] Fusion proteins comprising the Her2/neu chimeric antigen may be prepared by any suitable method, including, for example, cloning and restriction of appropriate sequences or direct chemical synthesis by methods discussed below. Alternatively, subsequences may be 20 cloned and the appropriate subsequences cleaved using appropriate restriction enzymes. The fragments may then be ligated to produce the desired DNA sequence. In one embodiment, DNA encoding the antigen can be produced using DNA amplification methods, for example polymerase chain reaction (PCR). First, the segments of the native DNA on either side of the new terminus are amplified separately. The 5' end of the one amplified sequence encodes the 25 peptide linker, while the 3' end of the other amplified sequence also encodes the peptide linker. Since the 5' end of the first fragment is complementary to the 3' end of the second fragment, the two fragments (after partial purification, e.g. on LMP agarose) can be used as an overlapping template in a third PCR reaction. The amplified sequence will contain codons, the segment on the carboxy side of the opening site (now forming the amino sequence), the linker, and the sequence on the amino side of the opening site (now forming the carboxyl 30 sequence). The antigen is ligated into a plasmid. Each method represents a separate embodiment of the present invention.

[00038] The results of the present invention demonstrate that administration of compositions of the present invention has utility for inducing formation of antigen-specific T cells (e.g. cytotoxic T cells) that recognize and kill tumor cells (Examples herein).

[00039] In one embodiment, the present invention provides a recombinant polypeptide comprising an N-terminal fragment of an LLO protein fused to a Her-2 chimeric protein or fused to a fragment thereof. In one embodiment, the present invention provides a recombinant polypeptide consisting of an N-terminal fragment of an LLO protein fused to a Her-2 chimeric protein or fused to a fragment thereof.

[00040] In another embodiment, the Her-2 chimeric protein of the methods and compositions of the present invention is a human Her-2 chimeric protein. In another embodiment, the Her-2 protein is a mouse Her-2 chimeric protein. In another embodiment, the Her-2 protein is a rat Her-2 chimeric protein. In another embodiment, the Her-2 protein is a primate Her-2 chimeric protein. In another embodiment, the Her-2 protein is a Her-2 chimeric protein of any other animal species or combinations thereof known in the art. Each possibility represents a separate embodiment of the present invention.

[00041] In another embodiment, a Her-2 protein is a protein referred to as "HER-2/neu," "Erbb2," "v-erb-b2," "c-erb-b2," "neu," or "cNeu." Each possibility represents a separate embodiment of the present invention.

[00042] In one embodiment, the Her2-neu chimeric protein, harbors two of the extracellular and one intracellular fragments of Her2/neu antigen showing clusters of MHC-class I epitopes of the oncogene, where, in another embodiment, the chimeric protein, harbors 3 H2Dq and at least 17 of the mapped human MHC-class I epitopes of the Her2/neu antigen (fragments EC1, EC2, and IC1) (See Fig. 1). In another embodiment, the chimeric protein harbors at least 13 of the mapped human MHC-class I epitopes (fragments EC2 and IC1). In another embodiment, the chimeric protein harbors at least 14 of the mapped human MHC-class I epitopes (fragments EC1 and IC1). In another embodiment, the chimeric protein harbors at least 9 of the mapped human MHC-class I epitopes (fragments EC1 and IC2). In another embodiment, the Her2-neu chimeric protein is fused to a non-hemolytic listeriolysin O (LLO). In another embodiment, the Her2-neu chimeric protein is fused to the first 441 amino acids of the *Listeria-monocytogenes* listeriolysin O (LLO) protein and expressed and secreted by the *Listeria monocytogenes* attenuated auxotrophic strain *LmddA*. In another embodiment, the expression and secretion of the fusion protein tLLO-ChHer2 from the

attenuated auxotrophic strain provided herein that expresses a chimeric Her2/neu antigen/LLO fusion protein is comparable to that of the *Lm*-LLO-ChHer2 in TCA precipitated cell culture supernatants after 8 hours of *in vitro* growth (See Figure 1B).

[00043] In one embodiment, no CTL activity is detected in naïve animals or mice injected with
5 an irrelevant *Listeria* vaccine (See Figure 2A). While in another embodiment, the attenuated auxotrophic strain (ADXS31-164) provided herein is able to stimulate the secretion of IFN- γ by the splenocytes from wild type FVB/N mice (Figure 2B).

[00044] In another embodiment, the metabolic enzyme of the methods and compositions provided herein is an amino acid metabolism enzyme, where, in another embodiment, the metabolic enzyme is an alanine racemase enzyme. In another embodiment, the metabolic enzyme is a D-amino acid transferase enzyme. In another embodiment, the metabolic enzyme catalyzes a formation of an amino acid used for a cell wall synthesis in the recombinant *Listeria* strain, where in another embodiment, the metabolic enzyme is an alanine racemase enzyme.
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15 [00045] In another embodiment, the gene encoding the metabolic enzyme is expressed under the control of the *Listeria* p60 promoter. In another embodiment, the inlA (encodes internalin) promoter is used. In another embodiment, the hly promoter is used. In another embodiment, the ActA promoter is used. In another embodiment, the integrase gene is expressed under the control of any other gram positive promoter. In another embodiment, the gene encoding the metabolic enzyme is expressed under the control of any other promoter that functions in
20 *Listeria*. The skilled artisan will appreciate that other promoters or polycistronic expression cassettes may be used to drive the expression of the gene. Each possibility represents a separate embodiment of the present invention.

[00046] In another embodiment, the Her-2 chimeric protein is encoded by the following
25 nucleic acid sequence set forth in SEQ ID NO:1

gagacccacacctggacatgtccggccaccttaccagggtgcagggtgtgcaggaaacctggaaactcacctacctgcccaccaatg
ccagcctgtcccttcgtcaggatattcaggagggtgcagggtctacgtgtcatcgccatcacaaccaagtgaggcagggtccactgcagag
gctgcggattgtgcgaggcaccgcacctttgaggacaactatgcctggccgtgctagacaatggagacccgctgaacaataccaccc
ctgtcacaggggcetccccaggaggcctgcggagctgcagttcgaagcctcacagagatcttggaggggtttgatccagc
30 ggaaccccccagctgtcaccaggacacgattttgttggagaataccaggagttgtggctgcaagaagatcttggagccctggcatt
tctgcccggagagcttggatggggaccctccaaacactgccccgtccagccagagcagctccaagtgttggagactcttggaaagaga

tcacagggtacctatacatctcagcatggccggacagcctgcctgacccctcagcgttccagaacctgcaagtaatccggggacgaattct
gcacaatggcgctactcgcgtgaccctgcaaggcgtggcatcagctggctgggctgcgtactgagggactggcagtggact
ggccctcatccaccataacacccacccctgtccgtgcacacggccctggaccagctttcgaaacccgaccaagctctgctcca
cactgccaacccggccagaggcagactgtgtggcgaggccctggccaccagctgtgcgcggcaggcagcagaagatccgg
5 aagtacacatgcggagactgctcaggaaacggagctggagccgctgacacccatggcgtggcgtacaccatggcgtggcagatccgg
gcggatccctgaaagagacggagctgaggaaggtaaggtgcttgatctggcgtttggcacagtctacaaggcatctggatccctg
atggggagaatgtgaaaattccagtggccatcaaagtgtgagggaaaacacatccccaaagccaacaaagaaatcttagacgaagc
atacgtgatggctgggtggctcccatatgtctccgccttggcatctgcctgacatccacggcgtcagctggtgcacacagcttgc
ccctatggcgtcccttagactaa (SEQ ID NO: 1).

10 [00047] In another embodiment, the Her-2 chimeric protein has the sequence:

E T H L D M L R H L Y Q G C Q V V Q G N L E L T Y L P T N A S L S
F L Q D I Q E V Q G Y V L I A H N Q V R Q V P L Q R L R I V R G T
Q L F E D N Y A L A V L D N G D P L N N T T P V T G A S P G G L R
15 E L Q L R S L T E I L K G G V L I Q R N P Q L C Y Q D T I L W K N I
Q E F A G C K K I F G S L A F L P E S F D G D P A S N T A P L Q P E
Q L Q V F E T L E E I T G Y L Y I S A W P D S L P D L S V F Q N L Q
V I R G R I L H N G A Y S L T L Q G L G I S W L G L R S L R E L G S
20 G L A L I H H N T H L C F V H T V P W D Q L F R N P H Q A L L H T
A N R P E D E C V G E G L A C H Q L C A R G Q Q K I R K Y T M R
R L L Q E T E L V E P L T P S G A M P N Q A Q M R I L K E T E L R
K V K V L G S G A F G T V Y K G I W I P D G E N V K I P V A I K V
L R E N T S P K A N K E I L D E A Y V M A G V G S P Y V S R L L G I C
25 L T S T V Q L V T Q L M P Y G C L L D (SEQ ID NO: 2).

25 [00048] In one embodiment, the Her2 chimeric protein or fragment thereof of the methods and compositions provided herein does not include a signal sequence thereof. In another embodiment, omission of the signal sequence enables the Her2 fragment to be successfully expressed in *Listeria*, due the high hydrophobicity of the signal sequence. Each possibility represents a separate embodiment of the present invention.

30 [00049] In another embodiment, the fragment of a Her2 chimeric protein of methods and compositions of the present invention does not include a transmembrane domain (TM) thereof. In one embodiment, omission of the TM enables the Her-2 fragment to be successfully expressed in *Listeria*, due the high hydrophobicity of the TM. Each possibility represents a separate embodiment of the present invention.

35 [00050] In one embodiment, the nucleic acid sequence of rat-Her2/neu gene is
CCGGAATCGCGGGCACCCAAAGTGTGTACCGGCACAGACATGAAGTTGCGGCTCC
CTGCCAGTCCTGAGACCCACCTGGACATGCTCCGCCACCTGTACCAGGGCTGTCA

GGTAGTGCAGGGCAACTTGGAGCTTACCTACGTGCCTGCCAATGCCAGCCTCTCA
TTCCTGCAGGACATCCAGGAAGTTACAGGGTTACATGCTCATCGCTACAACCAGG
TGAAGCGCGTCCCACTGCAAAGGGCTGCGCATCGTGAGAGGGACCCAGCTCTTG
AGGACAAGTATGCCCTGGCTGTGCTAGACAACCGAGATCCTCAGGACAATGTG
5 CCGCCTCCACCCAGGCAGAACCCCCAGAGGGGCTGCGGGAGCTGCAGCTCGAA
GTCTCACAGAGATCCTGAAGGGAGGAGTTTGATCCGTGGAACCCCTCAGCTCTG
CTACCAGGACATGGTTTGTGGAAGGACGTCTCCGCAAGAATAACCAACTGGCT
CCTGTCGATATAGACACCAATCGTCCCCGGCCTGTCCACCTTGTGCCCGCCT
GCAAAGACAATCACTGTTGGGTGAGAGTCCGGAAGACTGTCAGATCTGACTG
10 GCACCATCTGTACCAGTGGTTGTGCCCGTGCAAGGGCCGGCTGCCACTGACTG
CTGCCATGAGCAGTGTGCCGCAGGCTGCACGGGCCAACGATTCTGACTGCCTG
GCCTGCCTCCACTCAATCATAGTGGTATCTGTGAGCTGCACTGCCAGCCCTCGT
CACCTACAACACAGACACCTTGAGTCCATGCACAACCCCTGAGGGTCGCTACACC
TTTGGTGCCAGCTGCGTGACCACCTGCCCTACAACACTACCTGTCTACGGAAGTGG
15 GATCCTGCACTCTGGTGTGCCCGAATAACCAAGAGGTACAGCTGAGGACG
GAACACAGCGTTGTGAGAAATGCAGCAAGCCCTGTGCTCGAGTGTGCTATGGTCT
GGGCATGGAGCACCTCGAGGGCGAGGGCCATCACCAGTGACAATGTCCAGGA
GTTTGATGGCTGCAAGAAGATCTTGGGAGCCTGGCATTTGCCGGAGAGCTTT
GATGGGGACCCCTCCTCCGGCATTGCTCCGCTGAGGCCTGAGCAGCTCCAAGTGT
20 TCGAAACCCCTGGAGGAGATCACAGGTTACCTGTACATCTCAGCATGCCAGACA
GTCTCCGTGACCTCAGTGTCTCCAGAACCTCGAATCATTGGGGACGGATTCT
CCACGATGGCGCGTACTCATTGACACTGCAAGGCCTGGGATCCACTCGCTGGGG
CTGCGCTCACTGCGGGAGCTGGCAGTGGATTGGCTCTGATTCACCGCAACGCC
ATCTCTGCTTGTACACACTGTACCTTGGGACCAGCTCTCCGGAACCCACATCA
25 GCCCTGCTCACAGTGGAACCGGCCCGGAAGAGGATTGTGGTCTCGAGGGCTT
GGTCTGTAACTCACTGTGTGCCACGGCACTGCTGGGGCCAGGGCCCACCCAG
TGTGTCAACTGCAGTCATTCCCTCGGGGCCAGGAGTGTGAGTGACAAGCGCTGTGCCGTGTCA
TATGGAAGGGGCTCCCCGGGAGTATGTGAGTGACAAGCGCTGTGCCGTGTCA
CCCCGAGTGTAGCCTCAAAACAGCTCAGAGACCTGCTTGGATCGGAGGCTGAT
30 CAGTGTGCAGCCTGCGCCCACATACAAGGACTCGTCCCTCTGTGTGGCTCGCTGCC
CCAGTGGTGTGAAACCGGACCTCCTACATGCCCATCTGGAAGTACCCGGATGA
GGAGGGCATATGCCAGCCGTGCCCATCAACTGCACCCACTCCTGTGTGGATCTG
GATGAACGAGGCTGCCAGCAGAGCAGAGAGCCAGCCGGTACATTGATCATT
GCAACTGTAGTGGCGTCCTGCTGTTCTGATCTTAGTGGTGGTCGTTGGAATCCT

AATCAAACGAAGGAGACAGAAGAGATCCCGAAGTATACTGCGTAGGCTGCTGCA
GGAAACTGAGTTAGTGGAGCCGCTGACGCCAGCGGAGCAATGCCAACAGGC
TCAGATGCGGATCCTAAAAGAGAGACGGAGCTAAGGAAGGTGAAGGTGCTGGATC
AGGAGCTTGCACTGTCTACAAGGGCATCTGGATCCCAGATGGGAGAATGT
5 GAAAATCCCCGTGGCTATCAAGGTGTTGAGAGAAAACACATCTCCTAAAGCCAA
CAAAGAAATTCTAGATGAAGCGTATGTGATGGCTGGTGTGGTTCTCCGTATGTG
TCCCACCTCCTGGCATCTGCCTGACATCCACAGTACAGCTGGTACACAGCTTA
TGCCCTACGGCTGCCTCTGGACCATGTCCGAGAACACCCGAGGTGCGCTAGGCTC
CCAGGACCTGCTCAACTGGTGTTCAGATTGCCAAGGGATGAGCTACCTGGAG
10 GACGTGCGGCTTGTACACAGGGACCTGGCTGCCCGGAATGTGCTAGTCAAGAGT
CCCAACCACGTCAAGATTACAGATTTCGGGCTGGCTGGCTGCTGGACATTGATG
AGACAGAGTACCATGCAGATGGGGCAAGGTGCCATCAAATGGATGGCATTGG
AATCTATTCTCAGACGCCGGTTCACCCATCAGAGTGATGTGTGGAGCTATGGAGT
GACTGTGTGGAGCTGATGACTTTGGGCCAAACCTACGATGGAATCCCAGCC
15 CGGGAGATCCCTGATTGCTGGAGAACGGAGAACGCCAACCTCAGCCTCCAATCT
GCACCATTGATGTCTACATGATTATGGTCAAATGTTGGATGATTGACTCTGAATG
TCGCCCGAGATTCCGGGAGTTGGTGTCAAATTTCACGTATGGCGAGGGACCCC
CAGCGTTTGTGGTCATCCAGAACGAGGACTGGGCCATCCAGCCCCATGGACA
GTACCTCTACCGTTCACTGCTGGAAGATGATGACATGGTGACCTGGTAGACGC
20 TGAAGAGTATCTGGTCCCCAGCAGGGATTCTCTCCCCGGACCCCTACCCAGGC
ACTGGGAGCACAGCCATAGAAGGCACCGCAGCTCGTCCACCAGGAGTGGAGGT
GGTAGCTGACACTGGCCTGGAGCCCTCGGAAGAACGGCCCCCAGATCTCCA
CTGGCTCCCTCGGAAGGGCTGGCTCCGATGTGTTGATGGTACCTGGCAATGG
GGTAACCAAAGGGCTGCAGAGCCTCTCCACATGACCTCAGCCCTACAGCG
25 GTACAGCGAGGACCCACATTACCTCTGCCCGAGACTGATGGCTATGTTGCT
CCCCTGGCCTGCAGCCCCAGCCCGAGTATGTGAACCAATCAGAGGTTAGCCTC
AGCCTCTTAACCCCAGAGGGCTCTGCCTCCTGTCGGCTGCTGGTACT
CTAGAAAGACCAAGACTCTCTCCTGGAAAGAATGGGTTGTCAGACGTT
TTGCCTCGGGGGTGCTGTGGAGAACCCCTGAATACTTAGTACCGAGAGAAGGCA
30 CTGCCTCTCCGCCCCACCCCTCTCCTGCCTCAGCCCAGCCTTGACAAACCTCTAT
TACTGGGACCAGAACTCATGGAGCAGGGCCTCCACCAAGTAACCTGAAGGG
ACCCCCACTGCAGAGAACCCCTGAGTACCTAGGCCTGGATGTACCTGTA (SEQ ID
NO:45).

[00051] In one embodiment, the nucleic acid sequence encoding the rat/her2/neu EC1 fragment is

CCCAGGCAGAACCCAGAGGGGCTGCGGGAGCTGCAGCTCGAAGTCTCACAGA
GATCCTGAAGGGAGGAGTTGATCCGTGGAACCCCTCAGCTCTGCTACCAGGAC
5 ATGGTTTGTGGAAGGACGTCTCCGCAAGAATAACCAACTGGCTCCTGTCGATA
TAGACACCAATCGITCCCAGGGCTGTCCACCTTGTGCCCGGCCTGCAAAGACAA
TCACTGTTGGGTGAGAGTCCGGAAGACTGTCAGATCTGACTGGCACCATCTGT
ACCAGTGGTTGTGCCCGTGCAAGGGCCGGCTGCCACTGACTGCTGCCATGAGC
AGTGTGCCGCAGGCTGCACGGCCCCAAGCA (SEQ ID NO:46).

10 [00052] In another embodiment, the nucleic acid sequence encoding the rat her2/neu EC2 fragment is:

GGTCACAGCTGAGGACGGAACACACAGCGTTGTGAGAAATGCAGCAAGCCCTGTGC
TCGAGTGTGCTATGGTCTGGCATGGAGCACCTCGAGGGCGAGGGCCATCAC
CAGTGACAATGTCCAGGAGTTGATGGCTGCAAGAAGATCTTGGAGCCTGGC
15 ATTTTGCCGGAGAGCTTGATGGGACCCCTCCCGCATTGCTCCGCTGAGG
CCTGAGCAGCTCCAAGTGTGAAACCCCTGGAGGAGATCACAGGTTACCTGTACA
TCTCAGCATGGCCAGACAGTCTCCGTGACCTCAGTGTCTCCAGAACCTCGAAT
CATTGGGGACGGATTCTCCACGATGGCGCGTACTCATTGACACTGCAAGGCCTG
GGGATCCACTCGCTGGGCTCGCTCACTGCGGGAGCTGGCAGTGGATTGGCTC
20 TGATTCACCGAACGCCATCTCTGCTTGTACACACTGTACCTGGACCAGCTC
TTCCGGAACCCACATCAGGCCCTGCTCCACAGTGGAACCGGCCGGAAAGAGGAT
TGTGGTCTCGAGGGCTGGTCTGTAACTCACTGTGTGCCACGGGACTGCTGGG
GCCAGGGCCCACCCA (SEQ ID NO:47).

25 [00053] In another embodiment, the nucleic acid sequence encoding the rat her2/neu IC1 fragment is:

[00054] CGCCCAGCGGAGCAATGCCAACCAACCAGGCTCAGATGCGGATCCTAAAAGAG
ACGGAGCTAAGGAAGGTGAAGGTGCTGGATCAGGAGCTTGGCACTGTCTAC
AAGGGCATCTGGATCCCAGATGGGAGAATGTGAAAATCCCCGTGGCTATCAAG
GTGTTGAGAGAAAACACATCTCCTAAAGCCAACAAAGAAATTCTAGATGAAGCG
30 TATGTGATGGCTGGTGTGGGTCTCCGTATGTGTCCCGCCTCCTGGCATTGCT
GACATCCACAGTACAGCTGGTACACAGCTTATGCCCTACGGCTGCCCTCTGGAC
CATGTCCGAGAACACCGAGGTGCGCTAGGCTCCAGGACCTGCTCAACTGGTGTG

TTCAGATTGCCAAGGGATGAGCTACCTGGAGGACGTGCGGCTTGTACACAGGG
 ACCTGGCTGCCCGGAATGTGCTAGTCAAGAGTCCCAACCACGTCAAGATTACAG
 ATTTGGGCTGGCTCGGCTGCTGGACATTGATGAGACAGAGTACCATGCAGATGG
 GGGCAAGGTGCCCATCAAATGGATGGCATTGGAATCTATTCTCAGACGCCGGTTC
 5 ACCCATCAGAGTGATGTGTGGAGCTATGGAGTGACTGTGTGGAGCTGATGACTT
 TTGGGGCAAACCTTACGATGGAATCCCAGCCGGAGATCCCTGATTGCTGGA
 GAAGGGAGAACGCCTACCTCAGCCTCCAATCTGCACCATTGATGTCTACATGATT
 ATGGTCAAATGTTGGATGATTGACTCTGAATGTCGCCAGATTCCGGAGTTGG
 TGTCAGAATTTCACGTATGGCGAGGGACCCCCAGCGTTTGTGGTCATCCAGAA
 10 CGAGGACTTGGGCCATCCAGCCCCATGGACAGTACCTCTACCGTTCACTGCTG
 GAA (SEQ ID NO:48).

[00055] In one embodiment, the nucleic acid sequence of human-Her2/neu gene is:

ATGGAGCTGGCGGCCTTGTGCCGCTGGGGCTCCTCCTCGCCCTCTGCCCGG
 GAGCCCGAGCACCCAAAGTGTGCACCGGCACAGACATGAAGCTGCGGCTCCCTG
 15 CCAGTCCCAGAACCCACCTGGACATGCTCCGCCACCTCTACCAGGGCTGCCAGGT
 GGTGCAGGGAAACCTGGAACTCACCTACCTGCCAACCAATGCCAGCCTGTCCTTC
 CTGCAGGATATCCAGGAGGTGCAGGGCTACGTGCTCATCGCTACAACCAAGTG
 AGGCAGGGTCCCAC TGCA GAGGGTGCAGGGATTGTGCGAGGGACCCAGCTTTGAG
 GACAACATGCCCTGGCGTGCTAGACAATGGAGACCCGCTGAACAATACCACC
 20 CCTGTCACAGGGGCCTCCCCAGGAGGCCTGCCGGAGCTGCAGCTCGAACGCTC
 ACAGAGATCTTGTGAAAGGAGGGTCTTGATCCAGCGAACCCCCAGCTCTGCTAC
 CAGGACACGATTGTGGAAGGACATCTTCCACAAGAACAAACCAGCTGGCTCTCA
 CACTGATAGACACCAACCGCTCTGGGCCTGCCACCCCTGTTCTCCGATGTGTAA
 GGGCTCCCGCTGCTGGGAGAGAGTTCTGAGGATTGTCA GAGGCCTGACCGC
 25 TGTCTGTGCCGGTGGCTGTGCCGCTGCAAGGGCCACTGCCACTGACTGCTGC
 CATGAGCAGTGTGCTGCCGGCTGCACGGGCCAACGCACTCTGACTGCCTGGCCT
 GCCTCCACTTCAACCACAGTGGCATCTGTGAGCTGCACTGCCAGCCCTGGTCAC
 CTACAACACAGACACGTITGAGTCCATGCCAATCCCAGGGCCGGTATACATT
 GGCGCCAGCTGTGACTGCCTGTCCTACAACACTACCTTCTACGGACGTGGAT
 30 CCTGCACCCCTCGTCTGCCCTGCACAACCAAGAGGTGACAGCAGAGGATGGAA
 CACAGCGGTGTGAGAAGTGCAGCAAGCCCTGTGCCAGTGCTATGGCTGG
 GCATGGAGCACTGCGAGAGGTGAGGGCAGTTACCAAGTGCCTGGAGAGCTTGA
 TTGCTGGCTGCAAGAAGATCTTGGAGCCTGGCATTTCTGCCGGAGAGCTTGA

TGGGGACCCAGCCTCCAACACTGCCCGCTCCAGCCAGAGCAGCTCCAAGTGT
GAGACTCTGGAAGAGATCACAGGTTACCTATACATCTCAGCATGGCCGGACAGC
CTGCCTGACCTCAGCGTCTTCCAGAACCTGCAAGTAATCCGGGACGAATTCTGC
ACAATGGCGCCTACTCGCTGACCC TGCAAGGGCTGGGCATCAGCTGGCTGGGCT
5 GCGCTCACTGAGGGA ACTGGCAGTGGACTGGCCCTCATCCACCATAACACCCA
CCTCTGCTTCGTGCACACGGTGCCCTGGGACCAGCTCTTCGGAACCCGCCACCAA
GCTCTGCTCCACACTGCCAACCGGCCAGAGGACGAGTGTGTGGCGAGGGCCTG
GCCTGCCACCAGCTGTGCGCCGAGGGCACTGCTGGGTCCAGGGCCCACCCAG
TGTGTCAACTGCAGCCAGTTCCCTCGGGGCCAGGAGTGCCTGGAGGAATGCCGA
10 GTACTGCAGGGCTCCCCAGGGAGTATGTGAATGCCAGGCACTGTTGCCGTGCC
ACCTGAGTGTCA GCCCCAGAATGGCTCAGTGACCTGTTGGACCGGAGGGCTGA
CCAGTGTGTGGCCTGTGCCCACTATAAGGACCCCTCCCTGCCTGGGCCGCTGC
CCCAGCGGTGTGAAACCTGACCTCTCCTACATGCCCATCTGGAAGTTCCAGATG
AGGAGGGCGATGCCAGCCTGCCCATCAACTGCACCCACTCCTGTGTGGACCT
15 GGATGACAAGGGCTGCCCGCCGAGCAGAGAGCCAGCCCTCTGACGTCCATCGT
CTCTCGGGTGGTGGCATTCTGCTGGTCGTGGCTTGGGGGTGGCTTGGATCC
TCATCAAGCGACGGCAGCAGAAGATCCGAAGTACACGATGCCGAGACTGCTGC
AGGAAACGGAGCTGGTGGAGCCGCTGACACCTAGCGGAGCGATGCCAACCA
CGCAGATGCCGATCCTGAAAGAGACGGAGCTGAGGAAGGTGAAGGTGCTGGAT
20 CTGGCGCTTGGCACAGTCTACAAGGCATCTGGATCCCTGATGGGAGAATGT
GAAAATTCCAGTGGCCATCAAAGTGTGAGGGAAAACACATCCCCAAAGCCAA
CAAAGAAATCTTAGACGAAGCATACTGATGGCTGGTGTGGCTCCCCATATGTC
TCCCCTCTGGCTGCCTTAGACCATGTCCGGAAAACCGCGGACGCCCTGGCTC
TGCCCTATGGCTGCCTTAGACCATGTCCGGAAAACCGCGGACGCCCTGGCTC
25 CCAGGACCTGCTGAACGGTGTATGCAGATTGCCAAGGGGATGAGCTACCTGGA
GGATGTGCGGCTCGTACACAGGGACTTGGCCGCTCGGAACGTGCTGGTCAAGAG
TCCCAACCATGTCAAAATTACAGACTTCGGCTGGCTGGCTGGCTGGACATTGAC
GAGACAGAGTACCATGCAGATGGGGCAAGGTGCCCATCAAGTGGATGGCGCTG
GAGTCCATTCTCCGCCGGCGGTTACCCACCAAGAGTGTGTGGAGTTATGGT
30 TGACTGTGTGGAGCTGATGACTTTGGGCCAAACCTTACGATGGGATCCCAGC
CCGGGAGATCCCTGACCTGCTGGAAAAGGGGAGCGGCTGCCAGCCCCCAT
CTGCACCATTGATGTCTACATGATCATGGTCAAATGTTGGATGATTGACTCTGAA
TGTGGCCAAGATTCCGGAGTTGGTGTCTGAATTCTCCGCATGCCAGGGACC
CCCAGCGCTTGTGGTCATCCAGAATGAGGACTTGGGCCAGCCAGTCCCTGGGA

CAGCACCTTCTACCGCTCACTGCTGGAGGACGATGACATGGGGACCTGGTGGAT
 GCTGAGGAGTATCTGGTACCCCAGCAGGGCTTCTGTCCAGACCTGCCCGG
 GCGCTGGGGCATGGTCCACCACAGGCACCGCAGCTCATCTACCAGGAGTGGCG
 GTGGGGACCTGACACTAGGGCTGGAGCCCTCTGAAGAGGGAGGCCAGGTCTC
 5 CACTGGCACCCCTCCGAAGGGCTGGCTCCGATGTATTGATGGTGACCTGGGAAT
 GGGGGCAGCCAAGGGCTGCAAAGCCTCCCCACACATGACCCAGCCCTCTACA
 GCGGTACAGTGAGGACCCCACAGTACCCCTGCCCTGTGAGACTGATGGCTACGTT
 GCCCCCTGACCTGCAGCCCCAGCCTGAATATGTGAACCAGCCAGATGTTGGC
 CCCAGCCCCCTCGCCCCGAGAGGGCCCTGCCTGCTGCCGACCTGCTGGTGC
 10 CACTCTGGAAAGGGCAAGACTCTCTCCCAGGGAAGAATGGGTCGTCAAAGA
 CGTTTTGCCTTGGGGTGCCGTGGAGAACCCGAGTACTTGACACCCAGGGA
 GGAGCTGCCCTCAGCCCCACCCCTCCTGCCTCAGCCCAGCCTGACAACC
 TCTATTACTGGGACCAGGACCCACCAGAGCGGGGGCTCCACCCAGCACCTCA
 AAGGGACACCTACGGCAGAGAACCCAGAGTACCTGGGTCTGGACGTGCCAGTGT
 15 GAACCAGAAGGCCAAGTCCGCAGAAGCCCTGA (SEQ ID NO:49).

[00056] In another embodiment, the nucleic acid sequence encoding the human her2/neu EC1 fragment implemented into the chimera spans from 120-510 bp of the human EC1 region and is set forth in (SEQ ID NO: 50).

GAGACCCACCTGGACATGCTCCGCCACCTCTACCAGGGCTGCCAGGTGGTGCAG
 20 GGAAACCTGGAACTCACCTACCTGCCACCAATGCCAGCCTGTCCTCCTGCAGG
 ATATCCAGGAGGTGCAGGGCTACGTGCTCATCGCTACAACCAAGTGAGGCAGG
 TCCCAC TGCAAGGGCTCGGATTGTGCGAGGCACCCAGCTTTGAGGACAACCA
 TGCCCTGGCCGTGCTAGACAATGGAGACCCGCTGAACAATACCACCCCTGTCACA
 GGGGCCTCCCCAGGAGGCCTGCCGGAGCTGCAGCTCGAAGCCTCACAGAGATC
 25 TTGAAAGGAGGGTCTGATCCAGCGGAACCCCCAGCTCTGCTACCAGGACACG
 ATTTGTGGAAG (SEQ ID NO:50).

[00057] In one embodiment, the complete EC1 human her2/neu fragment spans from (58-979 bp of the human her2/neu gene and is set forth in (SEQ ID NO:54).

GCCGCGAGCACCCAAAGTGTGCACCGGCACAGACATGAAGCTGGCTCCCTGCC
 30 AGTCCCGAGACCCACCTGGACATGCTCCGCCACCTCTACCAGGGCTGCCAGGTGG
 TGCAGGGAAACCTGGAACTCACCTACCTGCCACCAATGCCAGCCTGTCCTCCT
 GCAGGATATCCAGGAGGTGCAGGGCTACGTGCTCATCGCTACAACCAAGTGAG

GCAGGTCCCCTGCAGAGGCTGCGGATTGTGCGAGGCACCCAGCTTTGAGGAC
 AACTATGCCCTGGCCGTGCTAGACAATGGAGACCCGCTGAACAATACCACCCCTG
 TCACAGGGGCCTCCCCAGGAGGCCCTGCGGGAGCTGCAGCTCGAAGCCTCACAG
 AGATCTTGAAAGGAGGGTCTTGATCCAGCGGAACCCCCAGCTCTGCTACCAGGA
 5 CACGATTTGTGGAAGGACATCTTCCACAAGAACAAACCAGCTGGCTCTCACACTG
 ATAGACACCAACCGCTCTCGGGCCTGCCACCCCTGTTCTCGATGTGTAAGGGCT
 CCCGCTGCTGGGGAGAGAGTTCTGAGGATTGTCAGAGCCTGACGCGCACTGTCTG
 TGCCGGTGGCTGTGCCGCTGCAAGGGGCCACTGCCACTGACTGCTGCCATGAG
 CAGTGTGCTGCCGGCTGCACGGGCCAACGCACTCTGACTGCCTGGCCTGCCTCC
 10 ACTTCAACCACAGTGGCATCTGTGAGCTGCACTGCCAGCCCTGGTCACCTACAA
 CACAGACACGTTGAGTCCATGCCAATCCGAGGGCCGGTATACATTGGCGCC
 AGCTGTGTGACTGCCTGTCCCTACAACCTACCTTCTACGGACGTGGATCCTGCAC
 CCTCGTCTGCCCTGCACAACCAAGAGGTGACAGCAGAGGAT (SEQ ID NO:54).

15 [00058] In another embodiment, the nucleic acid sequence encoding the human her2/neu EC2 fragment implemented into the chimera spans from 1077-1554 bp of the human her2/neu EC2 fragment and includes a 50 bp extension, and is set forth in (SEQ ID NO:51).

AATATCCAGGAGTTGCTGGCTGCAAGAACGATCTTGGGAGCCTGGCATTCTGCC
 GGAGAGCTTGATGGGGACCCAGCCTCCAACACTGCCCGCTCCAGCCAGAGCAG
 20 CTCCAAGTGTGAGACTCTGGAAGAGATCACAGGTTACCTATACATCTCAGCATG
 GCCGGACAGCCTGCCTGACCTCAGCGTCTCCAGAACCTGCAAGTAATCCGGGA
 CGAATTCTGCACAATGGCGCCTACTCGCTGACCCCTGCAAGGGCTGGCATCAGCTG
 GCTGGGGCTGCGCTCACTGAGGAACTGGCAGTGGACTGCCCTCATCCACCAT
 AACACCCACCTCTGCTCGACACCGGTGCCCTGGGACCCAGCTCTCGGAACCC
 25 GCACCAAGCTCTGCTCCACACTGCCAACCGGCCAGAGGACGAGTGTGTGGCGAG
 GGCCTGGCCTGCCACCAGCTGCGCCCGAGGG (SEQ ID NO:51).

[00059] In one embodiment, complete EC2 human her2/neu fragment spans from 907-1504 bp of the human her2/neu gene and is set forth in (SEQ ID NO:55).

TACCTTCTACGGACGTGGATCCTGCACCCCTCGTCTGCCCTGCACAACCAAG
 30 AGGTGACAGCAGAGGATGGAACACACAGCGGTGTGAGAAGTGCAGCAAGCCCTGTG
 CCCGAGTGTGCTATGGTCTGGCATGGAGCAGCTGCGAGAGGTGAGGGCAGTTAC
 CAGTGCCAATATCCAGGAGTTGCTGGCTGCAAGAACGATCTTGGGAGCCTGGCA

TTTCTGCCGGAGAGCTTGATGGGGACCCAGCCTCCAACACTGCCCGCTCCAGC
CAGAGCAGCTCCAAGTGTGAGACTCTGGAAGAGATCACAGGTTACCTATACAT
CTCAGCATGGCCGGACAGCCTGCCTGACCTCAGCGTCTCCAGAACCTGCAAGTA
ATCCGGGGACGAATTCTGCACAATGGCGCCTACTCGCTGACCCTGCAAGGGCTGG
5 GCATCAGCTGGCTGGGCTCGCCTCACTGAGGAACTGGCAGTGGACTGGCCCT
CATCCACCATAACACCCACCTCTGCTCGACACGGTGCCCTGGGACCAGCTC
TTTCGGAACCCGCACCAAGCTCTGCTCCACACTGCCAACCGGCCAGAG (SEQ ID
NO:55).

10 [00060] In another embodiment, the nucleic acid sequence encoding the human her2/neu IC1
fragment implemented into the chimera is set forth in (SEQ ID NO:52).

CAGCAGAAGATCCGGAAGTACACGATGCGGAGACTGCTGCAGGAAACGGAGCTG
GTGGAGCCGCTGACACCTAGCGGAGCGATGCCAACCAGGCGCAGATGCGGATC
CTGAAAGAGACGGAGCTGAGGAAGGTGAAGGTGCTGGATCTGGCGCTTTGGC
15 ACAGTCTACAAGGGCATCTGGATCCCTGATGGGGAGAATGTGAAAATTCCAGTG
GCCATCAAAGTGTGAGGGAAAACACATCCCCAAAGCCAACAAAGAAATCTTA
GACGAAGCATACTGATGGCTGGTGTGGGCTCCCCATATGTCTCCGCCTCTGG
GCATCTGCCTGACATCCACGGTGCAGCTGGTACACAGCTTATGCCCTATGGCTG
CCTCTTAGACT (SEQ ID NO:52).

20 [00061] In another embodiment, the nucleic acid sequence encoding the complete human
her2/neu IC1 fragment spans from 2034-3243 of the human her2/neu gene and is set forth in
(SEQ ID NO:56).

CAGCAGAAGATCCGGAAGTACACGATGCGGAGACTGCTGCAGGAAACGGAGCTG
GTGGAGCCGCTGACACCTAGCGGAGCGATGCCAACCAGGCGCAGATGCGGATC
25 CTGAAAGAGACGGAGCTGAGGAAGGTGAAGGTGCTGGATCTGGCGCTTTGGC
ACAGTCTACAAGGGCATCTGGATCCCTGATGGGGAGAATGTGAAAATTCCAGTG
GCCATCAAAGTGTGAGGGAAAACACATCCCCAAAGCCAACAAAGAAATCTTA
GACGAAGCATACTGATGGCTGGTGTGGGCTCCCCATATGTCTCCGCCTCTGG
GCATCTGCCTGACATCCACGGTGCAGCTGGTACACAGCTTATGCCCTATGGCTG
30 CCTCTTAGACCATGTCCGGAAAACCGCGGACGCCTGGCTCCAGGACCTGCTG
AACTGGTGTATGCAGATTGCCAAGGGATGAGCTACCTGGAGGATGTGCGGCTC
GTACACAGGGACTTGGCCGCTCGGAACGTGCTGGTCAAGAGTCCAACCATGTCA

AAATTACAGACTTCGGGCTGGCTCGCTGGACATTGACGAGACAGAGTACCA
TGCAGATGGGGCAAGGTGCCATCAAGTGGATGGCGCTGGAGTCCATTCTCCGC
CGGCAGGTTCACCCACCAGAGTGTGAGTTATGGTGTGACTGTGTGGAGC
TGATGACTTTGGGCAAACCTTACGATGGGATCCCAGCCCCGGAGATCCCTGA
5 CCTGCTGGAAAAGGGGAGCGCTGCCCCAGCCCCCATCTGCACCATTGATGTC
TACATGATCATGGTCAAATGTTGGATGATTGACTCTGAATGTCGGCCAAGATTCC
GGGAGTTGGTGTCTGAATTCTCCCGATGCCAGGGACCCCCAGCGCTTGTGGT
CATCCAGAATGAGGACTTGGCCCAGCCAGTCCCTGGACAGCACCTCTACCGC
TCACTGCTGGAGGACGATGACATGGGGACCTGGATGCTGAGGAGTATCTG
10 GTACCCCAGCAGGGCTTCTCTGTCCAGACCCCTGCCCGCGCTGGGGCATGG
TCCACCACAGGCACCGCAGCTCATCTACCAGGAGTGGCGGTGGGGACCTGACACT
AGGGCTGGAGCCCTCTGAAGAGGAGGCCAGGTCTCCACTGGCACCCCTCCGA
AGGGGCT (SEQ ID NO:56).

15 [00062] The LLO utilized in the methods and compositions provided herein is, in one embodiment, a *Listeria* LLO. In one embodiment, the *Listeria* from which the LLO is derived is *Listeria monocytogenes* (LM). In another embodiment, the *Listeria* is *Listeria ivanovii*. In another embodiment, the *Listeria* is *Listeria welshimeri*. In another embodiment, the *Listeria* is *Listeria seeligeri*. In another embodiment, the LLO protein is a non-*Listerial* LLO protein.
20 In another embodiment, the LLO protein is a synthetic LLO protein. In another embodiment it is a recombinant LLO protein.

[00063] In one embodiment, the LLO protein is encoded by the following nucleic acid sequence set forth in (SEQ ID NO:3)

atgaaaaaaaaataatgttagtttattacacttatattatgttagtctaccaattgcgcacaaactgaagcaaaggatgcattgcattcaataa
25 agaaaattcaatttcatccatggcaccaccaggcatctccgcctgcaagtccataagacgcacatcgaaaagaaacacgcggatgaaatcg
ataagtatatacaggatggattacaataaaaacaatgttagtataccacggagatgcagtgcacaaatgtgcgcacaaaaggtta
caaagatgaaatgaatatattgtgtggagaaaaagaagaaatccatcaatcaaataatgcagacattcaagttgtgaatgcaattcga
gcctaacctatccaggatgcctcgaaaagcgaattcggattagtagaaaatcaaccagatgtccctgtaaaacgtgattcattaacac
30 tcagcattgtattgccaggatgactaatcaagacaataaaatgttaaaaatgccactaatcaacgttaacaacgcagtaatacat
tagtggaaagatggaatgaaaaatgtcaagcttaccaaatgttaagtgcaaaaattgattatgtgacgaaatggcttacagtgaatca
caattaaatgcggaaatttgtacagcattaaagctgtaaataatagctgaatgtaaacttcggcgaatcagtgaagggaaaatgcaaga
agaagtcaatgtttaaacaaatttactataacgtgaatgttaatgaacccatacaagacccatgtttcggcaagctgttactaaagac
agttgcaagcgcgtggagtgaaatgcagaaaatcctctgcataatctcaagtgtggcgtatggccgtcaagttattgaaatttcaacta

attcccatagtaactaaagtaaaagctgtttgatgtgtccgtaaaggcgaaaatctgttcaggtgatgtagaactaacaatcatcaaaa
 attttccttcaaaggcgtaattacggagggtccgcaaaagatgaagttcaaatcatgcacggcaacctcgagacttacgcgatatttga
 aaaaaggcgctactttaatcgagaaacaccaggagttccattgtttatacaacaacttctaaaagacaatgaatttagctgttattaaaa
 acaactcagaatataattgaaacaacttcaaagcttatacagatgaaaaattaacatcgatcactctggaggatacgttgcattcaaca
 5 ttcttggatgaagtaattatgat (SEQ ID NO:3).

[00064] In another embodiment, the LLO protein has the sequence SEQ ID NO:4

M K K I M L V F I T L I L V S L P I A Q Q T E A K D A S A F N K E
 N S I S S M A P P A S P P A S P K T P I E K K H A D E I D K Y I Q G
 L D Y N K N N V L V Y H G D A V T N V P P R K G Y K D G N E Y I
 10 V V E K K K K S I N Q N N A D I Q V V N A I S S L T Y P G A L V K
 A N S E L V E N Q P D V L P V K R D S L T L S I D L P G M T N Q D
 N K I V V K N A T K S N V N N A V N T L V E R W N E K Y A Q A Y
 P N V S A K I D Y D D E M A Y S E S Q L I A K F G T A F K A V N N
 15 S L N V N F G A I S E G K M Q E E V I S F K Q I Y Y N V N V N E P
 T R P S R F F G K A V T K E Q L Q A L G V N A E N P P A Y I S S V
 A Y G R Q V Y L K L S T N S H S T K V K A A F D A A V S G K S V
 S G D V E L T N I I K N S S F K A V I Y G G S A K D E V Q I I D G
 N L G D L R D I L K K G A T F N R E T P G V P I A Y T T N F L K D
 20 N E L A V I K N N S E Y I E T T S K A Y T D G K I N I D H S G G Y
 V A Q F N I S W D E V N Y D (SEQ ID NO:4)

The first 25 amino acids of the proprotein corresponding to this sequence are the signal sequence and are cleaved from LLO when it is secreted by the bacterium. Thus, in this embodiment, the full length active LLO protein is 504 residues long. In another embodiment, 25 the LLO protein has a sequence set forth in GenBank Accession No. DQ054588, DQ054589, AY878649, U25452, or U25452. In another embodiment, the LLO protein is a variant of an LLO protein. In another embodiment, the LLO protein is a homologue of an LLO protein. Each possibility represents a separate embodiment of the present invention.

30 [00065] In another embodiment, “truncated LLO” or “tLLO” refers to a fragment of LLO that comprises the PEST-like domain. In another embodiment, the terms refer to an LLO fragment that does not contain the activation domain at the amino terminus and does not include cystine 484. In another embodiment, the LLO fragment consists of a PEST sequence. In another embodiment, the LLO fragment comprises a PEST sequence. In another embodiment, 35 the LLO fragment consists of about the first 400 to 441 amino acids of the 529 amino acid full-length LLO protein. In another embodiment, the LLO fragment is a non-hemolytic form of the LLO protein.

[00066] In another embodiment of methods and compositions of the present invention, a polypeptide encoded by a nucleic acid sequence of methods and compositions of the present invention is a fusion protein comprising the chimeric Her-2/neu antigen and an additional polypeptide, where in another embodiment, the fusion protein comprises, *inter alia*, an LM 5 non-hemolytic LLO protein (Examples herein).

[00067] In one embodiment, the LLO fragment consists of about residues 1-25. In another embodiment, the LLO fragment consists of about residues 1-50. In another embodiment, the LLO fragment consists of about residues 1-75. In another embodiment, the LLO fragment consists of about residues 1-100. In another embodiment, the LLO fragment consists of about 10 residues 1-125. In another embodiment, the LLO fragment consists of about residues 1-150. In another embodiment, the LLO fragment consists of about residues 1175. In another embodiment, the LLO fragment consists of about residues 1-200. In another embodiment, the LLO fragment consists of about residues 1-225. In another embodiment, the LLO fragment consists of about residues 1-250. In another embodiment, the LLO fragment consists of about 15 residues 1-275. In another embodiment, the LLO fragment consists of about residues 1-300. In another embodiment, the LLO fragment consists of about residues 1-325. In another embodiment, the LLO fragment consists of about residues 1-350. In another embodiment, the LLO fragment consists of about residues 1-375. In another embodiment, the LLO fragment consists of about residues 1-400. In another embodiment, the LLO fragment consists of about 20 residues 1-425. Each possibility represents a separate embodiment of the present invention.

[00068] In another embodiment, a fusion protein of methods and compositions of the present invention comprises a PEST sequence, either from an LLO protein or from another organism, e.g. a prokaryotic organism.

[00069] The PEST-like AA sequence has, in another embodiment, a sequence selected from 25 SEQ ID NO: 5-9. In another embodiment, the PEST-like sequence is a PEST-like sequence from the LM ActA protein. In another embodiment, the PEST-like sequence is KTEEQPSEVNTGPR (SEQ ID NO: 5), KASVTDTSEGDLSSMQSADESTPQPLK (SEQ ID NO: 6), KNEEVNASDFPPPPTDEELR (SEQ ID NO: 7), or RGGIPTSEEFSSLNSGDFTDENSETTEEEIDR (SEQ ID NO: 8). In another embodiment, the PEST-like sequence is from Streptolysin O protein of *Streptococcus sp.* In another embodiment, the PEST-like sequence is from *Streptococcus pyogenes* Streptolysin O, e.g. 30 KQNTASTETTTNEQPK (SEQ ID NO: 9) at AA 35-51. In another embodiment, the PEST-

like sequence is from *Streptococcus equisimilis* Streptolysin O, e.g. KQNTANTETTTNEQPK (SEQ ID NO: 10) at AA 38-54. In another embodiment, the PEST-like sequence is another PEST-like AA sequence derived from a prokaryotic organism. In another embodiment, the PEST-like sequence is any other PEST-like sequence known in the art. Each possibility represents a separate embodiment of the present invention.

[00070] In one embodiment, fusion of an antigen to the PEST-like sequence of LM enhanced cell mediated and anti-tumor immunity of the antigen. Thus, fusion of an antigen to other PEST-like sequences derived from other prokaryotic organisms will also enhance immunogenicity of the antigen. PEST-like sequence of other prokaryotic organism can be identified in accordance with methods such as described by, for example Rechsteiner and Rogers (1996, Trends Biochem. Sci. 21:267-271) for LM. Alternatively, PEST-like AA sequences from other prokaryotic organisms can also be identified based by this method. Other prokaryotic organisms wherein PEST-like AA sequences would be expected to include, but are not limited to, other *Listeria* species. In another embodiment, the PEST-like sequence is embedded within the antigenic protein. Thus, in another embodiment, "fusion" refers to an antigenic protein comprising both the antigen and the PEST-like amino acid sequence either linked at one end of the antigen or embedded within the antigen.

[00071] In another embodiment, provided herein is a vaccine comprising a recombinant polypeptide of the present invention. In another embodiment, provided herein is a vaccine consisting of a recombinant polypeptide of the present invention.

[00072] In another embodiment, provided herein is a nucleotide molecule encoding a recombinant polypeptide of the present invention. In another embodiment, provided herein is a vaccine comprising the nucleotide molecule.

[00073] In another embodiment, provided herein is a nucleotide molecule encoding a recombinant polypeptide of the present invention.

[00074] In another embodiment, provided herein is a recombinant polypeptide encoded by the nucleotide molecule of the present invention.

[00075] In another embodiment, provided herein is a vaccine comprising a nucleotide molecule or recombinant polypeptide of the present invention.

[00076] In another embodiment, provided herein is an immunogenic composition comprising a nucleotide molecule or recombinant polypeptide of the present invention.

[00077] In another embodiment, provided herein is a vector comprising a nucleotide molecule or recombinant polypeptide of the present invention.

5 [00078] In another embodiment, provided herein is a recombinant form of *Listeria* comprising a nucleotide molecule of the present invention.

[00079] In another embodiment, provided herein is a vaccine comprising a recombinant form of *Listeria* of the present invention.

10 [00080] In another embodiment, provided herein is a culture of a recombinant form of *Listeria* of the present invention.

[00081] In one embodiment, the vaccine for use in the methods of the present invention comprises a recombinant *Listeria monocytogenes*, in any form or embodiment as described herein. In one embodiment, the vaccine for use in the present invention consists of a recombinant *Listeria monocytogenes* of the present invention, in any form or embodiment as described herein. In another embodiment, the vaccine for use in the methods of the present invention consists essentially of a recombinant *Listeria monocytogenes* of the present invention, in any form or embodiment as described herein. In one embodiment, the term “comprise” refers to the inclusion of a recombinant *Listeria monocytogenes* in the vaccine, as well as inclusion of other vaccines or treatments that may be known in the art. In another embodiment, the term “consisting essentially of” refers to a vaccine, whose functional component is the recombinant *Listeria monocytogenes*, however, other components of the vaccine may be included that are not involved directly in the therapeutic effect of the vaccine and may, for example, refer to components which facilitate the effect of the recombinant *Listeria monocytogenes* (e.g. stabilizing, preserving, etc.). In another embodiment, the term “consisting” refers to a vaccine, which contains the recombinant *Listeria monocytogenes*.

[00082] In another embodiment, the methods of the present invention comprise the step of administering a recombinant *Listeria monocytogenes*, in any form or embodiment as described herein. In one embodiment, the methods of the present invention consist of the step of administering a recombinant *Listeria monocytogenes* of the present invention, in any form or embodiment as described herein. In another embodiment, the methods of the present invention consist essentially of the step of administering a recombinant *Listeria*

monocytogenes of the present invention, in any form or embodiment as described herein. In one embodiment, the term “comprise” refers to the inclusion of the step of administering a recombinant *Listeria monocytogenes* in the methods, as well as inclusion of other methods or treatments that may be known in the art. In another embodiment, the term “consisting essentially of” refers to a methods, whose functional component is the administration of recombinant *Listeria monocytogenes*, however, other steps of the methods may be included that are not involved directly in the therapeutic effect of the methods and may, for example, refer to steps which facilitate the effect of the administration of recombinant *Listeria monocytogenes*. In one embodiment, the term “consisting” refers to a method of administering recombinant *Listeria monocytogenes* with no additional steps.

[00083] In another embodiment, the *Listeria* of methods and compositions of the present invention is *Listeria monocytogenes*. In another embodiment, the *Listeria* is *Listeria ivanovii*. In another embodiment, the *Listeria* is *Listeria welshimeri*. In another embodiment, the *Listeria* is *Listeria seeligeri*. Each type of *Listeria* represents a separate embodiment of the present invention.

[00084] In one embodiment, the *Listeria* strain of the methods and compositions of the present invention is the ADXS31-164 strain. In another embodiment, ADXS31-164 stimulates the secretion of IFN- γ by the splenocytes from wild type FVB/N mice. Further, the data presented herein show that ADXS31-164 is able to elicit anti-Her2/neu specific immune responses to human epitopes that are located at different domains of the targeted antigen.

[00085] In another embodiment, the present invention provides a recombinant form of *Listeria* comprising a nucleotide molecule encoding a Her-2 chimeric protein or a fragment thereof.

[00086] In one embodiment, the present invention provides a method of inducing an anti-Her-2 immune response in a subject, comprising administering to the subject a recombinant polypeptide comprising an N-terminal fragment of a LLO protein fused to a Her-2 chimeric protein or fused to a fragment thereof, thereby inducing an anti-Her-2 immune response in a subject.

[00087] In one embodiment, the fusion protein of methods and compositions of the present invention comprises an LLO signal sequence from LLO. In another embodiment, the two molecules of the protein (the LLO fragment and the antigen) are joined directly. In another embodiment, the two molecules are joined by a short spacer peptide, consisting of one or

more amino acids. In one embodiment, the spacer has no specific biological activity other than to join the proteins or to preserve some minimum distance or other spatial relationship between them. In another embodiment, the constituent amino acids of the spacer are selected to influence some property of the molecule such as the folding, net charge, or hydrophobicity.

5 In another embodiment, the two molecules of the protein (the LLO fragment and the antigen) are synthesized separately or unfused. In another embodiment, the two molecules of the protein are synthesized separately from the same nucleic acid. In yet another embodiment, the two molecules are individually synthesized from separate nucleic acids. Each possibility represents a separate embodiment of the present invention.

10 [00088] In another embodiment, provided herein is a method of inducing an anti-Her-2 immune response in a subject, comprising administering to the subject a recombinant nucleotide encoding a recombinant polypeptide comprising an N-terminal fragment of a LLO protein fused to a Her-2 chimeric protein or fused to a fragment thereof, thereby inducing an anti-Her-2 immune response in a subject.

15 [00089] In one embodiment, provided herein is a method of eliciting an enhanced immune response to a Her2/neu-expressing tumor in a subject, where in another embodiment the method comprises administering to the subject a composition comprising the recombinant *Listeria* vaccine strain provided herein. In another embodiment, the immune response against the Her-2-expressing tumor comprises an immune response to a subdominant epitope of the 20 Her-2 protein. In another embodiment, the immune response against the Her-2-expressing tumor comprises an immune response to several subdominant epitopes of the Her-2 protein. In another embodiment, the immune response against the Her-2-expressing tumor comprises an immune response to at least 1-5 subdominant epitopes of the Her-2 protein. In another embodiment, the immune response against the Her-2-expressing tumor comprises an immune response to at least 1-10 subdominant epitopes of the Her-2 protein. In another embodiment, the immune response against the Her-2-expressing tumor comprises an immune response to at least 1-17 subdominant epitopes of the Her-2 protein. In another embodiment, the immune response against the Her-2-expressing tumor comprises an immune response to at least 17 25 subdominant epitopes of the Her-2 protein.

30 [00090] Point mutations or amino-acid deletions in the oncogenic protein Her2/neu, have been reported to mediate treatment of resistant tumor cells, when these tumors have been targeted by small fragment *Listeria*-based vaccines or trastuzumab (a monoclonal antibody against an

epitope located at the extracellular domain of the Her2/neu antigen). Described herein is a chimeric Her2/neu based composition which harbors two of the extracellular and one intracellular fragments of Her2/neu antigen showing clusters of MHC-class I epitopes of the oncogene. This chimeric protein, which harbors 3 H2Dq and at least 17 of the mapped human 5 MHC-class I epitopes of the Her2/neu antigen was fused to the first 441 amino acids of the *Listeria-monocytogenes* listeriolysin O protein and expressed and secreted by the *Listeria monocytogenes* attenuated strain *LmddA*.

[00091] Previous reports have shown that when Her2/neu transgenic mice were immunized with *Listeria*-based vaccines expressing and secreting small fragments of the Her2/neu antigen separately (each of which harbored only one H2Dq epitope of the Her2/neu oncogene), Her2/neu over-expressing tumors could escape due to mutations in those epitopes of the Her2/neu antigen targeted by each vaccine (see Singh R, Paterson Y. Immunoediting sculpts tumor epitopes during immunotherapy. *Cancer Res* 2007;67: 1887-92). Demonstrated herein is the unexpected result that when three or more epitopes of the Her2/neu protein are incorporated in a chimeric vaccine, it can eliminate the selection and escape of these tumors by escape mutations. Immunization with the novel Her2/neu chimeric *Listeria* vaccines did not result in any escape mutations that could be associated with point mutations or amino acid deletions in the Her2/neu antigen (see Example 4 herein).

[00092] In one embodiment, provided herein is a method of engineering a *Listeria* vaccine 20 strain to express a Her-2 chimeric protein or recombinant polypeptide expressing the chimeric protein, the method comprising transforming a *Listeria* strain with a nucleic acid molecule. In another embodiment, the nucleic acid molecule comprises a first open reading frame encoding a polypeptide, wherein the polypeptide comprises a Her2/neu chimeric antigen. In another embodiment, the nucleic acid molecule further comprises a second open reading 25 frame encoding a metabolic enzyme, and wherein said metabolic enzyme complements an endogenous gene that is lacking in the chromosome of the recombinant *Listeria* strain, thereby engineering a *Listeria* vaccine strain to express a Her-2 chimeric protein.

[00093] In one embodiment, the methods and compositions provided herein further comprise an adjuvant, where in another embodiment, the adjuvant comprises a granulocyte/macrophage 30 colony-stimulating factor (GM-CSF) protein, a nucleotide molecule encoding a GM-CSF protein, saponin QS21, monophosphoryl lipid A, or an unmethylated CpG-containing oligonucleotide.

[00094] In one embodiment, attenuated *Listeria* strains, such as LM delta-actA mutant (Brundage et al, 1993, Proc. Natl. Acad. Sci., USA, 90:11890-11894), *L. monocytogenes* delta-plcA (Camilli et al, 1991, J. Exp. Med., 173:751-754), or delta-ActA, delta INL-b (Brockstedt et al, 2004, PNAS, 101:13832-13837) are used in the present invention. In 5 another embodiment, attenuated *Listeria* strains are constructed by introducing one or more attenuating mutations, as will be understood by one of average skill in the art when equipped with the disclosure herein. Examples of such strains include, but are not limited to *Listeria* strains auxotrophic for aromatic amino acids (Alexander et al, 1993, Infection and Immunity 10 61:2245-2248) and mutant for the formation of lipoteichoic acids (Abachin et al, 2002, 10 Mol. Microbiol. 43:1-14) and those attenuated by a lack of a virulence gene (see examples herein).

[00095] In another embodiment, the nucleic acid molecule of methods and compositions of the present invention is operably linked to a promoter/regulatory sequence. In another embodiment, the first open reading frame of methods and compositions of the present 15 invention is operably linked to a promoter/regulatory sequence. In another embodiment, the second open reading frame of methods and compositions of the present invention is operably linked to a promoter/regulatory sequence. In another embodiment, each of the open reading frames are operably linked to a promoter/regulatory sequence. Each possibility represents a separate embodiment of the present invention.

20 [00096] The skilled artisan, when equipped with the present disclosure and the methods provided herein, will readily understand that different transcriptional promoters, terminators, carrier vectors or specific gene sequences (e.g. those in commercially available cloning vectors) can be used successfully in methods and compositions of the present invention. As is contemplated in the present invention, these functionalities are provided in, for example, the 25 commercially available vectors known as the pUC series. In another embodiment, non-essential DNA sequences (e.g. antibiotic resistance genes) are removed. Each possibility represents a separate embodiment of the present invention. In another embodiment, a commercially available plasmid is used in the present invention. Such plasmids are available from a variety of sources, for example, Invitrogen (La Jolla, CA), Stratagene (La Jolla, CA), 30 Clontech (Palo Alto, CA), or can be constructed using methods well known in the art.

[00097] Another embodiment is a plasmid such as pCR2.1 (Invitrogen, La Jolla, CA), which is a prokaryotic expression vector with a prokaryotic origin of replication and

promoter/regulatory elements to facilitate expression in a prokaryotic organism. In another embodiment, extraneous nucleotide sequences are removed to decrease the size of the plasmid and increase the size of the cassette that can be placed therein.

[00098] Such methods are well known in the art, and are described in, for example, Sambrook et al. (1989, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York) and Ausubel et al. (1997, *Current Protocols in Molecular Biology*, Green & Wiley, New York).

[00099] Antibiotic resistance genes are used in the conventional selection and cloning processes commonly employed in molecular biology and vaccine preparation. Antibiotic resistance genes contemplated in the present invention include, but are not limited to, gene products that confer resistance to ampicillin, penicillin, methicillin, streptomycin, erythromycin, kanamycin, tetracycline, cloramphenicol (CAT), neomycin, hygromycin, gentamicin and others well known in the art. Each gene represents a separate embodiment of the present invention.

[000100] Methods for transforming bacteria are well known in the art, and include calcium-chloride competent cell-based methods, electroporation methods, bacteriophage-mediated transduction, chemical, and physical transformation techniques (de Boer et al, 1989, *Cell* 56:641-649; Miller et al, 1995, *FASEB J.*, 9:190-199; Sambrook et al. 1989, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, New York; Ausubel et al., 1997, *Current Protocols in Molecular Biology*, John Wiley & Sons, New York; Gerhardt et al., eds., 1994, *Methods for General and Molecular Bacteriology*, American Society for Microbiology, Washington, DC; Miller, 1992, *A Short Course in Bacterial Genetics*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.) In another embodiment, the *Listeria* vaccine strain of the present invention is transformed by electroporation. Each method represents a separate embodiment of the present invention.

[000101] In another embodiment, conjugation is used to introduce genetic material and/or plasmids into bacteria. Methods for conjugation are well known in the art, and are described, for example, in Nikodinovic J et al. (A second generation ssp-derived *Escherichia coli*-*Streptomyces* shuttle expression vector that is generally transferable by conjugation. *Plasmid*. 2006 Nov;56(3):223-7) and Auchtung JM et al (Regulation of a *Bacillus subtilis* mobile genetic element by intercellular signaling and the global DNA damage response. *Proc Natl*

Acad Sci U S A. 2005 Aug 30;102 (35):12554-9). Each method represents a separate embodiment of the present invention.

[000102] “Transforming,” in one embodiment, is used identically with the term “transfecting,” and refers to engineering a bacterial cell to take up a plasmid or other heterologous DNA molecule. In another embodiment, “transforming” refers to engineering a bacterial cell to express a gene of a plasmid or other heterologous DNA molecule. Each possibility represents a separate embodiment of the present invention.

[000103] Plasmids and other expression vectors useful in the present invention are described elsewhere herein, and can include such features as a promoter/regulatory sequence, an origin of replication for gram negative and gram positive bacteria, an isolated nucleic acid encoding a fusion protein and an isolated nucleic acid encoding an amino acid metabolism gene. Further, an isolated nucleic acid encoding a fusion protein and an amino acid metabolism gene will have a promoter suitable for driving expression of such an isolated nucleic acid. Promoters useful for driving expression in a bacterial system are well known in the art, and include bacteriophage lambda, the bla promoter of the beta-lactamase gene of pBR322, and the CAT promoter of the chloramphenicol acetyl transferase gene of pBR325. Further examples of prokaryotic promoters include the major right and left promoters of bacteriophage lambda (PL and PR), the trp, recA, lacZ, lad, and gal promoters of *E. coli*, the alpha-amylase (Ulmanen et al, 1985. J. Bacteriol. 162:176-182) and the S28-specific promoters of *B. subtilis* (Gilman et al, 1984 Gene 32:11- 20), the promoters of the bacteriophages of *Bacillus* (Gryczan, 1982, In: The Molecular Biology of the *Bacilli*, Academic Press, Inc., New York), and *Streptomyces* promoters (Ward et al, 1986, Mol. Gen. Genet. 203:468-478). Additional prokaryotic promoters contemplated in the present invention are reviewed in, for example, Glick (1987, J. Ind. Microbiol. 1:277-282); Cenatiempo, (1986, Biochimie, 68:505-516); and Gottesman, (1984, Ann. Rev. Genet. 18:415-442). Further examples of promoter/regulatory elements contemplated in the present invention include, but are not limited to the *Listerial* prfA promoter, the *Listerial* hly promoter, the *Listerial* p60 promoter and the *Listerial* ActA promoter (GenBank Acc. No. NC_003210) or fragments thereof.

[000104] In another embodiment, a plasmid of methods and compositions of the present invention comprises a gene encoding a fusion protein. In another embodiment, subsequences are cloned and the appropriate subsequences cleaved using appropriate restriction enzymes.

The fragments are then, in another embodiment, ligated to produce the desired DNA sequence. In another embodiment, DNA encoding the antigen is produced using DNA amplification methods, for example polymerase chain reaction (PCR). First, the segments of the native DNA on either side of the new terminus are amplified separately. The 5' end of the 5 one amplified sequence encodes the peptide linker, while the 3' end of the other amplified sequence also encodes the peptide linker. Since the 5' end of the first fragment is complementary to the 3' end of the second fragment, the two fragments (after partial purification, e.g. on LMP agarose) can be used as an overlapping template in a third PCR reaction. The amplified sequence will contain codons, the segment on the carboxy side of the 10 opening site (now forming the amino sequence), the linker, and the sequence on the amino side of the opening site (now forming the carboxyl sequence). The antigen is ligated into a plasmid. Each method represents a separate embodiment of the present invention.

[000105] In another embodiment, the present invention further comprises a phage based chromosomal integration system for clinical applications. A host strain that is auxotrophic for 15 essential enzymes, including, but not limited to, d-alanine racemase will be used, for example Lmdal(-)dat(-). In another embodiment, in order to avoid a “phage curing step,” a phage integration system based on PSA is used (Lauer, et al., 2002 J Bacteriol, 184:4177-4186). This requires, in another embodiment, continuous selection by antibiotics to maintain the 20 integrated gene. Thus, in another embodiment, the current invention enables the establishment of a phage based chromosomal integration system that does not require selection with antibiotics. Instead, an auxotrophic host strain will be complemented.

[000106] The recombinant proteins of the present invention are synthesized, in another embodiment, using recombinant DNA methodology. This involves, in one embodiment, creating a DNA sequence that encodes the fusion protein, placing the DNA in an expression 25 cassette, such as the plasmid of the present invention, under the control of a particular promoter/regulatory element, and expressing the protein. DNA encoding the fusion protein (e.g. non-hemolytic LLO/antigen) of the present invention is prepared, in another embodiment, by any suitable method, including, for example, cloning and restriction of appropriate sequences or direct chemical synthesis by methods such as the phosphotriester 30 method of Narang et al. (1979, Meth. Enzymol. 68: 90-99); the phosphodiester method of Brown et al. (1979, Meth. Enzymol 68: 109-151); the diethylphosphoramide method of Beaucage et al. (1981, Tetra. Lett., 22: 15 1859-1862); and the solid support method of U.S. Pat. No. 4,458,066.

[000107] In another embodiment, chemical synthesis is used to produce a single stranded oligonucleotide. This single stranded oligonucleotide is converted, in various embodiments, into double stranded DNA by hybridization with a complementary sequence, or by polymerization with a DNA polymerase using the single strand as a template. One of skill in the art would recognize that while chemical synthesis of DNA is limited to sequences of about 100 bases, longer sequences can be obtained by the ligation of shorter sequences. In another embodiment, subsequences are cloned and the appropriate subsequences cleaved using appropriate restriction enzymes. The fragments are then ligated to produce the desired DNA sequence.

[000108] In another embodiment, DNA encoding the fusion protein or the recombinant protein of the present invention is cloned using DNA amplification methods such as polymerase chain reaction (PCR). Thus, the gene for non-hemolytic LLO is PCR amplified, using a sense primer comprising a suitable restriction site and an antisense primer comprising another restriction site, e.g. a non-identical restriction site to facilitate cloning. The same is repeated for the isolated nucleic acid encoding an antigen. Ligation of the non-hemolytic LLO and antigen sequences and insertion into a plasmid or vector produces a vector encoding non-hemolytic LLO joined to a terminus of the antigen. The two molecules are joined either directly or by a short spacer introduced by the restriction site.

[000109] In another embodiment, the molecules are separated by a peptide spacer consisting of one or more amino acids, generally the spacer will have no specific biological activity other than to join the proteins or to preserve some minimum distance or other spatial relationship between them. In another embodiment, the constituent AA of the spacer are selected to influence some property of the molecule such as the folding, net charge, or hydrophobicity. In another embodiment, the nucleic acid sequences encoding the fusion or recombinant proteins are transformed into a variety of host cells, including *E. coli*, other bacterial hosts, such as *Listeria*, yeast, and various higher eukaryotic cells such as the COS, CHO and HeLa cells lines and myeloma cell lines. The recombinant fusion protein gene will be operably linked to appropriate expression control sequences for each host. Promoter/regulatory sequences are described in detail elsewhere herein. In another embodiment, the plasmid further comprises additional promoter regulatory elements, as well as a ribosome binding site and a transcription termination signal. For eukaryotic cells, the control sequences will include a promoter and an enhancer derived from e.g. immunoglobulin genes, SV40,

cytomegalovirus, etc., and a polyadenylation sequence. In another embodiment, the sequences include splice donor and acceptor sequences.

[000110] In one embodiment, the term "operably linked" refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. A control sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the control sequences.

[000111] In another embodiment, in order to select for an auxotrophic bacterium comprising the plasmid, transformed auxotrophic bacteria are grown on a media that will select for expression of the amino acid metabolism gene. In another embodiment, a bacteria auxotrophic for D-glutamic acid synthesis is transformed with a plasmid comprising a gene for D-glutamic acid synthesis, and the auxotrophic bacteria will grow in the absence of D-glutamic acid, whereas auxotrophic bacteria that have not been transformed with the plasmid, or are not expressing the plasmid encoding a protein for D-glutamic acid synthesis, will not grow. In another embodiment, a bacterium auxotrophic for D-alanine synthesis will grow in the absence of D-alanine when transformed and expressing the plasmid of the present invention if the plasmid comprises an isolated nucleic acid encoding an amino acid metabolism enzyme for D-alanine synthesis. Such methods for making appropriate media comprising or lacking necessary growth factors, supplements, amino acids, vitamins, antibiotics, and the like are well known in the art, and are available commercially (Becton-Dickinson, Franklin Lakes, NJ). Each method represents a separate embodiment of the present invention.

[000112] In another embodiment, once the auxotrophic bacteria comprising the plasmid of the present invention have been selected on appropriate media, the bacteria are propagated in the presence of a selective pressure. Such propagation comprises growing the bacteria in media without the auxotrophic factor. The presence of the plasmid expressing an amino acid metabolism enzyme in the auxotrophic bacteria ensures that the plasmid will replicate along with the bacteria, thus continually selecting for bacteria harboring the plasmid. The skilled artisan, when equipped with the present disclosure and methods herein will be readily able to scale-up the production of the *Listeria* vaccine vector by adjusting the volume of the media in which the auxotrophic bacteria comprising the plasmid are growing.

[000113] The skilled artisan will appreciate that, in another embodiment, other auxotroph strains and complementation systems are adopted for the use with this invention.

[000114] In one embodiment, provided herein is a method of impeding a growth of a Her-2-expressing tumor in a subject, wherein and in another embodiment, the method comprises the 5 step of administering to the subject a composition comprising the recombinant *Listeria* vaccine strain described herein.

[000115] In another embodiment, provided herein is a method of impeding a growth of a Her-2-expressing tumor in a subject, wherein and in another embodiment, the method comprises the step of administering to the subject a composition comprising the recombinant *Listeria* 10 vaccine strain described herein.

[000116] In another embodiment, provided herein is a method of eliciting an enhanced immune response to a Her2/neu-expressing tumor in a subject, wherein and in another embodiment, the method comprises the step of administering to the subject a composition comprising the recombinant *Listeria* vaccine strain described herein. In yet another embodiment, the immune response against the Her2/neu-expressing tumor comprises an 15 immune response to at least one subdominant epitope of the Her2/neu protein.

[000117] In one embodiment, provided herein is a method of preventing an escape mutation in the treatment of Her2/neu over-expressing tumors, wherein and in another embodiment, the method comprises the step of administering to said subject a composition comprising the 20 recombinant *Listeria* vaccine strain provided herein.

[000118] In another embodiment, provided herein is a method of preventing the onset of a Her2/neu antigen-expressing tumor in a subject, wherein and in another embodiment, the method comprises the step of administering to the subject a composition comprising the recombinant *Listeria* vaccine strain provided herein.

[000119] In one embodiment, provided herein is a method of decreasing the frequency of intra-tumoral T regulatory cells, wherein and in another embodiment, the method comprises the step of administering to the subject a composition comprising the recombinant *Listeria* 25 vaccine strain provided herein.

[000120] In another embodiment, provided herein is a method of decreasing the frequency of 30 intra-tumoral T regulatory cells, wherein and in another embodiment, the method comprises

the step of administering to the subject a composition comprising the recombinant *Listeria* vaccine strain provided herein.

[000121] In one embodiment, provided herein is a method of decreasing the frequency of intra-tumoral myeloid derived suppressor cells, wherein and in another embodiment, the method comprises the step of administering to the subject a composition comprising the recombinant *Listeria* vaccine strain provided herein.

[000122] In another embodiment, provided herein is a method of decreasing the frequency of myeloid derived suppressor cells, wherein and in another embodiment, the method comprises the step of administering to the subject a composition comprising the recombinant *Listeria* vaccine strain provided herein.

[000123] In one embodiment, provided herein a method of preventing the formation of a Her2/neu-expressing tumor in a subject, wherein and in another embodiment, the method comprises the step of administering to the subject a composition comprising the recombinant *Listeria* vaccine strain provided herein.

[000124] In another embodiment, provided herein is a method of preventing the formation of a Her2/neu-expressing tumor in a subject, wherein and in another embodiment, the method comprises the step of administering to the subject a composition comprising the recombinant *Listeria* vaccine strain the provided herein.

[000125] In one embodiment, provided herein is a method of treating a Her2/neu-expressing tumor in a subject, wherein and in another embodiment, the method comprises the step of administering to the subject a composition comprising the recombinant *Listeria* vaccine strain provided herein.

[000126] In one embodiment, provided herein is a method of administering the composition of the present invention. In another embodiment, provided herein is a method of administering the vaccine of the present invention. In another embodiment, provided herein is a method of administering the recombinant polypeptide or recombinant nucleotide of the present invention. In another embodiment, the step of administering the composition, vaccine, recombinant polypeptide or recombinant nucleotide of the present invention is performed with an attenuated recombinant form of *Listeria* comprising the composition, vaccine, recombinant nucleotide or expressing the recombinant polypeptide, each in its own discrete embodiment. In another embodiment, the administering is performed with a different

attenuated bacterial vector. In another embodiment, the administering is performed with a DNA vaccine (e.g. a naked DNA vaccine). In another embodiment, administration of a recombinant polypeptide of the present invention is performed by producing the protein recombinantly, then administering the recombinant protein to a subject. Each possibility 5 represents a separate embodiment of the present invention.

[000127] In another embodiment, the immune response elicited by methods and compositions of the present invention comprises a CD8⁺ T cell-mediated response. In another embodiment, the immune response consists primarily of a CD8⁺ T cell-mediated response. In another embodiment, the only detectable component of the immune response is a CD8⁺ T cell-10 mediated response.

[000128] In another embodiment, the immune response elicited by methods and compositions provided herein comprises a CD4⁺ T cell-mediated response. In another embodiment, the immune response consists primarily of a CD4⁺ T cell-mediated response. In another embodiment, the only detectable component of the immune response is a CD4⁺ T cell-mediated response. In another embodiment, the CD4⁺ T cell-mediated response is accompanied by a measurable antibody response against the antigen. In another embodiment, the CD4⁺ T cell-mediated response is not accompanied by a measurable antibody response 15 against the antigen.

[000129] In another embodiment, the present invention provides a method of inducing a CD8⁺ 20 T cell-mediated immune response in a subject against a subdominant CD8⁺ T cell epitope of an antigen, comprising the steps of (a) fusing a nucleotide molecule encoding the Her2-neu chimeric antigen or a fragment thereof to a nucleotide molecule encoding an N-terminal fragment of a LLO protein, thereby creating a recombinant nucleotide encoding an LLO-antigen fusion protein; and (b) administering the recombinant nucleotide or the LLO-antigen 25 fusion to the subject; thereby inducing a CD8⁺ T cell-mediated immune response against a subdominant CD8⁺ T cell epitope of an antigen.

[000130] In one embodiment, provided herein is a method of increasing intratumoral ratio of 30 CD8+/T regulatory cells, wherein and in another embodiment, the method comprises the step of administering to the subject a composition comprising the recombinant polypeptide, recombinant *Listeria*, or recombinant vector of the present invention.

[000131] In another embodiment, provided herein is a method of increasing intratumoral ratio of CD8+/T regulatory cells, wherein and in another embodiment, the method comprises the step of administering to the subject a composition comprising the recombinant polypeptide, recombinant *Listeria*, or recombinant vector of the present invention.

5 [000132] In another embodiment, the immune response elicited by the methods and compositions provided herein comprises an immune response to at least one subdominant epitope of the antigen. In another embodiment, the immune response does not comprise an immune response to a subdominant epitope. In another embodiment, the immune response consists primarily of an immune response to at least one subdominant epitope. In another 10 embodiment, the only measurable component of the immune response is an immune response to at least one subdominant epitope. Each type of immune response represents a separate embodiment of the present invention.

15 [000133] Methods of measuring immune responses are well known in the art, and include, e.g. measuring suppression of tumor growth, flow cytometry, target cell lysis assays (e.g. chromium release assay), the use of tetramers, and others. Each method represents a separate embodiment of the present invention.

20 [000134] In another embodiment, the present invention provides a method of impeding a growth of a Her-2-expressing tumor in a subject, wherein and in another embodiment, the method comprises administering to the subject a recombinant polypeptide comprising an N-terminal fragment of a LLO protein fused to the Her-2 chimeric protein or a fragment thereof or a recombinant nucleotide encoding the recombinant polypeptide, wherein the subject mounts an immune response against the Her-2-expressing tumor, thereby impeding a growth of a Her-2-expressing tumor in a subject.

25 [000135] In another embodiment, the present invention provides a method of improving an antigenicity of a Her-2 chimeric protein, wherein and in another embodiment, the method comprises the step of fusing a nucleotide encoding an N-terminal fragment of a LLO protein to a nucleotide encoding the Her-2 protein or a fragment thereof to create a recombinant nucleotide, thereby improving an antigenicity of a Her-2 chimeric protein.

30 [000136] In another embodiment, provided herein is a method of improving an antigenicity of a Her-2 chimeric protein, wherein and in another embodiment, the method comprises engineering a *Listeria* strain to express the recombinant nucleotide. In another embodiment, a

different bacterial vector is used to express the recombinant nucleotide. In another embodiment, the bacterial vector is attenuated. In another embodiment, a DNA vaccine (e.g. a naked DNA vaccine) is used to express the recombinant nucleotide. In another embodiment, administration of the LLO-Her-2 chimera fusion peptide encoded by the nucleotide is 5 performed by producing the protein recombinantly, then administering the recombinant protein to a subject. Each possibility represents a separate embodiment of the present invention.

[000137] In one embodiment, the present invention provides a method for "epitope spreading" of a tumor. In another embodiment, the immunization using the compositions and methods 10 provided herein induce epitope spreading onto other tumors bearing antigens other than the antigen carried in the vaccine of the present invention.

[000138] In another embodiment, the dominant epitope or subdominant epitope is dominant or subdominant, respectively, in the subject being treated. In another embodiment, the dominant epitope or subdominant epitope is dominant or subdominant in a population being 15 treated.

[000139] In one embodiment, provided herein is a method of treating, suppressing, or inhibiting a cancer or a tumor growth in a subject by epitope spreading wherein and in another embodiment, said cancer is associated with expression of an antigen or fragment thereof comprised in the composition of the present invention. In another embodiment, the method 20 comprises administering to said subject a composition comprising the recombinant polypeptide, recombinant *Listeria*, or recombinant vector of the present invention. In yet another embodiment, the subject mounts an immune response against the antigen-expressing cancer or the antigen-expressing tumor, thereby treating, suppressing, or inhibiting a cancer or a tumor growth in a subject.

[000140] "Dominant CD8⁺ T cell epitope," in one embodiment, refers to an epitope that is 25 recognized by over 30% of the antigen-specific CD8⁺ T cells that are elicited by vaccination, infection, or a malignant growth with a protein or a pathogen or cancer cell containing the protein. In another embodiment, the term refers to an epitope recognized by over 35% of the antigen-specific CD8⁺ T cells that are elicited thereby. In another embodiment, the term refers 30 to an epitope recognized by over 40% of the antigen-specific CD8⁺ T cells. In another embodiment, the term refers to an epitope recognized by over 45% of the antigen-specific CD8⁺ T cells. In another embodiment, the term refers to an epitope recognized by over 50%

of the antigen-specific CD8⁺ T cells. In another embodiment, the term refers to an epitope recognized by over 55% of the antigen-specific CD8⁺ T cells. In another embodiment, the term refers to an epitope recognized by over 60% of the antigen-specific CD8⁺ T cells. In another embodiment, the term refers to an epitope recognized by over 65% of the antigen-specific CD8⁺ T cells. In another embodiment, the term refers to an epitope recognized by over 70% of the antigen-specific CD8⁺ T cells. In another embodiment, the term refers to an epitope recognized by over 75% of the antigen-specific CD8⁺ T cells. In another embodiment, the term refers to an epitope recognized by over 80% of the antigen-specific CD8⁺ T cells. In another embodiment, the term refers to an epitope recognized by over 85% of the antigen-specific CD8⁺ T cells. In another embodiment, the term refers to an epitope recognized by over 90% of the antigen-specific CD8⁺ T cells. In another embodiment, the term refers to an epitope recognized by over 95% of the antigen-specific CD8⁺ T cells. In another embodiment, the term refers to an epitope recognized by over 96% of the antigen-specific CD8⁺ T cells. In another embodiment, the term refers to an epitope recognized by over 97% of the antigen-specific CD8⁺ T cells. In another embodiment, the term refers to an epitope recognized by over 98% of the antigen-specific CD8⁺ T cells.

[000141] “Subdominant CD8⁺ T cell epitope,” in one embodiment, refers to an epitope recognized by fewer than 30% of the antigen-specific CD8⁺ T cells that are elicited by vaccination, infection, or a malignant growth with a protein or a pathogen or cancer cell containing the protein. In another embodiment, the term refers to an epitope recognized by fewer than 28% of the antigen-specific CD8⁺ T cells. In another embodiment, the term refers to an epitope recognized by over 26% of the antigen-specific CD8⁺ T cells. In another embodiment, the term refers to an epitope recognized by fewer than 24% of the antigen-specific CD8⁺ T cells. In another embodiment, the term refers to an epitope recognized by over 22% of the antigen-specific CD8⁺ T cells. In another embodiment, the term refers to an epitope recognized by fewer than 20% of the antigen-specific CD8⁺ T cells. In another embodiment, the term refers to an epitope recognized by over 18% of the antigen-specific CD8⁺ T cells. In another embodiment, the term refers to an epitope recognized by fewer than 16% of the antigen-specific CD8⁺ T cells. In another embodiment, the term refers to an epitope recognized by over 14% of the antigen-specific CD8⁺ T cells. In another embodiment, the term refers to an epitope recognized by over 12% of the antigen-specific CD8⁺ T cells. In another embodiment, the term refers to an epitope recognized by fewer than 10% of the antigen-specific CD8⁺ T cells. In another embodiment, the term refers to an epitope recognized by over 8% of the antigen-specific CD8⁺ T cells. In another embodiment, the term

refers to an epitope recognized by fewer than 6% of the antigen-specific CD8⁺ T cells. In another embodiment, the term refers to an epitope recognized by fewer than 5% of the antigen-specific CD8⁺ T cells. In another embodiment, the term refers to an epitope recognized by over 4% of the antigen-specific CD8⁺ T cells. In another embodiment, the term 5 refers to an epitope recognized by fewer than 3% of the antigen-specific CD8⁺ T cells. In another embodiment, the term refers to an epitope recognized by fewer than 2% of the antigen-specific CD8⁺ T cells. In another embodiment, the term refers to an epitope recognized by fewer than 1% of the antigen-specific CD8⁺ T cells. In another embodiment, the term refers to an epitope recognized by fewer than 0.5% of the antigen-specific CD8⁺ T 10 cells.

[000142] Each type of the dominant epitope and subdominant epitope represents a separate embodiment of the present invention.

[000143] The antigen in methods and compositions of the present invention is, in one embodiment, expressed at a detectable level on a non-tumor cell of the subject. In another 15 embodiment, the antigen is expressed at a detectable level on at least a certain percentage (e.g. 0.01%, 0.03%, 0.1%, 0.3%, 1%, 2%, 3%, or 5%) of non-tumor cells of the subject. In one embodiment, “non-tumor cell” refers to a cell outside the body of the tumor. In another embodiment, “non-tumor cell” refers to a non-malignant cell. In another embodiment, “non-tumor cell” refers to a non-transformed cell. In another embodiment, the non-tumor cell is a 20 somatic cell. In another embodiment, the non-tumor cell is a germ cell. Each possibility represents a separate embodiment of the present invention.

[000144] “Detectable level” refers, in one embodiment, to a level detectable by a standard assay. In one embodiment, the assay is an immunological assay. In one embodiment, the assay is enzyme-linked immunoassay (ELISA). In another embodiment, the assay is Western blot. In another embodiment, the assay is FACS. It is to be understood by a skilled artisan that 25 any other assay available in the art can be used in the methods provided herein. In another embodiment, a detectable level is determined relative to the background level of a particular assay. Methods for performing each of these techniques are well known to those skilled in the art, and each technique represents a separate embodiment of the present invention.

30 [000145] In one embodiment, vaccination with recombinant antigen-expressing LM induces epitope spreading. In another embodiment, vaccination with LLO-antigen fusions, even outside the context of Her2, induces epitope spreading as well. Each possibility represents a

separate embodiment of the present invention.

[000146] In another embodiment, the present invention provides a method of impeding a growth of an Her-2-expressing tumor in a subject, comprising administering to the subject a recombinant polypeptide comprising an N-terminal fragment of a LLO protein fused to a 5 Her-2 chimeric antigen, wherein the antigen has one or more subdominant CD8⁺ T cell epitopes, wherein the subject mounts an immune response against the antigen-expressing tumor, thereby impeding a growth of an Her-2-expressing tumor in a subject. In another embodiment, the antigen does not contain any of the dominant CD8⁺ T cell epitopes. In another embodiment, provided herein is a method of impeding a growth on a Her-2-expressing tumor in a subject, comprising administering to the subject a recombinant form of 10 *Listeria* comprising a recombinant nucleotide encoding the recombinant polypeptide provided herein.

[000147] In another embodiment, the present invention provides a method for inducing formation of cytotoxic T cells in a host having cancer, comprising administering to the host a 15 composition of the present invention, thereby inducing formation of cytotoxic T cells in a host having cancer.

[000148] In another embodiment, the present invention provides a method of reducing an incidence of cancer, comprising administering a composition of the present invention. In another embodiment, the present invention provides a method of ameliorating cancer, 20 comprising administering a composition of the present invention. Each possibility represents a separate embodiment of the present invention.

[000149] In one embodiment, the composition is administered to the cells of the subject *ex vivo*; in another embodiment, the composition is administered to the cells of a donor *ex vivo*; in another embodiment, the composition is administered to the cells of a donor *in vivo*, then is 25 transferred to the subject. Each possibility represents a separate embodiment of the present invention.

[000150] In one embodiment, the cancer treated by a method of the present invention is breast cancer. In another embodiment, the cancer is an Her2 containing cancer. In another embodiment, the cancer is a melanoma. In another embodiment, the cancer is pancreatic cancer. In another embodiment, the cancer is ovarian cancer. In another embodiment, the 30 cancer is gastric cancer. In another embodiment, the cancer is a carcinomatous lesion of the

pancreas. In another embodiment, the cancer is pulmonary adenocarcinoma. In another embodiment, the cancer is colorectal adenocarcinoma. In another embodiment, the cancer is pulmonary squamous adenocarcinoma. In another embodiment, the cancer is gastric adenocarcinoma. In another embodiment, the cancer is an ovarian surface epithelial neoplasm (e.g. a benign, proliferative or malignant variety thereof). In another embodiment, the cancer is an oral squamous cell carcinoma. In another embodiment, the cancer is non small-cell lung carcinoma. In another embodiment, the cancer is an endometrial carcinoma. In another embodiment, the cancer is a bladder cancer. In another embodiment, the cancer is a head and neck cancer. In another embodiment, the cancer is a prostate carcinoma. Each possibility represents a separate embodiment of the present invention.

[000151] In another embodiment of the methods of the present invention, the subject mounts an immune response against the antigen-expressing tumor or target antigen, thereby mediating the anti-tumor effects.

[000152] In another embodiment, the present invention provides an immunogenic composition for treating cancer, the composition comprising a fusion of a truncated LLO to a Her-2 chimeric protein. In another embodiment, the immunogenic composition further comprises a *Listeria* strain expressing the fusion. Each possibility represents a separate embodiment of the present invention. In another embodiment, the present invention provides an immunogenic composition for treating cancer, the composition comprising a *Listeria* strain expressing a Her-2 chimeric protein.

[000153] In one embodiment, a treatment protocol of the present invention is therapeutic. In another embodiment, the protocol is prophylactic. In another embodiment, the vaccines of the present invention are used to protect people at risk for cancer such as breast cancer or other types of Her2-containing tumors because of familial genetics or other circumstances that predispose them to these types of ailments as will be understood by a skilled artisan. In another embodiment, the vaccines are used as a cancer immunotherapy after debulking of tumor growth by surgery, conventional chemotherapy or radiation treatment. Following such treatments, the vaccines of the present invention are administered so that the CTL response to the tumor antigen of the vaccine destroys remaining metastases and prolongs remission from the cancer. In another embodiment, vaccines of the present invention are used to effect the growth of previously established tumors and to kill existing tumor cells. Each possibility represents a separate embodiment of the present invention.

[000154] In another embodiment, the vaccines and immunogenic compositions utilized in any of the methods described above have any of the characteristics of vaccines and immunogenic compositions of the present invention. Each characteristic represents a separate embodiment of the present invention.

5 [000155] Various embodiments of dosage ranges are contemplated by this invention. In one embodiment, in the case of vaccine vectors, the dosage is in the range of 0.4 LD₅₀/dose. In another embodiment, the dosage is from about 0.4-4.9 LD₅₀/dose. In another embodiment the dosage is from about 0.5-0.59 LD₅₀/dose. In another embodiment the dosage is from about 0.6-0.69 LD₅₀/dose. In another embodiment the dosage is from about 0.7-0.79 LD₅₀/dose. In another embodiment the dosage is about 0.8 LD₅₀/dose. In another embodiment, the dosage is 0.4 LD₅₀/dose to 0.8 of the LD₅₀/dose.

[000156] In another embodiment, the dosage is 10^7 bacteria/dose. In another embodiment, the dosage is 1.5×10^7 bacteria/dose. In another embodiment, the dosage is 2×10^7 bacteria/dose. In another embodiment, the dosage is 3×10^7 bacteria/dose. In another embodiment, the dosage is 4×10^7 bacteria/dose. In another embodiment, the dosage is 6×10^7 bacteria/dose. In another embodiment, the dosage is 8×10^7 bacteria/dose. In another embodiment, the dosage is 1×10^8 bacteria/dose. In another embodiment, the dosage is 1.5×10^8 bacteria/dose. In another embodiment, the dosage is 2×10^8 bacteria/dose. In another embodiment, the dosage is 3×10^8 bacteria/dose. In another embodiment, the dosage is 4×10^8 bacteria/dose. In another embodiment, the dosage is 6×10^8 bacteria/dose. In another embodiment, the dosage is 8×10^8 bacteria/dose. In another embodiment, the dosage is 1×10^9 bacteria/dose. In another embodiment, the dosage is 1.5×10^9 bacteria/dose. In another embodiment, the dosage is 2×10^9 bacteria/dose. In another embodiment, the dosage is 3×10^9 bacteria/dose. In another embodiment, the dosage is 5×10^9 bacteria/dose. In another embodiment, the dosage is 6×10^9 bacteria/dose. In another embodiment, the dosage is 8×10^9 bacteria/dose. In another embodiment, the dosage is 1×10^{10} bacteria/dose. In another embodiment, the dosage is 1.5×10^{10} bacteria/dose. In another embodiment, the dosage is 2×10^{10} bacteria/dose. In another embodiment, the dosage is 3×10^{10} bacteria/dose. In another embodiment, the dosage is 5×10^{10} bacteria/dose. In another embodiment, the dosage is 6×10^{10} bacteria/dose. In another embodiment, the dosage is 8×10^{10} bacteria/dose. In another embodiment, the dosage is 1×10^{11} bacteria/dose. In another embodiment, the dosage is 1.5×10^{11} bacteria/dose. In another embodiment, the dosage is 2×10^{11} bacteria/dose. In another embodiment, the dosage is 3×10^{11} bacteria/dose.

In another embodiment, the dosage is 5×10^{11} bacteria/dose. In another embodiment, the dosage is 6×10^{11} bacteria/dose. In another embodiment, the dosage is 8×10^{11} bacteria/dose. Each possibility represents a separate embodiment of the present invention.

[000157] In one embodiment, a vaccine or immunogenic composition of the present invention 5 is administered alone to a subject. In another embodiment, the vaccine or immunogenic composition is administered together with another cancer therapy. Each possibility represents a separate embodiment of the present invention.

[000158] The recombinant *Listeria* of methods and compositions of the present invention is, in one embodiment, stably transformed with a construct encoding an Her-2 chimeric antigen or 10 an LLO-Her-2 chimeric antigen fusion. In one embodiment, the construct contains a polylinker to facilitate further subcloning. Several techniques for producing recombinant *Listeria* are known.

[000159] In one embodiment, the construct or nucleic acid molecule is integrated into the 15 *Listerial* chromosome using homologous recombination. Techniques for homologous recombination are well known in the art, and are described, for example, in Baloglu S, Boyle SM, et al. (Immune responses of mice to vaccinia virus recombinants expressing either *Listeria monocytogenes* partial listeriolysin or *Brucella abortus* ribosomal L7/L12 protein. Vet Microbiol 2005, 109(1-2): 11-7); and Jiang LL, Song HH, et al., (Characterization of a mutant 20 *Listeria monocytogenes* strain expressing green fluorescent protein. Acta Biochim Biophys Sin (Shanghai) 2005, 37(1): 19-24). In another embodiment, homologous recombination is performed as described in United States Patent No. 6,855,320. In this case, a recombinant LM 25 strain that expresses E7 was made by chromosomal integration of the E7 gene under the control of the hly promoter and with the inclusion of the hly signal sequence to ensure secretion of the gene product, yielding the recombinant referred to as Lm-AZ/E7. In another embodiment, a temperature sensitive plasmid is used to select the recombinants. Each technique represents a separate embodiment of the present invention.

[000160] In another embodiment, the construct or nucleic acid molecule is integrated into the 30 *Listerial* chromosome using transposon insertion. Techniques for transposon insertion are well known in the art, and are described, *inter alia*, by Sun et al. (Infection and Immunity 1990, 58: 3770-3778) in the construction of DP-L967. Transposon mutagenesis has the advantage, in another embodiment, that a stable genomic insertion mutant can be formed but the disadvantage that the position in the genome where the foreign gene has been inserted is

unknown.

[000161] In another embodiment, the construct or nucleic acid molecule is integrated into the *Listerial* chromosome using phage integration sites (Lauer P, Chow MY et al, Construction, characterization, and use of two *Listeria monocytogenes* site-specific phage integration vectors. J Bacteriol 2002;184(15): 4177-86). In certain embodiments of this method, an integrase gene and attachment site of a bacteriophage (e.g. U153 or PSA listeriophage) is used to insert the heterologous gene into the corresponding attachment site, which may be any appropriate site in the genome (e.g. comK or the 3' end of the arg tRNA gene). In another embodiment, endogenous prophages are cured from the attachment site utilized prior to integration of the construct or heterologous gene. In another embodiment, this method results in single-copy integrants. Each possibility represents a separate embodiment of the present invention.

[000162] In another embodiment, one of various promoters is used to express the antigen or fusion protein containing same. In one embodiment, an LM promoter is used, e.g. promoters for the genes hly, actA, plca, plcB and mpl, which encode the *Listerial* proteins hemolysin, actA, phosphotidylinositol-specific phospholipase, phospholipase C, and metalloprotease, respectively. Each possibility represents a separate embodiment of the present invention.

[000163] In another embodiment, methods and compositions of the present invention utilize a homologue of a Her-2 chimeric protein or LLO sequence of the present invention. In another embodiment, the methods and compositions of the present invention utilize a Her-2 chimeric protein from a non-human mammal. The terms "homology," "homologous," etc, when in reference to any protein or peptide, refer in one embodiment, to a percentage of amino acid residues in the candidate sequence that are identical with the residues of a corresponding native polypeptide, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent homology, and not considering any conservative substitutions as part of the sequence identity. Methods and computer programs for the alignment are well known in the art.

[000164] In another embodiment, the term "homology," when in reference to any nucleic acid sequence similarly indicates a percentage of nucleotides in a candidate sequence that are identical with the nucleotides of a corresponding native nucleic acid sequence.

[000165] Homology is, in one embodiment, determined by computer algorithm for sequence

alignment, by methods well described in the art. For example, computer algorithm analysis of nucleic acid sequence homology may include the utilization of any number of software packages available, such as, for example, the BLAST, DOMAIN, BEAUTY (BLAST Enhanced Alignment Utility), GENPEPT and TREMBL packages.

5 [000166] In another embodiment, "homology" refers to identity to a sequence selected from SEQ ID No: 1-4 of greater than 70%. In another embodiment, "homology" refers to identity to a sequence selected from SEQ ID No: 1-4 of greater than 72%. In another embodiment, the identity is greater than 75%. In another embodiment, the identity is greater than 78%. In another embodiment, the identity is greater than 80%. In another embodiment, the identity is greater than 82%. In another embodiment, the identity is greater than 83%. In another embodiment, the identity is greater than 85%. In another embodiment, the identity is greater than 87%. In another embodiment, the identity is greater than 88%. In another embodiment, the identity is greater than 90%. In another embodiment, the identity is greater than 92%. In another embodiment, the identity is greater than 93%. In another embodiment, the identity is greater than 95%. In another embodiment, the identity is greater than 96%. In another embodiment, the identity is greater than 97%. In another embodiment, the identity is greater than 98%. In another embodiment, the identity is greater than 99%. In another embodiment, the identity is 100%. Each possibility represents a separate embodiment of the present invention.

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20 [000167] In another embodiment, homology is determined via determination of candidate sequence hybridization, methods of which are well described in the art (See, for example, "Nucleic Acid Hybridization" Hames, B. D., and Higgins S. J., Eds. (1985); Sambrook et al., 2001, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Press, N.Y.; and Ausubel et al., 1989, Current Protocols in Molecular Biology, Green Publishing Associates and Wiley Interscience, N.Y.). For example methods of hybridization may be carried out under moderate to stringent conditions, to the complement of a DNA encoding a native caspase peptide. Hybridization conditions being, for example, overnight incubation at 42 °C in a solution comprising: 10-20 % formamide, 5 X SSC (150 mM NaCl, 15 mM trisodium citrate), 50 mM sodium phosphate (pH 7. 6), 5 X Denhardt's solution, 10 % dextran sulfate, and 20

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30 μ g/ml denatured, sheared salmon sperm DNA.

[000168] In one embodiment of the present invention, "nucleic acids" refers to a string of at least two base-sugar-phosphate combinations. The term includes, in one embodiment, DNA

and RNA. "Nucleotides" refers, in one embodiment, to the monomeric units of nucleic acid polymers. RNA may be, in one embodiment, in the form of a tRNA (transfer RNA), snRNA (small nuclear RNA), rRNA (ribosomal RNA), mRNA (messenger RNA), anti-sense RNA, small inhibitory RNA (siRNA), micro RNA (miRNA) and ribozymes. The use of siRNA and 5 miRNA has been described (Caudy AA et al, *Genes & Devel* 16: 2491-96 and references cited therein). DNA may be in form of plasmid DNA, viral DNA, linear DNA, or chromosomal DNA or derivatives of these groups. In addition, these forms of DNA and RNA may be single, double, triple, or quadruple stranded. The term also includes, in another embodiment, artificial nucleic acids that may contain other types of backbones but the same bases. In one 10 embodiment, the artificial nucleic acid is a PNA (peptide nucleic acid). PNA contain peptide backbones and nucleotide bases and are able to bind, in one embodiment, to both DNA and RNA molecules. In another embodiment, the nucleotide is oxetane modified. In another embodiment, the nucleotide is modified by replacement of one or more phosphodiester bonds with a phosphorothioate bond. In another embodiment, the artificial nucleic acid contains any 15 other variant of the phosphate backbone of native nucleic acids known in the art. The use of phosphothiorate nucleic acids and PNA are known to those skilled in the art, and are described in, for example, Neilsen PE, *Curr Opin Struct Biol* 9:353-57; and Raz NK et al *Biochem Biophys Res Commun.* 297:1075-84. The production and use of nucleic acids is known to those skilled in art and is described, for example, in *Molecular Cloning*, (2001), Sambrook 20 and Russell, eds. and *Methods in Enzymology: Methods for molecular cloning in eukaryotic cells* (2003) Purchio and G. C. Fareed. Each nucleic acid derivative represents a separate embodiment of the present invention.

[000169] Protein and/or peptide homology for any amino acid sequence listed herein is determined, in one embodiment, by methods well described in the art, including immunoblot 25 analysis, or via computer algorithm analysis of amino acid sequences, utilizing any of a number of software packages available, via established methods. Some of these packages may include the FASTA, BLAST, MPsrch or Scanps packages, and may employ the use of the Smith and Waterman algorithms, and/or global/local or BLOCKS alignments for analysis, for example. Each method of determining homology represents a separate embodiment of the 30 present invention.

[000170] In another embodiment, the present invention provides a kit comprising a reagent utilized in performing a method of the present invention. In another embodiment, the present invention provides a kit comprising a composition, tool, or instrument of the present

invention.

[000171] The terms “contacting” or “administering,” in one embodiment, refer to directly contacting the cancer cell or tumor with a composition of the present invention. In another embodiment, the terms refer to indirectly contacting the cancer cell or tumor with a composition of the present invention. In another embodiment, methods of the present invention include methods in which the subject is contacted with a composition of the present invention after which the composition is brought in contact with the cancer cell or tumor by diffusion or any other active transport or passive transport process known in the art by which compounds circulate within the body. Each possibility represents a separate embodiment of the present invention.

[000172] In another embodiment, the terms “gene” and “recombinant gene” refer to nucleic acid molecules comprising an open reading frame encoding a polypeptide of the invention. Such natural allelic variations can typically result in 1-5% variance in the nucleotide sequence of a given gene. Alternative alleles can be identified by sequencing the gene of interest in a number of different individuals or organisms. This can be readily carried out by using hybridization probes to identify the same genetic locus in a variety of individuals or organisms. Any and all such nucleotide variations and resulting amino acid polymorphisms or variations that are the result of natural allelic variation and that do not alter the functional activity are intended to be within the scope of the invention.

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Pharmaceutical Compositions

[000173] The pharmaceutical compositions containing vaccines and compositions of the present invention are, in another embodiment, administered to a subject by any method known to a person skilled in the art, such as parenterally, paracancerally, transmucosally, transdermally, intramuscularly, intravenously, intra-dermally, subcutaneously, intra-peritonealy, intra-ventricularly, intra-cranially, intra-vaginally or intra-tumorally.

[000174] In another embodiment of the methods and compositions provided herein, the vaccines or compositions are administered orally, and are thus formulated in a form suitable for oral administration, i.e. as a solid or a liquid preparation. Suitable solid oral formulations include tablets, capsules, pills, granules, pellets and the like. Suitable liquid oral formulations include solutions, suspensions, dispersions, emulsions, oils and the like. In another embodiment of the present invention, the active ingredient is formulated in a capsule. In

accordance with this embodiment, the compositions of the present invention comprise, in addition to the active compound and the inert carrier or diluent, a hard gelating capsule.

[000175] In another embodiment, the vaccines or compositions are administered by intravenous, intra-arterial, or intra-muscular injection of a liquid preparation. Suitable liquid 5 formulations include solutions, suspensions, dispersions, emulsions, oils and the like. In one embodiment, the pharmaceutical compositions are administered intravenously and are thus formulated in a form suitable for intravenous administration. In another embodiment, the pharmaceutical compositions are administered intra-arterially and are thus formulated in a form suitable for intra-arterial administration. In another embodiment, the pharmaceutical 10 compositions are administered intra-muscularly and are thus formulated in a form suitable for intra-muscular administration.

[000176] In one embodiment, the term "treating" refers to curing a disease. In another embodiment, "treating" refers to preventing a disease. In another embodiment, "treating" refers to reducing the incidence of a disease. In another embodiment, "treating" refers to 15 ameliorating symptoms of a disease. In another embodiment, "treating" refers to inducing remission. In another embodiment, "treating" refers to slowing the progression of a disease. The terms "reducing", "suppressing" and "inhibiting" refer in another embodiment to lessening or decreasing. Each possibility represents a separate embodiment of the present invention.

20 [000177] The term "about" as used herein means in quantitative terms plus or minus 5%, or in another embodiment plus or minus 10%, or in another embodiment plus or minus 15%, or in another embodiment plus or minus 20%.

[000178] The term "subject" refers in one embodiment to a mammal including a human in need of therapy for, or susceptible to, a condition or its sequelae. The subject may include 25 dogs, cats, pigs, cows, sheep, goats, horses, rats, and mice and humans. The term "subject" does not exclude an individual that is normal in all respects.

[000179] The following examples are presented in order to more fully illustrate the preferred embodiments of the invention. They should in no way be construed, however, as limiting the broad scope of the invention.

EXAMPLES

Materials and Methods

[000180] Oligonucleotides were synthesized by Invitrogen (Carlsbad, CA) and DNA sequencing was done by Genewiz Inc, South Plainfield, NJ. Flow cytometry reagents were 5 purchased from Becton Dickinson Biosciences (BD, San Diego, CA). Cell culture media, supplements and all other reagents, unless indicated, were from Sigma (St. Louise, MO). Her2/neu HLA-A2 peptides were synthesized by EZbiolabs (Westfield, IN). Complete RPMI 1640 (C-RPMI) medium contained 2mM glutamine, 0.1 mM non-essential amino acids, and 1mM sodium pyruvate, 10% fetal bovine serum, penicillin/streptomycin, Hepes (25mM). The 10 polyclonal anti-LLO antibody was described previously and anti-Her2/neu antibody was purchased from Sigma.

Mice and Cell Lines

[000181] All animal experiments were performed according to approved protocols by IACUC at the University of Pennsylvania or Rutgers University. FVB/N mice were purchased from 15 Jackson laboratories (Bar Harbor, ME). The FVB/N Her2/neu transgenic mice, which overexpress the rat Her2/neu onco-protein were housed and bred at the animal core facility at the University of Pennsylvania. The NT-2 tumor cell line expresses high levels of rat Her2/neu protein, was derived from a spontaneous mammary tumor in these mice and grown as described previously. DHFR-G8 (3T3/neu) cells were obtained from ATCC and were 20 grown according to the ATCC recommendations. The EMT6-Luc cell line was a generous gift from Dr. John Ohlfest (University of Minnesota, MN) and was grown in complete C-RPMI medium. Bioluminescent work was conducted under guidance by the Small Animal Imaging Facility (SAIF) at the University of Pennsylvania (Philadelphia, PA).

Listeria constructs and antigen expression

25 [000182] Her2/neu-pGEM7Z was kindly provided by Dr. Mark Greene at the University of Pennsylvania and contained the full-length human Her2/neu (hHer2) gene cloned into the pGEM7Z plasmid (Promega, Madison WI). This plasmid was used as a template to amplify three segments of hHer-2/neu, namely, EC1, EC2, and IC1, by PCR using pfx DNA polymerase (Invitrogen) and the oligos indicated in Table 1.

30 [000183] Table 1: Primers for cloning of Human her-2-Chimera

	DNA sequence	Base pair region	Amino acid region or
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			junctions
Her-2-Chimera (F)	TGAT <u>CTCGAGACCCACCTGGACATGCTC</u> (SEQ ID NO:)	120-510	40-170
HerEC1-EC2F (Junction)	CTACCAGGACACGATTTGTGGAAG-AATATCCA GGAGTTGCTGGCTGC (SEQ ID NO:)	510/1077	170/359
HerEC1-EC2R (Junction)	GCAGCCAGCAA <u>ACTCCTGGATATT-CTTCCACAA</u> AATCGTGTCC <u>TGGTAG</u> (SEQ ID NO:)		
HerEC2-ICIF (Junction)	CTGCCACC <u>AGCTGTGCGCCCGAGGG-</u> CAGCAGAAGATCC <u>GGAAAGTACACGA</u> (SEQ ID NO:)	1554/2034	518/679
HerEC2-ICIR (Junction)	TCGTGTACT <u>TCGGATCTTCTGCTG</u> CC <u>CTCGGGC GCACAGCTGGTGGCAG</u> (SEQ ID NO:)		
Her-2-Chimera (R)	GTGG <u>CCC</u> <u>GGGTCTAGATTAGTCTAAGAGGCAGCCATAGG</u> (SEQ ID NO:)	2034-2424	679-808

[000184] The Her-2/neu chimera construct was generated by direct fusion by the SOEing PCR method and each separate hHer-2/neu segment as templates. Primers are shown in Table 2.

5 [000185] Sequence of primers for amplification of different segments human Her2 regions

	DNA sequence	Base pair region	Amino acid region
Her-2-EC1(F)	<u>CCGCCTCGAGGCCCGAGCACCCAAGTG</u> (SEQ ID NO:)	58-979	20-326
Her-2-EC1(R)	<u>CGCGACTAGTTAATCCTCTGCTGTCACCTC</u> (SEQ ID NO:)		
Her-2-EC2(F)	<u>CCGCCTCGAGTACCTTCTACGGACGTG</u> (SEQ ID NO:)	907-1504	303-501
Her-2-EC2(R)	<u>CGCGACTAGTTACTCTGGCCGGTTGGCAG</u> (SEQ ID NO:)		
Her-2-Her-2-IC1(F)	<u>CCGCCTCGAGCAGCAGAAGATCCGGAAAGTAC</u> (SEQ ID NO:)	2034-3243	679-1081
Her-2-IC1(R)	<u>CGCGACTAGTTAAGCCCCTCGGAGGGTG</u> (SEQ ID NO:)		

[000186] ChHer2 gene was excised from pAdv138 using XhoI and SpeI restriction enzymes, and cloned in frame with a truncated, non-hemolytic fragment of LLO in the *Lmdd* shuttle vector, pAdv134. The sequences of the insert, LLO and *hly* promoter were confirmed by DNA sequencing analysis. This plasmid was electroporated into electro-competent *actA*, *dal*, *dat* mutant *Listeria monocytogenes* strain, *LmddA* and positive clones were selected on Brain Heart infusion (BHI) agar plates containing streptomycin (250 μ g/ml). In some experiments

similar *Listeria* strains expressing hHer2/neu (*Lm*-hHer2) fragments were used for comparative purposes. These have been previously described. In all studies, an irrelevant *Listeria* construct (*Lm*-control) was included to account for the antigen independent effects of *Listeria* on the immune system. *Lm*-controls were based on the same *Listeria* platform as 5 ADXS31-164, but expressed a different antigen such as HPV16-E7 or NY-ESO-1. Expression and secretion of fusion proteins from *Listeria* were tested. Each construct was passaged twice *in vivo*.

Cytotoxicity assay

[000187] Groups of 3-5 FVB/N mice were immunized three times with one week intervals 10 with 1×10^8 colony forming units (CFU) of *Lm*-LLO-ChHer2, ADXS31-164, *Lm*-hHer2 ICI or *Lm*-control (expressing an irrelevant antigen) or were left naïve. NT-2 cells were grown *in vitro*, detached by trypsin and treated with mitomycin C (250 µg/ml in serum free C-RPMI medium) at 37°C for 45 minutes. After 5 washes, they were co-incubated with splenocytes harvested from immunized or naïve animals at a ratio of 1:5 (Stimulator: Responder) for 5 15 days at 37°C and 5% CO₂. A standard cytotoxicity assay was performed using europium labeled 3T3/neu (DHFR-G8) cells as targets according to the method previously described. Released europium from killed target cells was measured after 4 hour incubation using a spectrophotometer (Perkin Elmer, Victor²) at 590 nm. Percent specific lysis was defined as (lysis in experimental group-spontaneous lysis)/(Maximum lysis-spontaneous lysis).

20 Interferon-γ secretion by splenocytes from immunized mice

[000188] Groups of 3-5 FVB/N or HLA-A2 transgenic mice were immunized three times with one week intervals with 1×10^8 CFU of ADXS31-164, a negative *Listeria* control (expressing an irrelevant antigen) or were left naïve. Splenocytes from FVB/N mice were isolated one week after the last immunization and co-cultured in 24 well plates at 5×10^6 cells/well in the 25 presence of mitomycin C treated NT-2 cells in C-RPMI medium. Splenocytes from the HLA-A2 transgenic mice were incubated in the presence of 1µM of HLA-A2 specific peptides or 1µg/ml of a recombinant His-tagged ChHer2 protein, produced in *E. coli* and purified by a nickel based affinity chromatography system. Samples from supernatants were obtained 24 or 72 hours later and tested for the presence of interferon-γ (IFN-γ) using mouse IFN-γ Enzyme- 30 linked immunosorbent assay (ELISA) kit according to manufacturer's recommendations.

Tumor studies in Her2 transgenic animals

[000189] Six weeks old FVB/N rat Her2/neu transgenic mice (9-14/group) were immunized 6 times with 5×10^8 CFU of *Lm*-LLO-ChHer2, ADXS31-164 or *Lm*-control. They were observed twice a week for the emergence of spontaneous mammary tumors, which were measured using an electronic caliper, for up to 52 weeks. Escaped tumors were excised when 5 they reached a size 1cm² in average diameter and preserved in RNAlater at -20°C. In order to determine the effect of mutations in the Her2/neu protein on the escape of these tumors, genomic DNA was extracted using a genomic DNA isolation kit, and sequenced.

Effect of ADXS31-164 on regulatory T cells in spleens and tumors

[000190] Mice were implanted subcutaneously (s.c.) with 1×10^6 NT-2 cells. On days 7, 14 10 and 21, they were immunized with 1×10^8 CFUs of ADXS31-164, *LmddA*-control or left naïve. Tumors and spleens were extracted on day 28 and tested for the presence of CD3⁺/CD4⁺/FoxP3⁺ Tregs by FACS analysis. Briefly, splenocytes were isolated by homogenizing the spleens between two glass slides in C-RPMI medium. Tumors were minced 15 using a sterile razor blade and digested with a buffer containing DNase (12U/ml), and collagenase (2mg/ml) in PBS. After 60 min incubation at RT with agitation, cells were separated by vigorous pipetting. Red blood cells were lysed by RBC lysis buffer followed by several washes with complete RPMI-1640 medium containing 10% FBS. After filtration 20 through a nylon mesh, tumor cells and splenocytes were resuspended in FACS buffer (2% FBS/PBS) and stained with anti-CD3-PerCP-Cy5.5, CD4-FITC, CD25-APC antibodies followed by permeabilization and staining with anti-Foxp3-PE. Flow cytometry analysis was performed using 4-color FACS calibur (BD) and data were analyzed using cell quest software (BD).

Statistical analysis

[000191] The log-rank Chi-Squared test was used for survival data and student's *t*-test for the 25 CTL and ELISA assays, which were done in triplicates. A p-value of less than 0.05 (marked as *) was considered statistically significant in these analyzes. All statistical analysis was done with either Prism software, V.4.0a (2006) or SPSS software, V.15.0 (2006). For all FVB/N rat Her2/neu transgenic studies we used 8-14 mice per group, for all wild-type FVB/N studies we used at least 8 mice per group unless otherwise stated. All studies were repeated at 30 least once except for the long term tumor study in Her2/neu transgenic mouse model.

EXAMPLE 1**GENERATION OF *L. MONOCYTOGENES* STRAINS THAT SECRETE LLO FRAGMENTS FUSED TO Her-2 FRAGMENTS: CONSTRUCTION OF ADXS31-164.**

5 [000192] Construction of the chimeric *Her2/neu* gene (ChHer2) was described previously. Briefly, *ChHer2* gene was generated by direct fusion of two extracellular (aa 40-170 and aa 359-433) and one intracellular fragment (aa 678-808) of the *Her2/neu* protein by SOEing PCR method. The chimeric protein harbors most of the known human MHC class I epitopes of the protein. *ChHer2* gene was excised from the plasmid, pAdv138 (which was used to construct 10 *Lm*-LLO-ChHer2) and cloned into *LmddA* shuttle plasmid, resulting in the plasmid pAdv164 (Figure 1A). There are two major differences between these two plasmid backbones. 1) Whereas pAdv138 uses the chloramphenicol resistance marker (*cat*) for *in vitro* selection of recombinant bacteria, pAdv164 harbors the D-alanine racemase gene (*dal*) from *bacillus subtilis*, which uses a metabolic complementation pathway for *in vitro* selection and *in vivo* 15 plasmid retention in *LmddA* strain which lacks the *dal-dat* genes. This vaccine platform was designed and developed to address FDA concerns about the antibiotic resistance of the engineered *Listeria* vaccine strains. 2) Unlike pAdv138, pAdv164 does not harbor a copy of the *prfA* gene in the plasmid (see sequence below and Fig. 1A), as this is not necessary for *in vivo* complementation of the *Lmdd* strain. The *LmddA* vaccine strain also lacks the *actA* gene 20 (responsible for the intracellular movement and cell-to-cell spread of *Listeria*) so the recombinant vaccine strains derived from this backbone are 100 times less virulent than those derived from the *Lmdd*, its parent strain. *LmddA*-based vaccines are also cleared much faster (in less than 48 hours) than the *Lmdd*-based vaccines from the spleens of the immunized mice. The expression and secretion of the fusion protein tLLO-ChHer2 from this strain was 25 comparable to that of the *Lm*-LLO-ChHer2 in TCA precipitated cell culture supernatants after 8 hours of *in vitro* growth (Figure 1B) as a band of ~104 KD was detected by an anti-LLO antibody using Western Blot analysis. The *Listeria* backbone strain expressing only tLLO was used as negative control.

30 [000193] pAdv164 sequence (7075 base pairs) (see Figure 1):

cgaggagtatactggcttactatgttggcactgatgagggtgtcagtgaagtgttcatgtggcaggagaaaaaggctgcaccgggtgc
gtcagcagaatatgtgatacaggatatacccggttcctcgctcaactgactcgctacgctcggtcgactgcggcgagcggaaatgg
cttacgaacggggcggagattccttgaagatgccaggaagatacttaacagggaaatcgatggggccggcaaaaggccgtttcca
taggcctccgcggccctgacaaggcatcacgaaatctgacgctcaatcgtggggcggaaacccgacaggactataaagataaccaggc
35 gttccccctggggctccctcgctgcgtctccctgccttcggttaccgggtcattccgctgttatggccgcgttgtctcattcca

ggaaaacacatccccaaagccaacaaagaatcttagacgaagcatacgtatggctggctcccatatgtctccgct
tctgggcatctgcctgacatccacggcagctggcagacacagcttatgcctatggctgccttttagactaatctagacccggccact
aactcaacgcttagtggattaaatcccaaagccaaacagaaccagaacagaacaagtaacattggagttagaatgg
aagaagaaaaaagcaatgattcgtgtgaataatgcacgaaatcattgcttatttttaaaaagcgatatactagatataacgaaacaacg
5 aactgaataaagaatacaaaaaaaaagagccacgaccagttaaagcctgagaaactttaactgcgagccattgtattaccaccaatcaat
taaagaagtcgagacccaaaatttgtaaagtatttaattacttattaaatcagatacttaaatatctgtaaacccattatatcgggaaaa
gggattcaagtcttaagaagataccaggcaatcaattaagaaaaacttagttgattgccttttgtgattcaacttgcgtacttct
aactaatttaatttcgtaagaaaaggagaacagctgaatgaatcccttttgttagaaactgtgcctcatgcggctgttaaagtacaat
ttaaaaatagtaaaaattcgctcaatcactaccaagccaggtaaaagtaagggctattttgcgtatgcctaaaaaaaagcatgattgg
10 cgacgtggcgttctgacttccgaagaagcgattcacgaaaatcaagatacattacgcattggacacccaaacgttattcgttatgg
acgtatgcagacgaaaaccgttcatcacactaaaggacattctgaaaacaatttaagacaaatcaataccctttattgatttgcattcaca
cgaaaaaaagaaaactattcagcaagcgatatttaacaacagctattgatttaggtttatgcctacgtaattatcaaattgtataaggttat
caagcatatttttttagaaacgccagtctatgtacttcaaaatcagaatttaatctgtcaaagcagccaaataatctgcggaaaatc
cgagaatatttggaaagtcttgcctgatctacgtcaatcattttggattgtctgtatccaagaacggacaatgttagaatttttg
15 atcccaattaccgtattcttcaagaatggcaagattggctttcaaaacacagataataaggcattactcgtcaagtctacgggaaa
aagcggtacagaaggcaaaaaacaagtagatgaaccctggttaatctttattgcacgaaacgaaatttcaggagaaaagggttttagt
agggcgcaatagcgatattttaccctcttttagcctacttagttcaggctattcaatcgaaacgtgcgaatataatgtttgagtttaata
atcgatttagatcaacccttagaagaaaaaagaagtaatcaaaaattgttagaagtgcctattcagaaaactatcaagggctaatagggat
acattaccattttgcaaagcttgggtatcaagtgatttaaccagtaaagatttttgcgtcaagggctttaaattcaagaaaaaaag
20 aagcgaaacgtcaacgtttcatttgcagaatggaaagaagatttaatggcttatttagcgaaaaaaagcgatgtataacgccttatttag
cgacgaccaaaaaagagatttagagaagtgctaggcattctgaacggacattagataaattgcgtgaaggtactgaagggcaatcagga
aattttcttaagattaaaccaggaaagaaatggcattcaacttgcattgttaatcattgtgtctatcgatcattaaattaaaaaaagaa
gaacgagaaagctatataaaggcgtacagcttgcatttagtacgatacaggctgaaaataaaacccgactatgccattacatttatctatgatacgt
25 gttgttttcttgcgttagcttaattgcattttacctgcaataaaggattttactccattatactccattttccaaaaacatacggg
gaacacggaaactattgtacaggccacctcatgttaatggttcggccttgcattccatggaaatattcatccccctgc
cgcccttattatgtactttgtccggcgatattccatgcgtccagctccaccataattggccatgcggaaatattccggccggcaattttcag
gcgtttcccttcaaggatgcggcccttcaatttgcggagccagccgtccgcattgcctacaggcacccgtcccatgtgtctt
tttccgctgtactcggtccgtactcgatcgctcgcatttgcattgtgcgttttgcggatatttgcggatatttgcggatatttgcggatatttgcgg
30 cgacgctgaaacggatctcgccacatgtcagcagacggcgaaggccatacatgcgtccatgcggatatttgcgtactgcattaaaaaaag
cctttttcagccggatccaggcggcgatgttgcggcgatggaccattagattttacggcagccggatatttgcggatatttgcggatatttgcggatatttgcgg
caaactgcattaaagaaatagectttttcatccgtgtcgaaaaatggtaaaatcccttgcattttaaacggaggggttgcggatatttgcggatatttgcggatatttgcgg
gaatttgcattacgttgcatttgcggatatttgcggatatttgcggatatttgcggatatttgcggatatttgcggatatttgcggatatttgcggatatttgcggatatttgcgg
ttcgtgtggccggctcgtcaagccattcaacagaataacctgttaaggtcagtcactcagcagcgtccatcccttgcacccatgtgtctt

5 ggaaccgcgccaaggccaaatataggcgccitcaatccctttgcgcagtggaaatcgcttcatccaaaatggccacggccaagcatga
agcacctcggtcaagagcagccttgctgttctgcacccatgcggtaggcgttgcttcacaactgcacatcaagtggacatgtca
ccgatatgttttcatattgtcacatttcccttatcgccgacaagtcaattccgccccacgtatctgtaaaaaggtttgctcatggaa
aactcctctttttcagaaaatcccagttacgttaattttgagaattttatattgattaactaagttacccagtttccacctaaa
aaacaaatgatgagataatagctccaaaggctaaaggaggactataccaactattgttaattaa (SED ID NO: 53)

EXAMPLE 2: ADXS31-164 IS AS IMMUNOGENIC AS LM-LLO-ChHER2.

[000194] Immunogenic properties of ADXS31-164 in generating anti-Her2/neu specific cytotoxic T cells were compared to those of the *Lm*-LLO-ChHer2 vaccine in a standard CTL assay. Both vaccines elicited strong but comparable cytotoxic T cell responses toward Her2/neu antigen expressed by 3T3/neu target cells. Accordingly, mice immunized with a 5 *Listeria* expressing only an intracellular fragment of Her2-fused to LLO showed lower lytic activity than the chimeras which contain more MHC class I epitopes. No CTL activity was detected in naïve animals or mice injected with the irrelevant *Listeria* vaccine (Figure 2A). ADXS31-164 was also able to stimulate the secretion of IFN- γ by the splenocytes from wild type FVB/N mice (Figure 2B). This was detected in the culture supernatants of these cells that 10 were co-cultured with mitomycin C treated NT-2 cells, which express high levels of Her2/neu antigen (Figure 5C).

[000195] Proper processing and presentation of the human MHC class I epitopes after immunizations with ADXS31-164 was tested in HLA-A2 mice. Splenocytes from immunized 15 HLA-A2 transgenics were co-incubated for 72 hours with peptides corresponding to mapped HLA-A2 restricted epitopes located at the extracellular (HLYQGCQVV SEQ ID NO: 11 or KIFGSLAFL SEQ ID NO: 12) or intracellular (RLLQETELV SEQ ID NO: 13) domains of the Her2/neu molecule (Figure 2C). A recombinant ChHer2 protein was used as positive control and an irrelevant peptide or no peptide as negative controls. The data from this 20 experiment show that ADXS31-164 is able to elicit anti-Her2/neu specific immune responses to human epitopes that are located at different domains of the targeted antigen.

**EXAMPLE 3: ADXS31-164 WAS MORE EFFICACIOUS THAN LM-LLO-ChHER2
IN PREVENTING THE ONSET OF SPONTANEOUS MAMMARY TUMORS.**

[000196] Anti-tumor effects of ADXS31-164 were compared to those of *Lm*-LLO-ChHer2 in Her2/neu transgenic animals which develop slow growing, spontaneous mammary tumors at 20-25 weeks of age. All animals immunized with the irrelevant *Listeria*-control vaccine developed breast tumors within weeks 21-25 and were sacrificed before week 33. In contrast, 5 *Listeria*-Her2/neu recombinant vaccines caused a significant delay in the formation of the mammary tumors. On week 45, more than 50% of ADXS31-164 vaccinated mice (5 out of 9) were still tumor free, as compared to 25% of mice immunized with *Lm*-LLO-ChHer2. At week 52, 2 out of 8 mice immunized with ADXS31-164 still remained tumor free, whereas all mice from other experimental groups had already succumbed to their disease (Figure 3). These 10 results indicate that despite being more attenuated, ADXS31-164 is more efficacious than *Lm*-LLO-ChHer2 in preventing the onset of spontaneous mammary tumors in Her2/neu transgenic animals.

**EXAMPLE 4: MUTATIONS IN HER2/NEU GENE UPON IMMUNIZATION WITH
ADXS31-164.**

15 [000197] Mutations in the MHC class I epitopes of Her2/neu have been considered responsible for tumor escape upon immunization with small fragment vaccines or trastuzumab (Herceptin), a monoclonal antibody that targets an epitope in the extracellular domain of Her2/neu. To assess this, genomic material was extracted from the escaped tumors in the transgenic animals and sequenced the corresponding fragments of the *neu* gene in tumors 20 immunized with the chimeric or control vaccines. Mutations were not observed within the Her-2/neu gene of any vaccinated tumor samples suggesting alternative escape mechanisms (data not shown).

**EXAMPLE 5: ADXS31-164 CAUSES A SIGNIFICANT DECREASE IN INTRA-
TUMORAL T REGULATORY CELLS.**

25 [000198] To elucidate the effect of ADXS31-164 on the frequency of regulatory T cells in spleens and tumors, mice were implanted with NT-2 tumor cells. Splenocytes and intra-tumoral lymphocytes were isolated after three immunizations and stained for Tregs, which were defined as CD3⁺/CD4⁺/CD25⁺/FoxP3⁺ cells, although comparable results were obtained 30 with either FoxP3 or CD25 markers when analyzed separately. The results indicated that immunization with ADXS31-164 had no effect on the frequency of Tregs in the spleens, as compared to an irrelevant *Listeria* vaccine or the naïve animals (See Figure 4). In contrast, immunization with the *Listeria* vaccines caused a considerable impact on the presence of Tregs in the tumors (Figure 5A). Whereas in average 19.0% of all CD3⁺ T cells in untreated

tumors were Tregs, this frequency was reduced to 4.2% for the irrelevant vaccine and 3.4% for ADXS31-164, a 5-fold reduction in the frequency of intra-tumoral Tregs (Figure 5B). The decrease in the frequency of intra-tumoral Tregs in mice treated with either of the *LmddA* vaccines could not be attributed to differences in the sizes of the tumors. In a representative 5 experiment, the tumors from mice immunized with ADXS31-164 were significantly smaller [mean diameter (mm) \pm SD, 6.71 ± 0.43 , n=5] than the tumors from untreated mice (8.69 ± 0.98 , n=5, p<0.01) or treated with the irrelevant vaccine (8.41 ± 1.47 , n=5, p=0.04), whereas 10 comparison of these last two groups showed no statistically significant difference in tumor size (p=0.73). The lower frequency of Tregs in tumors treated with *LmddA* vaccines resulted in an increased intratumoral CD8/Tregs ratio, suggesting that a more favorable tumor microenvironment can be obtained after immunization with *LmddA* vaccines. However, only the vaccine expressing the target antigen HER2/neu (ADXS31-164) was able to reduce tumor growth, indicating that the decrease in Tregs has an effect only in the presence on antigen-specific responses in the tumor.

15

EXAMPLE 6: NO ESCAPE MUTATIONS WERE INTRODUCED BY LISTERIA VACCINE EXPRESSING HER-2 CHIMERA

[000199] Tumor samples of the mice immunized with different vaccines such as Lm-LLO-138, LmddA164 and irrelevant vaccine Lm-LLO-NY were harvested. The DNA was purified 20 from these samples and the DNA fragments corresponding to Her-2/neu regions IC1, EC1 and EC2 were amplified and were sequenced to determine if there were any immune escape mutations. The alignment of sequence from each DNA was performed using CLUSTALW. The results of the analysis indicated that there were no mutations in the DNA sequences harvested from tumors. The detailed analysis of these sequences is shown below.

25 **Alignment of EC2 (975 -1029 bp of Her-2-neu)**

[000200]

Reference GGTACACAGCTGAGGACCGAACACAGCGTTGTGAGAAATGCAGCAAGCCCTGTGCT
(SEQ ID NO:14)

Lm-LLO-138-2 GGTACACAGCTGAGGACCGAACACAGCGTTGTGAGAAATGCAGCAAGCCCTGTGCT
30 Lm-LLO-138-3 GGTACACAGCTGAGGACCGAACACAGCGTTGTGAGAAATGCAGCAAGCCCTGTGCT
Lm-ddA-164-1 GGTACACAGCTGAGGACCGAACACAGCGTTGTGAGAAATGCAGCAAGCCCTGTGCT
LmddA164-2 GGTACACAGCTGAGGACCGAACACAGCGTTGTGAGAAATGCAGCAAGCCCTGTGCT
Lm-ddA-164-3 GGTACACAGCTGAGGACCGAACACAGCGTTGTGAGAAATGCAGCAAGCCCTGTGCT
35 LmddA164-4 GGTACACAGCTGAGGACCGAACACAGCGTTGTGAGAAATGCAGCAAGCCCTGTGCT
Lm-ddA-164-5 GGTACACAGCTGAGGACCGAACACAGCGTTGTGAGAAATGCAGCAAGCCCTGTGCT
LmddA-164-6 GGTACACAGCTGAGGACCGAACACAGCGTTGTGAGAAATGCAGCAAGCCCTGTGCT

[000201]

Reference

CGAGTGTGCTATGGTCTGGCATGGAGCACCTCGAGGGCGAGGCCATCACCAGTGAC (SEQ ID NO:15)

5 Lm-LLO-138-2

CGAGTGTGCTATGGTCTGGCATGGAGCACCTCGAGGGCGAGGCCATCACCAGTGAC

Lm-LLO-138-3

CGAGTGTGCTATGGTCTGGCATGGAGCACCTCGAGGGCGAGGCCATCACCAGTGAC

Lm-ddA-164-1

10 CGAGTGTGCTATGGTCTGGCATGGAGCACCTCGAGGGCGAGGCCATCACCAGTGAC

LmddA164-2

CGAGTGTGCTATGGTCTGGCATGGAGCACCTCGAGGGCGAGGCCATCACCAGTGAC

Lm-ddA-164-3

CGAGTGTGCTATGGTCTGGCATGGAGCACCTCGAGGGCGAGGCCATCACCAGTGAC

15 LmddA164-4

CGAGTGTGCTATGGTCTGGCATGGAGCACCTCGAGGGCGAGGCCATCACCAGTGAC

Lm-ddA-164-5

CGAGTGTGCTATGGTCTGGCATGGAGCACCTCGAGGGCGAGGCCATCACCAGTGAC

LmddA-164-6

20 CGAGTGTGCTATGGTCTGGCATGGAGCACCTCGAGGGCGAGGCCATCACCAGTGAC

[000202]

Reference

AATGCCAGGAGTTGATGGCTGCAAGAAGATCTTGGAGCCTGGCATTGGCCGGAG (SEQ ID NO:16)

25 Lm-LLO-138-2

AATGCCAGGAGTTGATGGCTGCAAGAAGATCTTGGAGCCTGGCATTGGCCGGAG

Lm-LLO-138-3

AATGCCAGGAGTTGATGGCTGCAAGAAGATCTTGGAGCCTGGCATTGGCCGGAG

Lm-ddA-164-1

30 AATGCCAGGAGTTGATGGCTGCAAGAAGATCTTGGAGCCTGGCATTGGCCGGAG

LmddA164-2

AATGCCAGGAGTTGATGGCTGCAAGAAGATCTTGGAGCCTGGCATTGGCCGGAG

Lm-ddA-164-3

AATGCCAGGAGTTGATGGCTGCAAGAAGATCTTGGAGCCTGGCATTGGCCGGAG

35 LmddA164-4

AATGCCAGGAGTTGATGGCTGCAAGAAGATCTTGGAGCCTGGCATTGGCCGGAG

Lm-ddA-164-5

AATGCCAGGAGTTGATGGCTGCAAGAAGATCTTGGAGCCTGGCATTGGCCGGAG

LmddA-164-6

40 AATGCCAGGAGTTGATGGCTGCAAGAAGATCTTGGAGCCTGGCATTGGCCGGAG

[000203]

Reference AGCTTGATGGGACCCCTCCGGCATTGCTCCGCTGAGGCCTGAGCAGCTCCAAGTG
 (SEQ ID No:17)

5 Lm-LLO-138-2
 AGCTTGATGGGACCCCTCCGGCATTGCTCCGCTGAGGCCTGAGCAGCTCCAAGTG
 Lm-LLO-138-3
 AGCTTGATGGGACCCCTCCGGCATTGCTCCGCTGAGGCCTGAGCAGCTCCAAGTG
 Lm-ddA-164-1
 AGCTTGATGGGACCCCTCCGGCATTGCTCCGCTGAGGCCTGAGCAGCTCCAAGTG
 LmddA164-2
 10 AGCTTGATGGGACCCCTCCGGCATTGCTCCGCTGAGGCCTGAGCAGCTCCAAGTG
 Lm-ddA-164-3
 AGCTTGATGGGACCCCTCCGGCATTGCTCCGCTGAGGCCTGAGCAGCTCCAAGTG
 LmddA164-4
 AGCTTGATGGGACCCCTCCGGCATTGCTCCGCTGAGGCCTGAGCAGCTCCAAGTG
 15 Lm-ddA-164-5
 AGCTTGATGGGACCCCTCCGGCATTGCTCCGCTGAGGCCTGAGCAGCTCCAAGTG
 LmddA-164-6
 AGCTTGATGGGACCCCTCCGGCATTGCTCCGCTGAGGCCTGAGCAGCTCCAAGTG
 [000204]

20 Reference
 TTCGAAACCTGGAGGAGATCACAGGTTACCTGTACATCTCAGCATGGCCAGACAGTCTC (SEQ ID
 NO: 18)
 Lm-LLO-138-2
 TTCGAAACCTGGAGGAGATCACAGGTTACCTGTACATCTCAGCATGGCCAGACAGTCTC
 25 Lm-LLO-138-3
 TTCGAAACCTGGAGGAGATCACAGGTTACCTGTACATCTCAGCATGGCCAGACAGTCTC
 Lm-ddA-164-1
 TTCGAAACCTGGAGGAGATCACAGGTTACCTGTACATCTCAGCATGGCCAGACAGTCTC
 LmddA164-2
 30 TTCGAAACCTGGAGGAGATCACAGGTTACCTGTACATCTCAGCATGGCCAGACAGTCTC
 Lm-ddA-164-3
 TTCGAAACCTGGAGGAGATCACAGGTTACCTGTACATCTCAGCATGGCCAGACAGTCTC
 LmddA164-4
 TTCGAAACCTGGAGGAGATCACAGGTTACCTGTACATCTCAGCATGGCCAGACAGTCTC
 35 Lm-ddA-164-5
 TTCGAAACCTGGAGGAGATCACAGGTTACCTGTACATCTCAGCATGGCCANACAGTCTC
 LmddA-164-6
 TTCGAAACCTGGAGGAGATCACAGGTTACCTGTACATCTCAGCATGGCCAGACAGTCTC
 [000205]

40 Reference
 CGTGACCTCAGTGTCTCCAGAACCTCGAATCATTGGGGACGGATTCTCCACGATGGC (SEQ ID

NO: 19)

Lm-LLO-138-2

CGTGACCTCAGTGTCTCCAGAACCTCGAATCATTGGGGACGGATTCTCCACGATGGC

Lm-LLO-138-3

5 CGTGACCTCAGTGTCTCCAGAACCTCGAATCATTGGGGACGGATTCTCCACGATGGC

Lm-ddA-164-1

CGTGACCTCAGTGTCTCCAGAACCTCGAATCATTGGGGACGGATTCTCCACGATGGC

LmddA164-2

CGTGACCTCAGTGTCTCCAGAACCTCGAATCATTGGGGACGGATTCTCCACGATGGC

10 Lm-ddA-164-3

CGTGACCTCAGTGTCTCCAGAACCTCGAATCATTGGGGACGGATTCTCCACGATGGC

LmddA164-4

CGTGACCTCAGTGTCTCCAAAACCTCGAATCATTGGGGACGGATTCTCCACGATGGC

Lm-ddA-164-5

15 CGTGACCTCAGTGTCTCCAAAACCTCGAATCATTGGGGACGGATTCTCCACGATGGC

LmddA-164-6

CGTGACCTCAGTGTCTCCAAAACCTCGAATCATTGGGGACGGATTCTCCACGATGGC

[000206]

Reference

20 GCGTACTCATTGACACTGCAAGGCCTGGGATCCACTCGCTGGGCTGCGCTCACTGCGG (SEQ ID NO: 20)

Lm-LLO-138-2

GCGTACTCATTGACACTGCAAGGCCTGGGATCCACTCGCTGGGCTGCGCTCACTGCGG

Lm-LLO-138-3

25 GCGTACTCATTGACACTGCAAGGCCTGGGATCCACTCGCTGGGCTGCGCTCACTGCGG

Lm-ddA-164-1

GCGTACTCATTGACACTGCAAGGCCTGGGATCCACTCGCTGGGCTGCGCTCACTGCGG

LmddA164-3

GCGTACTCATTGACACTGCAAGGCCTGGGATCCACTCGCTGGGCTGCGCTCACTGCGG

30 Lm-ddA-164-5

GCGTACTCATTGACACTGCAAGGCCTGGGATCCACTCGCTGGGCTGCGCTCACTGCGG

Lm-ddA-164-6

GCGTACTCATTGACACTGCAAGGCCTGGGATCCACTCGCTGGGCTGCGCTCACTGCGG

[000207]

35 Reference GAGCTGGCAGTGGATTGGCTCTGATTACCGCAACGCCCATCTCTGTTGTACACACT (SEQ ID NO: 21)

Lm-LLO-138-2

GAGCTGGCAGTGGATTGGCTCTGATTACCGCAACGCCCATCTCTGTTGTACACACT

Lm-LLO-138-3

40 GAGCTGGCAGTGGATTGGCTCTGATTACCGCAACGCCCATCTCTGTTGTACACACT

Lm-ddA-164-1

GAGCTGGGCAGTGGATTGGCTCTGATTCACCGAACGCCATCTCTGTTGTACACACT

LmddA164-3

GAGCTGGGCAGTGGATTGGCTCTGATTCACCGAACGCCATCTCTGTTGTACACACT

Lm-ddA-164-5

5 GAGCTGGGCAGTGGATTGGCTCTGATTCACCGAACGCCATCTCTGTTGTACACACT

Lm-ddA-164-6

GAGCTGGGCAGTGGATTGGCTCTGATTCACCGAACGCCATCTCTGTTGTACACACT

[000208]

Reference

10 GTACCTTGGGACCAGCTTCCGGAACCCACATCAGGCCCTGCTCCACAGTGGAACCGG (SEQ ID NO: 22)

Lm-LLO-138-2

GTACCTTGGGACCAGCTTCCGGAACCCACATCAGGCCCTGCTCCACAGTGGAACCGG

Lm-LLO-138-3

15 GTACCTTGGGACCAGCTTCCGGAACCCACATCAGGCCCTGCTCCACAGTGGAACCGG

Lm-ddA-164-1

GTACCTTGGGACCAGCTTCCGGAACCCACATCAGGCCCTGCTCCACAGTGGAACCGG

LmddA164-3

GTACCTTGGGACCAGCTTCCGGAACCCACATCAGGCCCTGCTCCACAGTGGAACCGG

20 Lm-ddA-164-5

GTACCTTGGGACCAGCTTCCGGAACCCACATCAGGCCCTGCTCCACAGTGGAACCGG

Lm-ddA-164-6

GTACCTTGGGACCAGCTTCCGGAACCCACATCAGGCCCTGCTCCACAGTGGAACCGG

[000209]

25 Reference

CCGGAAGAGGATTGTGGTCTCGAGGGCTTGGTCTGTAACTCACTGTGTGCCACGGGCAC (SEQ ID NO: 23)

Lm-LLO-138-2

CCGGAAGAGGATTGTGGTCTCGAGGGCTTGGTCTGTAACTCACTGTGTGCCACGGGCAC

30 Lm-LLO-138-3

CCGGAAGAGGATTGTGGTCTCGAGGGCTTGGTCTGTAACTCACTGTGTGCCACGGGCAC

Lm-ddA-164-1

CCGGAAGAGGATTGTGGTCTCGAGGGCTTGGTCTGTAACTCACTGTGTGCCACGGGCAC

LmddA164-3

35 CCGGAAGAGGATTGTGGTCTCGAGGGCTTGGTCTGTAACTCACTGTGTGCCACGGGCAC

Lm-ddA-164-6

CCGGAAGAGGATTGTGGTCTCGAGGGCTTGGTCTGTAACTCACTGTGTGCCACGGGCAC

[000210]

Reference

40 TGCTGGGGCCAGGGCCCACCCAGTGTGTCAACTGCAGTCATTCCCTGGGCCAGGAG (SEQ ID NO: 24)

Lm-LLO-138-2

TGCTGGGGCCAGGGCCCACCCAGTGTCAACTGCAGTCATTCCCTCGGGGCCAGGAG

Lm-LLO-138-3

TGCTGGGGCCAGGGCCCACCCAGTGTCAACTGCAGTCATTCCCTCGGGGCCAGGAG

5 Lm-ddA-164-1

TGCTGGGGCCAGGGCCCACCCAGTGTCAACTGCAGTCATTCCCTCGGGGCCAGGAG

LmddA164-3

TGCTGGGGCCAGGGCCCACCCAGTGTCAACTGCAGTCATTCCCTCGGGGCCAGGAG

Lm-ddA-164-6 TGCTGGGGCCAGGGCCCACCCCA-----

10 **Alignment of IC1 (2114-3042 bp of Her-2-neu)**

[000211]

Reference CGCCCAGCGGAGCAATGCCAACCAACCAGGCTCAGATGCGGATCCTAAAAGAGACGGAGC
(SEQ ID NO: 25)

Lm-LLO-NY-2

15 CGCCCAGCGGAGCAATGCCAACCAACCAGGCTCAGATGCGGATCCTAAAAGAGACGGAGC

Lm-LLO-138-4

CGCCCAGCGGAGCAATGCCAACCAACCAGGCTCAGATGCGGATCCTAAAAGAGACGGAGC

Lm-ddA-164-2

CGCCCAGCGGAGCAATGCCAACCAACCAGGCTCAGATGCGGATCCTAAAAGAGACGGAGC

20 Lm-ddA-164-3

CGCCCAGCGGAGCAATGCCAACCAACCAGGCTCAGATGCGGATCCTAAAAGAGACGGAGC

Lm-ddA164-6

CGCCCAGCGGAGCAATGCCAACCAACCAGGCTCAGATGCGGATCCTAAAAGAGACGGAGC

[000212]

25 Reference

TAAGGAAGGTGAAGGTGCTGGATCAGGAGCTTGGCACTGTCTACAAGGGCATCTGGA (SEQ ID NO: 26)

Lm-LLO-NY-1

TAAGGAAGGTGAAGGTGCTGGATCAGGAGCTTGGCACTGTCTACAAGGGCATCTGGA

30 Lm-LLO-NY-2

TAAGGAAGGTGAAGGTGCTGGATCAGGAGCTTGGCACTGTCTACAAGGGCATCTGGA

Lm-LLO-138-1

TAAGGAAGGTGAACGTGCTGGATCAGGAGCTTGGCACTGTCTACAAGGGCATCTGGA

Lm-LLO-138-2

35 TAAGGAAGGTGAAGGTGCTGGATCAGGAGCTTGGCACTGTCTACAAGGGCATCTGGA

Lm-LLO-138-3

TAAGGAAGGTGAAGGTGCTGGATCAGGAGCTTGGCACTGTCTACAAGGGCATCTGGA

Lm-LLO-138-4

TAAGGAAGGTGAAGGTGCTGGATCAGGAGCTTGGCACTGTCTACAAGGGCATCTGGA

40 Lm-ddA-164-1

TAAGGAAGGTGAAGGTGCTGGATCAGGAGCTTGGCACTGTCTACAAGGGCATCTGGA

Lm-ddA-164-2

TAAGGAAGGTGAAGGTGCTGGATCAGGAGCTTGGCACTGTCTACAAGGGCATCTGGA

Lm-ddA-164-3

TAAGGAAGGTGAAGGTGCTGGATCAGGAGCTTGGCACTGTCTACAAGGGCATCTGGA

5 Lm-ddA-164-4

TAAGGAAGGTGAAGGTGCTGGATCAGGAGCTTGGCACTGTCTACAAGGGCATCTGGA

Lm-ddA-164-5

TAAGGAAGGTGAAGGTGCTGGATCAGGAGCTTGGCACTGTCTACAAGGGCATCTGGA

Lm-ddA164-6

10 TAAGGAAGGTGAAGGTGCTGGATCAGGAGCTTGGCACTGTCTACAAGGGCATCTGGA

[000213]

Reference

TCCCAGATGGGGAGAATGTGAAAATCCCCGTGGCTATCAAGGTGTTGAGAGAAAACACAT (SEQ ID NO: 27)

15 Lm-LLO-NY-1

TCCCAGATGGGGAGAATGTGAAAATCCCCGTGGCTATCAAGGTGTTGAGAGAAAACACAT

Lm-LLO-NY-2

TCCCAGATGGGGAGAATGTGAAAATCCCCGTGGCTATCAAGGTGTTGAGAGAAAACACAT

Lm-LLO-138-1

20 TCCCAGATGGGGAGAATGTGAAAATCCCCGTGGCTATCAAGGTGTTGAGAGAAAACACAT

Lm-LLO-138-2

TCCCAGATGGGGAGAATGTGAAAATCCCCGTGGCTATCAAGGTGTTGAGAGAAAACACAT

Lm-LLO-138-3

TCCCAGATGGGGAGAATGTGAAAATCCCCGTGGCTATCAAGGTGTTGAGAGAAAACACAT

25 Lm-LLO-138-4

TCCCAGATGGGGAGAATGTGAAAATCCCCGTGGCTATCAAGGTGTTGAGAGAAAACACAT

Lm-ddA-164-1

TCCCAGATGGGGAGAATGTGAAAATCCCCGTGGCTATCAAGGTGTTGAGAGAAAACACAT

Lm-ddA-164-2

30 TCCCAGATGGGGAGAATGTGAAAATCCCCGTGGCTATCAAGGTGTTGAGAGAAAACACAT

Lm-ddA-164-3

TCCCAGATGGGGAGAATGTGAAAATCCCCGTGGCTATCAAGGTGTTGAGAGAAAACACAT

Lm-ddA-164-4

TCCCAGATGGGGAGAATGTGAAAATCCCCGTGGCTATCAAGGTGTTGAGAGAAAACACAT

35 Lm-ddA-164-5

TCCCAGATGGGGAGAATGTGAAAATCCCCGTGGCTATCAAGGTGTTGAGAGAAAACACAT

Lm-ddA164-6

TCCCAGATGGGGAGAATGTGAAAATCCCCGTGGCTATCAAGGTGTTGAGAGAAAACACAT

[000214]

Reference

CTCCTAAAGCCAACAAAGAAATTCTAGATGAAGCGTATGTGATGGCTGGTGGGTTCTC (SEQ ID NO: 28)

Lm-LLO-NY-1

5 CTCCTAAAGCCAACAAAGAAATTCTAGATGAAGCGTATGTGATGGCTGGTGGGTTCTC

Lm-LLO-NY-2

CTCCTAAAGCCAACAAAGAAATTCTAGATGAAGCGTATGTGATGGCTGGTGGGTTCTC

Lm-LLO-138-1

CTCCTAAAGCCAACAAAGAAATTCTAGATGAAGCGTATGTGATGGCTGGTGGGTTCTC

10 Lm-LLO-138-2

CTCCTAAAGCCAACAAAGAAATTCTAGATGAAGCGTATGTGATGGCTGGTGGGTTCTC

Lm-LLO-138-3

CTCCTAAAGCCAACAAAGAAATTCTAGATGAAGCGTATGTGATGGCTGGTGGGTTCTC

Lm-LLO-138-4

15 CTCCTAAAGCCAACAAAGAAATTCTAGATGAAGCGTATGTGATGGCTGGTGGGTTCTC

Lm-ddA-164-1

CTCCTAAAGCCAACAAAGAAATTCTAGATGAAGCGTATGTGATGGCTGGTGGGTTCTC

Lm-ddA-164-2

CTCCTAAAGCCAACAAAGAAATTCTAGATGAAGCGTATGTGATGGCTGGTGGGTTCTC

20 Lm-ddA-164-3

CTCCTAAAGCCAACAAAGAAATTCTAGATGAAGCGTATGTGATGGCTGGTGGGTTCTC

Lm-ddA-164-4

CTCCTAAAGCCAACAAAGAAATTCTAGATGAAGCGTATGTGATGGCTGGTGGGTTCTC

Lm-ddA-164-5

25 CTCCTAAAGCCAACAAAGAAATTCTAGATGAAGCGTATGTGATGGCTGGTGGGTTCTC

Lm-ddA-164-6

CTCCTAAAGCCAACAAAGAAATTCTAGATGAAGCGTATGTGATGGCTGGTGGGTTCTC

[000215]

Reference

30 CGTATGTGTCCCCTGGCATCTGCCTGACATCCACAGTACAGCTGGTACACAGC (SEQ ID NO: 29)

Lm-LLO-NY-1

CGTATGTGTCCCCTGGCATCTGCCTGACATCCACAGTACAGCTGGTACACAGC

Lm-LLO-NY-2

35 CGTATGTGTCCCCTGGCATCTGCCTGACATCCACAGTACAGCTGGTACACAGC

Lm-LLO-138-1

CGTATGTGTCCCCTGGCATCTGCCTGACATCCACAGTACAGCTGGTACACAGC

Lm-LLO-138-2

CGTATGTGTCCCCTGGCATCTGCCTGACATCCACAGTACAGCTGGTACACAGC

40 Lm-LLO-138-3

CGTATGTGTCCCCTGGCATCTGCCTGACATCCACAGTACAGCTGGTACACAGC

Lm-LLO-138-4
CGTATGTGTCCCCTGGCATCTGCCTGACATCCACAGTACAGCTGGTACACAGC
Lm-ddA-164-1
CGTATGTGTCCCCTGGCATCTGCCTGACATCCACAGTACAGCTGGTACACAGC
5 Lm-ddA-164-2
CGTATGTGTCCCCTGGCATCTGCCTGACATCCACAGTACAGCTGGTACACAGC
Lm-ddA-164-3
CGTATGTGTCCCCTGGCATCTGCCTGACATCCACAGTACAGCTGGTACACAGC
Lm-ddA-164-4
10 CGTATGTGTCCCCTGGCATCTGCCTGACATCCACAGTACAGCTGGTACACAGC
Lm-ddA-164-5
CGTATGTGTCCCCTGGCATCTGCCTGACATCCACAGTACAGCTGGTACACAGC
Lm-ddA-164-6
CGTATGTGTCCCCTGGCATCTGCCTGACATCCACAGTACAGCTGGTACACAGC
15 [000216]
Reference
TTATGCCCTACGGCTGCCTCTGGACCATGTCCGAGAACACCCGAGGTCGCCTAGGCTCCC (SEQ ID NO: 30)
Lm-LLO-NY-1
20 TTATGCCCTACGGCTGCCTCTGGACCATGTCCGAGAACACCCGAGGTCGCCTAGGCTCCC
Lm-LLO-NY-2
TTATGCCCTACGGCTGCCTCTGGACCATGTCCGAGAACACCCGAGGTCGCCTAGGCTCCC
Lm-LLO-138-1
TTATGCCCTACGGCTGCCTCTGGACCATGTCCGAGAACACCCGAGGTCGCCTAGGCTCCC
25 Lm-LLO-138-2
TTATGCCCTACGGCTGCCTCTGGACCATGTCCGAGAACACCCGAGGTCGCCTAGGCTCCC
Lm-LLO-138-3
TTATGCCCTACGGCTGCCTCTGGACCATGTCCGAGAACACCCGAGGTCGCCTAGGCTCCC
Lm-LLO-138-4
30 TTATGCCCTACGGCTGCCTCTGGACCATGTCCGAGAACACCCGAGGTCGCCTAGGCTCCC
Lm-ddA-164-1
TTATGCCCTACGGCTGCCTCTGGACCATGTCCGAGAACACCCGAGGTCGCCTAGGCTCCC
Lm-ddA-164-2
TTATGCCCTACGGCTGCCTCTGGACCATGTCCGAGAACACCCGAGGTCGCCTAGGCTCCC
35 Lm-ddA-164-3
TTATGCCCTACGGCTGCCTCTGGACCATGTCCGAGAACACCCGAGGTCGCCTAGGCTCCC
Lm-ddA-164-4
TTATGCCCTACGGCTGCCTCTGGACCATGTCCGAGAACACCCGAGGTCGCCTAGGCTCCC
Lm-ddA-164-5
40 TTATGCCCTACGGCTGCCTCTGGACCATGTCCGAGAACACCCGAGGTCGCCTAGGCTCCC

Lm-ddA164-6

TTATGCCCTACGGCTGCCTCTGGACCATGTCCGAGAACACCGAGGTCGCCTAGGCTCCC

[000217]

Reference

5 AGGACCTGCTCAACTGGTGTGTTCAGATTCCAAGGGGATGAGCTACCTGGAGGACGTGC (SEQ ID NO: 31)

Lm-LLO-NY-1

AGGACCTGCTCAACTGGTGTGTTCAGATTCCAAGGGGATGAGCTACCTGGAGGACGTGC

Lm-LLO-NY-2

10 AGGACCTGCTCAACTGGTGTGTTCAGATTCCAAGGGGATGAGCTACCTGGAGGACGTGC

Lm-LLO-138-1

AGGACCTGCTCAACTGGTGTGTTCAGATTCCAAGGGGATGAGCTACCTGGAGGACGTGC

Lm-LLO-138-2

AGGACCTGCTCAACTGGTGTGTTCAGATTCCAAGGGGATGAGCTACCTGGAGGACGTGC

15 Lm-LLO-138-3

AGGACCTGCTCAACTGGTGTGTTCAGATTCCAAGGGGATGAGCTACCTGGAGGACGTGC

Lm-LLO-138-4

AGGACCTGCTCAACTGGTGTGTTCAGATTCCAAGGGGATGAGCTACCTGGAGGACGTGC

Lm-ddA-164-1

20 AGGACCTGCTCAACTGGTGTGTTCAGATTCCAAGGGGATGAGCTACCTGGAGGACGTGC

Lm-ddA-164-2

AGGACCTGCTCAACTGGTGTGTTCAGATTCCAAGGGGATGAGCTACCTGGAGGACGTGC

Lm-ddA-164-3

AGGACCTGCTCAACTGGTGTGTTCAGATTCCAAGGGGATGAGCTACCTGGAGGACGTGC

25 Lm-ddA-164-4

AGGACCTGCTCAACTGGTGTGTTCAGATTCCAAGGGGATGAGCTACCTGGAGGACGTGC

Lm-ddA-164-5

AGGACCTGCTCAACTGGTGTGTTCAGATTCCAAGGGGATGAGCTACCTGGAGGACGTGC

Lm-ddA-164-6

30 AGGACCTGCTCAACTGGTGTGTTCAGATTCCAAGGGGATGAGCTACCTGGAGGACGTGC

[000218]

Reference

GGCTTGTACACAGGGACCTGGCTGCCCGGAATGTGCTAGTCAAGAGTCCCAACCACGTCA (SEQ ID NO: 32)

35 Lm-LLO-NY-1

GGCTTGTACACAGGGACCTGGCTGCCCGGAATGTGCTAGTCAAGAGTCCCAACCACGTCA

Lm-LLO-NY-2

GGCTTGTACACAGGGACCTGGCTGCCCGGAATGTGCTAGTCAAGAGTCCCAACCACGTCA

Lm-LLO-138-1

40 GGCTTGTACACAGGGACCTGGCTGCCCGGAATGTGCTAGTCAAGAGTCCCAACCACGTCA

Lm-LLO-138-2
GGCTTGTACACAGGGACCTGGCTGCCCGAATGTGCTAGTCAAGAGTCCAACCACGTCA
Lm-LLO-138-3
GGCTTGTACACAGGGACCTGGCTGCCCGAATGTGCTAGTCAAGAGTCCAACCACGTCA
5 Lm-LLO-138-4
GGCTTGTACACAGGGACCTGGCTGCCCGAATGTGCTAGTCAAGAGTCCAACCACGTCA
Lm-ddA-164-1
GGCTTGTACACAGGGACCTGGCTGCCCGAATGTGCTAGTCAAGAGTCCAACCACGTCA
Lm-ddA-164-2
10 GGCTTGTACACAGGGACCTGGCTGCCCGAATGTGCTAGTCAAGAGTCCAACCACGTCA
Lm-ddA-164-4
GGCTTGTACACAGGGACCTGGCTGCCCGAATGTGCTAGTCAAGAGTCCAACCACGTCA
Lm-ddA-164-3
GGCTTGTACACAGGGACCTGGCTGCCCGAATGTGCTAGTCAAGAGTCCAACCACGTCA
15 Lm-ddA-164-5
GGCTTGTACACAGGGACCTGGCTGCCCGAATGTGCTAGTCAAGAGTCCAACCACGTCA
Lm-ddA164-6
GGCTTGTACACAGGGACCTGGCTGCCCGAATGTGCTAGTCAAGAGTCCAACCACGTCA
[000219]

20 Reference
AGATTACAGATTCGGGCTGGCTCGGCTGCTGGACATTGATGAGACAGAGTACCATGCAG (SEQ ID
NO: 33)
Lm-LLO-NY-1
AGATTACAGATTCGGGCTGGCTCGGCTGCTGGACATTGATGAGACAGAGTACCATGCAG
25 Lm-LLO-NY-2
AGATTACAGATTCGGGCTGGCTCGGCTGCTGGACATTGATGAGACAGAGTACCATGCAG
Lm-LLO-138-1
AGATTACAGATTCGGGCTGGCTCGGCTGCTGGACATTGATGAGACAGAGTACCATGCAG
Lm-LLO-138-2
30 AGATTACAGATTCGGGCTGGCTCGGCTGCTGGACATTGATGAGACAGAGTACCATGCAG
Lm-LLO-138-3
AGATTACAGATTCGGGCTGGCTCGGCTGCTGGACATTGATGAGACAGAGTACCATGCAG
Lm-LLO-138-4
AGATTACAGATTCGGGCTGGCTCGGCTGCTGGACATTGATGAGACAGAGTACCATGCAG
35 Lm-ddA-164-1
AGATTACAGATTCGGGCTGGCTCGGCTGCTGGACATTGATGAGACAGAGTACCATGCAG
Lm-ddA-164-2
AGATTACAGATTCGGGCTGGCTCGGCTGCTGGACATTGATGAGACAGAGTACCATGCAG
Lm-ddA-164-3
40 AGATTACAGATTCGGGCTGGCTCGGCTGCTGGACATTGATGAGACAGAGTACCATGCAG

Lm-ddA-164-4

AGATTACAGATTCTGGCTGGCTGGCTGCTGGACATTGATGAGACAGAGTACCATGCAG

Lm-ddA-164-5

AGATTACAGATTCTGGCTGGCTGGCTGCTGGACATTGATGAGACAGAGTACCATGCAG

5 Lm-ddA164-6

AGATTACAGATTCTGGCTGGCTGGCTGCTGGACATTGATGAGACAGAGTACCATGCAG

[000220]

Reference

ATGGGGCAAGGTGCCATCAAATGGATGGCATTGGAATCTATTCTCAGACGCCGGTTCA (SEQ ID

10 NO: 34)

Lm-LLO-NY-1

ATGGGGCAAGGTGCCATCAAATGGATGGCATTGGAATCTATTCTCAGACGCCGGTTCA

Lm-LLO-NY-2

ATGGGGCAAGGTGCCATCAAATGGATGGCATTGGAATCTATTCTCAGACGCCGGTTCA

15 Lm-LLO-138-1

ATGGGGCAAGGTGCCATCAAATGGATGGCATTGGAATCTATTCTCAGACGCCGGTTCA

Lm-LLO-138-2

ATGGGGCAAGGTGCCATCAAATGGATGGCATTGGAATCTATTCTCAGACGCCGGTTCA

Lm-LLO-138-3

20 ATGGGGCAAGGTGCCATCAAATGGATGGCATTGGAATCTATTCTCAGACGCCGGTTCA

Lm-LLO-138-4

ATGGGGCAAGGTGCCATCAAATGGATGGCATTGGAATCTATTCTCAGACGCCGGTTCA

Lm-ddA-164-1

ATGGGGCAAGGTGCCATCAAATGGATGGCATTGGAATCTATTCTCAGACGCCGGTTCA

25 Lm-ddA-164-2

ATGGGGCAAGGTGCCATCAAATGGATGGCATTGGAATCTATTCTCAGACGCCGGTTCA

Lm-ddA-164-3

ATGGGGCAAGGTGCCATCAAATGGATGGCATTGGAATCTATTCTCAGACGCCGGTTCA

Lm-ddA-164-4

30 ATGGGGCAAGGTGCCATCAAATGGATGGCATTGGAATCTATTCTCAGACGCCGGTTCA

Lm-ddA-164-5

ATGGGGCAAGGTGCCATCAAATGGATGGCATTGGAATCTATTCTCAGACGCCGGTTCA

Lm-ddA-164-6

ATGGGGCAAGGTGCCATCAAATGGATGGCATTGGAATCTATTCTCAGACGCCGGTTCA

35 [000221]

Reference

CCCATCAGAGTGATGTGGAGCTATGGAGTGACTGTGTGGAGCTGATGACTTTGGGG (SEQ ID

NO: 35)

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 Lm-LLO-138-1
 CCCATCAGAGTGTGTGGAGCTATGGAGTGACTGTGTGGAGCTGATGACTTTGGGG
 5 Lm-LLO-138-2
 CCCATCAGAGTGTGTGGAGCTATGGAGTGACTGTGTGGAGCTGATGACTTTGGGG
 Lm-LLO-138-3
 CCCATCAGAGTGTGTGGAGCTATGGAGTGACTGTGTGGAGCTGATGACTTTGGGG
 Lm-LLO-138-4
 10 CCCATCAGAGTGTGTGGAGCTATGGAGTGACTGTGTGGAGCTGATGACTTTGGGG
 Lm-ddA-164-1
 CCCATCAGAGTGTGTGGAGCTATGGAGTGACTGTGTGGAGCTGATGACTTTGGGG
 Lm-ddA-164-2
 CCCATCAGAGTGTGTGGAGCTATGGAGTGACTGTGTGGAGCTGATGACTTTGGGG
 15 Lm-ddA-164-3
 CCCATCAGAGTGTGTGGAGCTATGGAGTGACTGTGTGGAGCTGATGACTTTGGGG
 Lm-ddA-164-4
 CCCATCAGAGTGTGTGGAGCTATGGAGTGACTGTGTGGAGCTGATGACTTTGGGG
 Lm-ddA-164-5
 20 CCCATCAGAGTGTGTGGAGCTATGGAGTGACTGTGTGGAGCTGATGACTTTGGGG
 Lm-ddA-164-6
 CCCATCAGAGTGTGTGGAGCTATGGAGTGACTGTGTGGAGCTGATGACTTTGGGG
 [000222]
 Reference
 25 CCAAACCTTACGATGGAATCCCAGCCGGAGATCCCTGATTGCTGGAGAACGGAGAA (SEQ ID
 NO: 36)
 Lm-LLO-NY-1
 CCAAACCTTACGATGGAATCCCAGCCGGAGATCCCTGATTGCTGGAGAACGGAGAA
 Lm-LLO-NY-2
 30 CCAAACCTTACGATGGAATCCCAGCCGGAGATCCCTGATTGCTGGAGAACGGAGAA
 Lm-LLO-138-1
 CCAAACCTTACGATGGAATCCCAGCCGGAGATCCCTGATTGCTGGAGAACGGAGAA
 Lm-LLO-138-3
 CCAAACCTTACGATGGAATCCCAGCCGGAGATCCCTGATTGCTGGAGAACGGAGAA
 35 Lm-LLO-138-4
 CCAAACCTTACGATGNAATCCCAGCCGGAGATCCCTGATTGCTGGAGAACGGAGAA
 Lm-ddA-164-6
 CCAAACCTTACGATGGAATCCCAGCCGGAGATCCCTGATTGCTGGAGAACGGAGAA
 Lm-ddA-164-2
 40 CCAAACCTTACGATGGAATCCCAGCCGGAGATCCCTGATTGCTGGAGAACGGAGAA

Lm-LLO-138-2

CCAAACCTTACGATGGAATCCCAGCCGGGAGATCCCTGATTGCTGGAGAAGGGAGAA

Lm-ddA-164-3

CCAAACCTTACGATGGAATCCCAGCCGGGAGATCCCTGATTGCTGGAGAAGGGAGAA

5 Lm-ddA-164-5

CCAAACCTTACGATGGAATCCCAGCCGGGAGATCCCTGATTGCTGGAGAAGGGAGAA

Lm-ddA-164-1

CCAAACCTTACGATGGAATCCCAGCCGGGAGATCCCTGATTGCTGGAGAAGGGAGAA

Lm-ddA-164-4

10 CCAAACCTTACGATGGAATCCCAGCCGGGAGATCCCTGATTGCTGGAGAAGGGAGAA

[000223]

Reference CGCCTACCTCAGCCTCCAATCTGCACCATTGATGTCTACATGATTATGGTCAAATGTT
(SEQ ID NO: 37)

Lm-LLO-NY-1

15 CGCCTACCTCAGCCTCCAATCTGCACCATTGATGTCTACATGATTATGGTCAAATGTT

Lm-LLO-NY-2

CGCCTACCTCAGCCTCCAATCTGCACCATTGATGTCTACATGATTATGGTCAAATGTT

Lm-LLO-138-1

CGCCTACCTCAGCCTCCAATCTGCACCATTGATGTCTACATGATTATGGTCAAATGTT

20 Lm-LLO-138-2

CGCCTACCTCAGCCTCCAATCTGCACCATTGATGTCTACATGATTATGGTCAAATGTT

Lm-LLO-138-3

CGCCTACCTCAGCCTCCAATCTGCACCATTGATGTCTACATGATTATGGTCAAATGTT

Lm-LLO-138-4

25 CGCCTACCTCAGCCTCCAATCTGCACCATTGATGTCTACATGATTATGGTCAAATGTT

Lm-ddA-164-1 CGCCTACCTCAGCCTCCAATCTGCACCATTGATGTCTACATGATTATGGTCAAATGTT

Lm-ddA-164-2 CGCCTACCTCAGCCTCCAATCTGCACCATTGATGTCTACATGATTATGGTCAAATGTT

Lm-ddA-164-3 CGCCTACCTCAGCCTCCAATCTGCACCATTGATGTCTACATGATTATGGTCAAATGTT

Lm-ddA-164-4 CGCCTACCTCAGCCTCCAATCTGCACCATTGATGTCTACATGATTATGGTCAAATGTT

30 Lm-ddA-164-5 CGCCTACCTCAGCCTCCAATCTGCACCATTGATGTCTACATGATTATGGTCAAATGTT

Lm-ddA-164-6 CGCCTACCTCAGCCTCCAATCTGCACCATTGATGTCTACATGATTATGGTCAAATGTT

[000224]

Reference GGATGATTGACTCTGAATGTCGCCCCGAGATTCCGGGAGTTGGTGTCAAATTT (SEQ ID NO: 38)

35 Lm-LLO-NY-1 GGATGATTGACTCTGAATGTCGCCCCGAGATTCCGGGAGTTGGTGTCAAATTT

Lm-LLO-NY-2 GGATGATTGACTCTGAATGTCGCCCCGAGATTCCGGGAGTTGGTGTCAAATTT

Lm-LLO-138-2 GGATGATTGACTCTGAATGTCGCCCCGAGATTCCGGGAGTTGGTGTCAAATTT

Lm-LLO-138-3 GGATGATTGACTCTGAATGTCGCCCCGAGATTCCGGGAGTTGGTGTCAAATTT

Lm-LLO-138-4 GGATGATTGACTCTGAATGTCGCCCCGAGATTCCGGGAGTTGGTGTCAAATTT

40 Lm-ddA-164-1 GGATGATTGACTCTGAATGTCGCCCCGAGATTCCGGGAGTTGGTGTCAAATTT

Lm-ddA-164-2 GGATGATTGACTCTGAATGTCGCCCCGAGATTCCGGGAGTTGGTGTCAAATTT

Lm-ddA-164-3 GGATGATTGACTCTGAATGTCGCCGAGATTCCGGAGTTGGTGTCAAGAATT
 Lm-ddA-164-5 GGATGATTGACTCTGAATGTCGCCGAGATTCCGGAGTTGGTGTCAAGAATT
 Lm-ddA-164-4 GGATGATTGACTCTGAATGTCGCCGAGATTCCGGAGTTGGTGTCAAGAATT
 Lm-ddA-164-6 GGATGATTGACTCTGAATGTCGCCGAGATTCCGGAGTTGGTGTCAAGAATT

5 [000225]

Reference CACGTATGGCGAGGGACCCCCAGCGTTTGTGGTCATCCAGAACGAGGACTT (SEQ ID NO: 39)

Lm-LLO-NY-1 CACGTATGGCGAGGGACCCCCAGCGTTTGTGGTCATCCAGAACGAGGACTT

Lm-LLO-NY-2 CACGTATGGCGAGGGACCCCCAGCGTTTGTGGTCATCCAGAACGAGGACTT

10 Lm-LLO-138-2 CACGTATGGCGAGGGACCCCCAGCGTTTGTGGTCATCCAGAACGAGGACTT

Lm-LLO-138-3 CACGTATGGCGAGGGACCCCCAGCGTTTGTGGTCATCCAGAACGAGGACTT

Lm-LLO-138-4 CACGTATGGCGAGGGACCCCCAGCGTTTGTGGTCATCCAGAACGAGGACTT

Lm-ddA-164-1 CACGTATGGCGAGGGACCCCCAGCGTTTGTGGTCATCCAGAACGAGGACTT

Lm-ddA-164-2 CACGTATGGCGAGGGACCCCCAGCGTTTGTGGTCATCCAGAACGAGGACTT

15 Lm-ddA-164-3 CACGTATGGCGAGGGACCCCCAGCGTTTGTGGTCATCCAGAACGAGGACTT

Lm-ddA-164-5 CACGTATGGCGAGGGACCCCCAGCGTTTGTGGTCATCCAGAACGAGGACTT

Lm-ddA-164-6 CACGTATGGCGAGGGACCCCCAGCGTTTGTGGTCATCCAGAACGAGGACTT

Alignment of EC1 (399-758 bp of Her-2-neu)

20 [000226]

Reference

CCCAGGCAGAACCCCCAGAGGGGCTGCGGGAGCTGCAGCTCGAACGTCTCACAGAGATCCT (SEQ ID NO: 40)

Lm-LLO-138-1

25 CCCAGGCAGAACCCCCAGAGGGGCTGCGGGAGCTGCAGCTCGAACGTCTCACAGAGATCCT

Lm-LLO-138-2

CCCAGGCAGAACCCCCAGAGGGGCTGCGGGAGCTGCAGCTCGAACGTCTCACAGAGATCCT

Lm-ddA-164-1

CCCAGGCAGAACCCCCAGAGGGGCTGCGGGAGCTGCAGCTCGAACGTCTCACAGAGATCCT

30 LmddA-164-2

CCCAGGCAGAACCCCCAGAGGGGCTGCGGGAGCTGCAGCTCGAACGTCTCACAGAGATCCT

LmddA-164-3

CCCAGGCAGAACCCCCAGAGGGGCTGCGGGAGCTGCAGCTCGAACGTCTCACAGAGATCCT

LmddA-164-4

35 CCCAGGCAGAACCCCCAGAGGGGCTGCGGGAGCTGCAGCTCGAACGTCTCACAGAGATCCT

[000227]

Reference GAAGGGAGGAGTTTGATCCGTGGAACCCCTCAGCTCTGCTACCAGGACATGGTTTG (SEQ ID NO: 41)

Lm-LLO-138-1

40 GAAGGGAGGAGTTTGATCCGTGGAACCCCTCAGCTCTGCTACCAGGACATGGTTTG

Lm-LLO-138-2

GAAGGGAGGAGTTTGATCCGTGGAACCCCTCAGCTCTGCTACCAGGACATGGTTTGTG

Lm-ddA-164-1

GAAGGGAGGAGTTTGATCCGTGGAACCCCTCAGCTCTGCTACCAGGACATGGTTTGTG

LmddA-164-2

5 GAAGGGAGGAGTTTGATCCGTGGAACCCCTCAGCTCTGCTACCAGGACATGGTTTGTG

LmddA-164-3

GAAGGGAGGAGTTTGATCCGTGGAACCCCTCAGCTCTGCTACCAGGACATGGTTTGTG

LmddA164-4

GAAGGGAGGAGTTTGATCCGTGGAACCCCTCAGCTCTGCTACCAGGACATGGTTTGTG

10 [000228]

Reference

CCGGGCCTGTCCACCTTGTGCCCGCCTGCAAAGACAATCACTGTTGGGTGAGAGTCC (SEQ ID NO: 42)

Lm-LLO-138-1

15 CCGGGCCTGTCCACCTTGTGCCCGCCTGCAAAGACAATCACTGTTGGGTGAGAGTCC

Lm-LLO-138-2

CCGGGCCTGTCCACCTTGTGCCCGCCTGCAAAGACAATCACTGTTGGGTGAGAGTCC

Lm-ddA-164-1

CCGGGCCTGTCCACCTTGTGCCCGCCTGCAAAGACAATCACTGTTGGGTGAGAGTCC

20 LmddA-164-2

CCGGGCCTGTCCACCTTGTGCCCGCCTGCAAAGACAATCACTGTTGGGTGAGAGTCC

LmddA-164-3

CCGGGCCTGTCCACCTTGTGCCCGCCTGCAAAGACAATCACTGTTGGGTGAGAGTCC

LmddA164-4

25 CCGGGCCTGTCCACCTTGTGCCCGCCTGCAAAGACAATCACTGTTGGGTGAGAGTCC

[000229]

Reference

GGAAGACTGTCAGATCTGACTGGCACCATCTGTACCAGTGGTTGTGCCGGTGCAAGGG (SEQ ID NO: 43)

30 Lm-LLO-138-1

GGAAGACTGTCAGATCTGACTGGCACCATCTGTACCAGTGGTTGTGCCGGTGCAAGGG

Lm-LLO-138-2

GGAAGACTGTCAGATCTGACTGGCACCATCTGTACCAGTGGTTGTGCCGGTGCAAGGG

Lm-ddA-164-1

35 GGAAGACTGTCAGATCTGACTGGCACCATCTGTACCAGTGGTTGTGCCGGTGCAAGGG

LmddA-164-2

GGAAGACTGTCAGATCTGACTGGCACCATCTGTACCAGTGGTTGTGCCGGTGCAAGGG

LmddA-164-3

GGAAGACTGTCAGATCTGACTGGCACCATCTGTACCAGTGGTTGTGCCGGTGCAAGGG

40 LmddA164-4

GGAAGACTGTCAGATCTGACTGGCACCATCTGTACCAGTGGTTGTGCCGGTGCAAGGG

[000230]

Reference CCGGCTGCCCACTGACTGCTGCCATGAGCAGTGTGCCGCAGGCTGCACGGGCCCCAAGCA (SEQ ID NO: 44)

Lm-LLO-138-1

5 CCGGCTGCCCACTGACTGCTGCCATGAGCAGTGTGCCGCAGGCTGCACGGGCCCCAAGCA

Lm-LLO-138-2

CCGGCTGCCCACTGACTGCTGCCATGAGCAGTGTGCCGCAGGCTGCACGGGCCCCAAGCA

Lm-ddA-164-1

CCGGCTGCCCACTGACTGCTGCCATGAGCAGTGTGCCGCAGGCTGCACGGGCCCCAAGCA

10 LmddA-164-2

CCGGCTGCCCACTGACTGCTGCCATGAGCAGTGTGCCGCAGGCTGCACGGGCCCCAAGTA

LmddA-164-3

CCGGCTGCCCACTGACTGCTGCCATGAGCAGTGTGCCGCAGGCTGCACGGGCCCCAAGTA

LmddA164-4

15 CCGGCTGCCCACTGACTGCTGCCATGAGCAGTGTGCCGCAGGCTGCACGGGCCCCAAGTA

EXAMPLE 7: PERIPHERAL IMMUNIZATION WITH ADXS31-164 CAN DELAY THE GROWTH OF A METASTATIC BREAST CANCER CELL LINE IN THE BRAIN.

20 [000231] Mice were immunized IP with ADXS31-164 or irrelevant *Lm*-control vaccines and then implanted intra-cranially with 5,000 EMT6-Luc tumor cells, expressing luciferase and low levels of Her2/neu (Figure 6C). Tumors were monitored at different times post-inoculation by *ex vivo* imaging of anesthetized mice. On day 8 post-tumor inoculation tumors were detected in all control animals, but none of the mice in ADXS31-164 group showed any detectable tumors (Figure 6A and B). ADXS31-164 could clearly delay the onset of these tumors, as on day 11 post-tumor inoculation all mice in negative control group had already succumbed to their tumors, but all mice in ADXS31-164 group were still alive and only showed small signs of tumor growth. These results strongly suggest that the immune responses obtained with the peripheral administration of ADXS31-164 could possibly reach 25 the central nervous system and that *LmddA*-based vaccines might have a potential use for treatment of CNS tumors.

30 [000232] While certain features of the invention have been illustrated and described herein, many modifications, substitutions, changes, and equivalents will now occur to those of ordinary skill in the art. It is, therefore, to be understood that the appended claims are intended 35 to cover all such modifications and changes as fall within the true spirit of the invention.

What is claimed is:

1. An immunogenic composition comprising a fusion polypeptide, wherein said fusion polypeptide comprises a Her2/neu chimeric antigen fused to an additional polypeptide, and wherein administering said fusion protein to a subject having an Her2/neu-expressing tumor prevents escape mutations within said tumor.
2. A recombinant *Listeria* vaccine strain comprising the immunogenic composition of claim 1.
3. A nucleic acid molecule comprising a first open reading frame encoding said immunogenic composition of claim 2, wherein said nucleic molecule resides within said recombinant *Listeria* vaccine strain.
4. The composition of claim 3, wherein said nucleic acid molecule further comprises a second open reading frame encoding a metabolic enzyme, and wherein said metabolic enzyme complements an endogenous gene that is lacking in the chromosome of said recombinant *Listeria* vaccine strain.
5. The composition of claim 1, wherein said Her2/neu chimeric antigen comprises at least 5, 9, 13, 14, or 17 of the mapped human MHC-class I epitopes.
6. The composition of claim 3, wherein said nucleic acid molecule is integrated into the *Listeria* genome.
7. The composition of claim 3, wherein said nucleic acid molecule is in a plasmid in said recombinant *Listeria* vaccine strain.
8. The composition of claim 7, wherein said plasmid is stably maintained in said recombinant *Listeria* vaccine strain in the absence of antibiotic selection.

9. The composition of claim 7, wherein said plasmid does not confer antibiotic resistance upon said recombinant *Listeria*.
10. The composition of claim 2, wherein said recombinant *Listeria* strain is attenuated.
11. The composition of claim 2, wherein said recombinant *Listeria* lacks the ActA virulence gene.
12. The recombinant *Listeria* strain of claim 1, wherein said additional polypeptide is selected from the group consisting of: a) non-hemolytic LLO protein or N-terminal fragment, b) a PEST sequence, or c) an ActA fragment, and further wherein said additional polypeptide is fused to said Her2/neu chimeric antigen.
13. The recombinant *Listeria* strain of claim 4, wherein said metabolic enzyme encoded by said second open reading frame is an amino acid metabolism enzyme.
14. The recombinant *Listeria* strain of claim 4, wherein said metabolic enzyme encoded by said second open reading frame is an alanine racemase enzyme or a D-amino acid transferase enzyme.
15. The recombinant *Listeria* strain of claim 4, wherein said recombinant *Listeria* further comprises a third open reading frame
16. The recombinant *Listeria* strain of claim 15, wherein said metabolic enzyme encoded by said third open reading frame is a D-amino acid transferase enzyme or an alanine racemase enzyme.
17. The recombinant *Listeria* strain of claim 2, wherein said recombinant *Listeria* strain has been passaged through an animal host.
18. The recombinant *Listeria* vaccine strain of claim 2, further comprising an adjuvant.

19. The recombinant *Listeria* vaccine strain of claim 18, wherein said adjuvant comprises a granulocyte/macrophage colony-stimulating factor (GM-CSF) protein, a nucleotide molecule encoding a GM-CSF protein, saponin QS21, monophosphoryl lipid A, or an unmethylated CpG-containing oligonucleotide.
20. A method of impeding a growth of a Her-2-expressing tumor in a subject, said method comprising the step of administering to said subject the composition of claim 1.
21. A method of impeding a growth of a Her-2-expressing tumor in a subject, said method comprising the step of administering to said subject a composition comprising the vaccine of claim 2.
22. A method of impeding a growth of a Her-2-expressing tumor in a subject, said method comprising the step of administering to said subject a composition comprising the vaccine of claim 4
23. A method of eliciting an enhanced immune response to a Her2/neu-expressing tumor in a subject, said method comprising administering to said subject a composition comprising the composition of claim 1.
24. The method of claim 23, wherein said immune response against said Her2/neu-expressing tumor comprises an immune response to a subdominant epitope of said Her2/neu protein.
25. The method of claim 24, wherein said immune response against said Her2/neu-expressing tumor comprises an immune response to a subdominant epitope of said Her2/neu protein.
26. The method of claim 23, wherein said immune response against said Her2/neu-expressing tumor comprises an immune response to a subdominant epitope of said Her2/neu protein.

Her2/neu protein.

27. A method of eliciting an enhanced immune response to a Her2/neu-expressing tumor in a subject, said method comprising administering to said subject a composition comprising the vaccine of claim 2.

28. The method of claim 27, wherein said immune response against said Her2/neu-expressing tumor comprises an immune response to a subdominant epitope of said Her2/neu protein.

29. The method of claim 28, wherein said immune response against said Her2/neu-expressing tumor comprises an immune response to a subdominant epitope of said Her2/neu protein.

30. The method of claim 27, wherein said immune response against said Her2/neu-expressing tumor comprises an immune response to a subdominant epitope of said Her2/neu protein.

31. A method of eliciting an enhanced immune response to a Her2/neu-expressing tumor in a subject, said method comprising administering to said subject a composition comprising the vaccine of claim 4.

32. The method of claim 31, wherein said immune response against said Her2/neu-expressing tumor comprises an immune response to a subdominant epitope of said Her2/neu protein.

33. The method of claim 32, wherein said immune response against said Her2/neu-expressing tumor comprises an immune response to a subdominant epitope of said Her2/neu protein.

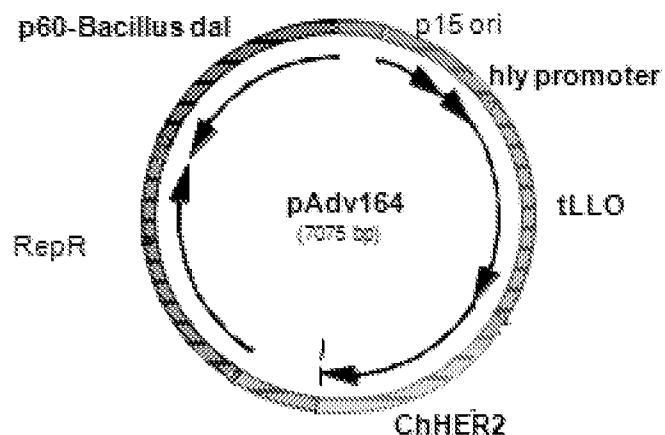
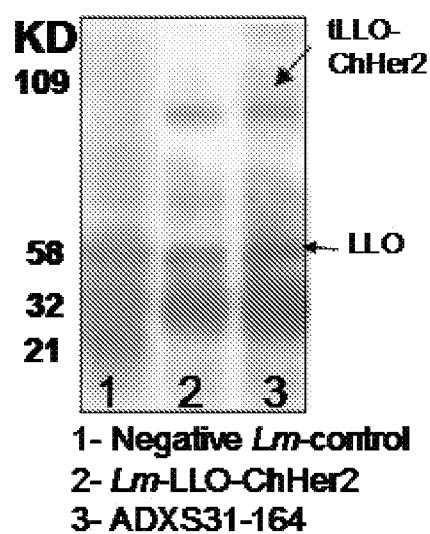
34. The method of claim 31, wherein said immune response against said Her2/neu-expressing tumor comprises an immune response to a subdominant epitope of said Her2/neu protein.
35. A method of preventing an escape mutation in the treatment of Her2/neu over-expressing tumors, comprising administering to a subject the composition of claim 1.
36. A method of preventing an escape mutation in the treatment of Her2/neu over-expressing tumors, comprising administering to a subject the recombinant *Listeria* vaccine strain of claim 2.
37. A method of preventing an escape mutation in the treatment of Her2/neu over-expressing tumors, comprising administering to a subject the recombinant *Listeria* vaccine strain of claim 4.
38. A method of preventing the onset of a Her2/neu antigen-expressing tumor in a subject, said method comprising the step of administering to said subject the composition of claim 1.
39. A method of preventing the onset of a Her2/neu antigen-expressing tumor in a subject, said method comprising the step of administering to said subject a composition comprising the vaccine of claim 2.
40. A method of preventing the onset of a Her2/neu antigen-expressing tumor in a subject, said method comprising the step of administering to said subject a composition comprising the vaccine of claim 4.
41. A method of decreasing the frequency of intra-tumoral T regulatory cells, said method comprising the step of administering to said subject the composition of claim 1.

42. A method of decreasing the frequency of intra-tumoral T regulatory cells, said method comprising the step of administering to said subject a composition comprising the vaccine of claims 2.
43. A method of decreasing the frequency of intra-tumoral T regulatory cells, said method comprising the step of administering to said subject a composition comprising the vaccine of claims 4.
44. A method of decreasing the frequency of intra-tumoral myeloid derived suppressor cells, said method comprising the step of administering to said subject the composition of claim 1.
45. A method of decreasing the frequency of myeloid derived suppressor cells, said method comprising the step of administering to said subject a composition comprising the vaccine of claims 2.
46. A method of decreasing the frequency of myeloid derived suppressor cells, said method comprising the step of administering to said subject a composition comprising the vaccine of claims 4.
47. A method of increasing intratumoral ratio of CD8+/T regulatory cells, said method comprising the step of administering to said subject the composition of claim 1.
48. A method of increasing intratumoral ratio of CD8+/T regulatory cells, said method comprising the step of administering to said subject a composition comprising the vaccine of claims 2.
49. A method of increasing intratumoral ratio of CD8+/T regulatory cells, said method comprising the step of administering to said subject a composition comprising the vaccine of claims 4.

50. A method of preventing the formation of a Her2/neu-expressing tumor in a subject, said method comprising the step of administering to said subject the composition of claim 1.
51. A method of preventing the formation of a Her2/neu-expressing tumor in a subject, said method comprising the step of administering to said subject a composition comprising the vaccine of claims 2.
52. A method of preventing the formation of a Her2/neu-expressing tumor in a subject, said method comprising the step of administering to said subject a composition comprising the vaccine of claims 4.
53. A method of treating a Her2/neu-expressing tumor in a subject, said method comprising the step of administering to said subject the composition of claim 1.
54. A method of treating a Her2/neu-expressing tumor in a subject, said method comprising the step of administering to said subject a composition comprising the vaccine of claims 2.
55. A method of treating a Her2/neu-expressing tumor in a subject, said method comprising the step of administering to said subject a composition comprising the vaccine of claims 4.

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Figure 1

A**B**

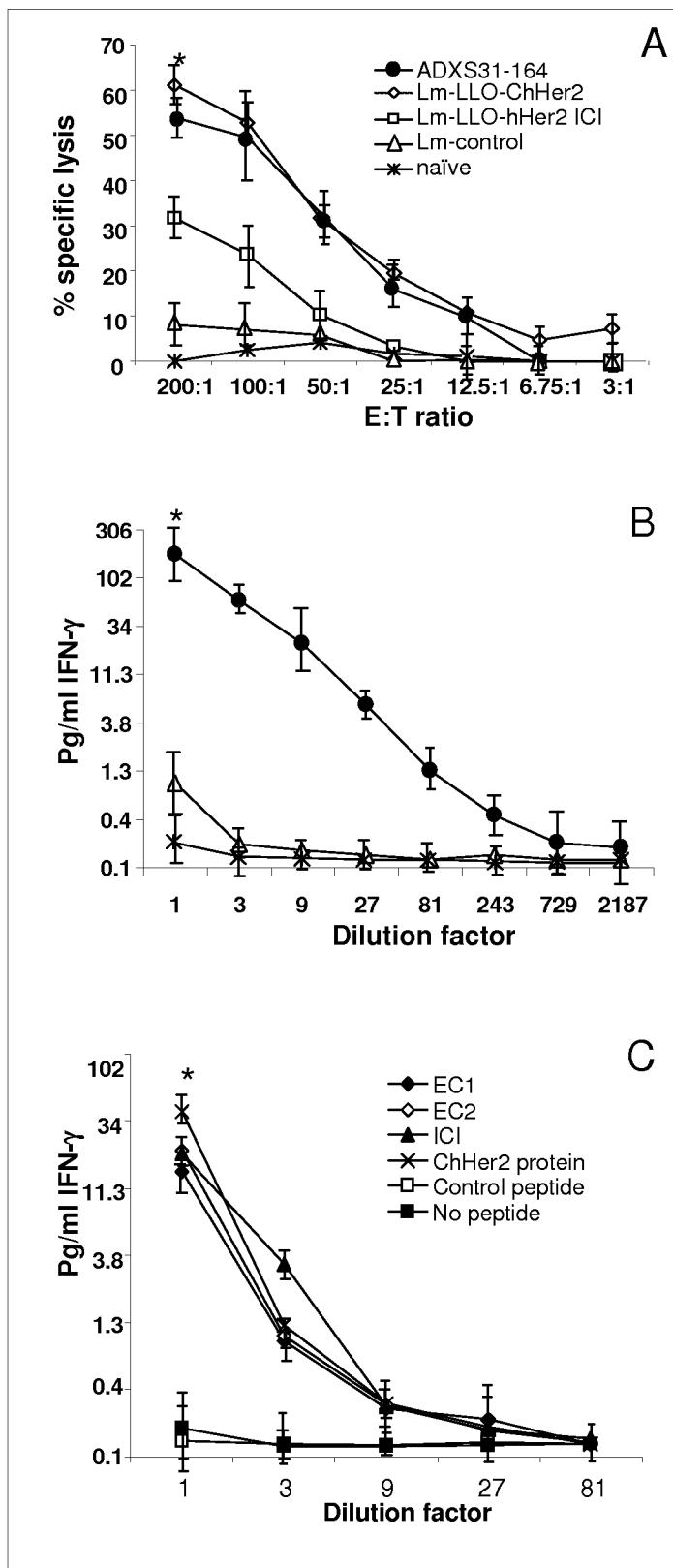


Figure 2

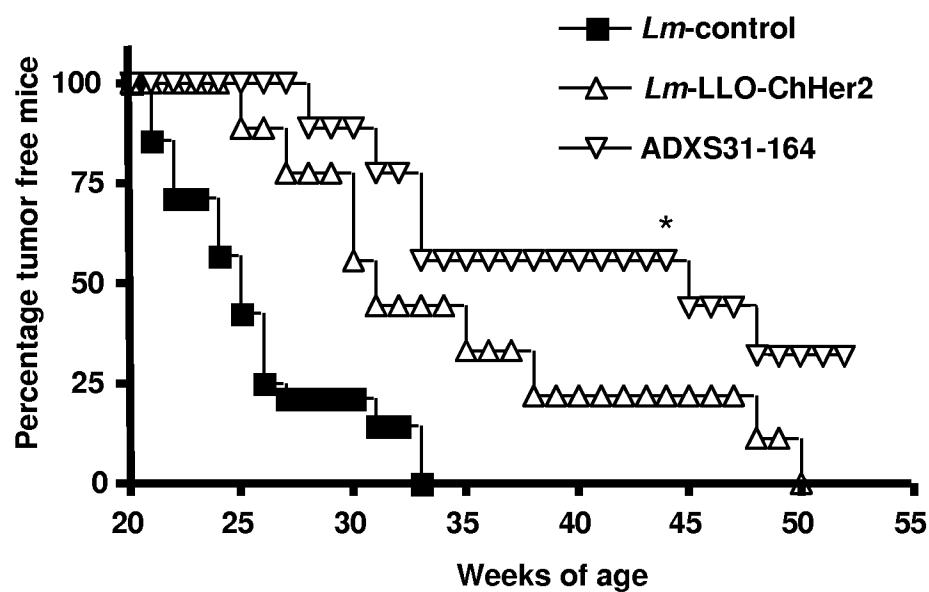
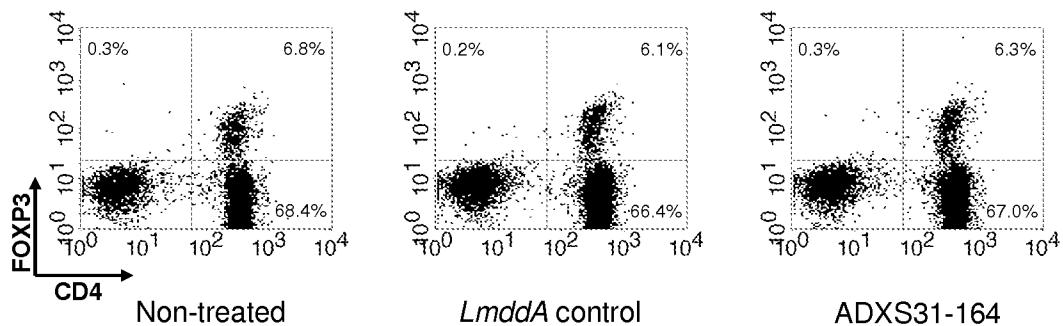


Figure 3

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Spleens

Gated on CD3⁺ T cells



Gated on CD3⁺CD4⁺ T cells

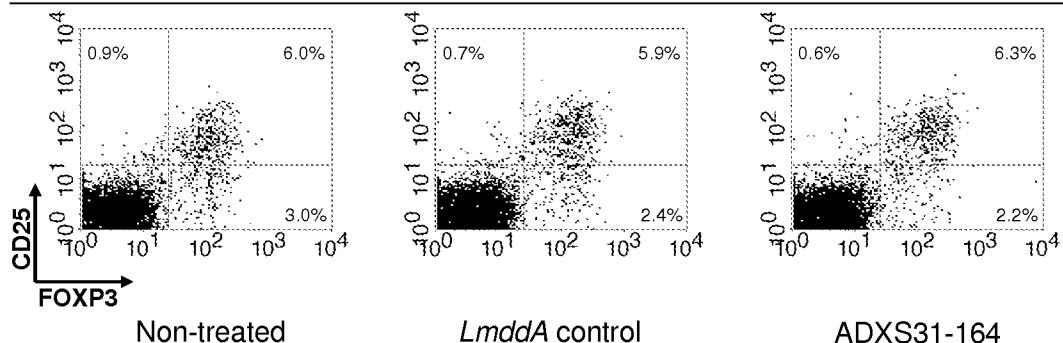


Figure 4

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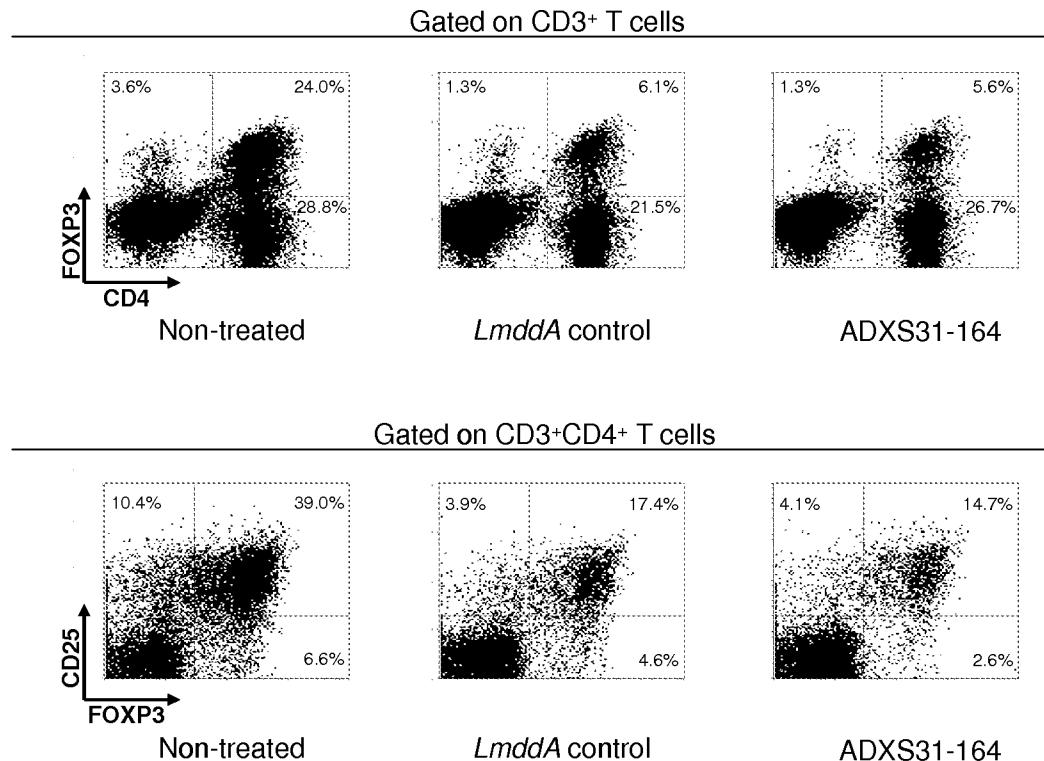
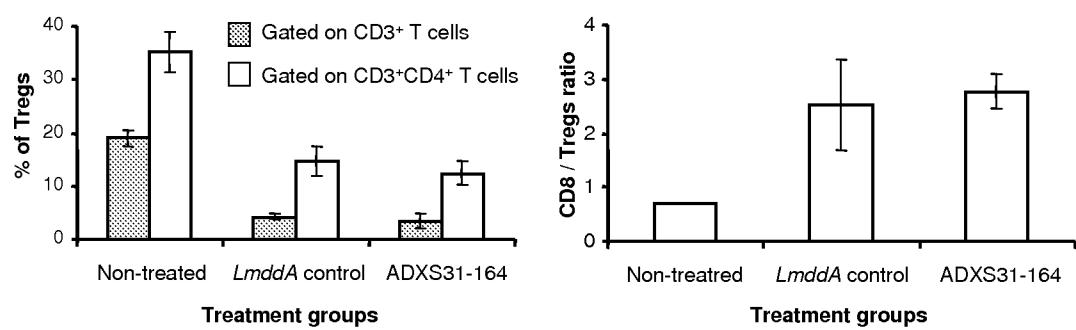
A**B**

Figure 5

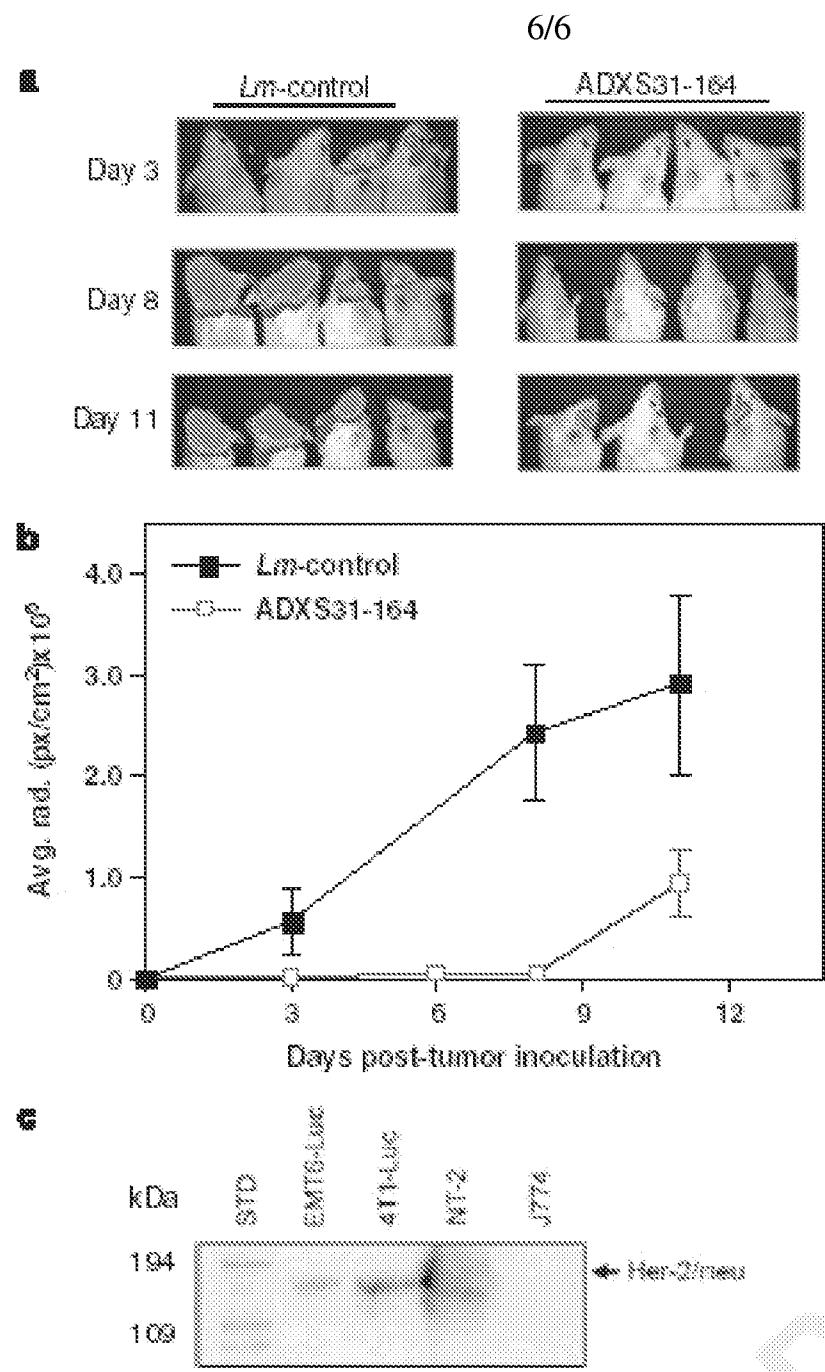


Figure 6