

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
3 November 2011 (03.11.2011)

(10) International Publication Number
WO 2011/136828 A1

(51) International Patent Classification:
A61K 38/19 (2006.01) *A61P 35/00* (2006.01)
A61P 35/02 (2006.01)

(74) Agents: CORLESS, Peter, F. et al.; Edwards Angell Palmer & Dodge LLP, P.O. Box 55874, Boston, MA 02205 (US).

(21) International Application Number:
PCT/US2010/052889

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(22) International Filing Date:
15 October 2010 (15.10.2010)

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/328,471 27 April 2010 (27.04.2010) US

(71) Applicant (for all designated States except US): THE JOHNS HOPKINS UNIVERSITY [US/US]; 3400 N. Charles Street, Baltimore, MD 21218 (US).

(72) Inventors; and

(75) Inventors/Applicants (for US only): KIM, Young, Jun [KR/KR]; 4940 Eastern Avenue, Baltimore, MD 21224 (US). PARDOLL, Drew, M. [US/US]; 1650 Orleans Street, Suite 424, Baltimore, MD 21231 (US). DRAKE, Charles, George [US/US]; 401 N. Broadway, Baltimore, MD 21231 (US). DAVIS, Meghan [US/US]; 1650 Orleans Street, Suite 424, Baltimore, MD 21231 (US). FU, Juan [CN/US]; 1650 Orleans Street, Suite 424, Baltimore, MD 21231 (US).

Published:

— with international search report (Art. 21(3))

(54) Title: IMMUNOGENIC COMPOSITIONS AND METHODS FOR TREATING NEOPLASIA

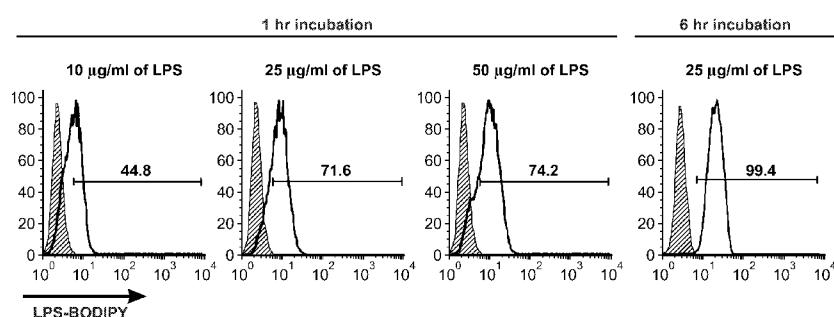


FIG. 1

(57) Abstract: The invention provides immunogenic compositions comprising neoplastic cells expressing a cytokine (GM-CSF) formulated with at least one TLR agonist and methods of using the composition to induce or enhance an immune response.

WO 2011/136828 A1

IMMUNOGENIC COMPOSITIONS AND METHODS FOR TREATING NEOPLASIA**CROSS-REFERENCE TO RELATED APPLICATION**

5 This application claims the benefit of the following U.S. Provisional Application No.: 61/328,471, filed April 27, 2010, the entire contents of which are incorporated herein by reference.

10 **STATEMENT OF RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH**

This work was supported by the following grants from the National Institutes of Health, Grant Nos: NIH K23-DE018464-02. The government has certain rights in the invention.

15

BACKGROUND OF THE INVENTION

Lethally irradiated tumor cell vaccines engineered to secrete GM-C SF (GVAX) showed promising efficacy in various models of melanoma, renal cell, prostate, and non- small cell lung, 20 pancreatic, as well as head and neck squamous cell carcinoma, but due to the multiple immunological checkpoint blockades, GVAX as a monotherapy is unlikely to be clinically effective in advanced disease. GVAX was recently found to have uncertain efficacy in a phase III trial for advanced hormone refractory prostate cancer. The failure to overcome critical mechanisms of immune evasion by the tumor has been the major limitation of past 25 immunotherapeutic strategies that were designed to primarily stimulate tumor-specific host immune responses. Among the many defined mechanisms for diminishing anti-tumor immune responses, regulatory T-cells (Treg), myeloid derived suppressor cells, and tolerizing dendritic cells (DCs) have been hypothesized as negative regulators that can prevent successful immunotherapy. One downstream consequence from some of these immune evasion checkpoint 30 pathways is the limited number of activated antigen presenting cells (APCs) in the afferent arm of the immune system. Improved tumor cell vaccines that are able to overcome critical mechanisms of immune evasion by the tumor cell are needed.

SUMMARY OF THE INVENTION

As described below, the present invention generally features immunogenic compositions comprising neoplastic cells expressing a cytokine (GM-CSF) formulated with at least one TLR agonist and methods of using the composition to induce or enhance an immune response.

5 In one aspect, the invention generally features an immunogenic composition containing one or more proliferation-incompetent genetically modified neoplastic cells expressing a recombinant immune stimulatory cytokine and at least one Toll-like receptor (TLR) agonist.

In another aspect, the invention generally features a vaccine for ameliorating a neoplasia in a subject, the vaccine containing an effective amount of proliferation-incompetent neoplastic 10 cells expressing a recombinant immune stimulatory cytokine and an effective amount of exogenous Toll-like receptor (TLR) agonist in a pharmaceutically acceptable excipient.

In yet another aspect, the invention generally features a method of inducing a neoplastic cell antigen-specific immune response in a subject having or having a propensity to develop a neoplasia, the method involving administering to the subject an effective amount of an 15 immunogenic composition containing one or more proliferation-incompetent neoplastic cells expressing a recombinant immune stimulatory cytokine and comprising an exogenous Toll-like receptor (TLR) agonist in an amount sufficient to induce a neoplastic cell antigen-specific immune response in said subject.

In a further aspect, the invention generally features a proliferation-incompetent 20 neoplastic cell comprising an expression vector encoding GM-CSF and at least one TLR agonist.

In yet another aspect, the invention generally features a method of treating or preventing a neoplasia in a subject having or having a propensity to develop a neoplasia, the method involving administering to the subject an effective amount of the immunogenic composition or 25 vaccine described herein in an amount sufficient to induce a neoplastic cell antigen-specific immune response in said subject, thereby treating the subject.

In yet another aspect, the invention generally features a method of treating or preventing tumor progression or metastasis in a subject having a neoplasia, the method comprising administering to the subject an effective amount of any of the immunogenic compositions or 30 vaccines described herein, thereby treating or preventing tumor progression or metastasis in the subject.

In yet another aspect, the invention generally features a method of treating an established tumor or preventing tumor formation in a subject, the method involving administering to the subject an effective amount of any of the immunogenic compositions or vaccines described herein, thereby treating an established tumor or preventing tumor formation in the subject.

5 In yet another aspect, the invention generally features a method of treating micrometastasis or residual disease in a subject having micrometastasis or residual disease, the method comprising administering to the subject an effective amount of any of the immunogenic compositions or vaccines described herein, thereby treating micrometastasis or residual disease in the subject.

10 In yet another aspect, the invention generally features a method of immunizing a subject, the method involving administering to the subject any of the immunogenic compositions or vaccines described herein.

In yet another aspect, the invention generally features a pharmaceutical composition for the treatment of neoplasia containing an effective amount of any of the immunogenic compositions or vaccines described herein and a pharmaceutically acceptable excipient.

15 In yet another aspect, the invention generally features a kit for the treatment of a neoplasia, the kit containing an effective amount of any of the immunogenic compositions or vaccines described herein, and directions for using the kit as in any of the methods described herein.

20 In yet another aspect, the invention generally features a method of treating or preventing cervical cancer in a subject, the method comprising administering to the subject an effective amount of any of the immunogenic compositions or vaccines described herein, thereby treating or preventing cervical cancer in the subject.

In yet another aspect, the invention generally features a method of treating polymorphic TLR deficiencies in a subject, the method comprising administering to the subject an effective amount of any of the immunogenic compositions or vaccines described herein, thereby treating the polymorphic TLR deficiencies in the subject.

In various embodiments of any of the above aspects or any other aspect of the invention delineated herein, the cytokine is granulocyte-macrophage colony-stimulating factor (GM-CSF).

30 In another embodiment the cell contains comparable amounts of at least one TLR agonist and GM-CSF. In a further embodiment, the TLR agonist is a TLR4, TLR5, TLR6, TLR7 or TLR8,

TLR9 agonist. In yet another embodiment the immunogenic composition contains a TLR4 agonist and a TLR7/8 agonist. In another embodiment the TLR4 agonist is LPS, an LPS fragment, or a synthetic glucopyranosyl lipid A analogue and the TLR7/8 analog is R848. In other embodiments the cells are rendered proliferation incompetent by irradiation. In certain 5 embodiments the neoplastic cell is selected from the group consisting of leukemia, chronic myeloid leukemia, prostate cancer, head and neck, Squamous Cell Carcinoma, tongue cancer, larynx cancer, tonsil cancer, hypopharynx cancer, nasopharynx cancer, breast cancer, colon cancer, lung cancer, melanoma, pancreatic cancer, glioblastoma and brain cancer. In other 10 embodiments the composition comprises at least about 1 ng of TLR4 agonist per 1×10^5 cells -10 ng of TLR4 agonist per 1×10^5 cells. In further embodiments the composition contains at least about 3-5 ng per 5×10^5 cells. In another embodiment the cell contains a TLR agonist in 15 association with Lipofectamine or other liposomal vectors. In further embodiments the cell contains LPS/liposomal micelles. In other embodiments the immunogenic composition contains cells that express one or more tumor antigens. In further embodiments the immunogenic composition contains cells that are autologous or allogeneic.

In various embodiments of any of the above aspects or any other aspect of the invention delineated herein, the neoplastic cell is derived from a cancer cell line or is derived from a tumor. In another embodiment the cancer cell line or tumor is selected from leukemia, chronic myeloid leukemia, prostate cancer, head and neck, Squamous Cell Carcinoma, tongue cancer, larynx cancer, tonsil cancer, hypopharynx cancer, nasopharynx cancer, breast cancer, colon cancer, lung cancer, melanoma, pancreatic cancer, glioblastoma and brain cancer. In another 20 embodiment the immunogenic composition is administered systemically or locally. In further embodiments the immunogenic composition is administered by intramuscular injection, intravenous injection, intratumoral injection, or peritumoral injection. In additional 25 embodiments the methods involve administering to the subject an effective amount of one or more chemotherapeutics. In certain embodiments the one or more chemotherapeutics is selected from abiraterone acetate, altretamine, anhydrovinblastine, auristatin, bexarotene, bicalutamide, BMS184476, 2,3,4,5,6-pentafluoro-N-(3-fluoro-4-methoxyphenyl)benzene sulfonamide, bleomycin, N,N-dimethyl-L-valyl-L-valyl-N-methyl-L-valyl-L-prolyl-L-proline-t-butylamide, 30 cachectin, cemadotin, chlorambucil, cyclophosphamide, 3',4'-didehydro-4'-deoxy-8'-norvincaleukoblastine, docetaxol, doxetaxel, cyclophosphamide, carboplatin, carmustine

(BCNU), cisplatin, cryptophycin, cyclophosphamide, cytarabine, dacarbazine (DTIC), dactinomycin, daunorubicin, decitabine dolastatin, doxorubicin (adriamycin), etoposide, 5-fluorouracil, finasteride, flutamide, hydroxyurea and hydroxyureataxanes, ifosfamide, liarozole, lonidamine, lomustine (CCNU), MDV3100, mechlorethamine (nitrogen mustard), melphalan, 5 mivobulin isethionate, rhizoxin, sertenef, streptozocin, mitomycin, methotrexate, taxanes, nilutamide, onapristone, paclitaxel, prednimustine, procarbazine, RPR109881, stramustine phosphate, tamoxifen, tasonermin, taxol, tretinoin, vinblastine, vincristine, vindesine sulfate, and vinflunine. In additional embodiments the methods enhance activation of dendritic cells and/or increases the number of AH1-specific cytotoxic T-cells or p15E-specific cytotoxic T-cells relative to the use of GVAX alone. In certain embodiments the methods reduce or stabilize tumor cell proliferation, tumor growth, or subject survival relative to the use of GVAX alone. In further embodiments the cell is derived from a leukemia, chronic myeloid leukemia, prostate cancer, head and neck, Squamous Cell Carcinoma, tongue cancer, larynx cancer, tonsil cancer, hypopharynx cancer, nasopharynx cancer, breast cancer, colon cancer, lung cancer, melanoma, 10 pancreatic cancer, glioblastoma and brain cancer or a cell line thereof. In additional 15 embodiments the cell comprises a TLR4 and a TLR7/8 agonist. In other embodiments the TLR agonist is in association with liposomal vector.

Definitions

20 By "cytokine" is meant a hormone that acts locally and that modulates an individual's immune response.

By "genetically modified neoplastic cell" is meant a cell or a population of cells that has been genetically modified to express a transgene, and that is administered to a patient as part of cancer therapy. An immunogenic composition or vaccine of the invention comprises neoplastic 25 (e.g., tumor) cells that are "autologous" or "allogeneic" to the patient undergoing treatment or "bystander cells" that are mixed with tumor cells taken from the patient. Generally, the genetically modified neoplastic cell is of the same general type of tumor cell as is afflicting the patient. For example, a patient suffering from melanoma will typically be administered a genetically modified cell derived from a melanoma. A GM-CSF-expressing genetically 30 modified tumor cell vaccine may be referred to herein as "GVAX". Autologous and allogeneic cancer cells that have been genetically modified to express a cytokine, e.g., GM-CSF, followed

by readministration to a patient for the treatment of cancer are described in U.S. Pat. Nos. 5,637,483, 5,904,920, 6,277,368 and 6,350,445, as well as in US Patent Publication No. 20100150946, each of which is expressly incorporated by reference herein. A form of GM-CSF-expressing genetically modified cancer cells or a "cytokine-expressing cellular vaccine" for 5 the treatment of pancreatic cancer is described in U.S. Pat. Nos. 6,033,674 and 5,985,290, both of which are expressly incorporated by reference herein. A universal immunomodulatory cytokine-expressing bystander cell line is described in U.S. Pat. No. 6,464,973, expressly incorporated by reference herein.

By "Granulocyte-macrophage colony stimulating factor (GM-CSF) polypeptide" is 10 meant a cytokine or fragment thereof having immunomodulatory activity and having at least about 85% amino acid sequence identity to GenBank Accession No. AAA52122.1.

An exemplary GM-CSF sequence (NCBI AAA52122.1) is provided below:

1 mwlqsllllg tvacsisapa rspspstqpw ehvnaiqear rllnlrsrdta aemnetvevi
61 semfdlqeqt clqtrlelyk qqlrgsltk1 kgpltmmash ykqhcpppte tscatqitf
121 esfkenlkdf llvipfdcwe pvqe

GM-CSF has been shown to induce the growth of hematopoietic cells of granulocyte and 20 macrophage lineages. In addition, it also activates the antigen processing and presenting function of dendritic cells, which are the major antigen presenting cells (APC) of the immune system. In one embodiment, an immunogenic composition comprises a GM-CSF coding sequence operatively linked to regulatory elements for expression in the cells of the vaccine.

By "GM-CSF nucleic acid molecule" is meant a polynucleotide encoding a GM-CSF polypeptide.

A GM-CSF nucleic acid molecule may encode a murine or human GM-CSF and may be 25 in the form of genomic DNA (See, US Patent Publication NO. 2006/0057127, which is hereby incorporated by reference in its entirety) or cDNA (See US Patent Publication NO. 2006/0057127, which is hereby incorporated by reference in its entirety). In one embodiment, the GM-CSF coding sequence encodes the amino acid sequence described in US Patent Publication NO. 2006/0057127, which is hereby incorporated by reference in its entirety. Other 30 examples of GM-CSF coding sequences are found in Genbank accession numbers:AF373868, AC034228, AC034216, M 10663 and NM000758.

An exemplary GM-CSF coding sequence (AF373868) is provided below:

1 gcagtctgtt tcctaccaga ctgggagcct caagggccaa atgtgagggc cagtggagg
61 gtcccgttt cctccccaga acaggtcctg gtgtggattt gaaagacttg ttgactgact

5	121	gtctgagcta	tgacaactca	tttcttaggag	gaaagtgacc	ttctctccca	gatgggcat
	181	acaggctctc	tgcctccctg	gccatcagct	gaaccactat	ctatggctcc	cttccctgccc
	241	ctccagcctc	cagggtgcta	tccaaacacat	gtgatatcta	catgtatgt	ccatgtcctc
	301	atctctcccc	cgagagctcc	ctggaaagag	ctgagccaag	gccttgcaaa	aaaggtggag
	361	aaagggccag	ggcctggaca	tttcatgttc	ccaccccccagc	ctggccacta	ggagtgttct
	421	acgcaggctc	agatggatgg	ggctggcctc	acagtggggt	ctggaggact	aaggtttgg
10	481	ttctctatgc	aaggtcagaa	aaactccac	agtacaggga	aactggccag	ggctgcagac
	541	tcagaccaca	gtgctaaagc	catgaactcc	acctgctctc	tgaaggctcg	ccaacctgag
	601	tccagcagaa	tgttctcgct	tgtgtccaaac	cccactgggt	taggctgaat	cagcctctag
	661	ggcccagagg	cactgcaccc	ggagtaggg	gcttctccag	tatcagagtc	accttcagag
	721	gcctggagcc	tttcataaaag	caggtaaagag	gactcaatag	atgcatctgc	atggaaaaca
	781	tcctccccc	taccaggcac	ctgtatgtac	aaccaatcac	agcagcacac	atacaccag
	841	aatgggcac	gtgtgggccc	acacccctt	agctatgaaa	cccaggcatg	gggcagcttg
15	901	agccagatac	cttgcataaa	cacaaactcg	tgctgtctc	tctgaactcc	attgtgaaaa
	961	tcaaacactt	gtcagcccc	caagagccct	tagatttct	actttccacac	ttccacagaa
	1021	aggcctctgg	agttggggga	tgctgggggtt	atgttaggaaa	ttaagcctgg	agggccttgc
	1081	tggggaaagcc	attgtccctg	tacctgagat	ggatgcagcc	acagccctgg	agccagctg
	1141	aagctcctgg	tgtcttctgg	gggctacata	taggagtgt	gtccgaacct	cagagggcga
20	1201	aacctgctct	gcagagggaa	tcaaggttca	cataaccaga	gaggggagtc	actcaggaag
	1261	gtggctccag	agccaagagt	cagactctgg	gtcccgaatt	gaccagcca	caccccccct
	1321	gaagcttgc	gagagtggct	gcagtcctgc	tgctggatgt	gcacatggtg	gtcattccct
	1381	ctgctcacag	gggcaggggt	ccccccctac	tggactgagg	ttgccccctg	ctccagggtcc
	1441	tgggtgggag	cccatgtgaa	ctgtcagttgg	ggcaggtctg	tgagagctcc	cctcacactc
	1501	aagtctctca	cagtggccag	agaagaggaa	ggctggagtc	agaatgaggc	accaggccgg
25	1561	gcatagcctg	cccaaaggcc	cctgggatta	cagggcaggat	ggggagccct	atctaagtgt
	1621	ctcccaacgc	ccacccccc	cattccaggc	caggaagtcc	aaactgtgcc	cctcagaggg
	1681	agggggcagc	ctcaggcccc	ttcagactgc	ccagggaggg	ctggagagcc	ctcaggaagg
	1741	cgggtgggt	ggctgtcggt	tcttggaaag	gttcatata	gaaaaccccc	aagcctgacc
30	1801	acctagggaa	aaggctcacc	gttccatgt	gtggctgata	agggccagga	gattccacag
	1861	ttcaggtagt	tcccccgcct	ccctggcatt	ttgtggtcac	cattaatcat	ttccctctgt
	1921	tatthaagag	ctttttgccc	agtgagccca	gtacacagag	agaaaaggct	aaggttctct
	1981	gaggatgtgg	ctgcagagcc	tgctgtctt	gggcactgtg	gcctgcagca	tctctgcacc
	2041	cggccgctcg	cccaagcccc	gcacgcagcc	ctgggagcat	gtgaatgcca	tccaggaggg
35	2101	ccggcgtctc	ctgaacctga	gtagagacac	tgctgtctgag	atgttaagtgt	agagaatgtg
	2161	ggcctgtgcc	taggccacc	agctggcccc	tgactggcca	cgcctgtcag	tttgataaca
	2221	tgacattttc	cttttctaca	gaatgaaaaca	gtagaagtca	tctcagaaat	gtttgaccc
	2281	caggtaaagat	gcttctctct	gacatagctt	tccagaagcc	cctggccctgg	ggtggaggtg
	2341	gggactccat	tttagatggc	accacacagg	gttgcact	ttctctccag	tcagctggct
40	2401	gcaggaggag	ggggtagcaa	ctgggtgctc	aagaggctgc	tggcctgtcc	cctatggcag
	2461	tcacatgagc	tcctttatca	gctgagccgc	catgggcaga	cctagcattc	aatggccagg
	2521	agtcaaccagg	ggacaggtgg	taaagtgggg	gtcacttcat	gagacaggag	ctgtgggtt
	2581	ggggcgctca	ctgtgcccc	agaccaagtc	ctgttgagac	agtgtctact	acagagaggc
	2641	acagaggggt	ttcaggaaca	acccttgc	accacagg	tccaggtgag	gccccacccc
45	2701	cctctccctg	aatgatgggg	tgagagtca	ctccttcct	aaggctggc	tcctctccag
	2761	gtgcccgtga	gggtggcctg	ggcggggcag	tgagaaggc	aggttcgtc	ctgcatgg
	2821	cagggcaggg	tctatgactg	gaccacgcct	gtgccccctcc	caagccctac	tcctggggcc
	2881	tgggggcagc	agcaaaaagg	agtgggtggag	agttcttgc	ccactgtgg	cacttggcc
	2941	ctgctcaccc	acgaacgaca	ttttccacag	gagccgaccc	gcctacagac	ccgcctggag
50	3001	ctgtacaaggc	agggcctgcg	gggcagccctc	accaagctca	agggccctt	gaccatgtat
	3061	gccagccact	acaaggcagca	ctgcctccca	accccggtga	gtgctacgg	cagggccctcc
	3121	agcaggaatg	tcttaatcta	gggggtgggg	tcgacatgg	gagagatcta	tggctgtggc
	3181	tgttcaggac	cccagggggt	ttctgtgcca	acagttatgt	aatgattagc	cctccagaga
	3241	ggaggcagac	agcccatttc	atcccaagga	gtcagagcc	cagagcgtcg	aagcccacag
	3301	tgctccccag	caggagctgc	tcctatcctg	gtcattatgt	tcattatgt	taatgaggtc
55	3361	agaggtgagg	gcaaaacccaa	ggaaacttgg	ggcctgccc	aggcccagag	gaagtgc
	3421	ggcccaagtg	ccaccttctg	gcaggacttt	cctctggccc	cacatgggt	gcttgaattg
	3481	caqaqqatca	aqqaqqqqq	qctacttqqa	atqqacaqq	acctcaqqca	tccttcctq

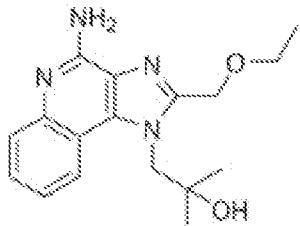
3541 cgggaaggga gcaaagttg tggccttgac tccactcctt ctgggtgccc agagacgacc
 3601 tcagccccgc tgccctgctc tgccctggga ccaaaaaggc aggcgttga ctgcccagaa
 3661 ggccaaaccc tc aggcgttgcac ttaagtctagg cccttgcactc tggtgtccac tggcagagct
 3721 atgcactcct tggggaaacac gtgggtggca gcagcgtcac ctgacccagg tcagtggtg
 3781 tgtcctggag tgggcctcctt ggcctcttag ttctaaaggagg cagtagagaa acatgtgtt
 3841 gcttccttcc cccacgttac ccacttgctt ggactcaagt gttttttatt tttcttttt
 3901 taaaggaaac ttccctgtca acccagatta tcaccttga aagtttcaaa gagaacctga
 3961 aggactttct gcttgcatacc ccccttgcact gctgggagcc agtccaggag tgagaccggc
 4021 cagatgaggc tggccaaagcc gggagactgc tctctcatga aacaagagct agaaacttag
 4081 gatgtcatac ttggaggggac caagggttgg ggcacagcca tgggtggagt ggcctggacc
 4141 tgccctgggc cacactgacc ctgatacagg catggcagaa gaatgggaat atttataact
 4201 gacagaaatc agtaatattt atatatttat atttttaaaat tatttattttt tttattttt
 4261 taagttcata ttccatattt attcaagatg ttttaccgtt ataattatta taaaaaatat
 4321 gcttctactt gtccagtggtt ctatgttgg ttttaccatg agcaaatgccc agtgggtgc
 4381 gcctccat gaggcagggg agggaggaaa cggggaggtg gagagggggc gggggccctcc
 4441 caggcgttgg gcactatcca aggccaaaca ctgtcagagc agagggggagg tgagagccgg
 4501 gcataagggtc ggaattctgc acacctggac gggcttcccg ggtatgtccca gggctccac
 4561 cccagagaat ggctctcaag ttcacctgga agtccaagtg accagcccaag ggaactctta
 4621 tccccagagaa gggcaccacc cttcctgggg aggccctgggg gttggctggg cactggctga
 4681 acagggccac tctggcatca ggcaaaacac ctgcctgtt gaggccttgg cccctgtgcc
 4741 ccacgcctg cccctcacac tctgagattt aaccatccg aaagtaaaca gaaaataga
 4801 ctaactgttc agggaaaag aaaccaaacc acagggtca cagtgcagcg tatttaccaa
 4861 acttgccccca aaatgggtga tcttaatctc tgagagtcg aatgtaaagg cataatttg
 4921 tggtacatgg ctgttagtgcc gcatgtttctt gaattgggtt ttattttac atgaaatttt
 4981 gaatctaactt aggcaacttc ccctaaaactt catggcctgc aggtaaaaaa caaagttaggc
 5041 ctcccttc tccttacttt gacagctggg ctcaaggcct ttttccctgaa cctgtccct
 5101 catctccctc caggactatg aggaagtggaa tggccccaa gtcttaggcg ggcagcagg
 5161 ccagcttctc cttgacaggt gggcttaagg aagctgctt gtggcagctt tagccctgc
 5221 ctggcactgt ctgcagtcat gcccccacca ccccttgc ttccctact tcagtcagca
 5281 cctgcagaca ggcgcaggcc tggccagaga cccactccat gtcatgcag aaagaccgtg
 5341 acttcagggtg tgattacaaa taagaagtca gggtaacgc tcaggatgaa gcctgagtgt
 5401 cagcacaggc aagaatccat gaagtgtgtc gtgggtgtt aaaaatgcatg aaaaatcacat
 5461 cttggcccaggc gataagggtcc tctctgtctt ccgcgttaagg cagtgtatgac tgataagagg
 5521 tttagcattt ccttagcctc acatataatag gtaccctct ccacagaaat gctgccaagc
 5581 ccaggcctcg gaccagttt ggtcacctt caagtaatac catgcacccctg tacgtgtcc
 5641 tggctcatgt gctctgggg tcagaaagcc attcttccca atgaaaggtag ccacgatatc
 5701 tccccacgaa aagtacacag cagtctgtgc tgacattcag aaaaactctt cggctgacaa
 5761 taacacacac aagataagtc tgggtctcca tcaaacgtta tttgtctt agtgcctt
 5821 tgtgctctg accaattttt ctggcttccg gggcccttc aataggcccc agaaaaccag
 5881 tgaggttaaga aacagctgcc ccggacctt tcataaccaca tttgaacagg gagagagaga
 5941 ttcaccagt cagtgcctc ggaagagata acaacaagggtt atagtggagt ga

By “Toll-like receptor agonist” is meant an agent that activates a member of the TLR receptor family.

By “glucopyranosyl lipid A” or “glycopyranosyl lipid A” or “glycopyranosyl lipid 45 adjuvant” or “GLA” is meant a novel, clinical-stage, human toll-like receptor-4 (TLR-4) agonist which is described in U.S. Patent Application No. 11/862,122 and U.S. Patent Application Publication No. 20080131466 which are incorporated herein in their entirety.

By “liposomal vector” is meant any composition that can be used to deliver agents to cells through formation of liposomes, a non-limiting example of which is lipofectamine.

By "R848" is meant an imidazoquinoline compound with a molecular weight of 314.17 which is a TLR agonist that activates immune cells via the TLR7/TLR8 MyD88-dependent signaling pathway. R848 has the following formula: C₁₇H₂₂N₄O₂, and structure:



5

By "TEGVAX" is meant a GVAX further formulated with a TLR agonist.

By "Toll like receptor (TLR)" is meant a member of the Toll-like receptor family of proteins or a fragment thereof that senses a microbial product and/or initiates an adaptive immune response. In one embodiment, a TLR activates a dendritic cell (DC).

10 By "Toll like receptor 4 (TLR4)" is meant a polypeptide having at least 85% sequence identity to NP_612564, or a fragment thereof having and having immunomodulatory activity. The sequence of human toll-like receptor 4 precursor NP_612564 is provided below:

15	1 mmsasrlagt lipamaflsc vrpeswepcv evvpnityqc melnfykipd nlpfstknld 61 lsfnpnrlhlg sysffsfpel qvldlsrcei qtiedgayqs lshlstlilt gnpiqslalg 121 afsglsslqk lvavetnlas lenfpighlk tlkelnvahn liqsfklpey fsnltnlehl 181 dlssnkiqsi yctdlrvlhq mpllnlsldl slnpmnfiqp gafkeirllhk ltlnnnfdsl 241 nvmktciqgl aglevhrlvl gefrnegnle kfdksalegl cnltieefrl ayldyylddi 301 idlfncltnv ssfsllvsvti ervkdfsynf gwqhlelvnc kfgqfpblk kslkrltfts 361 nkgganfsev dlpslefldl srnglsfkgc csqsdftts lkyldlssfng vitmssnflg 421 leqlehldfq hsnlkqmsef svfislrnli yldishthtr vafngifngl sslevlkmag 481 nsfqenflpd iftelrnltf ldlsqcqleq lsptafnsls slqvlnmshn nffsldtfpy 541 kclnslqvld yslnhimtsk kqelqhfppss laflnltqnd factcehqsf lqwikkdqrql 601 lvevermeca tpsdkqgmpv lslnitcqmnn ktiigvsvls vlvvsvvavl vykfyfhlml 661 lagcikygrg eniydafviy ssqdedwvrn elvknleegv ppfqclhyr dfipgvaiaa 721 nniheghks rkviivvvsqh fiqsrwcfie yeiaqtwqfl ssragiifiv lqkvektllr 781 qqvelyrls rntyleweds vlgrhifwrr lrkalldgks wnpegtvgtg cnwqeatsi
20	
25	

By "ameliorate" is meant decrease, suppress, attenuate, diminish, arrest, or stabilize the development or progression of a disease.

30 By "analog" is meant a molecule that is not identical, but has analogous functional or structural features. For example, a polypeptide analog retains the biological activity of a corresponding naturally-occurring polypeptide, while having certain biochemical modifications that enhance the analog's function relative to a naturally occurring polypeptide. Such

biochemical modifications could increase the analog's protease resistance, membrane permeability, or half-life, without altering, for example, ligand binding. An analog may include an unnatural amino acid.

In this disclosure, "comprises," "comprising," "containing" and "having" and the like can have the meaning ascribed to them in U.S. Patent law and can mean "includes," "including," and the like; "consisting essentially of" or "consists essentially" likewise has the meaning ascribed in U.S. Patent law and the term is open-ended, allowing for the presence of more than that which is recited so long as basic or novel characteristics of that which is recited is not changed by the presence of more than that which is recited, but excludes prior art embodiments.

By "disease" is meant any condition or disorder that damages or interferes with the normal function of a cell, tissue, or organ.

By "effective amount" is meant an amount sufficient to induce or enhance an immune response against a neoplastic cell or specific to a tumor antigen, to reduce or stabilize tumor growth, to enhance subject survival, or to otherwise ameliorate the symptoms of a disease relative to an untreated patient. The effective amount of active compound(s) used to practice the present invention for therapeutic treatment of a disease varies depending upon the manner of administration, the age, body weight, and general health of the subject. Ultimately, the attending physician or veterinarian will decide the appropriate amount and dosage regimen. Such amount is referred to as an "effective" amount.

By "fragment" is meant a portion of a polypeptide or nucleic acid molecule. This portion contains, preferably, at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% of the entire length of the reference nucleic acid molecule or polypeptide. A fragment may contain 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100, 200, 300, 400, 500, 600, 700, 800, 900, or 1000 nucleotides or amino acids. In one embodiment, a fragment of a TLR4 agonist comprises at least about 10, 25, 50, 100, 150, 200, 250, 300, 400, 500, 600 or 700 amino acids of LPS or another TLR4 agonist that is sufficient to enhance an immune response in a subject.

"Hybridization" means hydrogen bonding, which may be Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary nucleotide bases. For example, adenine and thymine are complementary nucleotide bases that pair through the formation of hydrogen bonds.

By "isolated" is meant a material that is free to varying degrees from components which normally accompany it as found in its native state. "Isolate" denotes a degree of separation from original source or surroundings.

By "isolated polynucleotide" is meant a nucleic acid (e.g., a DNA) that is free of the 5 genes which, in the naturally-occurring genome of the organism from which the nucleic acid molecule of the invention is derived, flank the gene. The term therefore includes, for example, a recombinant DNA that is incorporated into a vector; into an autonomously replicating plasmid or virus; or into the genomic DNA of a prokaryote or eukaryote; or that exists as a separate molecule (for example, a cDNA or a genomic or cDNA fragment produced by PCR or 10 restriction endonuclease digestion) independent of other sequences. In addition, the term includes an RNA molecule that is transcribed from a DNA molecule, as well as a recombinant DNA that is part of a hybrid gene encoding additional polypeptide sequence.

By an "isolated polypeptide" is meant a polypeptide of the invention that has been separated from components that naturally accompany it. Typically, the polypeptide is isolated 15 when it is at least 60%, by weight, free from the proteins and naturally-occurring organic molecules with which it is naturally associated. Preferably, the preparation is at least 75%, more preferably at least 90%, and most preferably at least 99%, by weight, a polypeptide of the invention. An isolated polypeptide of the invention may be obtained, for example, by extraction from a natural source, by expression of a recombinant nucleic acid encoding such a polypeptide; 20 or by chemically synthesizing the protein. Purity can be measured by any appropriate method, for example, column chromatography, polyacrylamide gel electrophoresis, or by HPLC analysis.

By "micrometastasis" is meant a form of metastasis in which secondary tumors are too minuscule to be clinically detected.

25 By "minimal residual disease" is meant the small numbers of tumor cells that remain in the patient during treatment or after treatment.

By "neoplasia" is meant a disease characterized by the pathological proliferation of a cell or tissue and its subsequent migration to or invasion of other tissues or organs. Neoplasia growth is typically uncontrolled and progressive, and occurs under conditions that would not 30 elicit, or would cause cessation of, multiplication of normal cells. Neoplasias can affect a variety of cell types, tissues, or organs, including but not limited to an organ selected from the group

consisting of bladder, bone, brain, breast, cartilage, esophagus, fallopian tube, gallbladder, heart, intestines, kidney, liver, lung, lymph node, nervous tissue, ovaries, pancreas, prostate, skeletal muscle, skin, spinal cord, spleen, stomach, testes, thymus, thyroid, trachea, urogenital tract, ureter, urethra, uterus, and vagina, or a tissue or cell type thereof. Neoplasias include cancers, 5 such as sarcomas, carcinomas, or plasmacytomas (malignant tumor of the plasma cells).

Neoplasia cells that invade surrounding tissue or enter the bloodstream or lymphatic vessels form secondary tumors, or metastases, at a distance from the original tumor. Neoplasia that has metastasized is more difficult to treat and often has a poorer prognosis. Depending on the severity of the neoplasia (i.e., tumor size and invasiveness), a stage number is assigned, I, II, 10 III, or IV. Stage I neoplasias are the least advanced and have the best prognosis. Stage II neoplasias typically include larger tumors and are associated with a somewhat poorer prognosis. Stage III and IV neoplasias have spread beyond their sites of origin and have the poorest prognosis.

As used herein, "obtaining" as in "obtaining an agent" includes synthesizing, 15 purchasing, or otherwise acquiring the agent.

As used herein, "recombinant" includes reference to a polypeptide produced using cells that express a heterologous polynucleotide encoding the polypeptide. The cells produce the recombinant polypeptide because they have been genetically altered by the introduction of the appropriate isolated nucleic acid sequence. The term also includes reference to a cell, or nucleic 20 acid, or vector, that has been modified by the introduction of a heterologous nucleic acid or the alteration of a native nucleic acid to a form not native to that cell, or that the cell is derived from a cell so modified. Thus, for example, recombinant cells express genes that are not found within the native (non-recombinant) form of the cell, express mutants of genes that are found within the native form, or express native genes that are otherwise abnormally expressed, under- 25 expressed or not expressed at all.

By "reduces" is meant a negative alteration of at least 10%, 25%, 50%, 75%, or 100%.

By "reference" is meant a standard or control condition.

A "reference sequence" is a defined sequence used as a basis for sequence comparison.

A reference sequence may be a subset of or the entirety of a specified sequence; for example, a 30 segment of a full-length cDNA or gene sequence, or the complete cDNA or gene sequence. For polypeptides, the length of the reference polypeptide sequence will generally be at least about

16 amino acids, preferably at least about 20 amino acids, more preferably at least about 25 amino acids, and even more preferably about 35 amino acids, about 50 amino acids, or about 100 amino acids. For nucleic acids, the length of the reference nucleic acid sequence will generally be at least about 50 nucleotides, preferably at least about 60 nucleotides, more 5 preferably at least about 75 nucleotides, and even more preferably about 100 nucleotides or about 300 nucleotides or any integer thereabout or there between.

By "specifically binds" is meant a compound or antibody that recognizes and binds a polypeptide of the invention, but which does not substantially recognize and bind other molecules in a sample, for example, a biological sample, which naturally includes a polypeptide 10 of the invention.

Nucleic acid molecules useful in the methods of the invention include any nucleic acid molecule that encodes a polypeptide of the invention or a fragment thereof. Such nucleic acid molecules need not be 100% identical with an endogenous nucleic acid sequence, but will typically exhibit substantial identity. Polynucleotides having "substantial identity" to an 15 endogenous sequence are typically capable of hybridizing with at least one strand of a double-stranded nucleic acid molecule. Nucleic acid molecules useful in the methods of the invention include any nucleic acid molecule that encodes a polypeptide of the invention or a fragment thereof. Such nucleic acid molecules need not be 100% identical with an endogenous nucleic acid sequence, but will typically exhibit substantial identity. Polynucleotides having 20 "substantial identity" to an endogenous sequence are typically capable of hybridizing with at least one strand of a double-stranded nucleic acid molecule. By "hybridize" is meant pair to form a double-stranded molecule between complementary polynucleotide sequences (e.g., a gene described herein), or portions thereof, under various conditions of stringency. (See, e.g., Wahl, G. M. and S. L. Berger (1987) Methods Enzymol. 152:399; Kimmel, A. R. (1987) 25 Methods Enzymol. 152:507).

By "substantially identical" is meant a polypeptide or nucleic acid molecule exhibiting at least 50% identity to a reference amino acid sequence (for example, any one of the amino acid sequences described herein) or nucleic acid sequence (for example, any one of the nucleic acid sequences described herein). Preferably, such a sequence is at least 60%, more preferably 80% 30 or 85%, and more preferably 90%, 95% or even 99% identical at the amino acid level or nucleic acid to the sequence used for comparison.

Sequence identity is typically measured using sequence analysis software (for example, Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, Wis. 53705, BLAST, BESTFIT, GAP, or PILEUP/Prettybox programs). Such software matches identical or similar 5 sequences by assigning degrees of homology to various substitutions, deletions, and/or other modifications. Conservative substitutions typically include substitutions within the following groups: glycine, alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid, asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine. In an exemplary approach to determining the degree of identity, a BLAST program may be used, with a 10 probability score between e^{-3} and e^{-100} indicating a closely related sequence.

By "subject" is meant a mammal, including, but not limited to, a human or non-human mammal, such as a bovine, equine, canine, ovine, or feline.

A "tumor," as used herein, refers to all neoplastic cell growth and proliferation, whether malignant or benign, and all precancerous and cancerous cells and tissues.

15 Ranges provided herein are understood to be shorthand for all of the values within the range. For example, a range of 1 to 50 is understood to include any number, combination of numbers, or sub-range from the group consisting 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50.

20 As used herein, the terms "treat," "treating," "treatment," and the like refer to reducing or ameliorating a disorder and/or symptoms associated therewith. It will be appreciated that, although not precluded, treating a disorder or condition does not require that the disorder, condition or symptoms associated therewith be completely eliminated.

25 Unless specifically stated or obvious from context, as used herein, the term "or" is understood to be inclusive. Unless specifically stated or obvious from context, as used herein, the terms "a", "an", and "the" are understood to be singular or plural.

30 Unless specifically stated or obvious from context, as used herein, the term "about" is understood as within a range of normal tolerance in the art, for example within 2 standard deviations of the mean. About can be understood as within 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.5%, 0.1%, 0.05%, or 0.01% of the stated value. Unless otherwise clear from context, all numerical values provided herein are modified by the term about.

The recitation of a listing of chemical groups in any definition of a variable herein includes definitions of that variable as any single group or combination of listed groups. The recitation of an embodiment for a variable or aspect herein includes that embodiment as any single embodiment or in combination with any other embodiments or portions thereof.

5 Any compositions or methods provided herein can be combined with one or more of any of the other compositions and methods provided herein.

BRIEF DESCRIPTION OF THE DRAWINGS

10 Figure 1 shows the delivery of TLR4 agonist into GVAX. Figure 1 is a set of FACS graphs showing the delivery of TLR4 agonists into GVAX. To optimize the absorption efficiency of LPS into the GVAX vaccine cells, the concentrations of Lipofectamine and LPS-BODIPY fluorophore conjugate were varied as noted. Cells were washed 5 times prior to the flow cytometry analysis. LPS was undetectable in the final wash using the LAL assay to less
15 than 0.125 EU/ml. Optimal absorption was at 6 hr incubation with 40 μ g/ml of Lipofectamine and 25 μ g/ml of LPS per 1×10^5 cells. These conditions were used for quantitation of LPS on a per cell basis.

Figures 2A, 2B, and 2C show that TEGVAX can induce an anti-tumor response *in vivo*. B16 inoculated C57BL/6 (Figure 2A), SCCFVII/SF inoculated C3H (Figure 2B), and CT26 20 inoculated Balb/c (Figure 2C) mice were treated with appropriate PBS, GVAX, or TEGVAX peritumorally typically from 3-5 days after the tumor injection. *In vivo* tumor progression in the TEGVAX group in all the murine models studied was statistically slower than those in the GVAX group. For the B16 and the SCCFVII/SF models, there were no differences between GVAX treated group and the PBS treated group. In the CT26 model, 40-60% of the mice in the 25 TEGVAX group experienced a regression of their palpable tumor to undetectable levels by day 18. Equimolar LPS formulated into 293T cells also showed no difference in growth rate in comparison to control treated mice. Mice that showed regression of tumor were rechallenged with CT26 tumor (2×10^6) and no tumor growth were noted. In all three murine models, the reduced tumor growth rate correlated with enhanced survival for mice in the TEGVAX group 30 compared to those in the GVAX group.

Figure 3 shows that the improved *in vivo* anti-tumor response noted for TEGVAX is MyD88 dependent. Using the B16 model, the *in vivo* tumor growth rate was performed with MyD88 null mice with the vaccine treatment performed intratumorally 3-5 days after tumor inoculation. The anti-tumor response in the TEGVAX treated group is abrogated in MyD88 null

5 mice.

Figures 4A and 4B show that TEGVAX treatment increases the lymphocytic and antigen presenting cell infiltration into the tumor microenvironment. Figure 4A is a set of photomicrographs of the immunohistochemical staining of CD4+, CD8+, CD86+, and CD45+ cells in CT26 tumor treated with control, GVAX, or TEGVAX. The yellow cells represent the

10 co-localized staining of CD4 or CD8 with CD45 in the first two panels. The third panel represents CD86 conjugate staining alone. These cells were formally quantitated in 10 randomly selected fields per slice at 40X magnification. Figure 4B is a graphical presentation of the quantitated data obtained by immunohistochemistry. Within the tumor treated with either GVAX or TEGVAX there were statistical differences in each of quantitated CD4, CD8,

15 CD86 and CD45 cells between the two groups (P<0.01).

Figure 5 shows that TEGVAX treatment increases activated dendritic cells (DC) in the draining lymph nodes (DLN). CT26 tumor bearing Balb/c mice were treated with PBS (not shown), GVAX, or TEGVAX peritumorally 3 days after the tumor injection. DCs were isolated from the DLN 5-7 days after treatment. B220+CD11c+ cells were gated and CD86, 20 MHCI, and CD80 staining were analyzed as shown. TEGVAX had increased number of CD86+MHCI+ as well as CD80+MHCI+ DCs in the DLN in comparison to GVAX treated group.

Figures 6A and 6B show that TEGVAX treatment increases the number of tumor specific CD8+ T-cells. CT26 bearing Balb/c mice were treated with PBS, GVAX, or TEGVAX 25 peritumorally 3 days, and 5 days later CD8+ cells were isolated and purified from the spleen and lymph nodes. ELISPOT assays were performed using 4T1 cells as APCs and AH1 peptide, and IFN-levels were measured. Figure 6A is a graph of the number of AH1 specific IFN-producing T-cells were statistically greater in the TEGVAX group compared to GVAX group in both the spleen and the draining LN (P<0.01). Figure 6B is a graph of AH1 specific CTL killing. *In* 30 *vivo* CTL assays were used to measure percent killing of AH1 specific CTLs in CT26 tumor bearing mice treated with or without TEGVAX.-gal CFSE labeled low and AH1 CFSE labeled

high cells were co-injected into naïve, tumor bearing or TEGVAX treated tumor bearing groups. The average mean specific tumor lysis was higher in the TEGVAX group compared to the untreated groups (P<0.07).

Figure 7 shows that GVAX with equimolar LPS injected separately without formulation 5 do not induce an anti -tumor response. B16 bearing mice were treated peritumorally with either GVAX, LPS, or with a mixture of LPS and GVAX without liposomal formulation. Equimolar amounts of LPS as used in Figure 2 were injected peritumorally in these experiments.

Figure 8 shows that TEGVAX can also induce a systemic anti-tumor response. Using the B16 tumor model, mice were treated with either TEGVAX peritumorally or TEGVAX 10 injected into the contralateral limb from the site of tumor inoculation. Both TEGVAX groups showed similar *in vivo* tumor growth rate.

Figure 9 is a graph showing that the combination of GVAX, R848, and GLA prevented 15 melanoma tumor cell growth. GVAX and its formulations with various TLR agonists were injected 3 days after B16 tumor inoculation. The tumor was not palpable at the time of vaccine treatments. The relative growth rate of the tumor was followed for each group. For the B16 tumor treated with GVAX, GLA, and R848, the tumor growth was initially noted to be less than the other groups, and in some of these mice, these tumor regressed.

Figure 10 is a graph showing that TLR agonist enhanced GVAX reduced the tumor 20 growth rate on established B16 tumor. B16 tumor cell was injected into the footpad, and once the tumor was palpable, the mice were treated with the vaccine formulations as note in the figure. The vaccines were injected into the contralateral limb.

Figure 11 provides several panels showing results of an ELISPOT assay and *in vivo* CTL 25 assay showing that mice treated with GVAX/GLA/R848 had the highest number of p15E-specific T-cells, and that this result correlated with the *in vivo* tumor growth rate. Mice treated with the vaccine formulations were harvested 1-2 weeks after treatment, and their spleen analyzed for tumor specific T-cells.

Figure 12 provides two panels showing that mice treated with GVAX/GLA/R848 had significantly enhanced activated dendritic cells.

Figure 13 provides FACS analysis showing that GVAX/GLA/R848 treated mice had 30 statistically significant numbers of CD11c+ cells that express IL-12.

DETAILED DESCRIPTION OF THE INVENTION

The invention provides immunogenic compositions comprising neoplastic cells expressing a cytokine (GM-CSF) formulated with at least one TLR agonist and methods of using the composition to induce or enhance an immune response.

5 The invention is based, at least in part, on the discovery that the addition of TLR agonists to a tumor cell-based vaccine increased the efficacy of the vaccine by overcoming host immune response avoidance mechanisms present in the tumor tissue. In particular, as reported in more detail below, a syngeneic GM-CSF secreting whole cell tumor vaccine (GVAX) with TLR4 agonist (TEGVAX for TLR-enhanced GVAX) was generated. The efficacy of this
10 vaccine was tested in three different therapeutic murine models, including the poorly immunogenic B16 murine melanoma model. Immunohistochemistry, flow cytometry analysis, ELISPOT, and *in vivo* CTL analysis was utilized to assess both innate and adaptive immune response against the tumor tissue. The intratumoral and systemic administration of TEGVAX resulted in an increased antitumor response *in vivo* in comparison to GVAX alone. Improved
15 antitumor efficacy of TEGVAX was not present in TLR signaling impaired MyD88-/- mice. In the CT26 murine model in Balb/c mice, 40-60% of the mice showed regression of the transplanted tumor. When rechallenged with CT26 tumor cells, these mice proved to be immunized against the tumor. TEGVAX treated tumors showed increased infiltrating CD4 and CD8 T-cells as well as increased numbers of CD86+ cells in the tumor tissue. Draining lymph
20 nodes from the TEGVAX treated mice had enhanced number of activated CD86+MHCII+ and CD80+MHCII+ dendritic cells in comparison to GVAX and mock treated groups. ELISPOT assay as well as *in vivo* CTL assay showed increased numbers of CTLs specific for the AH1 tumor antigen in mice treated with TEGVAX. Cell based vaccine can be formulated with TLR agonist with improved anti-tumor response *in vivo*.

25

TLR Agonists Enhance Antitumor Immune Responses

Use of tumor cell vaccines as a source of unbiased tumor antigens have shown to be safe in multiple clinical trials. However, the clinical efficacy of this approach has been limited by its modest upregulation of activated antigen presenting cells that can skew the T-cell response
30 towards an anti-tumor cytotoxic response. In the context of infection, TLR agonists have been shown to render dendritic cell activation immunogenic, whereas lack of TLR4 signaling can

lead to tolerance(14). Blander and Medzhitov showed phagocytosed microbial antigens can be more efficiently presented in the presence of LPS(17). Without TLR4 signaling in the antigen-containing phagolysosome, peptide-MHC class II complexes are inefficiently presented on the cell surface on DCs. The implication from these studies is that localized TLR4 stimulation can 5 enhance antitumor response when given as part of a combinatorial vaccine.

One concern for using any TLR agonists is its toxicity since the physiologic ligand for TLR4 is LPS, the macromolecule responsible for sepsis. TLR4 agonist activity of LPS has been isolated to the lipid A component of LPS, and various forms of non-toxic lipid A forms have been introduced into cancer patients(18). Others have used LPS at suprapticogenic doses 10 intradermally for cancer patients without any evidence of sepsis and anaphylaxis(19). Another concern is that TLR4 receptors expressed on tumor cells may promote carcinogenesis(20).

The GVAX vaccine, which comprises genetically modified tumor cells expressing the immunomodulatory cytokine GM-CSF, is currently undergoing clinical trials for melanoma, breast, pancreatic, and colon cancer. As reported herein, combining TLR stimulation 15 locoregionally with GVAX can significantly enhance the immunogenicity of the GVAX vaccine. To minimize pro-carcinogenic TLR4 stimulation and to minimize systemic TLR4 stimulation, LPS and other TLR agonists have been formulated into GVAX cells, and this novel combinatorial vaccine showed anti-tumor efficacy in several murine models.

Accordingly, the invention provides therapeutic compositions comprising TLR agonist 20 containing neoplasia cell-based vaccines, and methods of using such cell-based vaccines to prevent, reduce, or eliminate the invasiveness of neoplastic cells (e.g., breast cancer and melanoma cells) or to otherwise treat a neoplasia or symptom thereof.

In one particular embodiment, the invention provides an autologous cancer cell vaccine 25 consisting of patient-specific cancer cells genetically modified to secrete granulocyte-macrophage colony stimulating factor (GM-CSF) and further formulated to comprise a toll like receptor agonist. GM-CSF modulates the proliferation and differentiation of a variety of hematopoietic progenitor cells with some specificity towards stimulation of leukocyte production and may reverse treatment-induced neutropenias. This agent also promotes antigen presentation, up-regulates antibody-dependent cellular cytotoxicity (ADCC), and increases 30 interleukin-2-mediated lymphokine-activated killer cell function and may augment host antitumoral immunity. TLR agonists act as adjuvants and increase the efficacy of the vaccine.

Tumor cell-based vaccines formulated with TLR agonists increased the number of activated locoregional dendritic cells as well as increasing the tumor specific CTL response. For safety, cells are irradiated prior to vaccination.

5 Immunogenic Compositions

Immunogenic compositions of the invention, including cancer vaccines, are useful as therapeutics and prophylactics for the treatment of specific types of cancers. Advantageously, these vaccines may be tailored to treat the cancers of particular individuals, by generating vaccines that target specific tumor antigens expressed on a tumor in a subject. Vaccines of the 10 invention typically contain inactivated tumor cells or cells expressing tumor antigens that have been genetically modified to express GM-CSF, the cells further comprise a TLR4 agonist or fragment thereof. Cells of the invention are induced to take up the TLR4 agonist by contacting them with the TLR4 agonist in combination with Lipofectamine or other liposomal vehicles, for example, oil/water emulsion lipophilic molecules, anything that would solubilize these 15 lipophilic molecule, non-cytotoxic, passive absorption . Without wishing to be bound by theory, the TLR agonist, or a biologically active fragment thereof, is contacted with liposome under conditions sufficient to form TLR agonist-containing micelles. The micelles are absorbed non-specifically up by the GM-CSF expressing tumor cells and this TLR agonist formulated cellular vaccine may be used to stimulate a subject's immune system. The immune system responds to 20 this stimulation by generating immunoresponsive cells that target the neoplasia. In particular embodiments, cancer vaccines of the invention desirably target undetectable tumor cells, such as micrometastasis or residual disease, thereby preventing relapses of the neoplasia. Unlike vaccines for other disease that prevent the occurrence of the disease, cancer vaccines are typically administered after a subject has been identified as having a neoplasia.

25 Neoplastic cell vaccines are produced using the cellular compositions of the invention, which are generated as described herein. The cells are rendered proliferation-incompetent and injected into the patient where the tumor initiating cell antigens stimulate an immune response. Desirably, the immune system targets the small population of cancer cells that carry one or more antigens that was displayed on the lethally irradiated cells.

Recombinant Polypeptide Expression

The invention provides cells that have been genetically modified to express a recombinant GM-CSF polypeptide. GM-CSF polypeptides of the invention are produced using virtually any method known in the scientific community. Typically, recombinant polypeptides 5 are produced by transformation of a suitable host cell (e.g., a cell derived from a tumor or a neoplastic cell line) with all or part of a GM-CSF polypeptide-encoding nucleic acid molecule or fragment thereof in a suitable expression vehicle. Such nucleic acid molecules can be delivered to cells derived from a subject having a neoplasia or to a neoplastic cell line *in vitro*. The nucleic acid molecules must be delivered to the cells of a subject in a form in which they 10 can be taken up so that therapeutically effective levels of a GM-CSF protein or fragment thereof can be produced.

Transducing viral (e.g., retroviral, adenoviral, and adeno-associated viral) vectors can be used for gene delivery, especially because of their high efficiency of infection and stable integration and expression (see, e.g., Cayouette et al., Human Gene Therapy 8:423-430, 1997; 15 Kido et al., Current Eye Research 15:833-844, 1996; Bloomer et al., Journal of Virology 71:6641-6649, 1997; Naldini et al., Science 272:263-267, 1996; and Miyoshi et al., Proc. Natl. Acad. Sci. U.S.A. 94:10319, 1997). For example, a polynucleotide encoding a GM-CSF protein, variant, or a fragment thereof, can be cloned into a retroviral vector and expression can be driven from its endogenous promoter, from the retroviral long terminal repeat, or from a 20 promoter specific for a target cell type of interest. Other viral vectors that can be used include, for example, a vaccinia virus, a bovine papilloma virus, or a herpes virus, such as Epstein-Barr Virus (also see, for example, the vectors of Miller, Human Gene Therapy 15-14, 1990; Friedman, Science 244:1275-1281, 1989; Eglitis et al., BioTechniques 6:608-614, 1988; Tolstoshev et al., Current Opinion in Biotechnology 1:55-61, 1990; Sharp, The Lancet 25 337:1277-1278, 1991; Cornetta et al., Nucleic Acid Research and Molecular Biology 36:311-322, 1987; Anderson, Science 226:401-409, 1984; Moen, Blood Cells 17:407-416, 1991; Miller et al., Biotechnology 7:980-990, 1989; Le Gal La Salle et al., Science 259:988-990, 1993; and Johnson, Chest 107:77S-83S, 1995). Retroviral vectors are particularly well developed and have been used in clinical settings (Rosenberg et al., N. Engl. J. Med 323:370, 1990; Anderson 30 et al., U.S. Pat. No. 5,399,346). Most preferably, a viral vector is used to administer a GM-CSF

polynucleotide to a cell of the invention, prior to, concurrent with, or following the delivery of a TLR4 agonist to the cell.

Non-viral approaches can also be employed for the introduction of a vector encoding GM-CSF to a cell derived from a patient having a neoplasia or derived from a neoplastic cell line. For example, a nucleic acid molecule encoding GM-CSF can be introduced into a cell by administering the nucleic acid molecule in the presence of lipofection (Feigner et al., Proc. Natl. Acad. Sci. U.S.A. 84:7413, 1987; Ono et al., Neuroscience Letters 17:259, 1990; Brigham et al., Am. J. Med. Sci. 298:278, 1989; Staubinger et al., Methods in Enzymology 101:512, 1983), asialoorosomucoid-polylysine conjugation (Wu et al., Journal of Biological Chemistry 10 263:14621, 1988; Wu et al., Journal of Biological Chemistry 264:16985, 1989), or by micro-injection under surgical conditions (Wolff et al., Science 247:1465, 1990). Preferably the nucleic acids are administered in combination with a liposome and protamine. The cells may be treated with Lipofectamine in combination with a TLR4 agonist concurrently, prior to, or following transfection with the GM-CSF encoding vector.

15 Methods for accomplishing transfection *in vitro* include the use of calcium phosphate, DEAE dextran, electroporation, and protoplast fusion. Liposomes can also be potentially beneficial for delivery of DNA into a cell.

20 cDNA expression for use in the methods of the invention can be directed from any suitable promoter (e.g., the human cytomegalovirus (CMV), simian virus 40 (SV40), or metallothionein promoters), and regulated by any appropriate mammalian regulatory element. For example, if desired, enhancers known to preferentially direct gene expression in specific cell types can be used to direct the expression of a nucleic acid. The enhancers used can include, without limitation, those that are characterized as tissue- or cell-specific enhancers.

25 Alternatively, if a genomic clone is used as a therapeutic construct, regulation can be mediated by the cognate regulatory sequences or, if desired, by regulatory sequences derived from a heterologous source, including any of the promoters or regulatory elements described above.

30 Another therapeutic approach included in the invention involves administration of a recombinant therapeutic, such as a cell secreting a recombinant GM-CSF protein, variant, or fragment thereof, either directly to the site of a potential or actual disease-affected tissue (e.g., intertumorally, peritumorally) or systemically (for example, by any conventional recombinant protein administration technique). The dosage of the administered cells expressing the GM-CSF

protein depends on a number of factors, including the size and health of the individual patient. For any particular subject, the specific dosage regimes should be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions.

5

Autologous Cells

The use of autologous genetically modified cells expressing a cytokine, e.g. GM-CSF and comprising a TLR4 antigen, provide advantages since each patient's tumor expresses a unique set of tumor antigens that can differ from those found on histologically-similar, MHC-matched tumor cells from another patient. See, e.g., Kawakami et al., *J. Immunol.*, 148, 638-643 (1992); Darrow et al., *J. Immunol.*, 142, 3329-3335 (1989); and Hom et al., *J. Immunother.*, 10, 153-164 (1991). In contrast, MHC-matched tumor cells provide the advantage that the patient need not be taken to surgery to obtain a sample of their tumor for genetically modified tumor cell production.

15 In one embodiment, the method of treating a subject having a neoplasia involves obtaining tumor cells from the subject; contacting the cells with an expression vector encoding GM-CSF; contacting the cells with a TLR agonist under conditions that provide for the cell to phagocytose or otherwise take up the TLR agonist; rendering the cells proliferation incompetent (e.g., by irradiating the cells); and administering the cells to the subject from which they were 20 obtained. Preferably, the composition is administered intradermally, subcutaneously or intratumorally to the mammalian subject.

In some cases, a single autologous tumor cell may express GM-CSF alone or GM-CSF in combination with one or more tumor-associated antigens. In other cases, GM-CSF and the one or more tumor-associated antigens may be expressed by different autologous tumor cells. In 25 one aspect of the invention, an autologous tumor cell is modified by introduction of a vector comprising a nucleic acid sequence encoding GM-CSF, operatively linked to a promoter and expression/control sequences necessary for expression thereof. In another aspect, the same autologous tumor cell or a second autologous tumor cell can be modified by introduction of a vector comprising a nucleic acid sequence encoding one or more tumor-associated antigens 30 operatively linked to a promoter and expression/control sequences necessary for expression thereof. The nucleic acid sequence encoding the one or more tumor-associated antigens can be

introduced into the same or a different autologous tumor cell using the same or a different vector. The nucleic acid sequence encoding the one or more tumor-associated antigens may or may not further comprise a selectable marker sequence operatively linked to a promoter. Desirably, the autologous tumor cell expresses high levels of GM-CSF and/or the one or more 5 prostate tumor-associated antigens.

Allogeneic Cells

In one embodiment, genetically modified allogeneic cells containing a TLR4 agonist (e.g., comprising TLR agonists in association with liposomal vehicle) can enhance tumor 10 immunogenicity. As used herein, a "neoplastic cell line" comprises cells that were initially derived from a tumor or other neoplastic cell. Such cells typically exhibit indefinite growth in culture. In one aspect, the method for enhancing an immune response from a subject involves: (a) obtaining a neoplastic cell line; (b) genetically modifying the cell line to render the cells capable of producing an increased level of a cytokine, e.g., GM-CSF; (c) contacting the cells of 15 TLR agonists under conditions such that the cells take up the agonist; (d) rendering the modified neoplastic cell line proliferation incompetent; and (d) administering the neoplastic cell line to a subject having or having a propensity to develop a neoplasia of the same type as that from which the neoplastic cell line was obtained. In some embodiments, the administered cells are allogeneic and are or are not MHC-matched to the subject. Such allogeneic lines provide the 20 advantage that they can be prepared in advance, characterized, aliquoted in vials containing a known amount of a TLR4 agonist and producing a comparable amount of a cytokine are stored (i.e. frozen) such that well characterized cells are available for administration to a subject. Methods for the production of genetically modified allogeneic cells are described for example in WO 00/72686, expressly incorporated by reference herein.

25 Any suitable route of administration can be used to introduce an allogeneic cell line composition into the patient, preferably, the composition is administered intradermally, subcutaneously or intratumorally.

Administration

Neoplastic cells that have been genetically modified to express a cytokine (e.g., GM-30 CSF) and that comprise TLR agonists may be cryopreserved prior to administration. Preferably, such cells are treated to render them unable to proliferate ("proliferation-incompetent"). Most

preferably, such cells are rendered proliferation-incompetent by irradiation which allows proliferation incompetence, but still allows the production of GM-CSF prior to ultimate cell death. In one embodiment, a cell of the invention is irradiated at a dose of from about 50 to about 200 rads/minute or from about 120 to about 140 rads/min prior to administration to the 5 patient. Preferably, the cells are irradiated with a total dose sufficient to inhibit substantially 100% of the cells from further proliferation. Desirably the cells are irradiated with a total dose of from about 10,000 to 20,000 rads, optimally, with about 15,000 rads.

Typically more than one administration of cytokine (e.g., GM-CSF)-expressing cells comprising TLR agonists is delivered to the subject in a course of treatment. Dependent upon 10 the particular course of treatment, multiple injections may be given at a single time point with the treatment repeated at various time intervals. For example, an initial or "priming" treatment may be followed by one or more "booster" treatments. Such "priming" and "booster" treatments are typically delivered by the same route of administration and/or at about the same site. When 15 multiple doses are administered, the first immunization dose may be higher than subsequent immunization doses. For example, a $5 \times 10^{7-8}$ prime dose may be followed by several booster doses of 10^{7-8} to $3 \times 10^{7-9}$ GM-CSF and tumor antigen producing cells.

A single injection of genetically modified cells comprising TLR agonists is typically between at least about 10^5 to 10^9 cells, e.g., 1×10^6 , 2×10^6 , 3×10^6 , 4×10^6 , 5×10^6 , 6×10^6 , 7×10^6 , 8×10^6 , 9×10^6 , 1×10^7 , 5×10^7 , $x 10^8$, $x 10^9$ cells. The number of cytokine and tumor 20 antigen producing cells may be adjusted according, for example, to the level of cytokine and/or tumor antigen produced by a given cellular immunotherapy composition. In preferred embodiments the modified cells are injected either intramuscularly or intravenously.

In some embodiments, cytokine-producing cells of the cellular immunotherapy are administered in a dose that is capable of producing at least 1, 5, 10, 25, 75, 100, 200, 300, 400, 25 500 ng of GM-CSF per 24 hours per one million cells. In some embodiments, an immunogenic composition of the invention is administered in a dose that is capable of producing at least 500 ng of a particular tumor antigen per 24 hours per one million cells. Determination of optimal cell dosage and ratios is a matter of routine determination and within the skill of a practitioner of ordinary skill, in light of the disclosure provided herein.

30 Cells of the invention are processed to remove most additional components used in

preparing the cells. In particular, fetal calf serum, bovine serum components, or other biological supplements in the culture medium are removed. In one embodiment, the cells are washed, such as by repeated gentle centrifugation, into a suitable pharmacologically compatible excipient. Compatible excipients include various cell culture media, isotonic saline, with or without a 5 physiologically compatible buffer, for example, phosphate or hepes, and nutrients such as dextrose, physiologically compatible ions, or amino acids, particularly those devoid of other immunogenic components. A composition for administration *in vivo* can comprise appropriate carriers or diluents, which further can be pharmaceutically acceptable. For example, carrying reagents, such as albumin and blood plasma fractions and inactive thickening agents, may be 10 used. The means of making such a composition have been described in the art. See, e.g., Remington's Pharmaceutical Sciences 19th edition, Genarro, A. Ed. (1995).

In pharmaceutical dosage form, the immunogenic compositions described herein can be used alone or in appropriate association, as well as in combination, with other pharmaceutically active compounds as are known in the art.

15 **Pharmaceutical Therapeutics**

The invention includes neoplastic cell-based vaccines that are useful for the treatment of neoplasia (e.g., a proliferation-incompetent neoplastic cell expressing GM-CSF and comprising a TLR4 agonist or fragment thereof). In one particular embodiment, the immunogenic compositions of the invention are useful for inducing or enhancing an immune response against 20 a neoplasia or a tumor specific antigen. The phrase "enhanced immune response" as used herein means that a detectable increase of a specific immune activation is detectable (e.g. an increase in B-cell and/or T-cell response). An example of an enhanced immune response is an increase in the amount of an antibody that binds an antigen which is detected at a lower level prior to administration of a cytokine-expressing cellular vaccine of the invention. Another example, is 25 an increased cellular immune response. A cellular immune response involves T cells, and can be observed in vitro (e.g. measured by a Chromium release assay) or in vivo. An enhanced immune response is typically accompanied by an increase of a specific population of immune cells

In another embodiment, the immunogenic compositions of the invention are useful for 30 inhibiting neoplastic cell growth. The phrase "inhibiting neoplastic growth" refers to any

measurable decrease in tumor mass, tumor volume, amount of tumor cells or growth rate of the tumor. Measurable decreases in tumor mass can be detected by numerous methods known to those skilled in the art. These include direct measurement of accessible tumors, counting of tumor cells (e.g. present in blood), measurements of tumor antigens (e.g. Prostate Specific Antigen (PSA), Alphafetoprotein (AFP) and various visualization techniques (e.g. MRI, CAT-scan and X-rays). Decreases in the tumor growth rate typically correlates with longer survival time for a subject with cancer. In still other embodiments, cell-based vaccines of the invention are useful for preventing or reducing tumor growth and/or the propensity of a neoplastic cell to invade a surrounding tissue or to otherwise metastasize. For therapeutic uses, the vaccines disclosed herein may be administered systemically, for example, formulated in a pharmaceutically-acceptable buffer such as physiological saline, or locally. Preferable routes of administration include, for example, intertumoral, peritumoral, subcutaneous, intravenous, interperitoneally, intramuscular, or intradermal injections that are sufficient to induce or enhance an immune response. Treatment of human patients or other animals will be carried out using a therapeutically effective amount of a therapeutic identified herein in a physiologically-acceptable carrier. Suitable carriers and their formulation are described, for example, in Remington's Pharmaceutical Sciences by E. W. Martin. The amount of the therapeutic agent to be administered varies depending upon the manner of administration, the age and body weight of the patient, and with the clinical symptoms of the neoplasia. Generally, amounts will be in the range of those used for other agents used in the treatment of other diseases associated with neoplasia, although in certain instances lower amounts will be needed because of the increased specificity of the compound.

Formulation of Pharmaceutical Compositions

The administration of a cell-based vaccines for the treatment of neoplasia may be by any suitable means that results in a concentration of the vaccine that, combined with other components, is effective in ameliorating, reducing, or stabilizing a neoplasia. The vaccine may be contained in any appropriate amount in any suitable carrier substance, and is generally present in an amount of 1-95% by weight of the total weight of the composition. The cell-based vaccine may be provided in a dosage form that is suitable for parenteral (e.g., subcutaneously, intravenously, intramuscularly, or intraperitoneally) administration route. The preferred method

of administration is either intramuscularly, intratumoral, or intravenous infusion. The pharmaceutical compositions may be formulated according to conventional pharmaceutical practice (see, e.g., Remington: The Science and Practice of Pharmacy (20th ed.), ed. A. R. Gennaro, Lippincott Williams & Wilkins, 2000 and Encyclopedia of Pharmaceutical

5 Technology, eds. J. Swarbrick and J. C. Boylan, 1988-1999, Marcel Dekker, New York).

Parenteral Compositions

The pharmaceutical composition comprising the cell-based vaccine may be administered parenterally by injection, infusion or implantation (subcutaneous, intravenous, intramuscular, 10 intraperitoneal, or the like) in dosage forms, formulations, or via suitable delivery devices or implants containing conventional, non-toxic pharmaceutically acceptable carriers and adjuvants. The formulation and preparation of such compositions are well known to those skilled in the art of pharmaceutical formulation. Formulations can be found in Remington: The Science and Practice of Pharmacy, supra.

15 Compositions for parenteral use may be provided in unit dosage forms (e.g., in single-dose ampoules), or in vials containing several doses and in which a suitable preservative may be added (see below). The composition may be in the form of a solution, a suspension, an emulsion, an infusion device, or a delivery device for implantation, or it may be presented as a dry powder to be reconstituted with water or another suitable vehicle before use. Apart from the 20 active agent that reduces or ameliorates a neoplasia, the composition may include suitable parenterally acceptable carriers and/or excipients. The active therapeutic agent(s) may be incorporated into microspheres, microcapsules, nanoparticles, liposomes, or the like for controlled release. Furthermore, the composition may include suspending, solubilizing, stabilizing, pH-adjusting agents, tonicity adjusting agents, and/or dispersing, agents.

25 As indicated above, the pharmaceutical compositions according to the invention may be in the form suitable for sterile injection. To prepare such a composition, the suitable therapeutic(s) are dissolved or suspended in a parenterally acceptable liquid vehicle. Among acceptable vehicles and solvents that may be employed are water, water adjusted to a suitable pH by addition of an appropriate amount of hydrochloric acid, sodium hydroxide or a suitable 30 buffer, 1,3-butanediol, Ringer's solution, and isotonic sodium chloride solution and dextrose solution. The aqueous formulation may also contain one or more preservatives (e.g., methyl,

ethyl or n-propyl p-hydroxybenzoate). In cases where one of the compounds is only sparingly or slightly soluble in water, a dissolution enhancing or solubilizing agent can be added, or the solvent may include 10-60% w/w of propylene glycol or the like.

5 Combination Therapies

Optionally, cancer vaccine based therapeutics of the invention may be administered in combination with any other chemotherapeutic; such methods are known to the skilled artisan and described in Remington's Pharmaceutical Sciences by E. W. Martin. In particular, an immunogenic composition of the invention can be administered in combination with any one or 10 more of abiraterone acetate, altretamine, anhydrovinblastine, auristatin, bexarotene, bicalutamide, BMS184476, 2,3,4,5,6-pentafluoro-N-(3-fluoro-4-methoxyphenyl)benzene sulfonamide, bleomycin, N,N-dimethyl-L-valyl-L-valyl-N-methyl-L-valyl-L-prolyl-L-proline-t-butylamide, cachectin, cemadotin, chlorambucil, cyclophosphamide, 3',4'-didehydro-4'-deoxy-8'-norvincaleukoblastine, docetaxol, doxetaxel, cyclophosphamide, carboplatin, carmustine 15 (BCNU), cisplatin, cryptophycin, cyclophosphamide, cytarabine, dacarbazine (DTIC), dactinomycin, daunorubicin, decitabine dolastatin, doxorubicin (adriamycin), etoposide, 5-fluorouracil, finasteride, flutamide, hydroxyurea and hydroxyureataxanes, ifosfamide, liarozole, 20 lonidamine, lomustine (CCNU), MDV3100, mechlorethamine (nitrogen mustard), melphalan, mivobulin isethionate, rhizoxin, sertenef, streptozocin, mitomycin, methotrexate, taxanes, nilutamide, onapristone, paclitaxel, prednimustine, procarbazine, RPR109881, stramustine 25 phosphate, tamoxifen, tasonermin, taxol, tretinoin, vinblastine, vincristine, vindesine sulfate, and vinflunine.

In the combination therapies of the invention, the therapy components are administered simultaneously, or within 1, 3, 5, 7, 14, 21 or 28 days of each other, in amounts sufficient to 25 inhibit the growth of a neoplasm. Depending on the type of cancer and its stage of development, the combination therapy can be used to treat cancer, to slow the spreading of the cancer, to slow the cancer's growth, to kill or arrest cancer cells that may have spread to other parts of the body from the original tumor, to relieve symptoms caused by the cancer, or to prevent cancer in the first place. Combination therapy can also help people live more 30 comfortably by eliminating cancer cells that cause pain or discomfort.

The administration of a combination of the present invention allows for the

administration of lower doses of each compound, providing similar efficacy and lower toxicity compared to administration of either compound alone. Alternatively, such combinations result in improved efficacy in treating neoplasms with similar or reduced toxicity.

5 Kits

The invention provides kits for the treatment or prevention of neoplasia. In one embodiment, the kit includes a therapeutic or prophylactic composition containing an effective amount of a tumor cell-based vaccine in unit dosage form. In some embodiments, the kit comprises a sterile container which contains a therapeutic or prophylactic cellular composition; 10 such containers can be boxes, ampoules, bottles, vials, tubes, bags, pouches, blister-packs, or other suitable container forms known in the art. Such containers can be made of plastic, glass, laminated paper, metal foil, or other materials suitable for holding medicaments.

If desired a tumor cell-based vaccine of the invention is provided together with instructions for administering the vaccine to a subject having or at risk of developing cancer 15 (e.g., melanoma, breast cancer). The instructions will generally include information about the use of the composition for the treatment or prevention of neoplasia. In other embodiments, the instructions include at least one of the following: description of the therapeutic agent; dosage schedule and administration for treatment or prevention of ischemia or symptoms thereof; precautions; warnings; indications; counter-indications; overdosage information; adverse 20 reactions; animal pharmacology; clinical studies; and/or references. The instructions may be printed directly on the container (when present), or as a label applied to the container, or as a separate sheet, pamphlet, card, or folder supplied in or with the container.

The practice of the present invention employs, unless otherwise indicated, conventional 25 techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry and immunology, which are well within the purview of the skilled artisan. Such techniques are explained fully in the literature, such as, "Molecular Cloning: A Laboratory Manual", second edition (Sambrook, 1989); "Oligonucleotide Synthesis" (Gait, 1984); "Animal Cell Culture" (Freshney, 1987); "Methods in Enzymology" "Handbook of Experimental 30 Immunology" (Weir, 1996); "Gene Transfer Vectors for Mammalian Cells" (Miller and Calos, 1987); "Current Protocols in Molecular Biology" (Ausubel, 1987); "PCR: The Polymerase

Chain Reaction”, (Mullis, 1994); “Current Protocols in Immunology” (Coligan, 1991). These techniques are applicable to the production of the polynucleotides and polypeptides of the invention, and, as such, may be considered in making and practicing the invention. Particularly useful techniques for particular embodiments will be discussed in the sections that follow.

5 The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the assay, screening, and therapeutic methods of the invention, and are not intended to limit the scope of what the inventors regard as their invention.

10

EXAMPLES

Example 1: Formulation of TLR Agonist Enhanced GVAX (TEGVAX)

In order to enhance locoregional innate immune cell activation as well as minimize systemic toxicity of Toll like receptor 4 (TLR4) stimulation and minimize TLR4R stimulation on tumor cells, lipopolysaccharide (LPS) was formulated into GVAX (TEGVAX - TLR4 enhanced GVAX). A commercially available liposome vector – Lipofectamine – was used to optimize absorption of LPS into GVAX cells prior to lethal irradiation. In order to quantitate the extent of absorption, LPS- DIPYrromethene BOron Difluoride (BODIPY) fluorophore was used to test various conditions for optimization to have 98% of the cells formulated with LPS-BODIPY (Figure 1). In order to quantitate the total amount of LPS absorbed per cell, *Limulus* 15 *Amebocyte Lysate (LAL)* assay (Cambrex) was used to demonstrate that 4.73+- 0.2 ng of LPS was absorbed into 5×10^5 cells with the lipofectamine method that optimized LPS formulation. For each of the *in vivo* murine tumor experiments, aliquots of TEGVAX were tested using LAL assay as well as GM-CSF ELISA assay to ensure comparable amount of LPS and GM-CSF in the TEGVAX formulation. The typical GM -CSF secreted ranged from 20 25 50- 200 ng/ml/ 10^6 cells/day.

Example 2: TEGVAX induces *in vivo* anti-tumor response in multiple murine cancer models.

The efficacy of TEGVAX was initially tested in a B16 melanoma murine model 30 whereby TEGVAX was delivered intratumorally 3 to 5 days after tumor inoculation in a therapeutic model. Part of the rationale for intratumoral injection was to ensure that

locoregional antigen presenting cells (APCs) that circulate between the tumor and the draining lymph nodes were targeted. These time-points were selected because anergic and tolerant tumor specific T-cells were present as early as 3 days after B16 injection. As shown in Figure 2, TEGVAX decreased the growth rate of the B16 tumor in comparison to GVAX treatment.

5 These statistically significant differences in growth rate translated to survival curve differences (Figure 2). However, all the C57Bl/6 mice treated with TEGVAX eventually developed large tumors at the site of injection. Given that 4.73ng of LPS per 5×10^5 GVAX cells was measured, control experiments were performed whereby an equimolar amount of LPS was injected peritumorally as in the TEGVAX treatment group. These mice had no differences in
10 growth rate or survival as tumor-bearing mice treated with GVAX or PBS (Figure 7). Equimolar LPS injected with GVAX without liposomal formulation also did not demonstrate an anti-tumor response in comparison to the PBS control group, suggesting that the intracellular formulation of LPS is important for its anti-tumor response (Figure 7). TEGVAX also induced a systemic anti-tumor response (Figure 8). Using the B16 melanoma model, mice were treated
15 with TEGVAX either peritumorally or in the contralateral limb from the site of tumor inoculation, both treated groups showed similar *in vivo* tumor growth rate (Figure 8).

To test if TEGVAX can also demonstrate an anti-tumor response in other murine tumor models, SCCFVII/SF cells were subcutaneously injected into the flanks of syngeneic C3H/HeOUJ mice with a wild-type TLR4R (Figure 2). Once the tumor was palpable, the
20 tumor was treated with either TEGVAX or GVAX prepared from murine SCCFVII/SF squamous cell carcinoma cells. Comparable to the TEGVAX experiments with B16 model, TEGVAX showed significant anti-tumor response. Once again, all the mice treated with TEGVAX eventually developed bulky tumors that required euthanasia. Orthotopic injection was not performed due to inaccuracies inherent in measuring tongue and floor of mouth tumor
25 in SCCFVII/SF mice models.

Lastly, TEGVAX was also tested in CT26 colon carcinoma model, which also showed an anti-tumor response whereby the growth rate of the transplanted tumor was statistically slower than CT26 tumor treated with GVAX alone (Figure 2). For CT26, 40-60% of the mice treated with TEGVAX actually had regression of tumor. CT26 cells were harvested from the
30 mice that survived and the tumors were re -transplanted, and none grew out any tumor, demonstrating a complete immunotherapeutic cure. For the CT26 model, LPS formulated with

lethally irradiated HEK 293 cells in equimolar amounts as TEGVAX was also tested. LPS formulated into cell based vaccine without an identical set of tumor antigens and GMCSF did not elicit an anti-tumor response in the CT26 model.

5 **Example 3: Anti-tumor response to TEGVAX is MyD88 dependent.**

In order to verify that the *in vivo* anti-tumor effects noted above were in fact due to the introduction of TLR4 signaling, B16 tumor was inoculated into C57Bl/6 mice having MyD88 -/- genotype. The tumor was then treated with TEGVAX or GVAX. Figure 3 shows that the enhanced anti-tumor response with the addition of TLR4 agonist formulated into GVAX 10 cells are, in fact, secondary to TLR4 stimulation. A mild difference noted early in the GVAX group eventually overlapped with the growth curve of the PBS treated group (Figure 7). MyD88 is an essential intracellular mediator of TLR4 signaling, and the absence of TLR4 signaling in MyD88 null mice no longer demonstrated the enhanced *in vivo* anti-tumor response noted consistently in wild type C57Bl/6 mice.

15

Example 4: TEGVAX induces enhancement of T-cell infiltration and APCs in the tumor microenvironment.

In order to examine the potential mechanism of the *in vivo* anti-tumor responses from TEGVAX treatment, the tumor tissue was harvested after treatments and analyzed for 20 lymphocytic infiltrate. As shown in Figure 4, tumor treated with TEGVAX had quantitatively increased CD4 and CD8 infiltration compared to the control treated and the GVAX treated tumors. Moreover, given the hypothesis that TLR4 agonist stimulates the locoregional professional APCs to mature, immunostaining was performed with antibodies against CD86 and a quantitative enhancement of CD86+ cells in the tumor microenvironment treated with 25 TEGVAX was noted. Whether macrophages were increased in the tumor tissue was also examined, but no significant differences in the infiltration of F4+ macrophages between the treatment groups was found.

30 **Example 5: Augmentation of dendritic cell activation in the locally draining lymph nodes.**

The immunostaining data from Figure 4 were consistent with the hypothesis that TLR4 agonist formulated cell vaccines can increase the number of activated locoregional dendritic cells. DCs are important primary antigen processing cells that can dictate whether effector cells become tolerogenic or cytotoxic. Without wishing to be bound by theory, it is likely that LPS 5 absorbed into GVAX is phagocytosed into the infiltrating DCs as cellular debris and micelles with tumor antigens. Thus, it seemed likely that there would be increased number of activated locoregional DC population with TEGVAX treatment. In order to test this hypothesis, dendritic cells were purified from the draining lymph nodes from tumor bearing mice treated with either PBS, GVAX, or TEGVAX and gated for the conventional DC population with B220 and CD1 10 1c. Multiparametric staining of DC activation marker CD86, CD80, and MHCII from these gated cells shows that dendritic cells from the TEGVAX treated group has greater population of activated DCs (Figure 5) in comparison to the GVAX treated group or the control treated group.

Example 6: Tumor specific cytotoxic T-cells are expanded in mice treated with 15 TEGVAX.

One rationale for studying the CT26 model in conjunction with SCCVII/SF and B16 model was the availability of reagents for quantitation of AH1-specific cytotoxic T-cells. To test if the *in vivo* anti-tumor responses for TEGVAX that activates locoregional dendritic cells can increase the downstream population of tumor specific cytotoxic effector cells, ELISPOT 20 assays were performed. Using the immunogenic MHC class I L^d restricted AH1 peptides, T-cells from the draining lymph node and spleen were harvested and IFN-producing cytotoxic T-cells screened in the presence of AH1 peptides (the immunodominant peptide for CT26 cells pulsed with APC in the ELISPOT assay. While minimal AH1 specific T- cells were detected on day 5 after treatment with TEGVAX, by day 8, there were statistically significant AH1 25 specific T-cells in the TEGVAX group in both the draining lymph nodes and the spleen as shown in Figure 6A. *In vivo* cytotoxic T-lymphocyte (CTL) assays also demonstrated enhanced cytotoxic T-cell priming for the AH1 peptide in the TEGVAX treated group in comparison to the untreated group (Figure 6B). *In vivo* CTL assays with p15E specific T-cell from the B16 model also demonstrated enhanced p15E-specific T- cells in the TEGVAX 30 group.

Example 7: TLR4 agonist in combination with GVAX reduced tumor cell growth and increased the number of AH1-specific cytotoxic T-cells

As reported above, TEGVAX improved *in vivo* tumor responses in three separate murine models. In the B16 melanoma model, the CT26 colon carcinoma models, as well as the SCCVII tongue squamous model, TEGVAX injected intratumorally into established tumors reduced tumor growth in comparison to GVAX alone. Systemic treatment using TEGVAX after tumor inoculation also reduced B16 growth rate *in vivo*. In order to examine the immunological mechanism inducing the anti-tumor responses, the CT26 murine model was used to study the anti-tumor cytotoxic T-cell (CTL) responses. Using the well characterized Elispot assay and *in vivo* CTL assay, TEGVAX treated mice showed increased number of AH1-specific cytotoxic T-cells (CTL) by day 8 after treatment. In these experiments, the rate of tumor growth rate in the preclinical models was reduced by combining TLR4 agonist with GVAX.

15 Example 8: A synthetic TLR4 agonist - glycopyranosyl lipid A analogue (GLA) - in combination with GVAX and a TLR7 and TLR8 agonist (R848) significantly reduced the growth of melanoma cells

To determine whether more than one type of TLR signaling in the tumor microenvironment can improve upon the anti-tumor response another form of TLR4 agonist, GLA, a synthetic glycopyranosyl lipid A analogue, was used with the GVAX. GLA formulated 20 GVAX was also combined with R848, a TLR7 and TLR8 agonist. *In vivo* tumor treatment experiments were performed with B16 melanoma mice. Using the B16 melanoma model, the combination of GVAX, GLA, and R848 prevented the growth of the aggressive B16 tumor in wildtype mice (Figure 9).

The vaccine formulations were injected 2-4 days after tumor inoculation. Typically, 25 GVAX by itself does not prevent the growth of the B16 *in vivo* in these poorly immunogenic tumor cells. When these mice were re-injected with B16 melanoma cell lines, the challenge tumor cells did not grow. Both ELISPOT and *in vivo* CTL assays were performed to quantitate p15E specific CD8+ T-cells, and the mice treated with GVAX, GLA, R848 had significantly more p15E specific CD8+ T-cells than mice treated with GVAX alone.

30

Example 9: A combination of GVAX/GLA/R848 enhanced dendritic cell activation

In order to treat an organized tumor tissue, the GVAX/GLA/R848 formulation was used to treat palpable B16 tumor. The vaccine formulation was injected 7-10 days after tumor inoculation as site distant from the palpable tumor tissue. Once again the TLR agonist enhanced GVAX reduced the tumor growth rate (Figure 10).

5 ELISPOT and *in vivo* CTL assays were performed in each of the groups to quantitate the level of p15E specific CTL, and the results demonstrated that the mice treated with GVAX/GLA/R848 had the highest number of p15E-specific T-cells that correlated with the *in vivo* tumor growth rate (Figure 11).

Given these anti-tumor responses with the GVAX/GLA/R848 formulation, it was 10 hypothesized that the TLR agonist enhanced vaccines were stimulating the dendritic cells. Therefore, draining lymph nodes and the spleen from each treatment group was harvested, and the activation status of the dendritic cells was examined. Using CD80 and CD86 activation markers on CD11c+ and B220+ cells, the GVAX/GLA/R848 treated group showed significantly enhanced activated dendritic cells (Figure 12).

15 The tumor tissue from each treatment group was analysed using immunohistochemistry. A higher number of CD4+, CD8+, and CD86+ cells was found in tumors treated with GVAX/GLA/R848. Lastly, the cytokine milieu was examined in each of the treatment groups to determine whether IL-12 was elevated in the mice treated with the TLR agonists. CD11c positive cells were harvested in both draining lymph nodes and the spleen, and the level of IL-12 20 cytokines was examined for the ability to direct the T-cell repertoire towards an anti-tumor Th1 response (Figure 13). GVAX/GLA/R848 treated mice had statistically significant number of CD11c+ cells that express IL-12, which is the primary cytokine that directs the T-cell milieu towards a Th1 response. This was noted in both the draining lymph nodes and the spleen.

In summary, these results showed that a combinatorial approach using GVAX platform 25 with multiple TLR agonists had increased efficacy in the treatment of solid tumors. A combination of GVAX with TLR4 and TLR7/8 agonists improves the anti-tumor response *in vivo*. The improved anti-tumor response is correlated with increased number of activated dendritic cells, increased number of tumor specific CTLs, and increased number of CD11c+ that can secrete IL-12 in the draining lymph nodes and the spleen.

30

The results described above were obtained using the following methods and materials.

Murine Tumor Cell Lines

The SCCFVII/SF head and neck squamous cell carcinoma, B16-F0 melanoma and B16-F0 transduced to secrete GM-CSF cell lines were cultured in RPMI 1640 media containing 10%
5 heat -inactivated fetal calf serum, penicillin (100 U/ml) and streptomycin (100 U/ml). CT26 colorectal carcinoma cell line was cultured similarly with the addition of MEM nonessential amino acids (Sigma, St. Louis), 1 mM sodium pyruvate, and 2 mM of L-glutamine. The bystander B78HI cells transduced with GM-CSF were cultured in the same media has CT26 but with the addition of Hygromycin B from Streptomyces hygroscopicus (1g/L) (Roche Applied
10 Science, Indianapolis, IN). All cells were incubated at 37°C in 5% humidified CO₂. The GVAX vaccine cell lines tested in vaccination models whereby the vaccines were injected 10 days prior to tumor inoculation showed no tumor growth for all three GVAX lines.

Mice

15 Adult (>50 days of age) female C57BL/6, Balb/C, and C3H/HeOUJ mice were purchased from Jackson Laboratory and housed according to the Johns Hopkins Animal Care and Use Committee. C57BL/6 MyD88 -/- mice were obtained from Dr. Franck Housseau (Johns Hopkins University).

20 *TEGVAX formulation*

LipofectAMINE Reagent (120 μ g/ml) and LPS from *Escherichia coli* 026:B6 (10-25 μ g/ml) were combined into liposome complex. Cells for lipopolysaccharide (LPS) formulation were seeded at a density of 5×10^5 cells and after 24 hrs they were incubated with the liposome-LPS complex for 6 hrs, washed 5 times with PBS, and then injected into mice. To quantitate LPS 25 incorporated into cells *Limulus Amebocyte Lysate* (LAL) assay (Cambrex) was performed as directed by the manufacturer. LPS (23 EU/ng) from 0 to 3.0 EU/ml were used as standards. Prior to lethal irradiation, the TEGVAX was cultured to ensure viable cell growth. Annexin staining verified no evidence of apoptosis after formulation.

30 *In vivo vaccine treatment assay*

C57BL/6 mice were injected subcutaneously in the right flank with 5×10^4 B16-F0 cells. Three to five days post inoculation 10^6 lethally irradiated (150 Gy) B16 GM-CSF (GVAX), 10^6 lethally irradiated (150 Gy) LPS formulated GVAX (TEGVAX formulation) or LPS absorbed 293 cells were injected peritumorally. In some cases, the vaccines were injected in the 5 contralateral limb from the tumor-inoculated limb. C3H/HeOuJ mice and Balb/c mice were used with SCCFVII/SF cells and CT26 cells, respectively with comparable methods.

Immunohistochemistry

Frozen CT26 tumor from mice was cut in 10 μ m thickness and blocked with 1% BSA 30 minutes at room temperature. Anti-mouse CD45 (eBioscience) and anti-mouse CD4-Fluorescein isothiocyanate (FITC), CD8-FITC, CD86-FITC antibodies (BD Pharmingen) incubated at 4°C for 1 hour were used as primary antibodies. Anti-CD45-Cy3 (Invitrogen) was used as secondary in some case. DAPI was used as counterstain for 10 minutes. Cells in 10 randomly selected fields at 40x magnification were counted. Nikon Eclipse F800 was the 15 microscope, Nikon DS-Qi1mc was the camera, and NIS-Element AR 3.0 was the software used for this experiments. Spleens and draining lymph nodes from tumor challenged BL/6 mice was harvested five days post GVAX and TEGVAX treatment. Sliced spleens were enzymatically digested in media containing DNase I (Roche) and a commercially available blend of enzymes 20 for tissue dissociation (LIBERASE BLENDZYME 2) (20,000 Mandl U/ml) (Roche). DC-enriched populations were obtained by depleting CD3+ and CD19+, and stained for CD11c+ and B220+. CD11c+B220+ gated DC's were evaluated by multicolored FACS analysis using 25 CD80, CD86, and MHCII antibodies from BD Biosciences.

ELISPOT assay

25 Enzyme-linked immunosorbent spot (ELISPOT) plates (MultiScreen_{HTS} filter plate, Millipore) were coated with a mouse interferon (IFN)- γ antibody (Ab) (MabTech) for 24 hours and 4T1 breast cancer cells were pulsed with 10 μ g/ml of AH1 peptide overnight. The following day, spleens and lymph nodes were harvested from mice. 10^6 CD8a+ cells were plated in triplicates to be co-cultured with 10^5 unpulsed 4T1 cells, co-cultured with 10^5 pulsed 30 4T1 cells, or stimulated with 1 μ M of PMA and 10ng/ml of ionomycin for a positive control. On the third day, the plate was incubated with a biotinylated anti-mouse IFN- γ Ab (MabTech)

followed by incubation with Streptavidin-HRP for ELISPOT (BD). The plate was developed using AEC Substrate Reagent Set for ELISPOT (BD) and analyzing using an ELISPOT Plate Reader (Immunospot).

5 *In vivo CTL assay*

A commercially available reagent for *in vivo* tracing CellTrace™ CFSE Cell Proliferation Kit (Molecular Probes) was used to test the percent killing of CTLs in CT26 tumor challenged mice treated with or without TEGVAX. Splenocytes were processed and pulsed with either β -galactosidase (β -gal) or AH1 peptide at a concentration of 10 μ g/ml for 90 minutes.

10 The β -gal population was CFSE labeled low (0.5 μ M) and the AH1 population was carboxyfluorescein diacetate succinimidyl ester (CFSE) labeled (5 μ M). After a 10 minute incubation, both CFSE labeled cells were injected 10⁷ cells/mouse into the three groups of mice. Twenty-four hours post-injection, splenic cells were harvested and analyzed.

15 *Statistical Analysis*

Paired *t*-test was used to calculate two-tailed *p* value to estimate statistical significance of difference between two treatment groups. Statistically significant *p* values were labeled as follow: ***p*<0.01 and **p*<0.05. Data were analyzed using Excel software.

20 **Other Embodiments**

From the foregoing description, it will be apparent that variations and modifications may be made to the invention described herein to adopt it to various usages and conditions. Such embodiments are also within the scope of the following claims.

25 The recitation of a listing of elements in any definition of a variable herein includes definitions of that variable as any single element or combination (or subcombination) of listed elements. The recitation of an embodiment herein includes that embodiment as any single embodiment or in combination with any other embodiments or portions thereof.

30 All patents and publications mentioned in this specification are herein incorporated by reference to the same extent as if each independent patent and publication was specifically and individually indicated to be incorporated by reference.

What is claimed is:

1. An immunogenic composition comprising one or more proliferation-incompetent genetically modified neoplastic cells expressing a recombinant immune stimulatory cytokine and comprising at least one Toll-like receptor (TLR) agonist.
5
2. The immunogenic composition of claim 1, wherein the cytokine is granulocyte-macrophage colony-stimulating factor (GM-CSF).
- 10 3. The immunogenic composition of claim 1, wherein the cell comprises comparable amounts of at least one TLR agonist and GM-CSF .
4. The immunogenic composition claim 1, wherein the TLR agonist is a TLR4, TLR5, TLR6, TLR7 or TLR8, TLR9 agonist.
15
5. The immunogenic composition claim 1, wherein the composition comprises a TLR4 agonist and a TLR7/8 agonist.
- 20 6. The immunogenic composition of claim 5, wherein the TLR4 agonist is LPS, an LPS fragment, or a synthetic glycopyranosyl lipid A analogue and the TLR7/8 analog is R848.
7. The immunogenic composition of claim 1, wherein the cells are rendered proliferation incompetent by irradiation.
25
8. The immunogenic composition of claim 1, wherein the neoplastic cell is selected from the group consisting of leukemia, chronic myeloid leukemia, prostate cancer, head and neck, Squamous Cell Carcinoma, tongue cancer, larynx cancer, tonsil cancer, hypopharynx cancer, nasopharynx cancer, breast cancer, colon cancer, lung cancer, melanoma, pancreatic cancer, glioblastoma and brain cancer.
30

9. The immunogenic composition of claim 1, wherein the composition comprises at least about 1 ng of TLR4 agonist per 1×10^5 cells -10 ng of TLR4 agonist per 1×10^5 cells.

10. The immunogenic composition of claim 1, wherein the composition comprises at least 5 about 3-5 ng per 5×10^5 cells.

11. The immunogenic composition of claim 1, wherein the cell comprises a TLR agonist in association with Lipofectamine or other liposomal vectors.

10 12. The immunogenic composition of claim 11, wherein the cell comprises LPS/liposomal micelles.

13. The immunogenic composition of claim 1, further comprising cells that express one or more tumor antigens.

15

14. The immunogenic composition of any one of claims 1-13, wherein the cells are autologous or allogeneic.

20 15. A vaccine for ameliorating a neoplasia in a subject, the vaccine comprising an effective amount of proliferation-incompetent neoplastic cells expressing a recombinant immune stimulatory cytokine and comprising an effective amount of exogenous Toll-like receptor (TLR) agonist in a pharmaceutically acceptable excipient.

25 16. The vaccine of claim 15, wherein the cells are genetically modified to express GM-CSF.

17. The vaccine of claim 15, wherein the composition comprises a TLR4 agonist and a TLR7/8 agonist.

30 18. The vaccine of claim 17, wherein the TLR4 agonist is LPS, an LPS fragment, or a synthetic glucopyranosyl lipid A analogue and the TLR7/8 analog is R848.

19. The vaccine of claim 15, wherein the cells comprise LPS/liposomal micelles.

20. The vaccine of claim 12, further comprising a cell that expresses one or more tumor antigens.

5

21. The vaccine of any of claims 12-20, wherein the cells are autologous or allogeneic.

22. A method of inducing a neoplastic cell antigen-specific immune response in a subject having or having a propensity to develop a neoplasia, the method comprising administering to 10 the subject an effective amount of an immunogenic composition comprising one or more proliferation-incompetent neoplastic cells expressing a recombinant immune stimulatory cytokine and comprising an exogenous Toll-like receptor (TLR) agonist in an amount sufficient to induce a neoplastic cell antigen-specific immune response in said subject.

15 23. A method of treating or preventing a neoplasia in a subject having or having a propensity to develop a neoplasia, the method comprising administering to the subject an effective amount of the immunogenic composition or vaccine of any one of claims 1-21 in an amount sufficient to induce a neoplastic cell antigen-specific immune response in said subject, thereby treating the subject.

20

24. A method of treating or preventing tumor progression or metastasis in a subject having a neoplasia, the method comprising administering to the subject an effective amount of the immunogenic composition or vaccine of any one of claims 1-21, thereby treating or preventing tumor progression or metastasis in the subject.

25

25. A method of treating an established tumor or preventing tumor formation in a subject, the method comprising administering to the subject an effective amount of the immunogenic composition or vaccine of any one of claims 1-21, thereby treating an established tumor or preventing tumor formation in the subject.

30

26. A method of treating micrometastasis or residual disease in a subject having micrometastasis or residual disease, the method comprising administering to the subject an effective amount of the immunogenic composition or vaccine of any one of claims 1-21, thereby treating micrometastasis or residual disease in the subject.

5

27. A method of immunizing a subject, the method comprising administering to the subject an immunogenic composition or vaccine of any of claims 1-21.

28. The method of any of claims 14-27, wherein the neoplastic cell is derived from a cancer cell line or is derived from a tumor.

10 29. The method of claim 17, wherein the cancer cell line or tumor is selected from the group consisting of leukemia, chronic myeloid leukemia, prostate cancer, head and neck, Squamous Cell Carcinoma, tongue cancer, larynx cancer, tonsil cancer, hypopharynx cancer, nasopharynx cancer, breast cancer, colon cancer, lung cancer, melanoma, pancreatic cancer, glioblastoma and brain cancer.

15 30. The method of any of claims 14-16, wherein the immunogenic composition is administered systemically or locally.

20

31. The method of claim 19, wherein the immunogenic composition is administered by intramuscular injection, intravenous injection, intratumoral injection, or peritumoral injection

25 32. The method of claim 8 or 9, further comprising administering to the subject an effective amount of one or more chemotherapeutics.

33. The method of claim 12, wherein the one or more chemotherapeutics is selected from the group consisting of abiraterone acetate, altretamine, anhydrovinblastine, auristatin, bexarotene, bicalutamide, BMS184476, 2,3,4,5,6-pentafluoro-N-(3-fluoro-4-methoxyphenyl)benzene sulfonamide, bleomycin, N,N-dimethyl-L-valyl-L-valyl-N-methyl-L-valyl-L-prolyl-L-proline-t-butylamide, cachectin, cemadotin, chlorambucil, cyclophosphamide, 3',4'-didehydro-4'-deoxy-

8'-norvin- caleukoblastine, docetaxol, doxetaxol, cyclophosphamide, carboplatin, carmustine (BCNU), cisplatin, cryptophycin, cyclophosphamide, cytarabine, dacarbazine (DTIC), dactinomycin, daunorubicin, decitabine dolastatin, doxorubicin (adriamycin), etoposide, 5-fluorouracil, finasteride, flutamide, hydroxyurea and hydroxyureataxanes, ifosfamide, liarozole,

5 ionidamine, lomustine (CCNU), MDV3100, mechlorethamine (nitrogen mustard), melphalan, mivobulin isethionate, rhizoxin, sertenef, streptozocin, mitomycin, methotrexate, taxanes, nilutamide, onapristone, paclitaxel, prednimustine, procarbazine, RPR109881, stramustine phosphate, tamoxifen, tasonermin, taxol, tretinoin, vinblastine, vincristine, vindesine sulfate, and vinflunine.

10

34. The method of any of claims 12-31, wherein the method enhances activation of dendritic cells and/or increases the number of AH1-specific cytotoxic T-cells or p15E-specific cytotoxic T-cells relative to the use of GVAX alone.

15 35. The method of any of claims 12-31, wherein the method reduces or stabilizes tumor cell proliferation, tumor growth, or subject survival relative to the use of GVAX alone.

36. A proliferation-incompetent neoplastic cell comprising an expression vector encoding GM-CSF and at least one TLR agonist.

20

37. The neoplastic cell of claim 34, wherein the cell is derived from a leukemia, chronic myeloid leukemia, prostate cancer, head and neck, Squamous Cell Carcinoma, tongue cancer, larynx cancer, tonsil cancer, hypopharynx cancer, nasopharynx cancer, breast cancer, colon cancer, lung cancer, melanoma, pancreatic cancer, glioblastoma and brain cancer or a cell line thereof.

25 38. The neoplastic cell of claim 34, wherein the cell comprises a TLR4 and a TLR7/8 agonist.

30 39. The neoplastic cell of claim 34, wherein the TLR agonist is in association with liposomal vector.

40. A pharmaceutical composition for the treatment of neoplasia comprising an effective amount of the immunogenic composition or vaccine of any of claims 1-21 and a pharmaceutically acceptable excipient.

5

41. A kit for the treatment of a neoplasia, the kit comprising an effective amount of the immunogenic composition or vaccine of any one of claims 1-21, and directions for using the kit in a method of any one of claims 22-33.

10 42. A method of treating or preventing cervical cancer in a subject, the method comprising administering to the subject an effective amount of the immunogenic composition or vaccine of any one of claims 1-21, thereby treating or preventing cervical cancer in the subject.

15 43. A method of treating polymorphic TLR deficiencies in a subject, the method comprising administering to the subject an effective amount of the immunogenic composition or vaccine of any one of claims 1-21, thereby treating the polymorphic TLR deficiencies in the subject.

20

1/14

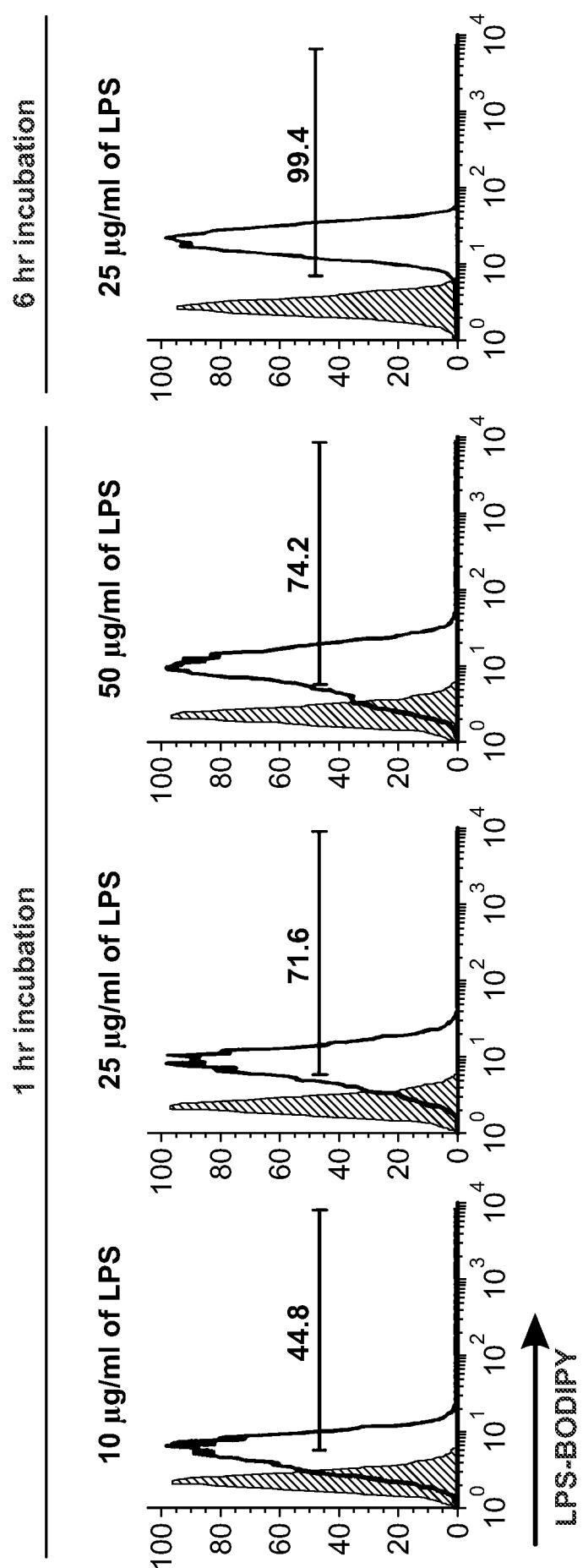
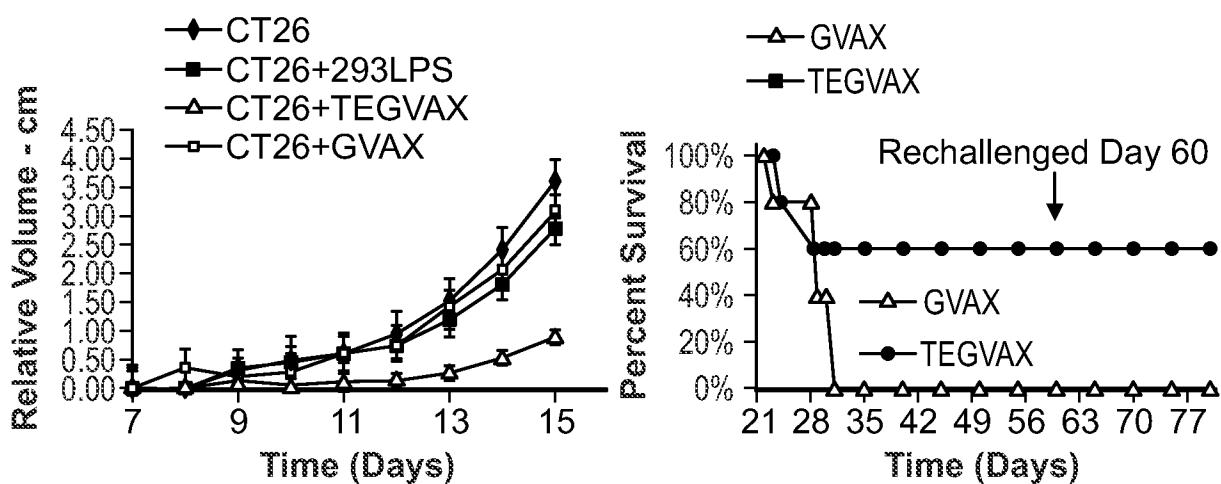
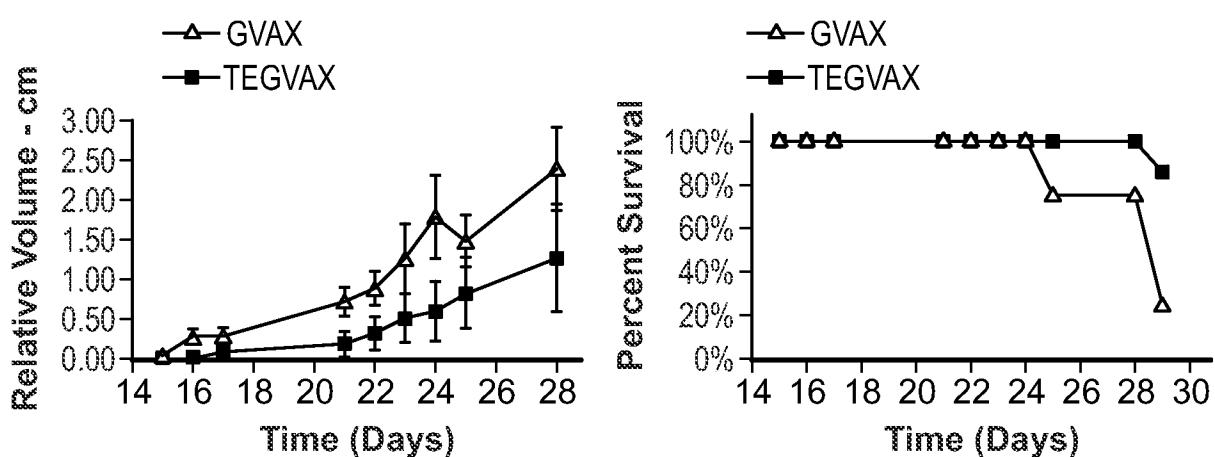
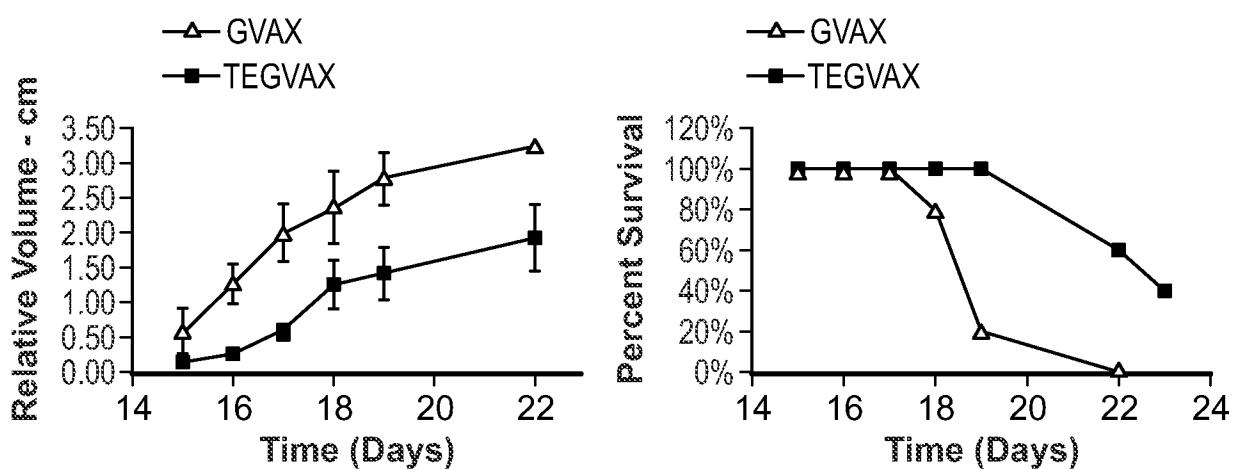


FIG. 1



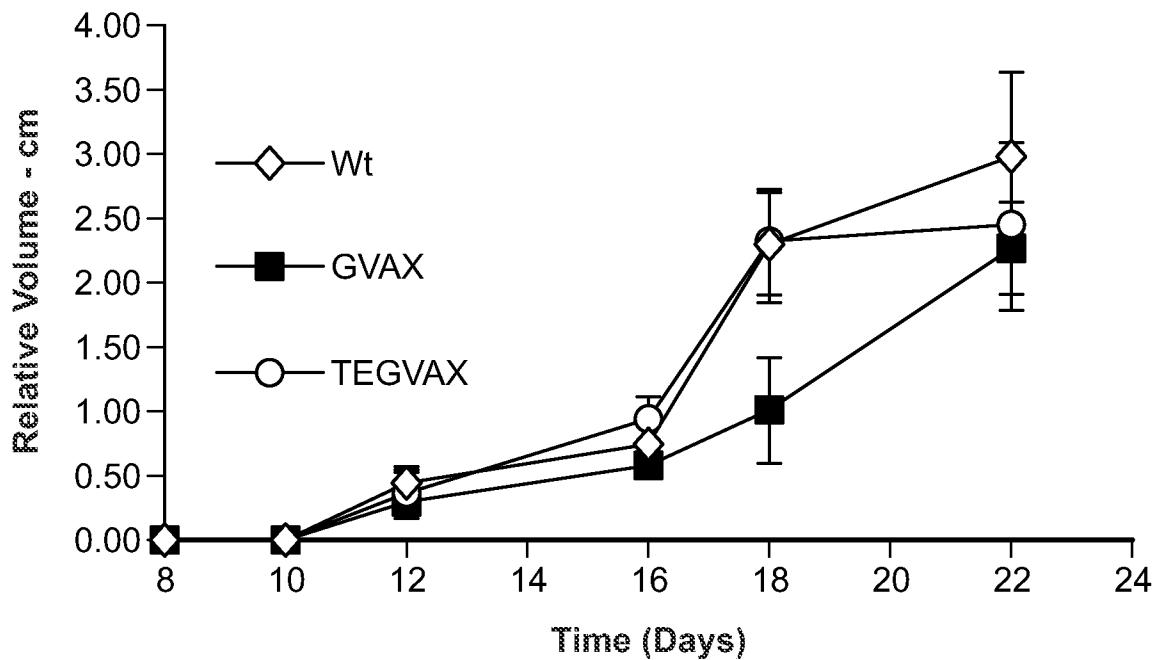


FIG. 3

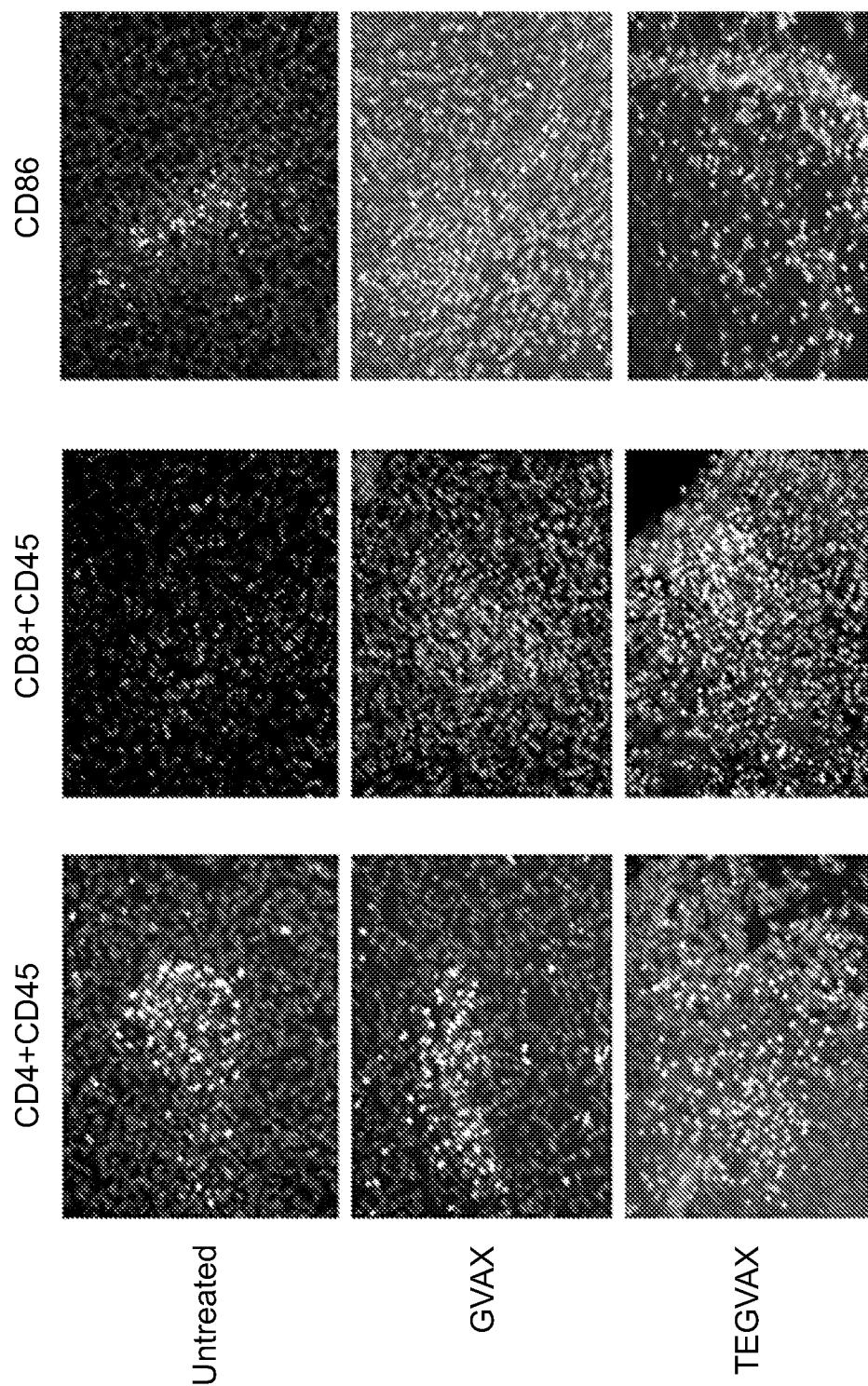


FIG. 4A

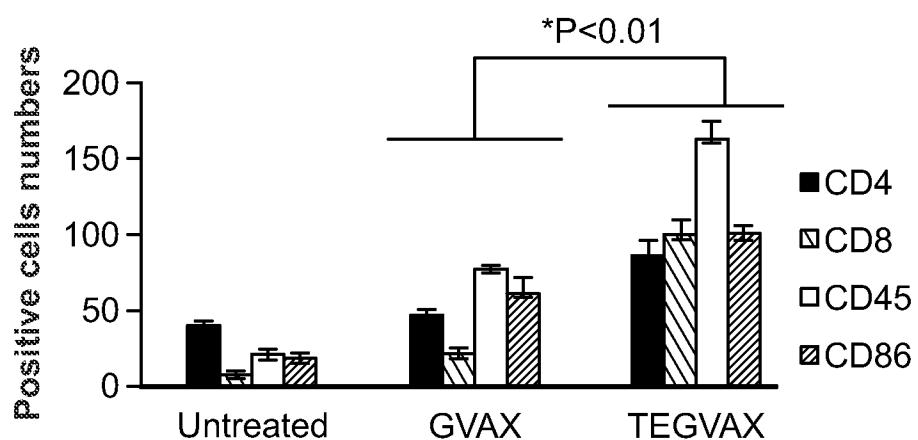


FIG. 4B

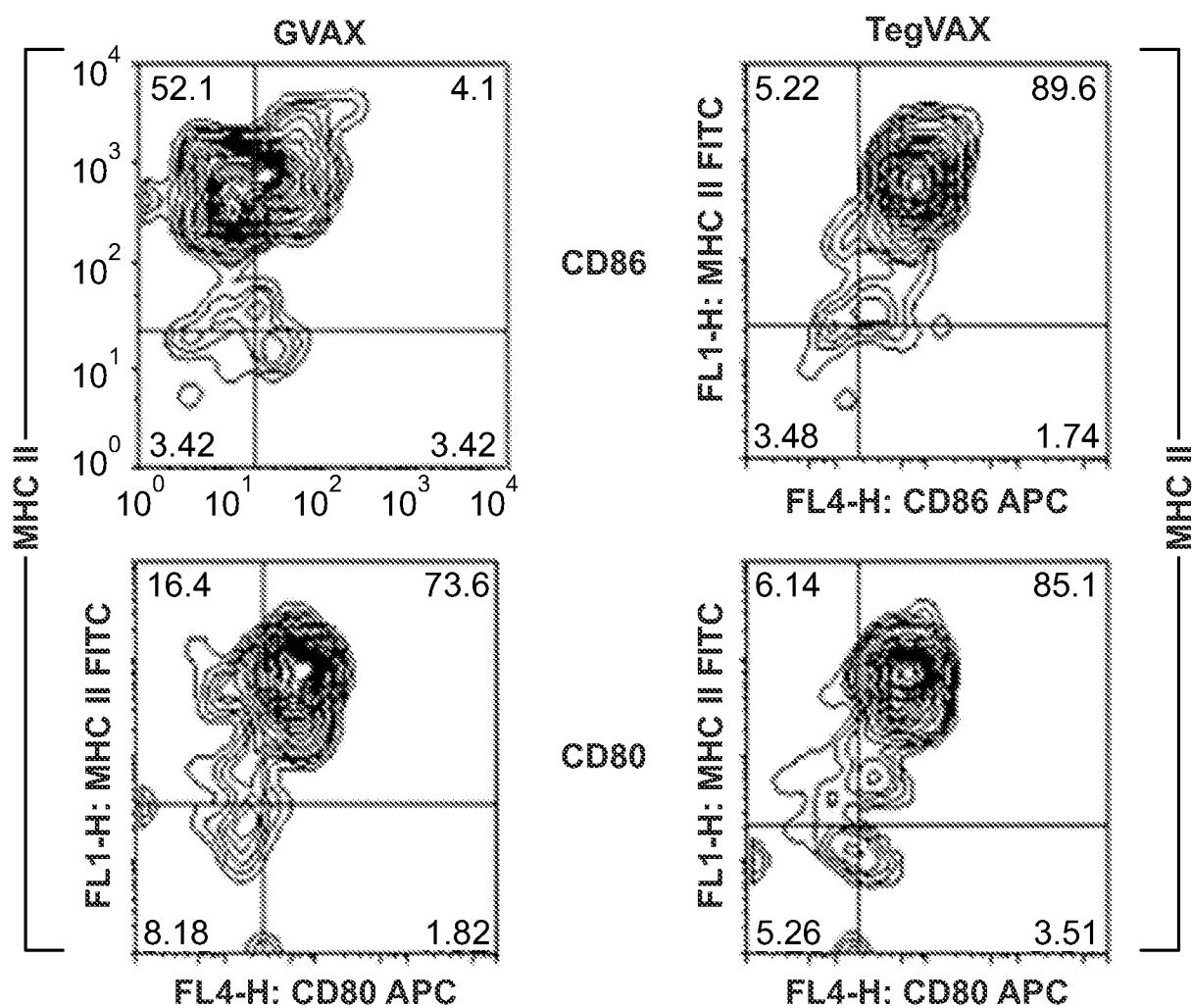


FIG. 5

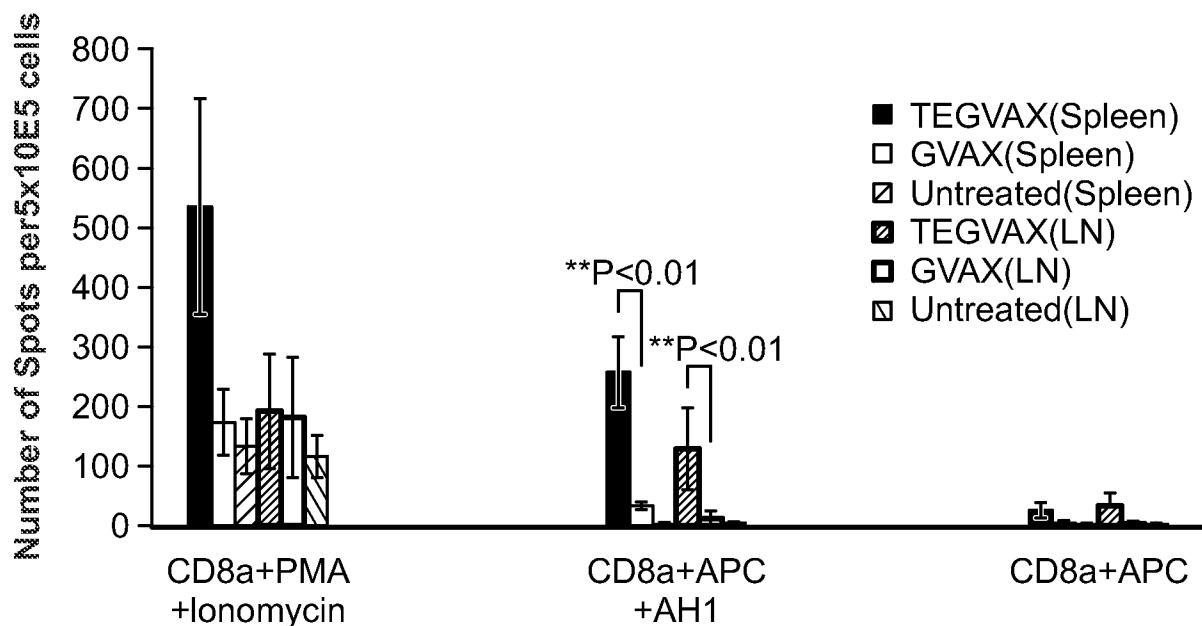


FIG. 6A

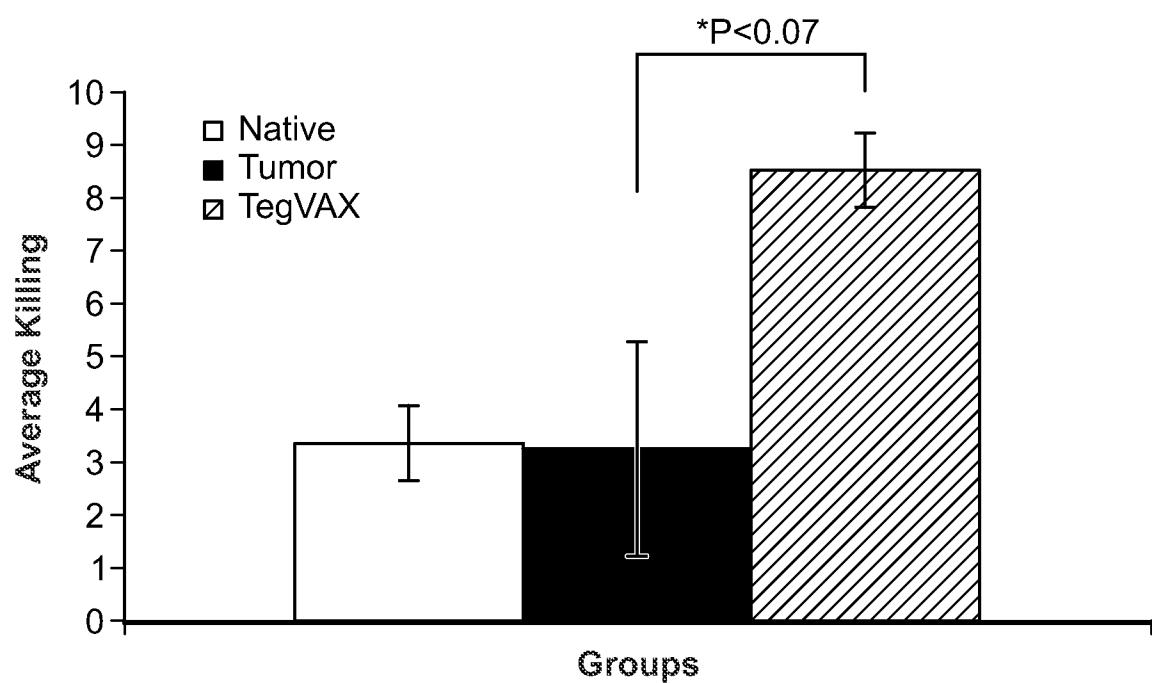


FIG. 6B

7/14

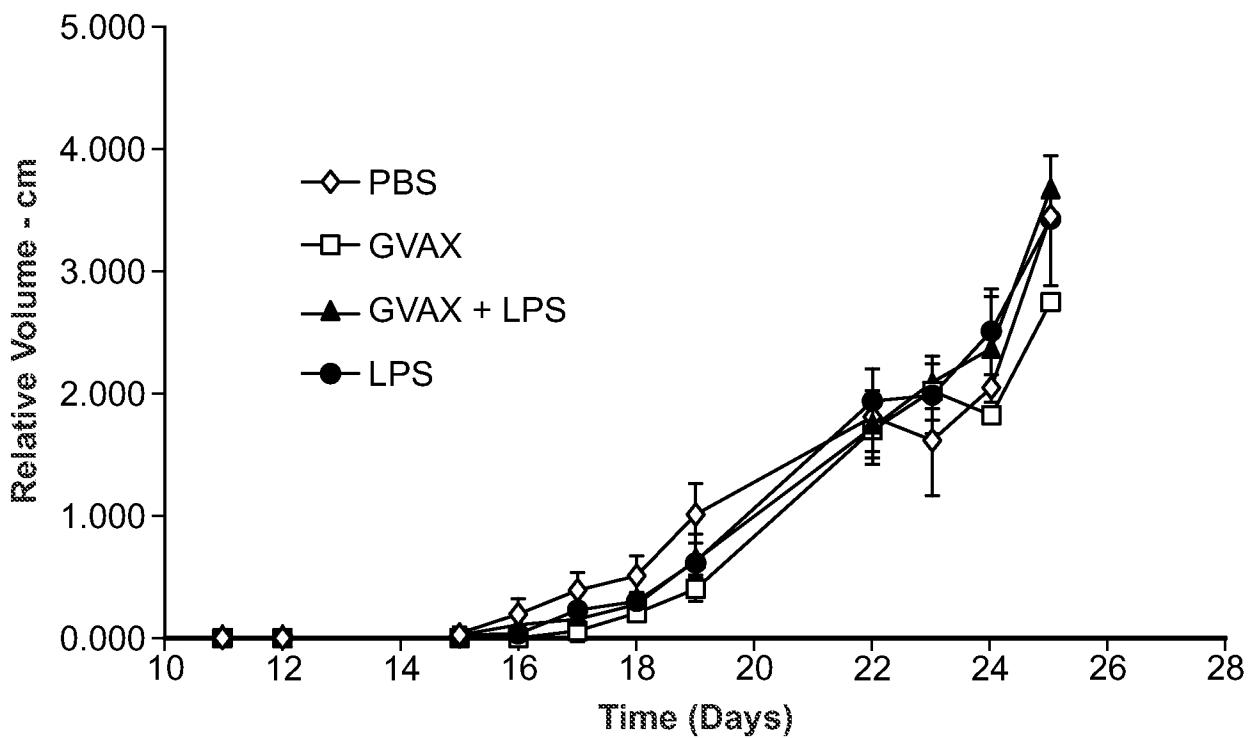


FIG. 7

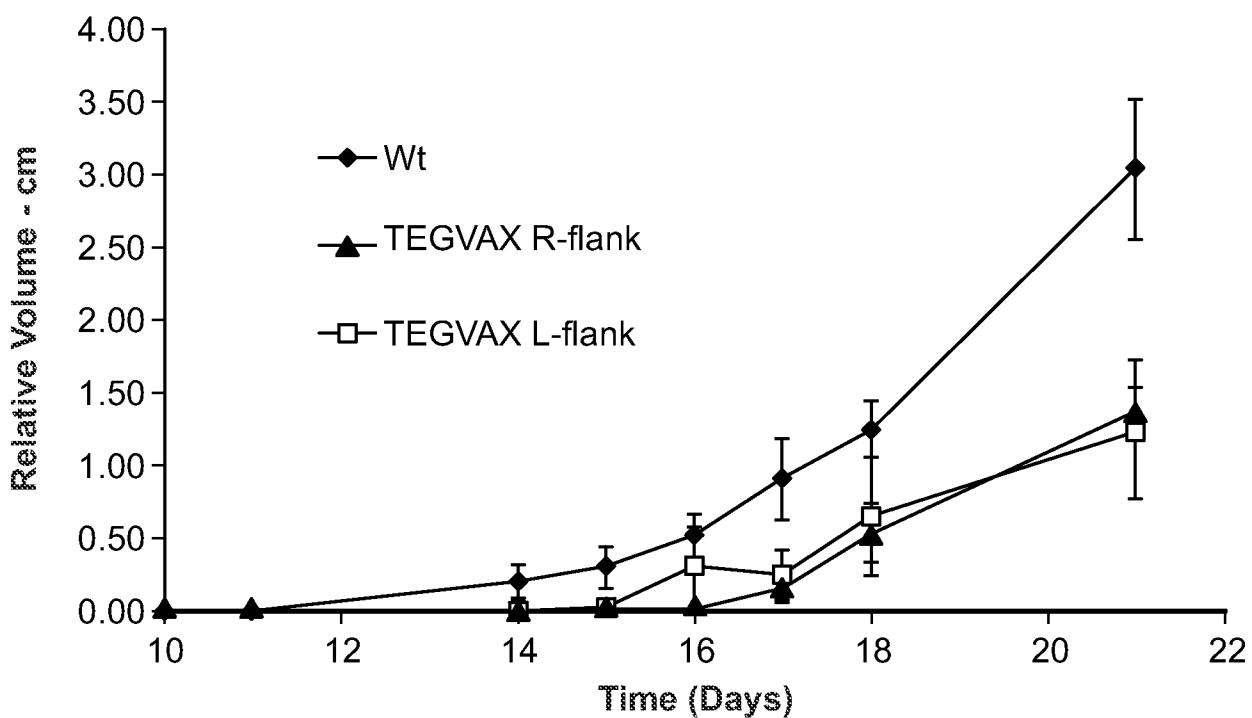


FIG. 8

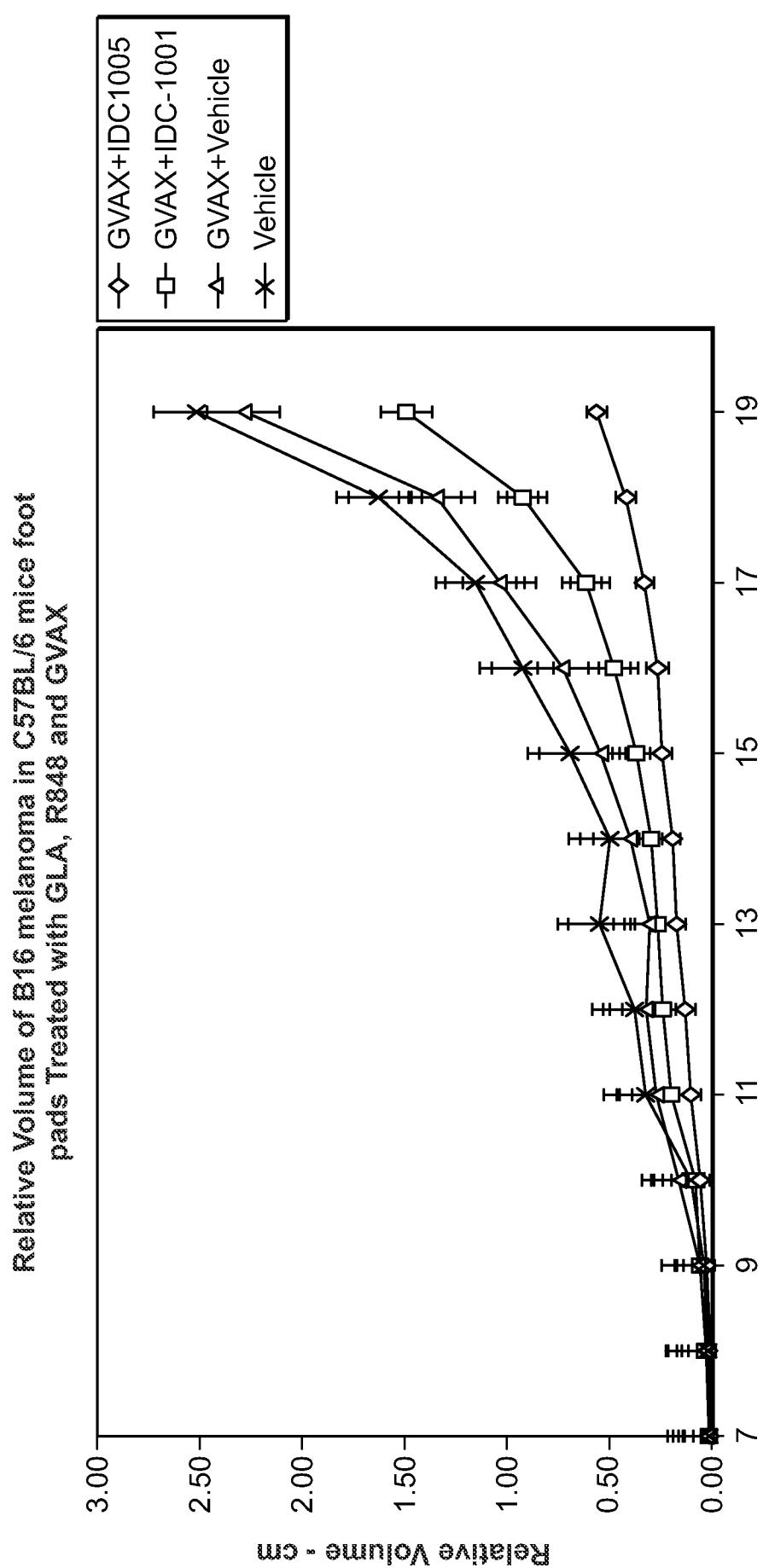


FIG. 9

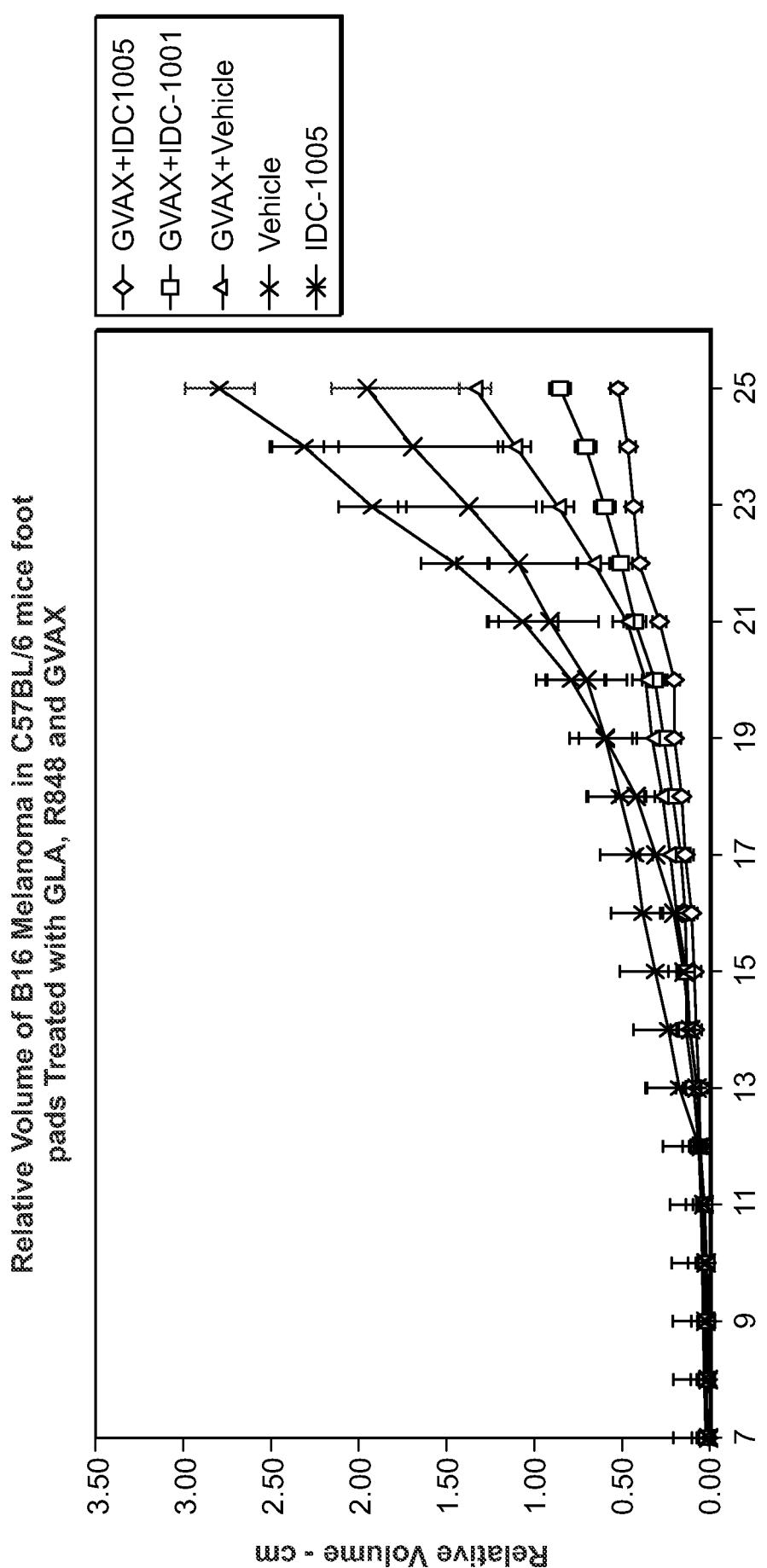
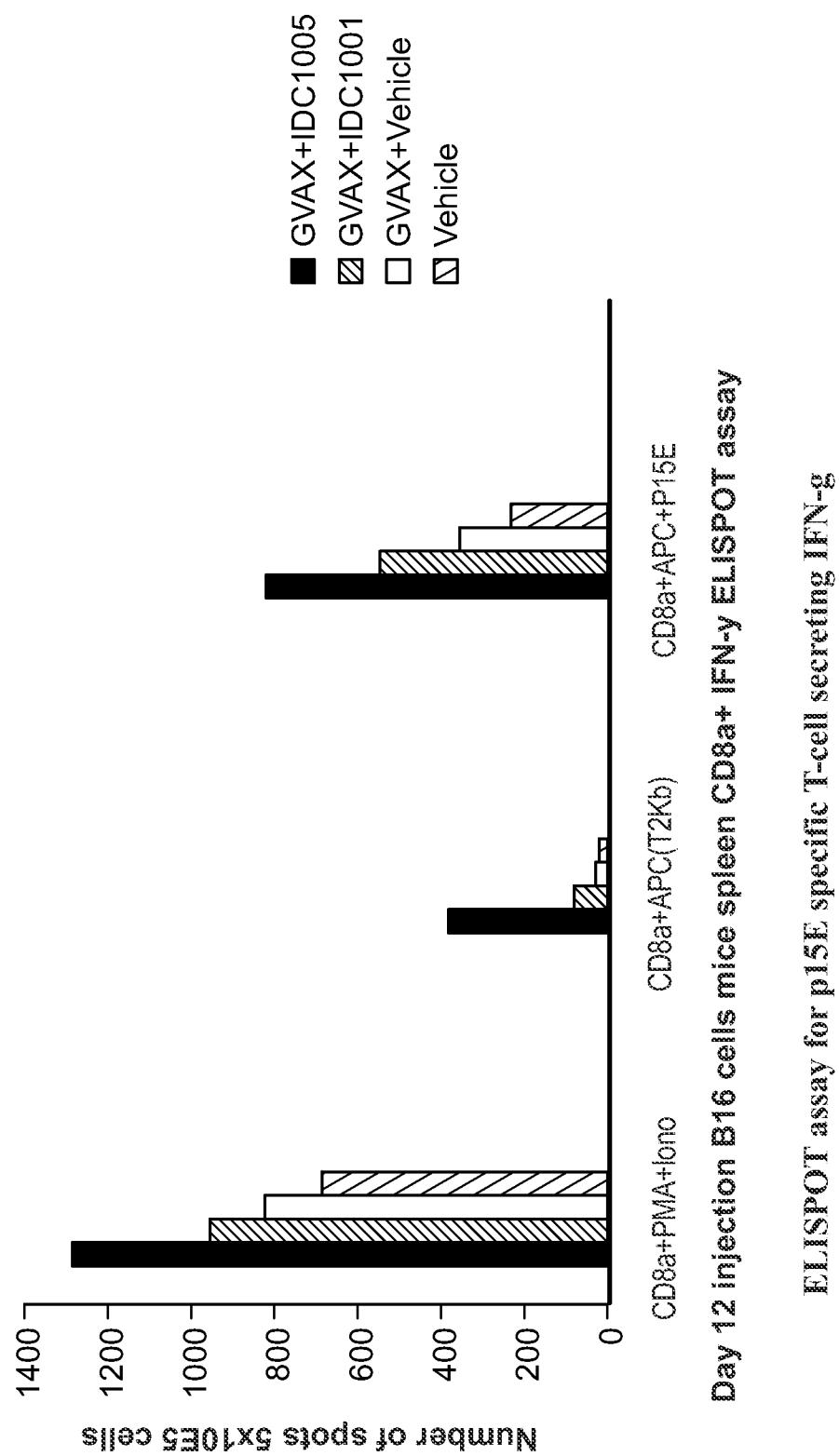


FIG. 10

FIG. 11



11/14

FIG. 11 (continued)

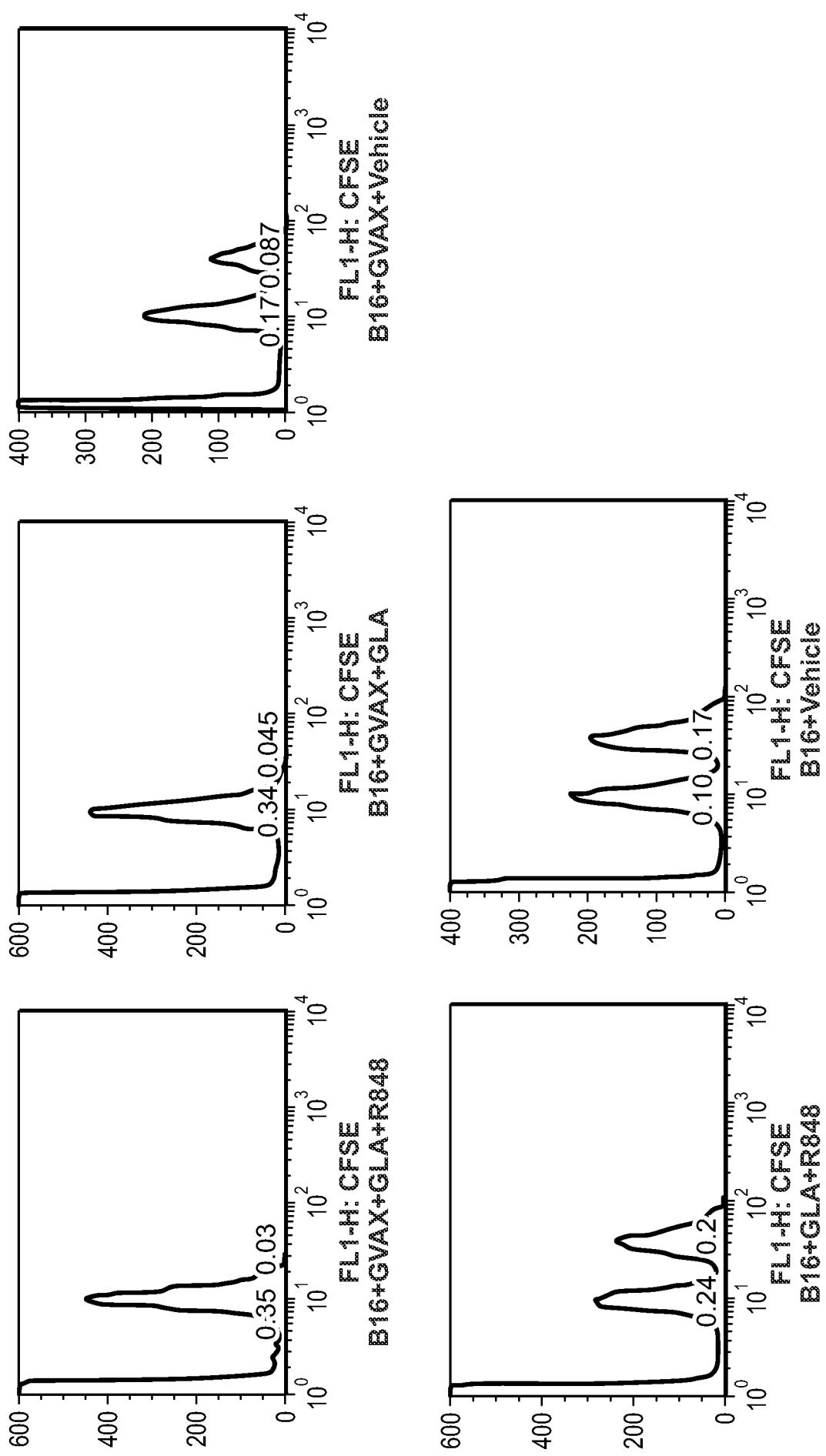
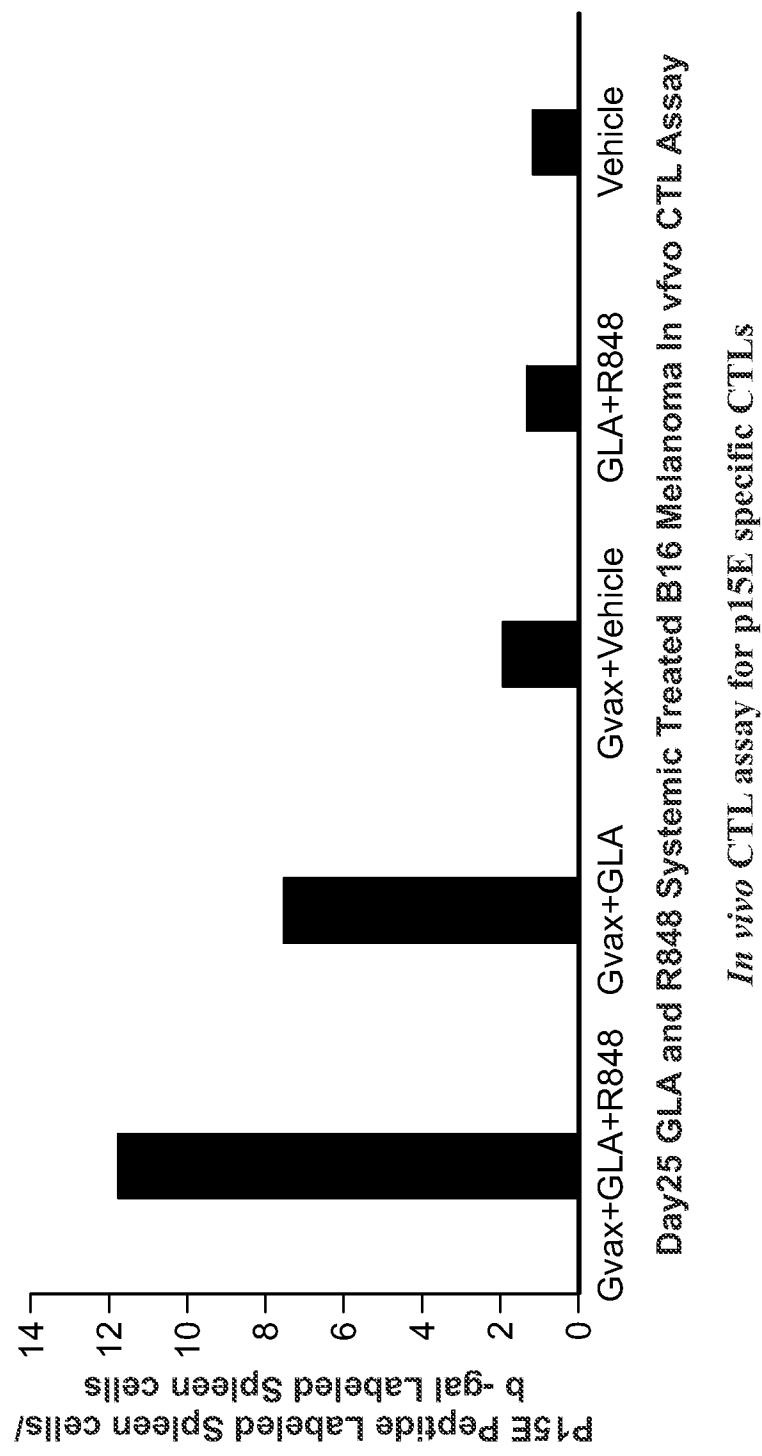


FIG. 11 (continued)



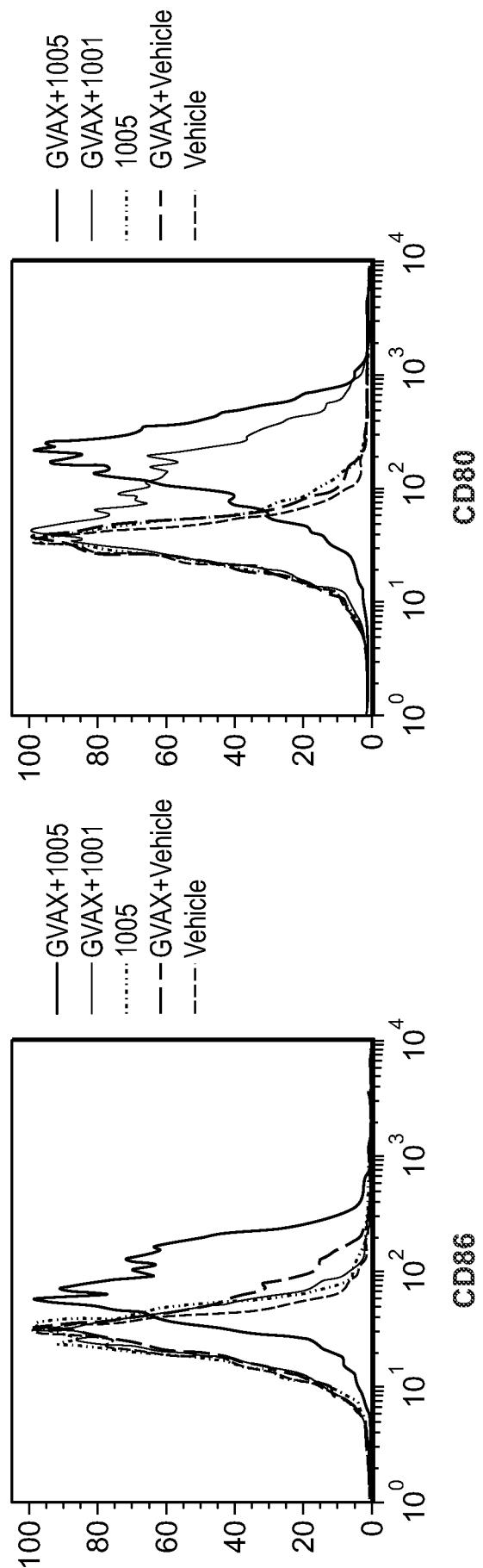


FIG. 12

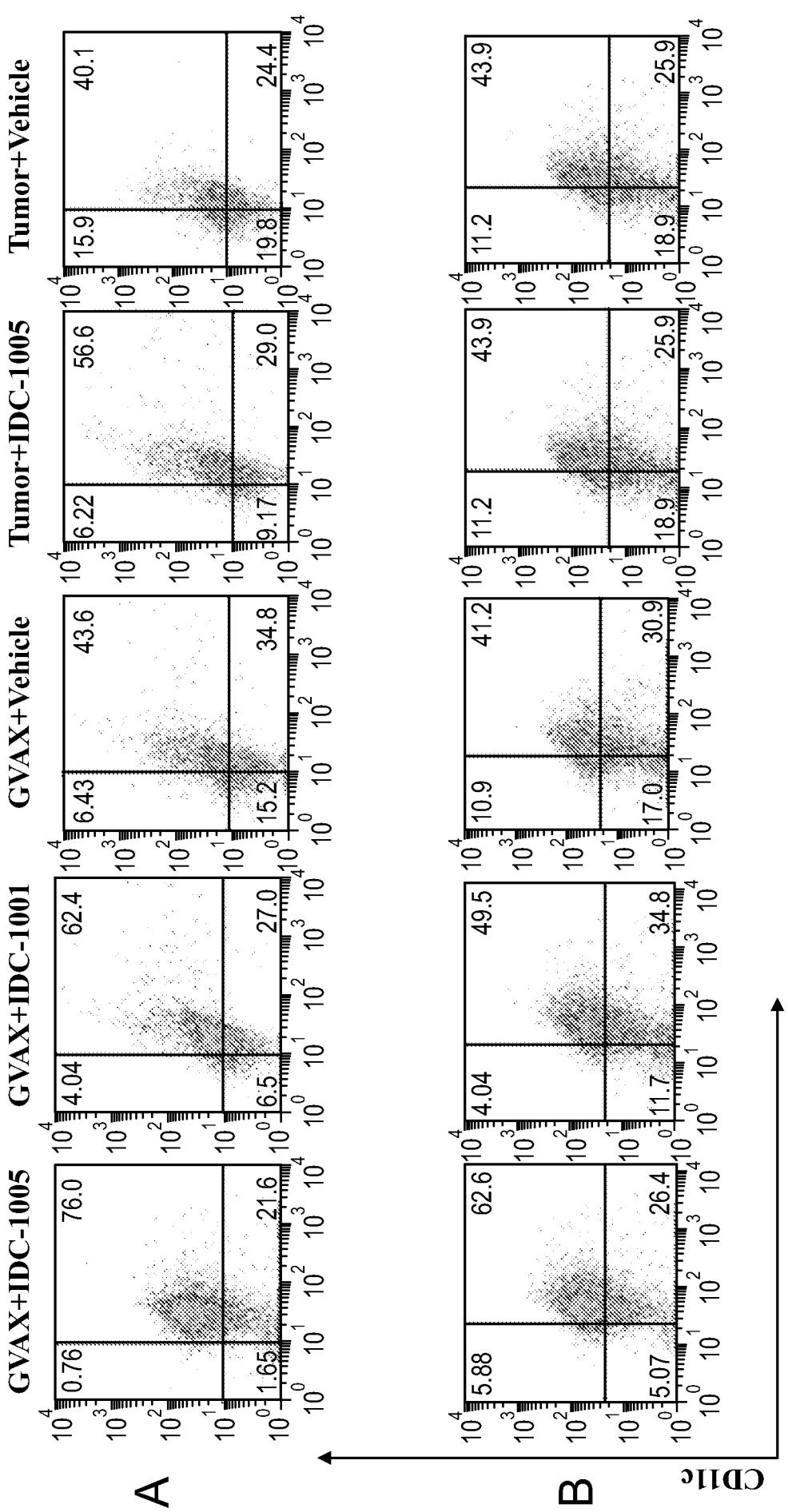


FIG. 13

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2010/052889

A. CLASSIFICATION OF SUBJECT MATTER

A61K 38/19(2006.01)i, A61P 35/02(2006.01)i, A61P 35/00(2006.01)i

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K 38/19; A61P 37/00; A61K 35/12; A61K 39/39; A61K 39/00; A61P 35/00; C07K 14/535; A61P 37/04; A61K 38/20

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

Korean utility models and applications for utility models
Japanese utility models and applications for utility modelsElectronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal), PubMed

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2008-0075705 A1 (TRAN, A-C) 27 March 2008 See claims 27, 30, 31, 33; paragraph [0010], [0034]- [0046].	1-19,36
Y	US 2008-0145375 A1 (BEMBRIDGE, G.P. et al.) 19 June 2008 See claims 1, 7, 14, 47, 49, 60; abstract; paragraphs [0014]-[0098].	1-19,36
Y	WO 2010-024897 A2 (CELL GENESYS) 04 March 2010 See claims 1-5, 7-9, 11-16; paragraphs [0001]-[0049].	1-19,36
Y	US 2009-0081157 A1 (KORNBLUTH, R.S. et al.) 26 March 2009 See claims 1, 11, 15, 19, 22, 23; paragraphs [0016]-[0037].	1-19,36
A	KORNBLUTH, R.S., et al. Immunostimulatory combinations: designing the next generation of vaccine adjuvants, Journal of Leukocyte Biology, 2006, Vol. 80, pages 1084-1102. See the whole document.	1-19,36

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

* Special categories of cited documents:
 "A" document defining the general state of the art which is not considered to be of particular relevance
 "E" earlier application or patent but published on or after the international filing date
 "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)
 "O" document referring to an oral disclosure, use, exhibition or other means
 "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
 "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
 "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
 "&" document member of the same patent family

Date of the actual completion of the international search
25 JULY 2011 (25.07.2011)Date of mailing of the international search report
26 JULY 2011 (26.07.2011)

Name and mailing address of the ISA/KR

Korean Intellectual Property Office
Government Complex-Daejeon, 189 Cheongsa-ro,
Seo-gu, Daejeon 302-701, Republic of Korea

Facsimile No. 82-42-472-7140

Authorized officer

Lee, Soo-Jung

Telephone No. 82-42-481-8466



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2010/052889**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.: 22-28, 34-35, 42-43

because they relate to subject matter not required to be searched by this Authority, namely:

Claims 22-28, 34-35, 42-43 pertain to methods for treatment of the human body by therapy, and thus relate to a subject matter which this International Searching Authority is not required, under Article 17(2)(a)(i) of the PCT and Rule 39.1(iv) of the Regulations under the PCT, to search.

2. Claims Nos.: 20-21, 23-35, 37-43

because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

The claims do not clearly define the matter for which protection is sought. See the extra sheet.

3. Claims Nos.: 21, 23-28, 30, 34-35, 40-43

because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

No protest accompanied the payment of additional search fees.

Continuation of Box No. II Observations where certain claims were found unsearchable.

2. Claims Nos: **20-21, 23-35, 37-43**

because they relate to part of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

Claims 20-21 relate to a vaccine, but the referred claims relate to an immunogenic composition.

Claims 23-28, 34-35, 40, 42-43 are referring to claims 20-21. **Claims 28-35** relate to a method, but the referred claims, at least some of them, relate to an immunogenic composition and/or a vaccine.

Claim 34 relates to a method, but **claims 37-39**, dependant on claim 34, relate to neoplastic cells.

Claim 41 is referring to claims 20-21, 23-33. As **claims 20-21, 23-35, 37-43** do not clearly define the matter for which protection is sought, these claims do not meet the requirement of PCT Article 6.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/US2010/052889

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2008-0075705 A1	27.03.2008	US 2003-044393 A1	06.03.2003
US 2008-0145375 A1	19.06.2008	AU 2004-271726 A1 BR P10414381A CA 2538197 A1 CN 1878567 A EP 1682175 A2 GB 0321615 D0 IL174131D0 IS8363A JP 2007-505827 A JP 2007-505827 T KR20070029111A MX PA06002969A NO20061242A NZ545948A RU 2006106848 A RU 2370537 C2 SG145767A1 WO 2005-025614 A2 WO 2005-025614 A3 ZA200602156A	24.03.2005 21.11.2006 24.03.2005 13.12.2006 26.07.2006 15.10.2003 01.08.2006 20.03.2006 15.03.2007 15.03.2007 13.03.2007 26.03.2007 01.06.2006 31.07.2009 27.10.2007 20.10.2009 29.09.2008 24.03.2005 06.10.2005 25.07.2007
WO 2010-024897 A2	04.03.2010	CA 2735502 A1 WO 2010-024897 A3	04.03.2010 04.03.2010
US 2009-0081157 A1	26.03.2009	AU 2007-239095 A1 CA 2636424 A1 EP 1979488 A2 EP 1979488 A4 JP 2009-522372 T US 2009-081157 A1 WO 2007-120368 A2 WO 2007-120368 A3 WO 2007-120368 A8	25.10.2007 25.10.2007 15.10.2008 27.05.2009 11.06.2009 26.03.2009 25.10.2007 12.06.2008 14.02.2008