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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
28 March 2013 (28.03.2013)

WIPO | PCT

(10) International Publication Number
WO 2013/043647 A1

(51) International Patent Classification:
A61K 39/395 (2006.01) *A61K 38/18* (2006.01)
A61K 38/19 (2006.01) *A61P 35/00* (2006.01)

(21) International Application Number:
PCT/US2012/055992

(22) International Filing Date:
19 September 2012 (19.09.2012)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/536,115 19 September 2011 (19.09.2011) US

(71) Applicant: **THE JOHNS HOPKINS UNIVERSITY** [US/US]; Johns Hopkins Technology Transfer, 100 N. Charles St., 5th Floor, Baltimore, Maryland 21201 (US).

(72) Inventors: **KIM, Young Jun**; 3302 Governor Carroll Court, Ellicott City, Maryland 21043 (US). **PARDOLL, Drew M.**; 19400 James Creek Ct., Brookville, Maryland 20833 (US). **FU, Juan**; 6313 Holly Lane, Apt. B, Baltimore, Maryland 21212 (US).

(74) Agent: **KAGAN, Sarah A.**; BANNER & WITCOFF, LTD., 1100 13th Street, N.W., Suite 1200, Washington, DC 20005-4051 (US).

(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, BN, 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, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, 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.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, 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).

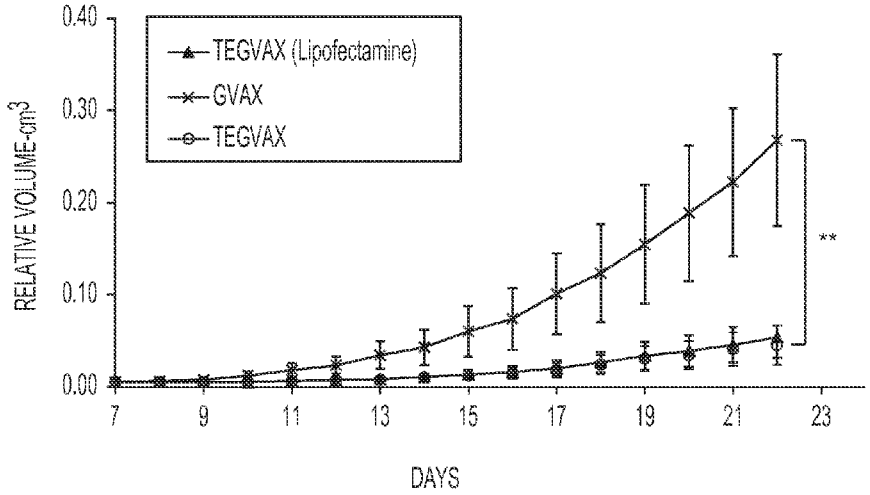
Published:

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

[Continued on next page]

(54) **Title**: CANCER IMMUNOTHERAPY

(SUPPLEMENTAL FIGURE 4)



(57) **Abstract**: We formulated multiple TLR agonists into GVAX (lethally irradiated tumor cell vaccines engineered to secrete GM-CSF). Specifically, GLA and R848, TLR4 and TLR7/8 agonists found to be safe in patients, were formulated with GVAX (TEGVAX - for TLR agonists enhanced GVAX), and this formulation was effective in producing anti-tumor responses in 3 different preclinical models, including palpable B16. These anti-tumor responses were correlated with increased CD4 and CD8 T-cells that can secrete IFN circulating in the tumor microenvironment as well as significantly higher level of p15E specific CTL mediated cell killing in mice treated with TEGVAX in comparison to controls. When combined with anti-PD-1 antibody, TEGVAX was able to induce regression of established B16 tumors.

WO 2013/043647 A1

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

CANCER IMMUNOTHERAPY

[01] This invention was made with support from the National Institutes of Health. Therefore the U.S. government retains certain rights under the terms of grant no. NIH K23-DE018464-02.

TECHNICAL FIELD OF THE INVENTION

[02] This invention is related to the area of cancer therapy. In particular, it relates to cancer immunotherapy.

BACKGROUND OF THE INVENTION

[03] Due to its extensive history of safety as well as availability in multiple tumor types, lethally irradiated tumor cell vaccines engineered to secrete GM-CSF (GVAX) is one vaccine platform that has potential for combinatorial therapy with immune checkpoint blockade antibodies. However, local GM-CSF secreted by GVAX can mobilize myeloid precursors into macrophages and dendritic cells, but this cytokine may not induce their activation. Thus, a major limitation of GVAX is in the activation of antigen presenting cells (APC) necessary for optimal tumor antigen presentation in the afferent arm of the immune system. One simple strategy that phenocopies the robust immunological responses seen in vaccines against infectious agents is to combine multiple TLR agonists with a cancer cell-based vaccine. Clinically, multiple adjuvants have been developed for cancer patients to augment the potency of cancer vaccines, and many of these adjuvants are typically TLR agonists.

[04] One real concern with non-targeted TLR stimulation is the procarcinogenic consequences of chronic TLR stimulation in the tumor cells. Stimulation of TLR4 receptors expressed on tumor cells has shown to promote carcinogenesis. TLR signaling in the hematopoietic compartment, however, has been shown to elicit anti-tumor responses, which have translated into multiple clinical trials. In order to target the dendritic cells in the tumor microenvironment and test whether TLR4 stimulation in the tumor microenvironment can induce a procarcinogenic effect *in vivo*, our group

injected LPS formulated GVAX intratumorally and found that intratumoral injection of TLR4 ligand absorbed GVAX improved the local anti-tumor response *in vivo* in three different murine models.

[05] There is a continuing need in the art to obtain safer and more effective treatments of tumors.

SUMMARY OF THE INVENTION

[06] According to one aspect of the invention a composition is provided which may be used for treating cancer patients. The composition comprises (a) cytokine-expressing, proliferation incompetent, whole cancer cells; (b) an anti-PD-1 antibody that specifically binds to human Programmed Death 1 (PD-1); and (c) a TLR (toll like receptor) agonist; wherein the whole cancer cells are formulated with the TLR agonist.

[07] According to another aspect of the invention a method is provided. Agents are administered to a cancer patient. The agents are : (a) cytokine-expressing, proliferation incompetent, whole cancer cells; (b) an anti-PD-1 antibody that specifically binds to human Programmed Death 1 (PD-1); and (c) a TLR (toll like receptor) agonist; wherein the whole cancer cells are formulated with the TLR agonist. The whole cancer cells are formulated with the TLR agonist.

[08] According to another aspect of the invention a kit is provided which comprises the agents: (a) cytokine-expressing, proliferation incompetent, whole cancer cells; (b) an anti-PD-1 antibody that specifically binds to human Programmed Death 1 (PD-1); and (c) a TLR (toll like receptor) agonist; wherein the whole cancer cells are formulated with the TLR agonist. Optionally the whole cancer cells are formulated with the TLR agonist.

[09] These and other embodiments which will be apparent to those of skill in the art upon reading the specification provide the art with

BRIEF DESCRIPTION OF THE DRAWINGS

[10] Fig. 1A-1D. TEGVAX can increase activated plasmacytoid (pDC) and conventional DC (cDC) in the draining lymph nodes. Fig. 1A. From day 3 after vaccine injection in non-tumor bearing mice, pDC and cDC gated populations from vaccine draining LN were quantitated for CD86. IDC-1005 is GLA and R848 in 10% (w/v) squalene oil-in-water vehicle and TEGVAX is IDC-1005 formulated with irradiated GVAX. Fig. 1B. TEGVAX induced increased CD86 and CD80 activation markers on the pDC from the draining lymph nodes in comparison to GVAX day 3 after vaccine injection (*P<0.05). Fig. 1C. TEGVAX has increased CD86 and CD80 activation markers on the cDC from the draining lymph nodes in comparison to GVAX day 3 after vaccine injection (*P<0.05). Fig. 1D. CD86+ pDC and cDC from DLN from day 3 to 7 after injection with TEGVAX or GVAX.

[11] **Fig. 2A-2D.** TEGVAX induces significant *in vivo* anti-tumor response in comparison to GVAX in multiple animal models. All these *in vivo* treatment experiments are representative of at least 3 separate experiments, and each group in all experiments consists of 10 mice/group. **Fig. 2**A. Mice with palpable B16 melanoma were treated with GVAX, TEGVAX (GVAX/IDC-1005), and IDC-1005 in the contralateral limb and the volume of tumor measured daily. IDC-1005 is GLA and R848 in 10% (w/v) squalene oil-in-water emulsion. **Fig. 2**B. Palpable B16 melanomas treated with multiple injections of vaccines and TLR agonists on day7, day14 and day28 (arrow). **Fig. 2**C. Palpable SCCFVII tongue cancer was treated with 10^6 SCCFVII-GVAX in the contralateral limb and the tumor was measured daily. C3H/HeOUJ mice treated with TEGVAX regressed, and these mice were rechallenged with SCCFVII $5x10^4$ and $1x10^5$ on day22 and day27, respectively at a different site (arrowhead) (* P<0.05; ** P<0.01). No tumor grew at these rechallenge sites. **Fig. 2**D. B16 treatment assay were performed with TEGVAX (1 hour incubation of GVAX and IDC-1005) vs. GVAX and IDC-1005 mixture without incubation. The incubation group had significantly anti-tumor effect in comparison to the no incubation group (* P<0.05).

[12] **Fig. 3A-3D.** TEGVAX treatment increased tumor necrosis and tumor infiltrating lymphocytes and APC. **Fig. 3**A. B16 tumor tissue from each of the treatment groups were harvested and stained with H&E. TEGVAX treated tumor showed increased

number of necrotic foci (circled areas, 20x). **Fig. 3**B. Necrotic foci were quantified in 10 separate regions under 20x magnification in tumor tissue treated with TEGVAX and GVAX (**P<0.01). **Fig. 3**C. Frozen tumor tissue was stained with αCD4 and αCD8 FITC conjugates and counterstained with DAPI in each of the treated groups. TEGVAX treated B16 melanoma had increased infiltration of CD4 and CD8 cells in the tumor microenvironment tumor tissue. **Fig. 3**D. The tumor infiltrating CD4, CD8, and CD86 cells were randomly counted in 10 different fields with immunofluorescent microscope. TEGVAX treated B16 tumors had significantly increased infiltrating lymphocytes in comparison to GVAX and IDC-1005 treated tumors (* P<0.05; ** P<0.01).

[13] **Fig. 4A-4D.** TEGVAX induces T_H1 response as well as upregulation of B7-H1 expression in the tumor. **Fig. 4**A. Tumor draining lymph nodes were harvested from the treated mice and intracellular staining of IFNγ from both CD4 and CD8 populations were performed. **Fig. 4**B. Mean fluorescent intensity of CD4+ IFNγ+ and CD8+ IFNγ+ were quantitated in each of the treated groups. **Fig. 4**C. Treated tumor tissue were harvested and stained with rat anti-mouse B7-H1 antibodies and anti-rat Cy3 conjugate were used to assess B7-H1 positive cells. Blinded quantitation was performed in 10 different fields at 40x magnification and is summarized in **Fig. 4D.**.

[14] **Fig. 5A-5D.** The *in vivo* anti-tumor response of TEGVAX is both CD4 and CD8 T-cell dependent. **Fig. 5**A. Both GK1.5 (CD4 depleting) and 2.43 (CD8 depleting) antibodies were injected intraperitoneally during the vaccine treatment. **Fig. 5**B. CD4, CD8 cells were depleted individually in the TEGVAX and control treatment assay. **Fig. 5**C. The anti-tumor response of TEGVAX is abrogated in Rag2-/- mice. **Fig. 5**D. The anti-tumor response of TEGVAX is abrogated in MyD88-/- and TRIF-/- double knockout mice. The *in vivo* effect of TEGVAX is identical to GVAX in these TLR signaling defective mice (**P<0.01).

[15] **Fig. 6A-B.** TEGVAX treated mice has increased number of tumor specific CTL cells. *In vivo* CTL and ELISPOT assay with P15E peptides were performed in B16 tumor bearing mice treated with vaccines and adjuvants. **Fig. 6**A. *In vivo* CTL assay was performed on day 25 after vaccine treatment from each of the treated groups. P15E

peptide-specific killing was calculated using the following formula: (1-% of CFSE $_{peptide}$/% of CFSE $_{no\ peptide}$) x 100. **Fig. 6**B. ELISPOT assay on splenocytes from each of the treated groups on day 23. P15E peptide was pulsed into T2kb APC (** P<0.01).

[16] **Fig. 7A-7C.** TEGVAX/anti-PD-1 treatment can induce regression of established B16 tumors. **Fig.** 7A. Right panel shows tumor growth rate of established B16 tumor treated with various combinations of vaccines and anti-PD-1. **Fig.** 7B. Left panel shows two mice – right mice is one of the mice treated with TEGVAX/anti-PD-1, left mice is one of the mice treated with GVAX. TEGVAX treated mice did not show any regression. Circles show the site of tumor inoculation. Arrow shows the site of vitiligo. **Fig.** 7C. Tumor draining lymph nodes were harvested and TH1 cytokines were quantitated from CD4, CD8, and CD11c+ DC cells.

[17] **Fig. 8 (Supplemental Figure 1)**. Increased IL-12, TNF-α, and IFN-α in tumor draining LN of TEGVAX treated mice. Tumor DLN were harvested and Th1 cytokine profiles on CD11c+ cells were analyzed in each of the treated groups. TEGVAX treated mice had significantly increased IL12, TNF-α and IFN-α positive CD11c+ cells in comparison to GVAX or the control treated groups.

[18] **Fig. 9 (Supplemental Figure 2**.) TEGVAX induces anti-tumor response in established CT26 model. 10^5 CT26 colon cancer cells were injected into the footpad, and mice with palpable tumors were treated with the vaccines. CT26-GVAX were prepared as described in Yoshimura et.al. (11). TEGVAX significantly decreased mice CT26 colon cancer growth in comparison to CT26-GVAX alone or with IDC-1005 alone (**P<0.01).

[19] **Fig. 10A-B (Supplemental Figure 3**.) Multiple TLR agonists formulated into TEGVAX had the greatest anti-tumor response *in vivo*. **Fig. 10**A. Mice bearing palpable B16 tumor was treated with TEGVAX, GVAX, GVAX formulated with R848, or GVAX formulated with GLA. All formulation consisted of incubating B16-GVAX with adjuvants in 10% (w/v) squalene oil-in-water emulsion vehicle for 1 hr at

4 deg C prior to vaccine injection (**P<0.01). **Fig. 10**B. Individual adjuvants with vehicle without GVAX had no *in vivo* anti-tumor effects.

[20] **Fig. 11 (Supplemental Figure 4)**. TEGVAX formulated with absorbed GLA and R848 using Lipofectamine ™ 2000 (Invitrogen) had comparable *in vivo* anti-tumor effect as TEGVAX formulated with oil-in-water emulsion vehicle (**P<0.01).

[21] **Fig. 12. (Supplemental Figure 5)**. TEGVAX increased the number of activated DC that have endogenously endocytosed Q-dot, a surrogate tumor antigen, from the vaccine. GVAX and TEGVAX were labeled with Qtracker 655 (Invitrogen) and inoculated into mice footpads. After 3 days the draining popliteal LNs were harvested, and lymphocytes depleted with CD3 and CD19 antibodies. FACS analysis quantified endogenous pDC and cDC labeled with Q-dot from the vaccines.

DETAILED DESCRIPTION OF THE INVENTION

[22] We have developed *in vivo* evidence that optimally formulated cancer vaccines combined with PD-1 blockade can be used therapeutically for treating tumors. We have collected evidence demonstrating that the combination regimen can be effective against established tumors that are poorly immunogenic. All the components of this combinatorial regimen have been individually tested in patients and found to be clinically safe. The disclosed treatment strategy may work by adaptive immune evasion, although applicants do not intend to be bound by any proposed mechanism of action.

[23] Patients having a variety of **cancers** may be treated with the combination regimen. Such cancers include colorectal cancer, an aero-digestive squamous cancer, a lung cancer, a brain cancer, a liver cancer, a stomach cancer, a sarcoma, a leukemia, a lymphoma, a multiple myeloma, head-and-neck cancer, an ovarian cancer, cervical cancer, a uterine cancer, a breast cancer, a melanoma, a prostate cancer, a pancreatic carcinoma, and a renal carcinoma. This list is meant to be illustrative rather than limiting.

[24] Whole cancer cells may be allogeneic, syngeneic, or **autologous** to the treatment recipient. Typically they may be treated to make them **proliferation incompetent** by a technique which preserves preserve their immunogenicity and their metabolic activity. One typically used technique is irradiation. Such cells. Typically the same general type of tumor cell is used that the patient bears. For example, a patient suffering from melanoma will typically be administered proliferation incompetent melanoma cells. The cells may express and secrete a **cytokine naturally or by transfection** with a nucleic acid which directs such expression and secretion. One suitable cytokine is GM-CSF. For example, the tumor cell may express a transgene encoding GM-CSF as 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. One example of a GM-CSF-expressing,genetically modified cancer cell for 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. Other cytokines can be used. Suitable **cytokines** which may be used include cytokines which stimulate dendritic cell induction, recruitment, and/or maturation. Such cytokines include, but are not limited to, one or more of GM-CSF, CD40 ligand, IL-12, CCL3, CCL20, and CCL21. Granulocyte-macrophage colony stimulating factor (GM-CSF) polypeptide is a cytokine or fragment having immunomodulatory activity and having at least about 85% amino acid sequence identity to GenBank Accession No. AAA52122.1.

[25] According to one alternative embodiment, cytokines are delivered by inactivated bystander cells which express and secrete one or more cytokines. The bystander cells may provide all of the cytokines which stimulate dendritic cell induction, recruitment, and/or maturation, or may supplement cytokines secreted by the inactivated tumor cells. Immunomodulatory cytokine-expressing bystander cell lines are described in U.S. Pat. Nos. 6,464,973, and 8,012,469, Dessureault et al., Ann. Surg. Oncol. 14: 869-84, 2007, and Eager and Nemunaitis, Mol. Ther. 12: 18-27, 2005, each of which is expressly incorporated by reference.

[26] **Antibodies** which are suitable for use in the treatment regimen and compositions and kits include any which specifically bind to **Programmed Death 1 (PD-1).**

Exemplary types of antibodies which may be employed include without limitation human, humanized, chimeric, monolclonal, polyclonal, single chain, antibody binding fragments, and diabodies. Typically antibodies are substantially encoded by an immunoglobulin gene or immunoglobulin genes, or fragments thereof. Antibodies are capable of specifically binding an antigen or epitope. See, e.g. Fundamental Immunology, 3rd Edition, W.E. Paul, ed., Raven Press, N.Y. (1993); Wilson (1994; J. Immunol. Methods 175:267-273; Yarmush (1992) J. Biochem. Biophys. Methods 25:85-97. An antibody typically specifically binds to an antigen or epitope. Specific binding occurs to the corresponding antigen or epitope even in the presence of a heterogeneous population of proteins and other biologics. Specific binding of an antibody indicates that it binds to its target antigen or epitope with an affinity that is substantially greater than binding to irrelevant antigens The relative difference in affinity is often at least 25% greater, more often at least 50% greater, most often at least 100%. The relative difference can be at least 2x, at least 5x, at least 10x, at least 25x, at least 50x, at least 100x, at least 1000x, for example.

[27] Toll like receptors (**TLR**) are a family of proteins that sense a microbial product and/or initiates an adaptive immune response. TLR activate a dendritic cell (DC). TLRs are conserved membrane spanning molecules containing an ectodomain of leucine-rich repeats, a transmembrane domain and an intracellular TIR. (Toll/IL-1R) domain. TLRs recognize distinct structures in microbes, often referred to as "PAMPs" (pathogen associated molecular patterns). Ligand binding to TLRs invokes a cascade of intra-cellular signaling pathways that induce the production of factors involved in inflammation and immunity.

[28] Exemplary **agonists** which may be used for these receptors include, without limitation lipoproteins, lipopolypeptides, peptidoglycans, zymosan, lipopolysaccharide, neisserial porins, flagellin, profillin, galactoceramide, muramyl dipeptide, glucopyranosyl lipid A (GLA), and resiquimod (R848). Peptidoglycans, lipoproteins, and lipoteichoic acids are cell wall components of Gram-positive. Lipopolysaccharides are expressed by most bacteria. Flagellin is the structural component of bacterial flagella that is secreted by pathogenic and commensal bacterial. A Galactosylceramide (α-GalCer) is an activator of natural killer T (NKT)

cells. Muramyl dipeptide is a bioactive peptidoglycan motif common to all bacteria. Such agonists mediate innate immune activation via Toll-like Receptors. Specific binding of an agonist for its cognate receptor is often expressed in terms of an affinity. The ligands of the present invention may bind with affinities of between about 10^4 M^{-1} and about 10^8 M^{-1}. Affinity is calculated as $K_d = k_{off}/k_{on}$ (k_{off} is the dissociation rate constant, K_{on} is the association rate constant and K_d is the equilibrium constant). Single or multiple agonists may be used.

[29] In humans, ten TLR have been identified. TLRs that are expressed on the surface of cells include TLR-1,-2,-4,-5, and -6, while TLR-3, -7/8, and -9 are expressed with the ER compartment. Human dendritic cell subsets can be identified on the basis of distinct TLR expression patterns. By way of example, the myeloid or "conventional" subset of DC (mDC) expresses TLRs 1–8 when stimulated, and a cascade of activation markers (e.g. CD80, CD86, MHC class I and II, CCR7), pro-inflammatory cytokines, and chemokines are produced. A result of this stimulation and resulting expression is antigen-specific CD4+ and CD8+ T cell priming. These DCs acquire an enhanced capacity to take up antigens and present them in an appropriate form to T cells. In contrast, the plasmacytoid subset of DC (pDC) expresses only TLR7 and TLR9 upon activation, with a resulting activation of NK cells as well as T-cells. As dying tumor cells may adversely affect DC function, it has been suggested that activating DC with TLR agonists may be beneficial for priming anti-tumor immunity in an immunotherapy approach to the treatment of cancer. It has also been suggested that successful treatment of breast cancer using radiation and chemotherapy requires TLR4 activation.

[30] TLR agonists known in the art and useful in the present invention include, but are not limited to, the following:

Pam3Cys, a TLR-1/2 agonist;

CFA, a TLR-2 agonist;

MALP2, a TLR-2 agonist;

Pam2Cys, a TLR-2 agonist;

FSL-1, a TLR-2 agonist;

Hib-OMPC, a TLR-2 agonist;

polyribosinic:polyribocytidic acid (Poly I:C), a TLR-3 agonist;

polyadenosine-polyuridylic acid (poly AU), a TLR-3 agonist;

Polyinosinic-Polycytidylic acid stabilized with poly-L-lysine and carboxymethylcellulose (Hiltonol®), a TLR-3 agonist;

monophosphoryl lipid A (MPL), a TLR-4 agonist;

LPS, a TLR-4 agonist;

bacterial flagellin, a TLR-5 agonist;

sialyl-Tn (STn), a carbohydrate associated with the MUC1 mucin on a number of human

cancer cells and a TLR-4 agonist;

imiquimod, a TLR-7 agonist;

resiquimod, a TLR-7/8 agonist;

loxoribine, a TLR-7/8 agonist; and

unmethylated CpG dinucleotide (CpG-ODN), a TLR-9 agonist.

[31] Formulation of the whole cancer cells with the TLR agonist appears to be a contributing factor to enhanced efficacy. Formulations can be incubated together for periods of times such as ¼, ½, 1, 2, 3, 5, 10, 24 hours, at temperatures such as 4

degrees C. Alternatively, binding in the presence of a lipophilic agent or an emulsifying agent can be employed. Such agents are well known in the art.

[32] Various dosing schedules may be envisioned, with simultaneous or staggered timing, with single or multiple agents, single cycle or multiple cycles.

[33] Methods of **administering** treatment agents to cancer patients vary. Exemplary methods include without limitation subcutaneous, intravenous, intramuscular, intraarterial, intradermal, intrathecal, intratumoral, intraperitoneal, sublingual, and epidural administrations. Administration may be to a human, mammal, mammalian subject, animal, veterinary subject, placebo subject, research subject, or experimental subject. Typically an agent such as an exogenous ligand, reagent, placebo, small molecule, pharmaceutical agent, therapeutic agent, diagnostic agent, or composition is contacted with the subject in an appropriate anatomical location. Administration may be for the purposes of therapy, pharmacokinetic study, diagnostic assay, research, placebo, or experimental method. Agents according to the invention may be, but not need not be, administered as a single composition. Although administration as a single composition is contemplated by the present invention, agents may be delivered to a single subject as separate administrations, which may be at the same or different time, and which may be by the same route or different routes of administration. In some cases, the agents may in fact contact each other within the subject's body, forming a composition *in vivo*.

[34] The above disclosure generally describes the present invention. All references disclosed herein are expressly incorporated by reference. A more complete understanding can be obtained by reference to the following specific examples which are provided herein for purposes of illustration only, and are not intended to limit the scope of the invention.

EXAMPLES

[35] In order to evaluate the capacity of multiple TLR agonists to induce systemic anti-tumor immunity in the clinical setting, we formulated glucopyranosyl lipid A (GLA-TLR4 agonist) and resiquimod (TLR7/8 agonist) with GVAX and studied their anti-

tumor effects *in vivo* in palpable B16 model. GLA is a synthetic hexa-acylated molecule that was shown to have stronger TLR4 agonist than the monophosphyl lipid A, which is currently undergoing clinical trials as a TLR4 agonist adjuvant in cancer vaccines. R848 is a TLR7/8 agonist that was found to produce 50-100 fold cytokines response compared to imiquimod (TLR7 agonist), and this agent has also passed phase I trials for safety in patients. We combined these adjuvants that have been tested in patients to be clinically safe individually to formulate TLR Agonist Enhanced GVAX (TEGVAX), and examined their potential to activate APC and to increase the anti-tumor T-cells in the context of established tumors.

[36] Both Li et.al. and Curran et.al. showed that anti-PD-1 and cellular vaccines can be combined to induce an anti-tumor response *in vivo*, but this report utilized a non-palpable tumor inoculation method which may not model clinical scenarios with established tumor tissue. Curran et.al. also combined anti-CTLA-4 with anti-PD-1 and vaccine, but we chose anti-PD-1 blockade due to the toxicity associated with ipilimumab in both preclinical and clinical studies. With our focus on developing safe vaccines that can generate significant interferon responses in the tumor microenvironment, we utilized a stringent model to test the combinatorial therapy of TEGVAX and anti-PD-1 on palpable, established B16 tumors.

EXAMPLE 1

Material and Methods

[37] **Mice and reagents:** 6-8 weeks old female C57BL/6, Balb/c, and C3H/HeOUJ mice (Jackson Lab) were housed according to the Johns Hopkins Hospital (JHH) Animal Care and Use Committee. C57BL/6 MyD88$^{-/-}$TRIF$^{-/-}$, and B6 (Cg) Rag2tml (Rag2$^{-/-}$) mice were obtained from Drs. Franck Housseau and Fan Pan, respectively (JHH). B16 and B16 GVAX cells were cultured in RPMI1640 media containing 10% ΔFCS, penicillin (100U/ml) and streptomycin (100U/ml). The Cytofix/Cytoperm reagent kit and antibodies were purchased from BD Bioscience. CD11c+ cells were isolated by anti-mouse CD11c microBeads (MACS, Miltenyi Biotec). CD4 depleting GK1.5 antibody and CD8 depleting 2.43 (Bio X Cell) at 200μg/dose, were injected

intraperitoneally every 2 days (total 5 times). Hybridoma expressing blocking anti-PD-1 antibody (clone G4) was obtained from Dr. Charles Drake (JHH).

[38] Glucopyranosyl lipid A (GLA) at 1mg/ml and resiquimod (R848) at 0.2mg/ml resiquimod were prepared in 10% (w/v) squalene oil-in-water emulsion (Immune Design). IDC-1005 is the mixture of 1mg/ml GLA and 0.2mg/ml R848 in emulsion vehicle. IDC-1005 was incubated with irradiated GVAX cells at 4 deg C for 0.5-2 hours prior to inoculation. GVAX formulated with IDC-1005 is labeled as TEGVAX. In some cases, GLA and R848 without emulsion vehicles were absorbed into GVAX cells with Lipofectamine and washed 4 times to remove non-absorbed TLR agonists and transfectants.

[39] **Tumor treatment assay**: C57BL/6 mice were injected with 1-5x10^4 B16 in the footpads. Once palpable tumor developed (5-10 days), 100 µl of 10^6 B16 GVAX formulated with or without IDC-1005 were injected subcutaneously into the contralateral limb. The control groups were injected with vehicle. For all these experiments, 10 mice were used per group. C3H/HeOUJ mice and Balb/c mice were used with SCCFVII/SF cells and CT26 cells, respectively with comparable methods (28). Previously prepared irradiated 10^6 SCCFVII/SF-GVAX was used for SCCFVII model in a same manner as B16 (3). For CT26 model, CT26 cells transduced with GM-CSF were used as CT26 GVAX (11). For all the vaccines, GM-CSF was titered to ensure that GM-CSF expression level ranged from 50-500ng/10^6 cells/24 hours. Tumor was measured daily. In some cases, GVAX were labeled with Qtracker 655 (Invitrogen) prior to inoculation. For the PD-1 experiments, 100 µg/mice/injection was injected intraperitoneally twice a week once tumor was palpable in conjunction with vaccine treatments.

[40] **DC activation assay:** Spleens and draining lymph nodes (DLN) from tumor-bearing or naive mice were harvested 3-7 days post-vaccine treatments. Crushed spleens were digested in media containing DNAse I (Roche) and Liberase Blendzyme 2 (20,000 Mandl U/ml) (Roche). DC-enriched populations were obtained by depleting $CD3^+$ and $CD19^+$, and gated for $CD11c^+$ and $B220^+$. These were evaluated by a multicolored FACS analysis using CD80, CD86, CD40, and MHCII antibodies.

[41] **ELISPOT assay:** ELISPOT plates (MultiScreen$_{HTS}$ filter plate, Millipore) were coated with a mouse IFN-γ Ab (MabTech) for 24 hours and T2kb cells were pulsed with 10 μg/ml of P15E (KSPWFTTL) peptide overnight. 10^6 splenic CD8 cells from spleen were plated in triplicates to be co-cultured with pulsed or unpulsed 10^5 T2kb cells or stimulated with 1μM of PMA and 10ng/ml of Ionomycin as positive controls. On day 3, biotinylated anti-mouse IFN-γ Ab (MabTech) and Strepavidin-HRP were added. AEC Substrate Reagent (BD) was used to develop spots and analyzed using ELISPOT Plate Reader (Immunospot).

[42] **In vivo CTL assay**: Splenocytes were labeled with 0.5μM and 5μM CFSE (Molecular Probes). The 5μM CFSE labeled cells were pulsed with 10μg/ml P15E (KSPWFTTL) peptide. The 0.5μM CFSE labeled cells pulsed with β-gal (TPHPARIGL) peptide. Mice were injected intravenously with a 1:1 mixture of these cells, and splenocytes were isolated after 24 hours and analyzed by flow cytometry. Antigen-specific killing was calculated using the following formula: (1-% of $CFSE_{P15E}$/% of $CFSE_{\beta\text{-gal}}$) X 100.

[43] **Immunohistochemistry:** 10μm thick frozen sections were fixed with acetone, and blocked with 1% BSA for 30 minutes at RT. For paraffin embedded tissue, the sections were fixed in 4% paraformaldehyde prior to 1% BSA blocking as note above. αCD4, αCD8, αCD86 FITC conjugates and αCD45 and αB7-H1 primary antibodies were incubated 1 hour and 4°C. Cy3 conjugate antibody was used as secondary antibody in some cases. DAPI was used as the nuclear counterstain. Positive cells in 10 randomly selected fields at x40 magnification were quantitated. Quantitation of positive staining was blindly performed (LH) after the IHC slides were stained and marked randomly (JF). The microscope was Nikon, Eclipse E800. The camera was Nikon, DS-Qi1Mc. The software was NIS-Element AR 3.0.

[44] **Cytokines analysis:** Harvested DC was cultured with Golgistop™ (BD) protein transport monensin and LPS 0.1μg/ml 5hrs. DC was stained for anti-mouse CD11c, CD86, MHCII, IL-12, IFNα and TNFα expression, and harvested lymphocytes were stained for anti-mouse IL-2, IFNα, IFNγ, TNFα expression after membrane

permeabilization with Cytokit. Data were acquired by FACS analysis after rinsing out the antibodies.

EXAMPLE 2

Multiple TLR agonists enhanced GVAX (TEGVAX) increased both activated conventional and plasmacytoid dendritic cells in the draining lymph nodes in comparison to GVAX

[45] To further increase the anti-tumor response generated by GVAX, we combined GLA and R848 with GVAX, and tested whether this TEGVAX formulation would increase the number of activated dendritic cells in the DLN of non-tumor bearing mice. Compared to GVAX, TEGVAX was able to enhance the activation phenotype of dendritic cells from the DLN of the vaccine inoculation site (Figure 1). Both plasmacytoid DCs (pDC) and the conventional DCs (cDC) were analyzed, and both population showed enhanced expression of CD80 and CD86 activation markers for the TEGVAX treated group (Figure 1A-C). Gated DCs showed increased activation markers peaking on day 3 after adjuvant injection and this persisted until day 7 (Figure 1D). Lastly, to assess whether these activated DC provide the appropriate cytokine milieu for an anti-tumor response, cytokine profiles from CD11c+ cells from the draining lymph nodes also demonstrated increase amount of IL-12, IFNα, and TNFα that can skew the T-cell repertoire towards T_H1 response (Supplemental Figure 1).

EXAMPLE 3

TEGVAX treatment of established tumors significantly reduced tumor growth rate *in vivo*.

[46] We initially tested our TEGVAX with established B16 tumors in a therapeutic model. When the inoculated B16 tumor was palpable (typically on day 7-10), we treated the tumor bearing mice subcutaneously with TEGVAX, GVAX, GLA/R848 (IDC-1005), and vehicle controls in the contralateral limb. As shown in Figure 2A, mice that received TEGVAX displayed significantly lower tumor growth rate with only a single treatment. Both GVAX alone and TLR agonists alone had some modest benefit, but

the combination treatment produced the best *in vivo* anti-tumor response. When we compared TEGVAX with GVAX formulation with GLA alone or with R848 alone, we found that the combined GLA/R848 formulation into GVAX had the best anti-tumor response (Supplemental Figure 2A).

[47] To further improve the anti-tumor response, we performed the experiment with multiple TEGVAX treatments, and found that multiple TEGVAX treated mice did not display the exponential growth seen in untreated mice (Figure 2B). TEGVAX was injected every 7 days because of the decreased level of activated DC at this point (Figure 1D). These TEGVAX treated mice with smoldering tumors were inoculated with 10^5 B16 cells at a different site from the primary tumor site, and, in these mice, no tumor grew at the second site, demonstrating *in vivo* immunity against subsequent B16 challenges (data not shown). We also tested TEGVAX in the SCCFVII squamous cell carcinoma model in C3H mice as well as CT26 colon carcinoma model in Balb/c mice with comparable results, demonstrating that this anti-tumor response applies to multiple tumor histologies and is not dependent on murine background (Figure 2C)(Supplemental Figure 3). For the SCCFVII model, single TEGVAX treatment actually induced complete regression of the tumor. Once again, a re-challenge with SCCFVII tumor cells in these mice showed no evidence of tumor growth at the secondary site.

[48] The anti-tumor effect of TEGVAX was dependent on the formulation of the TEGVAX. When we treated the tumor bearing mice with GVAX and GLA/R848 without 1 hour of co-incubation prior to injection, no anti-tumor effect was noted (Fig. 2D). We believe that the co-incubation time can allow the hydrophobic TLR agonists to be absorbed into the GVAX with the squalene oil emulsion vehicle, and that this formulation is necessary for the anti-tumor response of TEGVAX. To test this, we absorbed GLA and R848 into GVAX with Lipofectamine and washed the cells to remove non-absorbed adjuvants/transfectant, and we found that TEGVAX generated from transfection method is comparable to the TEGVAX generated from prolonged incubation method with lipophilic vehicle in terms of their anti-tumor efficacy (Supplemental Figure 4).

EXAMPLE 4

TEGVAX increased lymphocytic infiltration in the tumor microenvironment, and this was associated with induction of tumor necrosis, locoregional T_H1 response, and increased B7-H1 expression on the tumor.

[49] We examined the tumor tissue harvested at the completion of our treatment assays, and examined the tumor microenvironment for lymphocytes. Histological analysis showed significantly increased regions of focal necrosis in those mice treated with TEGVAX in comparison to those treated with GVAX or controls (Figure 3A,B). Immunohistochemistry with CD4 and CD8 antibodies showed that TEGVAX treated tumor had abundant infiltrating CD4 and CD8 cells within the tumor tissue (Figure 3C,D). When we examined the tumor draining lymph nodes, we noted that TEGVAX treated mice had increased IFNγ+ CD4 and CD8 in comparison to GVAX or control treatments. To test whether these increased T_H1 cells is associated with the upregulation of B7-H1 on the tumor cells, we stained the tumor tissue and found that TEGVAX also also significantly increased the expression of B7-H1 in comparison to GVAX and vehicle treated mice (Figure 4C).

EXAMPLE 5

TEGVAX's anti-tumor response was dependent on both CD4 and CD8 cells, as well as MyD88/TRIF signaling.

[50] In order to assess whether T-cells were important for TEGVAX's anti-tumor response, we performed vaccine treatment assays with CD4 and CD8 depletions. TEGVAX treatment effects were abrogated upon depletion of either CD4 or CD8 T-cells, demonstrating the importance of both CD4 and CD8 lymphocytes for the anti-tumor immune response. Experiments using Rag2 KO mice confirmed these findings (Figure 5A, B, C).

[51] Since multiple TLR agonists were used as the formulation component for TEGVAX, we sought to determine whether TLR signaling was critical for the vaccine effect. TEGVAX treatments of B16 tumor-bearing mice were performed with MyD88/TRIF double knockout mice. Once again, the anti-tumor effect of TEGVAX was completely abrogated in these mice, confirming that TEGVAX's antitumor effect is dependent on TLR signaling (Figure 5D).

EXAMPLE 6

TEGVAX increases the number of tumor specific p15E specific cytotoxic T-cells

[52] With clear *in vivo* evidence of anti-tumor effect with TEGVAX that correlated with activated DC, we also examined the effector arm of immune system. Based on the expression of the immunodominant p15E antigen in B16 tumors, we performed *in vivo* CTL assay to quantitate the level of p15E specific IFNγ secreting CTLs (Figure 6). As shown in Figure 6, at day 21 when there were clear separations in the growth rates of TEGVAX treatment group to the other control groups (Figure 1), there was increased killing activity of p15E specific CTL in the spleen of the mice treated with TEGVAX in comparison to GVAX or other control mice (Figure 6A). ELISPOT assays were also performed from CD8 T-cells from each of the treated and control groups with T2kb cells pulsed with p15E peptide as the APC. These two independent assays demonstrated increased numbers and activity of tumor specific (anti-p15E) CTLs in the mice treated with TEGVAX (Figure 6B).

EXAMPLE 7

TEGVAX combined with anti-PD-1 antibody can induce regression of established B16 tumor

[53] Because of TEGVAX's ability to increase the number of p15E-specific cytotoxic T-cells as well as IFNγ producing CD8 cells, we combined TEGVAX with the blockade of immune checkpoint pathway B7-H1/PD-1. PD-1 is expressed on activated T-cells and the co-localization of its ligand B7-H1, which is upregulated with IFNγ secreting

T-cells, on the tumor cells is correlated with clinical efficacy of anti-PD-1 blockade. The implication of these findings that supported the adaptive immune resistance mechanism is that anti-PD-1 blockade is ideally suited to be combined with a vaccine that can generate a powerful T_H1 response. In our murine model, TEGVAX increased the both IFNγ secreting T-cells as wells as upregulated the expression of B7-H1 on the tumor cells (Figure 4). When the anti-PD-1 antibody treatment was combined with TEGVAX, established B16 tumors were noted to regress in 50% of the mice treated (Figure 7A). In these mice that responded with the combined treatment, vitiligo was noted as shown in Figure 7B. Mice treated with anti-PD-1 alone or with GVAX had no improvement in tumor growth rate over GVAX treatment alone. These *in vivo* tumor treatment assays correlated with synergistic effects of increasing T_H1 cytokines harvested from the tumor DLN from mice treated with TEGVAX and anti-PD-1 in comparison to the TEGVAX or anti-PD-1 alone. (Figure 7C).

EXAMPLE 8

Discussion

[54] In this report, we formulated GVAX with TLR4 (GLA) and TLR7/8 (R848) agonists and demonstrated significant augmentation of *in vivo* anti-tumor response in comparison to GVAX alone or TLR agonists alone in 3 different murine models. We showed that these adjuvants increased the number of activated plasmacytoid DC (pDC) as well as conventional DC (cDC). These *in vivo* anti-tumor responses were T-cell dependent, and tumor specific p15E specific T-cells in the B16 model were elevated in the TEGVAX treated group in comparison to GVAX treated group. For these experiments, we used a therapeutic model on established B16 tumors, a poorly immunogenic tumor that cannot be treated with known formulations of vaccines once they become palpable. With repeated TEGVAX treatments, the tumor tend to display a "smoldering" growth rate as shown in Figure 2. For SCCFVII model, only a single treatment with TEGVAX induced regression of the palpable tumor. For CT26 colon carcinoma model, TEGVAX treatment on palpable lesions also demonstrated *in vivo* anti-tumor effects (Suppl Fig 3). TEGVAX treatment was associated with increased IFNγ+ CD4 and CD8 cells in the tumor draining lymph node as well as increased expression of B7-H1 on the tumor cells. When TEGVAX was combined with anti-

PD-1 blockade, we observed regression of established B16 tumors as shown in Figure 7.

[55] GLA and R848 were initially selected because these adjuvants significantly induced enhanced anti-tumor cytokine profiles compared to other TLR agonists, and both have been tested in patients to be safe. GLA, a TLR4 agonist, was compared to monophosphoryl lipid A (MPL) in it ability to activate murine and human dendritic cells, and GLA was found to be more potent in terms of increasing multiple T_H1 type cytokines both *in vivo* and *in vitro*. R848, a TLR7/8 agonist, was also found have significant improvements in increasing type I IFN profile over imiquimod. Our *in vivo* results on established tumors, therefore, demonstrated the high translational potential of TEGVAX as a cancer vaccine. Interestingly, when we examined the tumor microenvironment of the treated mice, we noted increased IFNγ level from CD4 and CD8 cells from tumor draining lymph nodes as well as increased B7-H1 expression from the tumor in TEGVAX treated mice in comparison to GVAX (Figure 4).

[56] Our preclinical studies, therefore, was an excellent venue to test the combinatorial therapy of vaccine with anti-PD-1 blockade. The importance of B7-H1 induction on tumor cells and its engagement with PD-1 on lymphocytes as a critical immune evasion mechanism was demonstrated in preclinical models, and B7-H1 expression was clinically correlated with worse prognosis in multiple cancer types. For patients with advanced melanoma, lung cancer, and renal carcinoma, monotherapy with blocking anti-PD-1 antibody resulted in significant survival benefit, and concurrent histological analysis demonstrated strong correlation between B7-H1 and IFNγ expression and response to PD-1 blockadeIn epithelial tissues as well as B16-F10 tumor cells, IFNγ can induce B7-H1 expression. Cumulatively, these findings support the adaptive resistance mechanism whereby anti-PD-1 blockade can unleash its anti-tumor efficacy of resident tumor specific cytotoxic T-cells that induce its own suppression by inducing B7-H1 on the tumor partly via IFNγ. Anti-PD-1 blockade is therefore ideally suited for combinatorial therapy with IFNγ inducing vaccine. The regression of established B16 with TEGVAX/anti-PD-1 treatment shown in Figure 7 provide *in vivo* preclinical justification for clinical trial combining anti-PD-1 therapy with an appropriate vaccine.

[57] Previous reports showed that the combination of anti-PD-1, anti-CTLA-4, and vaccine may promote anti-tumor responses, but their *in vivo* assay involved initiating treatment on non-palpable, non-established B16 tumor. Our treatment assay was much more stringent in that we initiated treatment 7-10 days after B16 inoculation, at which point most immunotherapy fail. Moreover, we did not need to combine anti-PD-1 with other immune checkpoint blockade antibodies to demonstrate regression of established tumor.

[58] The synergistic effect of combining TEGVAX and anti-PD-1 was corroborated when we examined the draining lymph nodes from the tumor. Tumor infiltrating lymphocyte (TIL) collection was not feasible because of the regressed tumor or the small tumor size to sample sufficient cells for flow cytometry. However, both CD4 and CD8 lymphocytes and CD11c+ dendrititic cells from the popliteal lymph nodes displayed several fold increase in IL-2, IL-12, and IFNγ expression level in comparison to the modest elevation in the TEGVAX or anti-PD-1 treated groups alone (Figure 7C). These T_H1 cytokines correlated with the increased number of p15E specific CTL in the TEGVAX/anti-PD-1 treated group in comparison to the TEGVAX and the PD-1 treated groups (data not shown).

[59] From a translational standpoint, ipilimumab is associated with grade 3-4 toxicity and isolated reports of therapy related mortality, so the addition of anti-CTLA-4 antibody may not be the first option to improve upon our *in vivo* results. However, because of significant redundancy in the many families of immune checkpoint molecules as well as the availability of clinical grade checkpoint blocking antibodies, there are potentials to add other immune checkpoint blocking antibodies to anti-PD-1 treatment. Because of the likelihood that increased IFNγ can potentially upregulate B7-H1 in the tumor microenvironment, an alternative approach would be to increase the level of tumor specific CTLs using a safe vaccine that can be combined with anti-PD-1. For the purpose of this study, we chose two TLR agonists with TLR4 and TLR7/8 activity, but it is unclear whether other TLR agonists (flagellin, CpG, etc) or other PAMP molecules can further augment the anti-tumor response. Lastly, it should be reiterated that all the components of TEGVAX have been tested in patients to be safe. With its demonstration as a potent anti-tumor agent by itself, TEGVAX in

combination with anti-PD-1 antibody has high translational potential for clinical trials.

[60] We previously demonstrated an *in vivo* response with intratumoral injection using GVAX formulated with LPS, but now we report a much more potent response with GVAX formulated with both TLR4 and TLR7/8 agonist as a therapeutic. The mechanisms that underlie the intratumoral injection in comparison to the systemic injection in these treatment models are different, but cumulatively, these experiments demonstrate that TLR agonists formulated with a cellular vaccine injected intratumorally or systemically will unlikely have a pro-carcinogenic effect. MyD88-NF-κB signaling pathways in tumor cells have been shown to have pro-carcinogenic consequences. However, TLR agonists have been clearly shown to activate MyD88-TRIF pathways in dendritic cells to prime the adaptive immune system for anti-tumor responses. In this report, the TLR agonists formulated into GVAX can stimulate the DC in the draining lymph nodes, and any untoward pro-carcinogenic effects could not be demonstrated in our systemic treatment models.

[61] GVAX, and in some models, the TLR agonists themselves, has an early modest effect in the tumor growth rate, but there is clear separation between the TEGVAX group and control groups in all the murine models studied. We also compared GLA/GVAX and R848/GVAX against TEGVAX, and it is clearly TEGVAX that combines both TLR4 and TLR7/8 activities that has the best *in vivo* anti-tumor response (Supplemental Figure 2). For B16 experiments, viable B16 tumor cells were injected subcutaneously distant from the site of initial tumor inoculation or vaccine inoculation, and no tumor growth were noted. Similarly for SCCFVII model, a rechallenged experiment showed no outgrowth of tumor after only a single TEGVAX treatment (Figure 2C).

[62] We feel that it is the combination of both TLR4 and TLR7/8 stimulation that can increase both cDC as well as pDC to induce robust priming of T-cells into anti-tumor T-cells that is responsible for the *in vivo* anti-tumor effect of TEGVAX. TEGVAX treatment assay was performed with MyD88-TRIF double knockout mice, and the anti-tumor effect was completely abrogated (Figure 4C). MyD88 is the critical

mediator for TLR7/8 signaling, and both TRIF and MyD88 is the critical downstream mediator of TLR4 signaling. When we examined the draining lymph node CD11c+ cells, we noted increase T_H1 cytokines expression for TEGVAX treated group in comparison to controls (Figure 5). Dot plot of CD11c and these T_H1 cytokines were informative in that the increase in the double positives are derived predominantly from the shift in the CD11c+ population, suggesting that the addition of TLR agonists primarily induced increased trafficking of APCs to the lymph nodes rather than increase the production of inflammatory cytokines on a per cell basis. To this end, we labeled TEGVAX with Q-dot, and quantitated the endogenous APC's that was labeled via cell-to-cell transfer with Q-dot in the draining lymph node (Supplemental Figure 5). For both pDC and cDC population, there were quantitatively more activated DCs that were labeled with Q-dot, consistent with our hypothesis that the TLR adjuvants increase the number of activated DC that circulate between the tumor and the draining lymph nodes where they can prime the T-cells.

[63] Our previous work with LPS absorbed GVAX demonstrated that the absorption of TLR4 agonist into a cellular vaccine was critical for *in vivo* anti-tumor efficacy. We surmised that the absorption of GLA and R848 into GVAX is also critical for the enhanced anti-tumor response observed with TEGVAX. When we simply co-inject the GLA and R848 adjuvants with GVAX without any incubation, there was no *in vivo* anti-tumor response (Figure 2D). Only when the TLR agonists and GVAX are incubated with lipophilic vehicles for an hour did we obtain a TEGVAX formulation that was able to induce a partial response for the B16 model as well as induce regression for the SCCFVII model. To further test this, we formulated GVAX with GLA and R848 using Lipofectamine without squalene oil-in-water emulsion, and demonstrated comparable *in vivo* anti-tumor response in the B16 model (Supplemental Figure 4). One aspect of the future work, therefore, involves further optimization of TEGVAX formulation.

[64] Cumulatively, this report demonstrates that TEGVAX offers a significant improvement over GVAX as a therapeutic vaccine that can significantly augment the priming of tumor specific T-cells, even for established tumors, and it is an excellent

candidate to be added in a combinatorial therapy with anti-PD-1 blocking antibody or other forms of immune checkpoint blockade in cancer patients.

CLAIMS

1. A composition comprising:

a cytokine-expressing, proliferation incompetent, whole cancer cells;

an anti-PD-1 antibody that specifically binds to human Programmed Death 1 (PD-1); and

a TLR (toll like receptor) agonist;

wherein the whole cancer cells are formulated with the TLR agonist.

2. The composition of claim 1 wherein the cytokine is GM-CSF (granulocyte moncyte cell stimulating factor).
3. The composition of claim 1 wherein the whole cancer cells are autologous to the patient.
4. The composition of claim 1 wherein the whole cancer cells are formulated with a TLR7/8 agonist.
5. The composition of claim 1 wherein the whole cancer cells are formulated with a TLR4 agonist.
6. The composition of claim 1 wherein the whole cancer cells are formulated with a TLR4 and a TLR7/8 agonist.
7. The composition of claim 1 wherein the TLR agonist is selected from the group consisting of GLA, R848, and a combination thereof.
8. The composition of claim 1 wherein the cancer cells are melanoma cells.
9. The composition of claim 1 wherein the TLR agonist and whole tumor cells are formulated with an emulsion vehicle.
10. The composition of claim 1 wherein the TLR agonist and whole tumor cells are formulated with Lipofectamine™.
11. The composition of claim 1 wherein the TLR agonist and whole tumor cells are formulated with a cationic lipid.
12. A method comprising:

administering to a cancer patient immunotherapeutic agents:

cytokine-expressing, proliferation incompetent, whole cancer cells;

an anti-PD-1 antibody that specifically binds to human Programmed Death 1 (PD-1); and

a TLR (toll like receptor) agonist;

wherein the whole cancer cells are formulated with the TLR agonist.

13. The method of claim 12 wherein the cytokine is GM-CSF (granulocyte moncyte cell stimulating factor).
14. The method of claim 12 wherein the whole cancer cells are autologous to the patient.
15. The method of claim 12 wherein the whole cancer cells are formulated with a TLR7/8 agonist.
16. The method of claim 12 wherein the whole cancer cells are formulated with a TLR4 agonist.
17. The method of claim 12 wherein the whole cancer cells are formulated with a TLR4 and a TLR7/8 agonist.
18. The method of claim 12 wherein the TLR agonist is selected from the group consisting of GLA, R848, and a combination thereof.
19. The method of claim 12 wherein the whole cancer cells are melanoma cells.
20. The method of claim 12 wherein the TLR agonist and whole tumor cells are formulated with an emulsion vehicle.
21. The method of claim 12 wherein the TLR agonist and whole tumor cells are formulated with Lipofectamine™.
22. The method of claim 12 wherein the TLR agonist and whole tumor cells are formulated with a cationic lipid.
23. A kit comprising agents:

    cytokine-expressing, proliferation incompetent, whole cancer cells;

    an anti-PD-1 antibody that specifically binds to human Programmed Death 1 (PD-1); and

    a TLR (toll like receptor) agonist.
24. The kit of claim 23 further comprising instructions for administering and/or formulating the agents.
25. The kit of claim 23 further comprising an emulsion vehicle.
26. The kit of claim 23 further comprising Lipofectamine™.
27. The kit of claim 23 further comprising a cationic lipid.

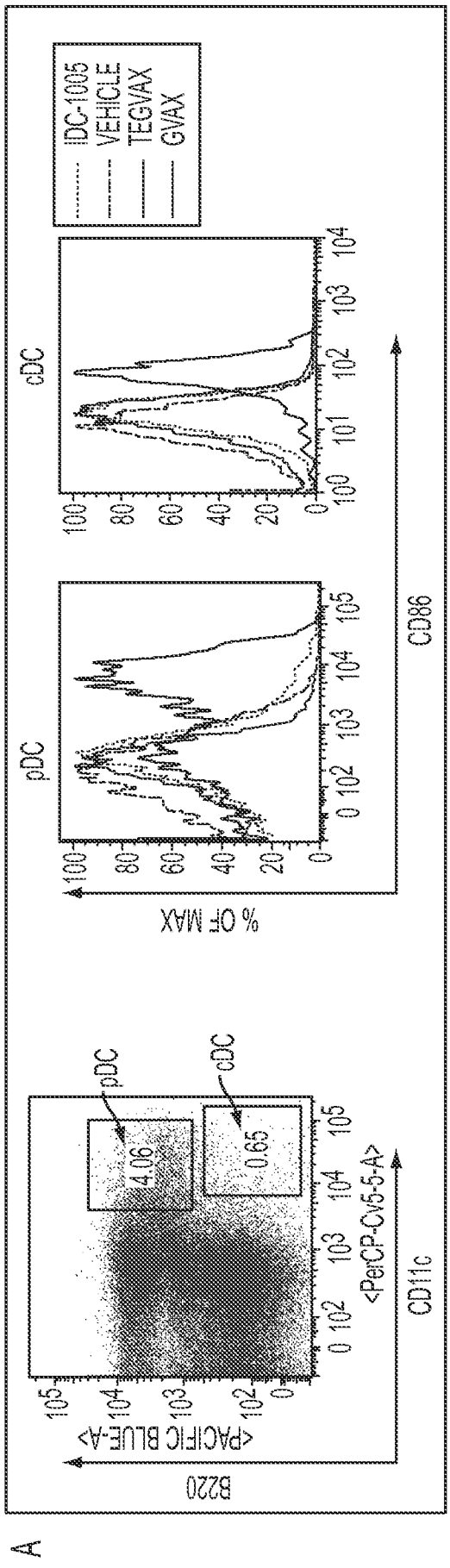

A
B220
<PACIFIC BLUE-A>
CD11c
<PerCP-Cy5-5-A>
pDC
cDC
4.06
0.65
% OF MAX
CD86
IDC-1005
VEHICLE
TEGVAX
GVAX

FIG. 1

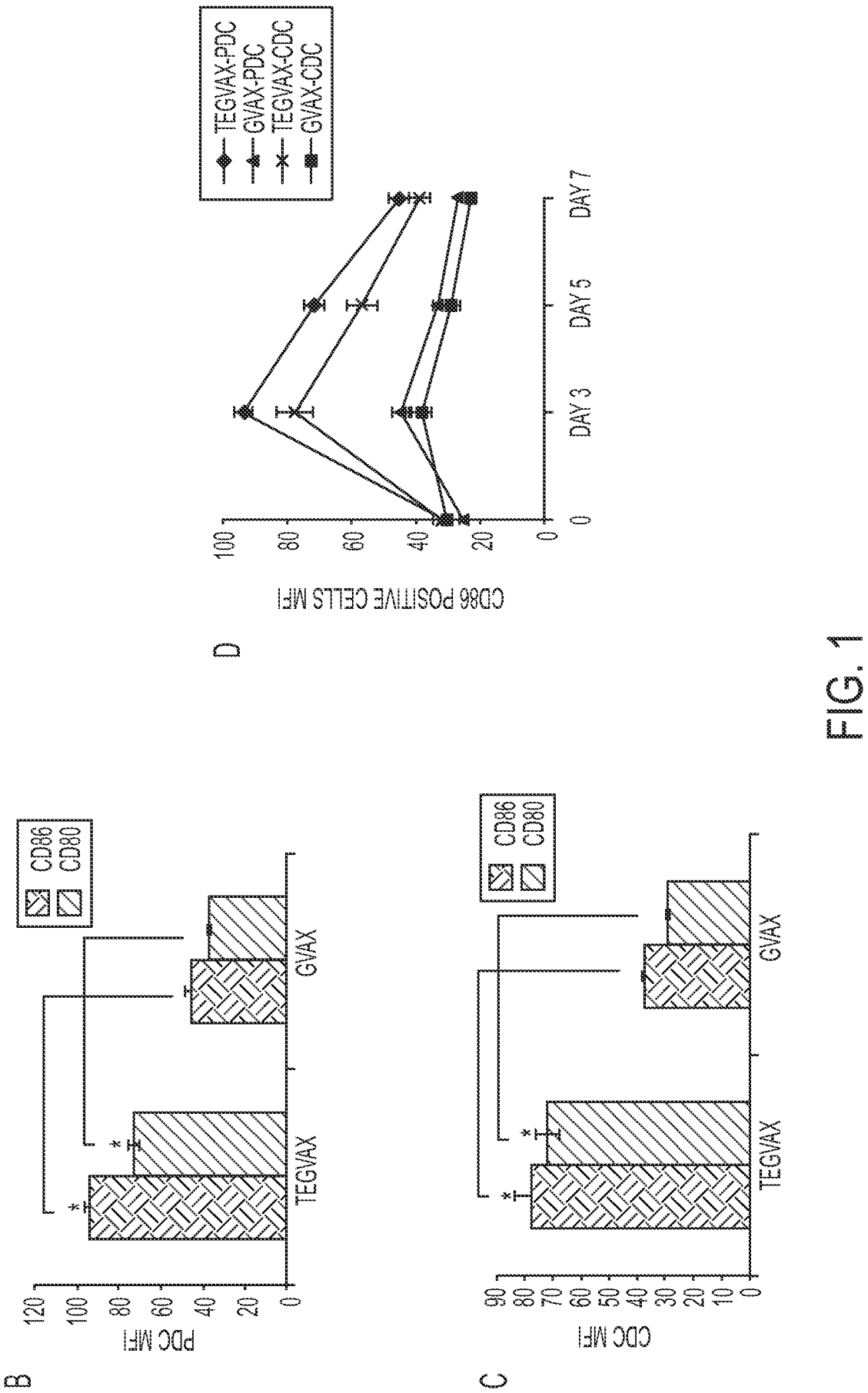

D
CD86 POSITIVE CELLS MFI
100
80
60
40
20
0
DAY 3
DAY 5
DAY 7
TEGVAX-PDC
GVAX-PDC
TEGVAX-CDC
GVAX-CDC
B
PDC MFI
120
100
80
60
40
20
0
CD86
CD80
TEGVAX
GVAX
C
CDC MFI
90
80
70
60
50
40
30
20
10
0
CD86
CD80
TEGVAX
GVAX

FIG. 1

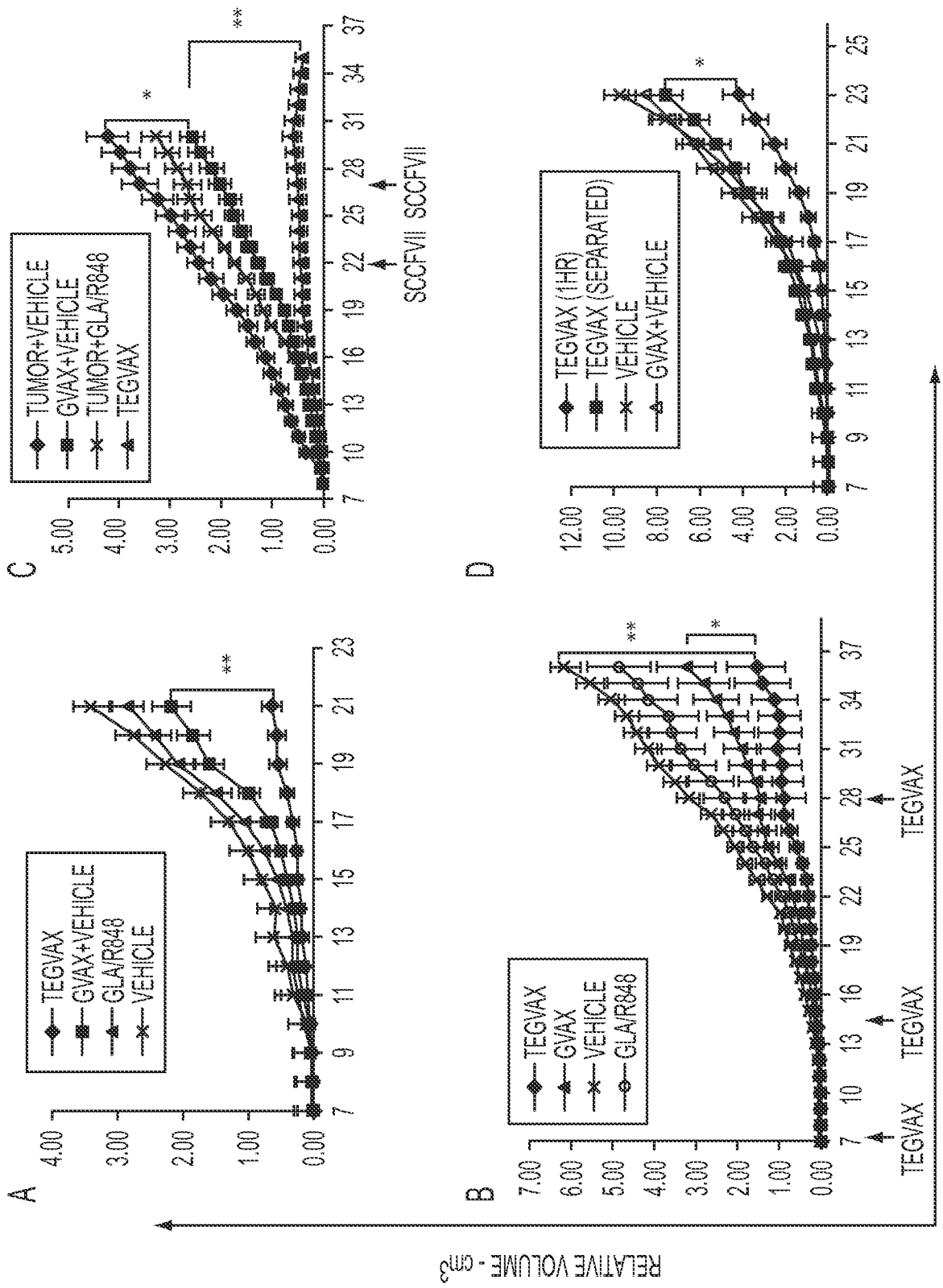

A
TEGVAX
GVAX+VEHICLE
GLA/R848
VEHICLE
**
B
TEGVAX
GVAX
VEHICLE
GLA/R848
**
*
TEGVAX
TEGVAX
TEGVAX
C
TUMOR+VEHICLE
GVAX+VEHICLE
TUMOR+GLA/R848
TEGVAX
*
**
SCCFVII
SCCFVII
D
TEGVAX (1HR)
TEGVAX (SEPARATED)
VEHICLE
GVAX+VEHICLE
*
RELATIVE VOLUME - cm3
DAYS

FIG. 2

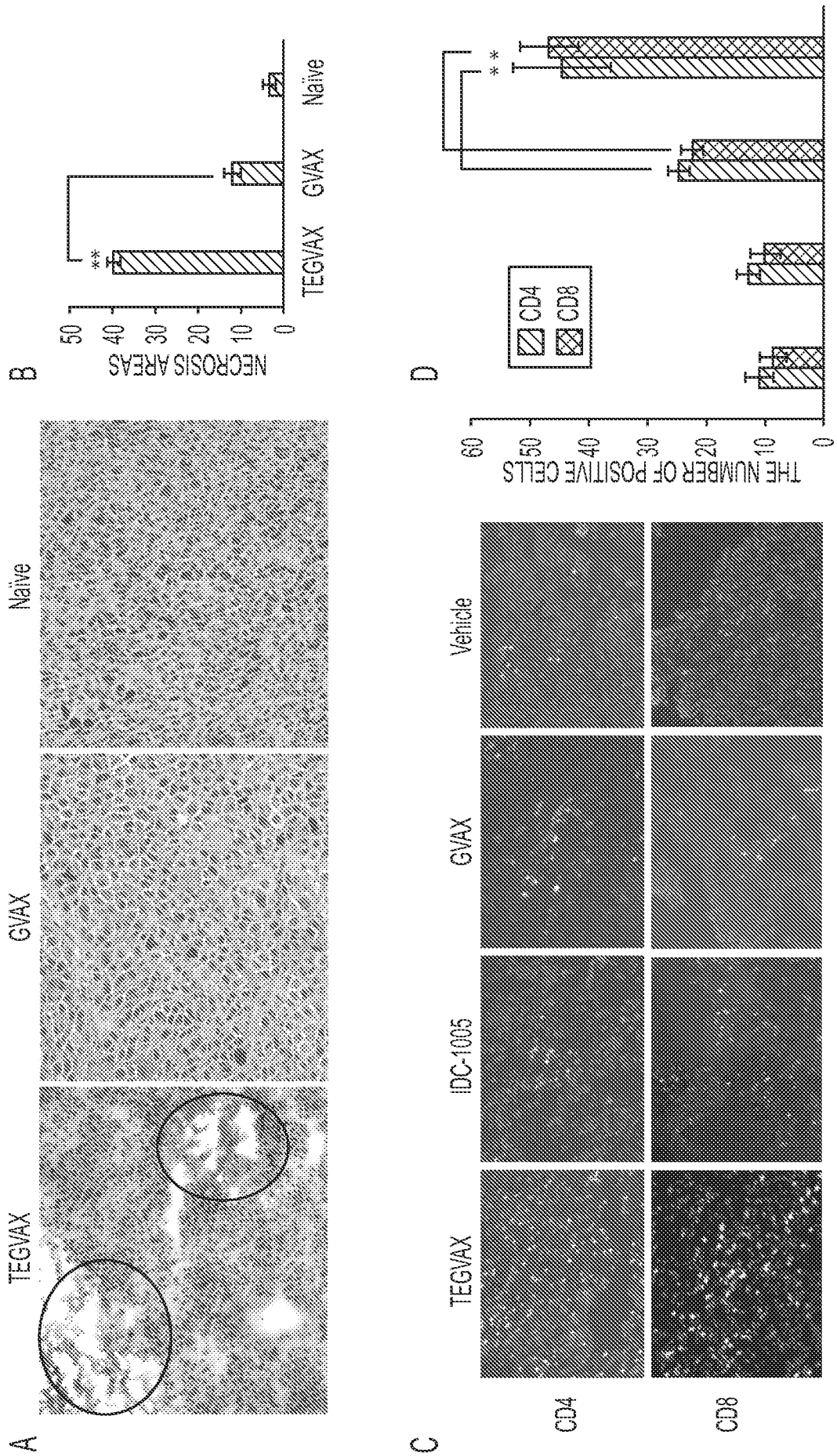

A
TEGVAX
GVAX
Naive
B
NECROSIS AREAS
50
40
30
20
10
0
**
TEGVAX
GVAX
Naive
C
TEGVAX
IDC-1005
GVAX
Vehicle
CD4
CD8
D
THE NUMBER OF POSITIVE CELLS
60
50
40
30
20
10
0
CD4
CD8
**
UNTREATED IDC-1005
GVAX
TEGVAX

FIG. 3

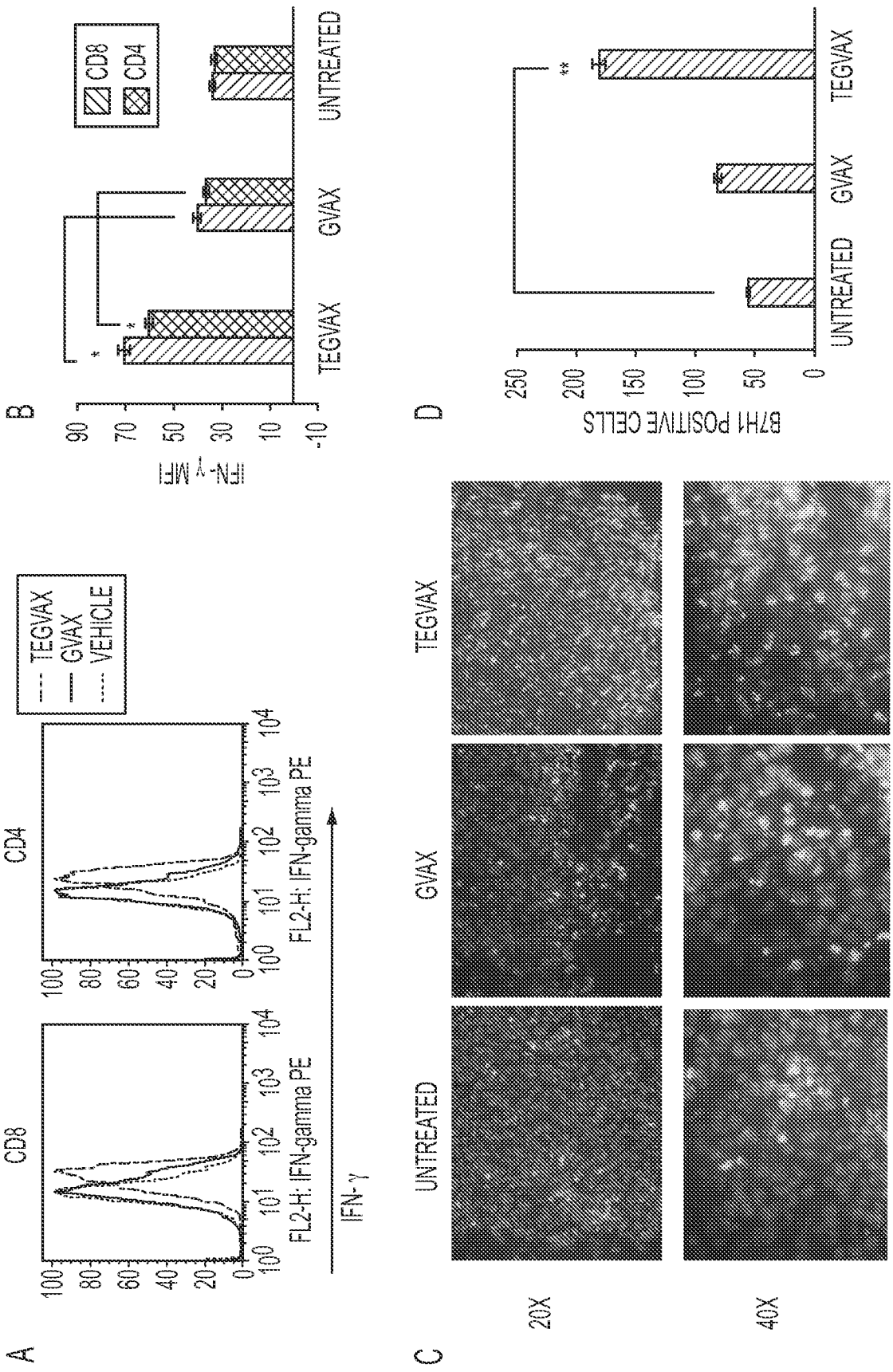

A
CD8
CD4
TEGVAX
GVAX
VEHICLE
FL2-H: IFN-gamma PE
IFN-γ
B
IFN-γ MFI
CD8
CD4
TEGVAX
GVAX
UNTREATED
C
UNTREATED
GVAX
TEGVAX
20X
40X
D
B7H1 POSITIVE CELLS
UNTREATED
GVAX
TEGVAX

FIG. 4

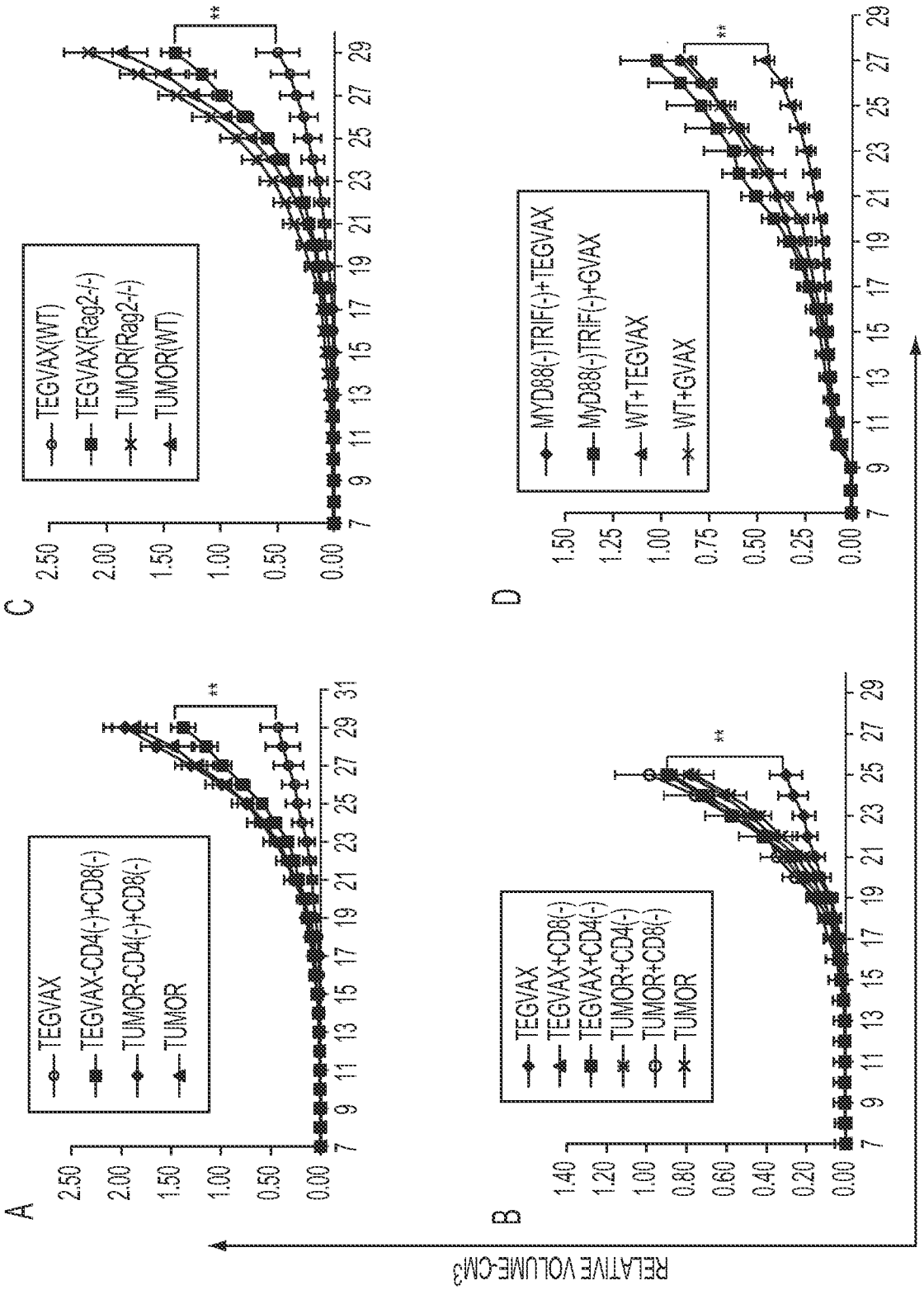

A
TEGVAX
TEGVAX-CD4(-)+CD8(-)
TUMOR-CD4(-)+CD8(-)
TUMOR
B
TEGVAX
TEGVAX+CD8(-)
TEGVAX+CD4(-)
TUMOR+CD4(-)
TUMOR+CD8(-)
TUMOR
C
TEGVAX(WT)
TEGVAX(Rag2-/-)
TUMOR(Rag2-/-)
TUMOR(WT)
D
MYD88(-)TRIF(-)+TEGVAX
MyD88(-)TRIF(-)+GVAX
WT+TEGVAX
WT+GVAX
RELATIVE VOLUME-CM3
DAYS

FIG. 5

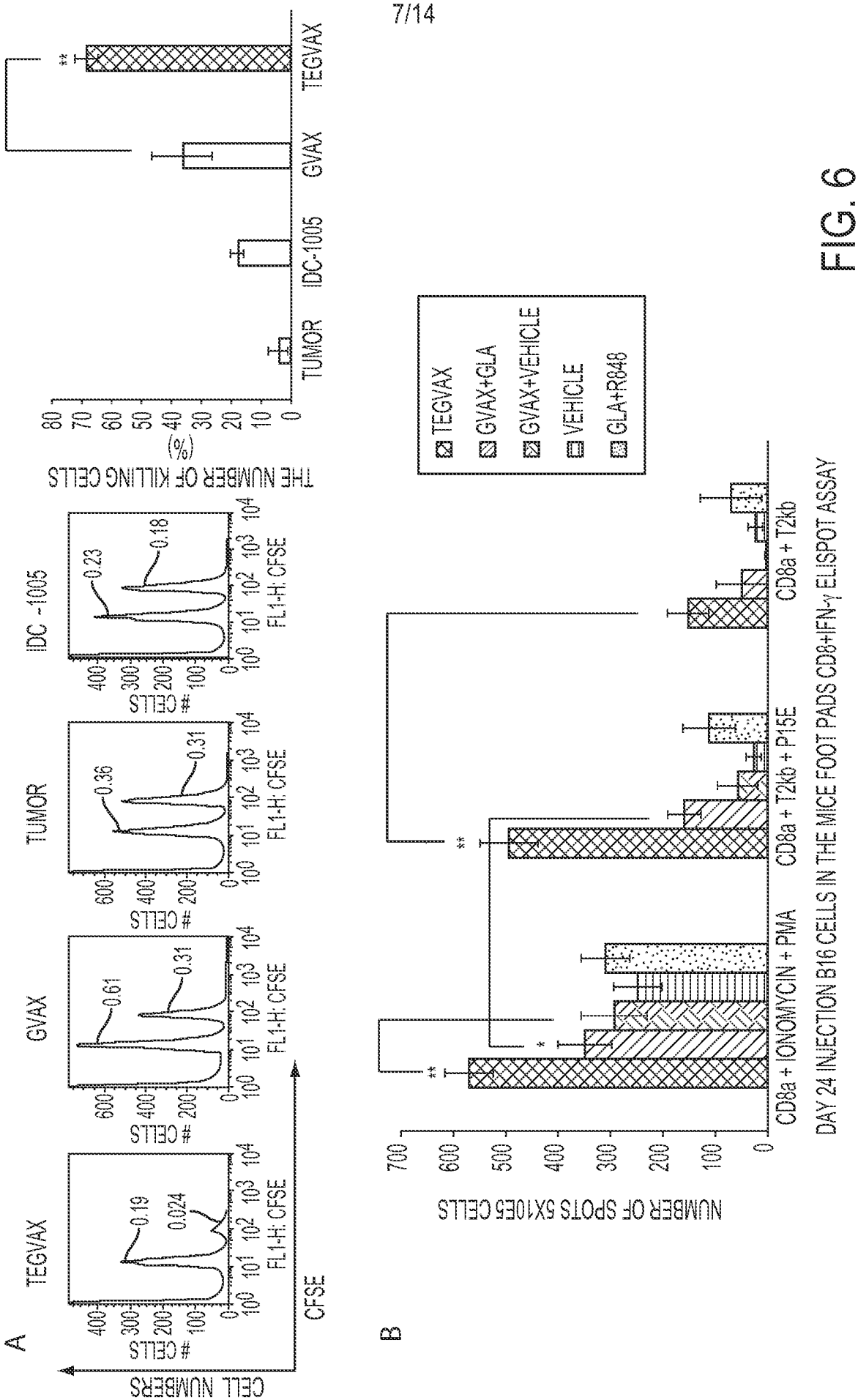
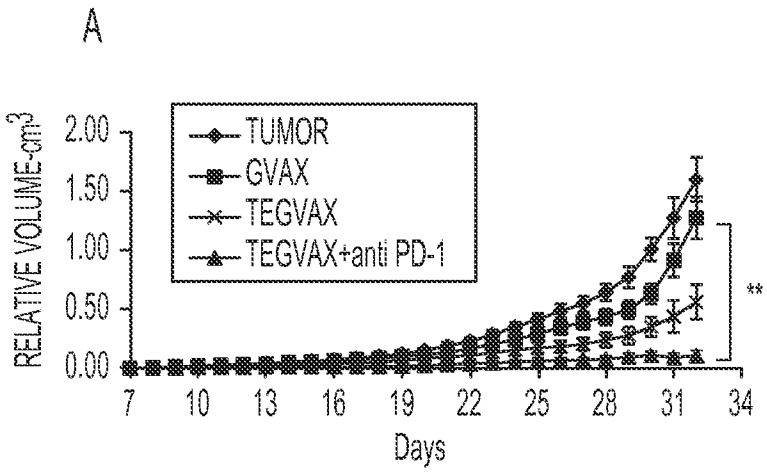

A
TEGVAX
GVAX
TUMOR
IDC -1005
CELL NUMBERS
CELLS
FL1-H: CFSE
CFSE
0.19
0.024
0.61
0.31
0.36
0.31
0.23
0.18
THE NUMBER OF KILLING CELLS (%)
TUMOR
IDC-1005
GVAX
TEGVAX
B
TEGVAX
GVAX+GLA
GVAX+VEHICLE
VEHICLE
GLA+R848
NUMBER OF SPOTS 5X10E5 CELLS
CD8a + IONOMYCIN + PMA
CD8a + T2kb + P15E
CD8a + T2kb
DAY 24 INJECTION B16 CELLS IN THE MICE FOOT PADS CD8+IFN-γ ELISPOT ASSAY

FIG. 6


A
RELATIVE VOLUME-cm3
2.00
1.50
1.00
0.50
0.00
TUMOR
GVAX
TEGVAX
TEGVAX+anti PD-1
**
7
10
13
16
19
22
25
28
31
34
Days

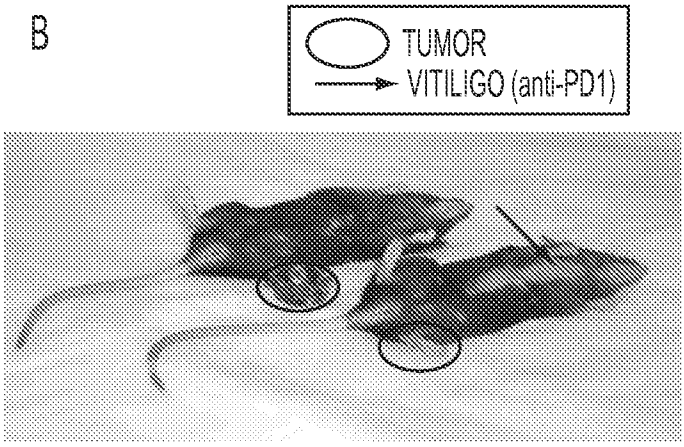

B
TUMOR
VITILIGO (anti-PD1)

FIG. 7

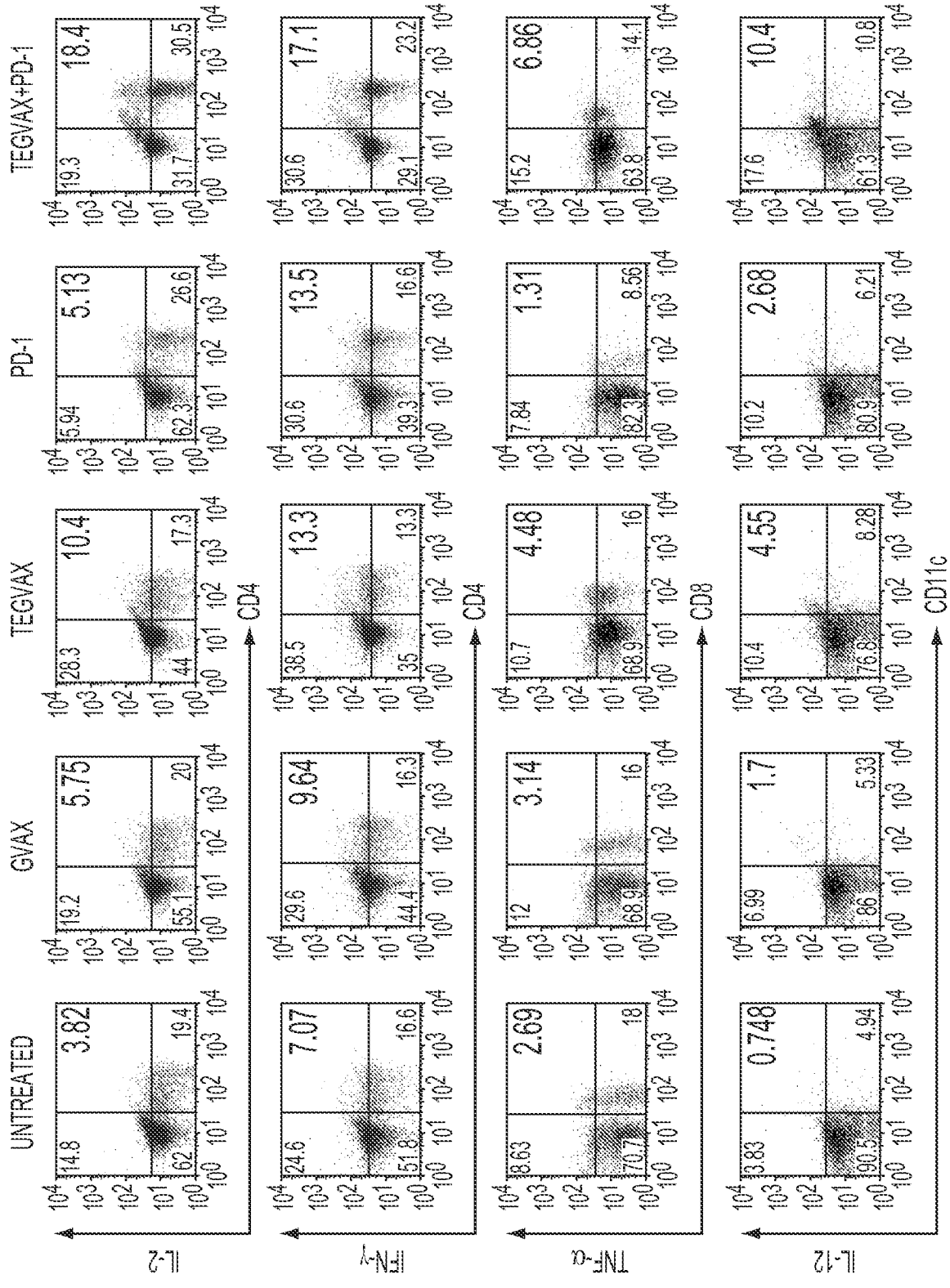

C
UNTREATED
GVAX
TEGVAX
PD-1
TEGVAX+PD-1
IL-2
IFN-γ
TNF-α
IL-12
CD4
CD4
CD8
CD11c
14.8
3.82
19.2
5.75
28.3
10.4
5.94
5.13
19.3
18.4
62
19.4
55.1
20
44
17.3
62.3
26.6
31.7
30.5
24.6
7.07
29.6
9.64
38.5
13.3
30.6
13.5
30.6
17.1
51.8
16.6
44.4
16.3
35
13.3
39.3
16.6
29.1
23.2
8.63
2.69
12
3.14
10.7
4.48
7.84
1.31
15.2
6.86
70.7
18
68.9
16
68.9
16
82.3
8.56
63.8
14.1
3.83
0.748
6.99
1.7
10.4
4.55
10.2
2.68
17.6
10.4
90.5
4.94
86
5.33
76.8
8.28
80.9
6.21
61.3
10.8

FIG. 7

(SUPPLEMENTAL FIGURE 1)

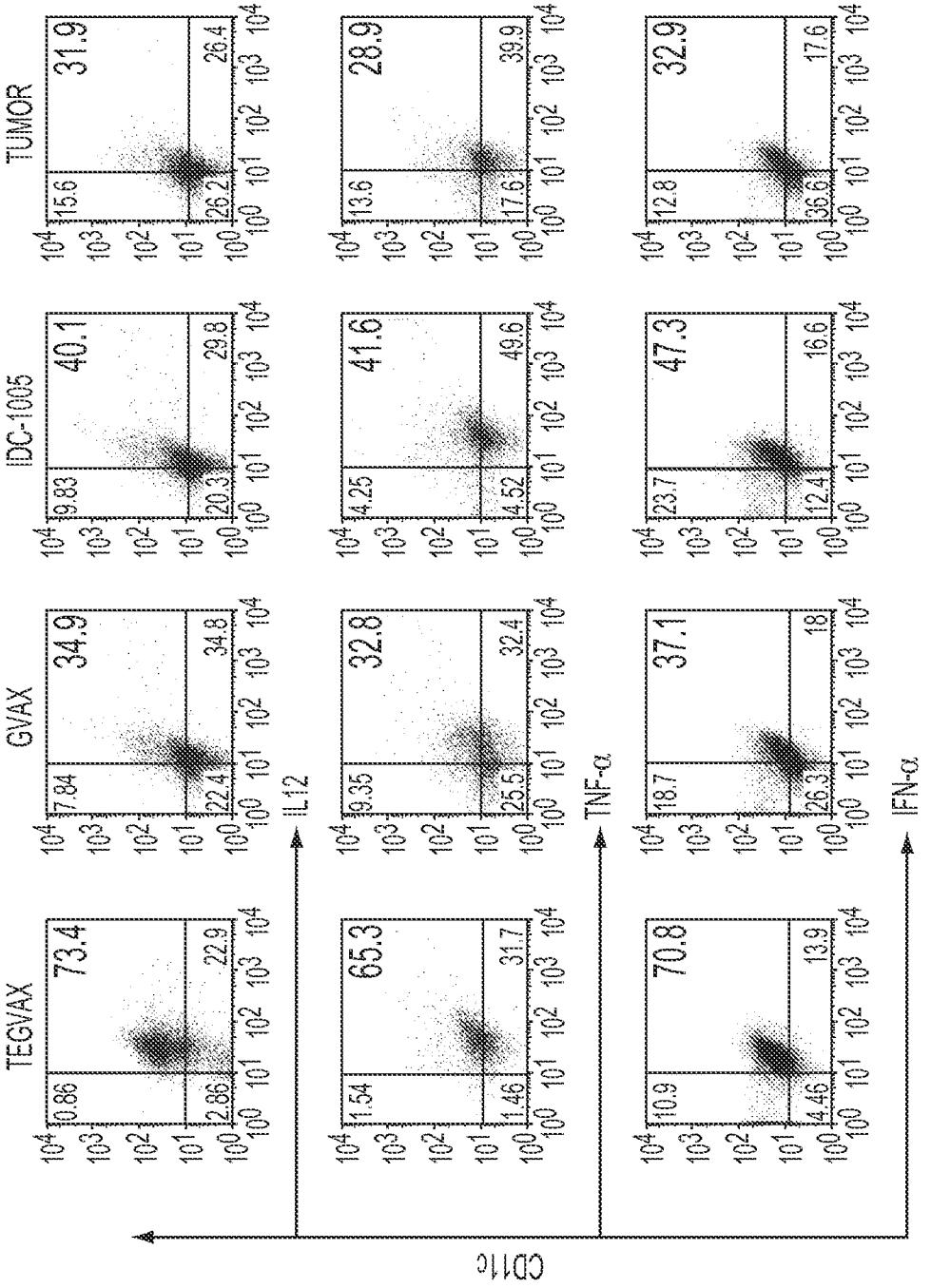


FIG. 8

(SUPPLEMENTAL FIGURE 2)

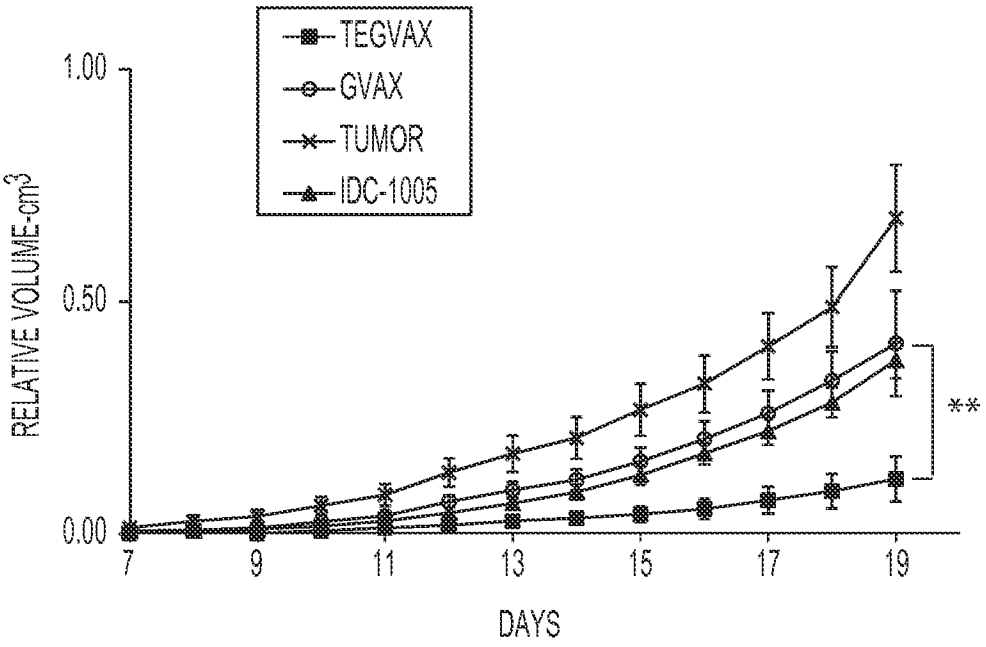


FIG. 9

(SUPPLEMENTAL FIGURE 3)

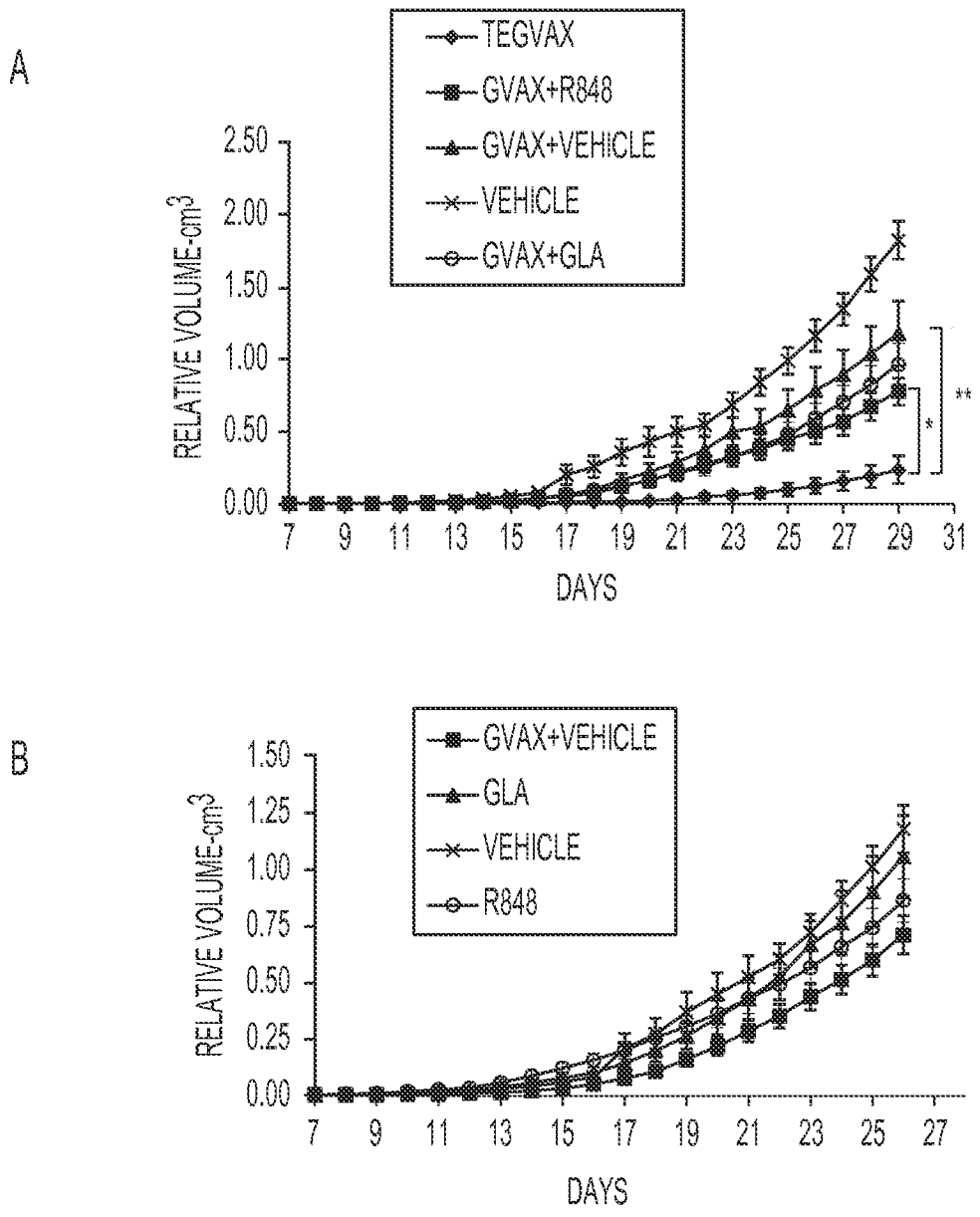


FIG. 10

(SUPPLEMENTAL FIGURE 4)

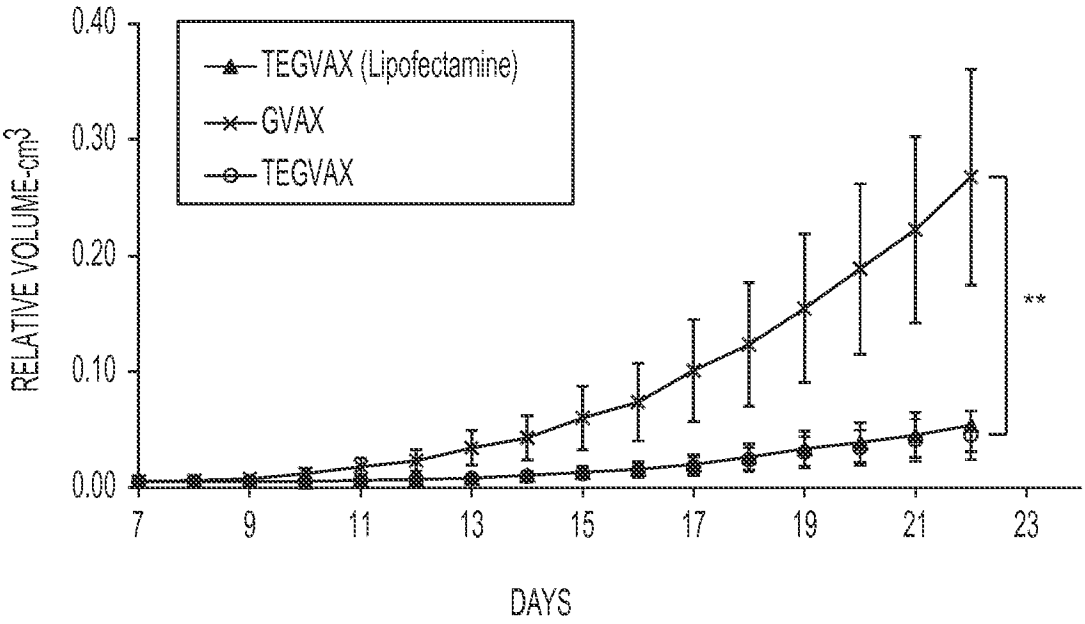


FIG. 11

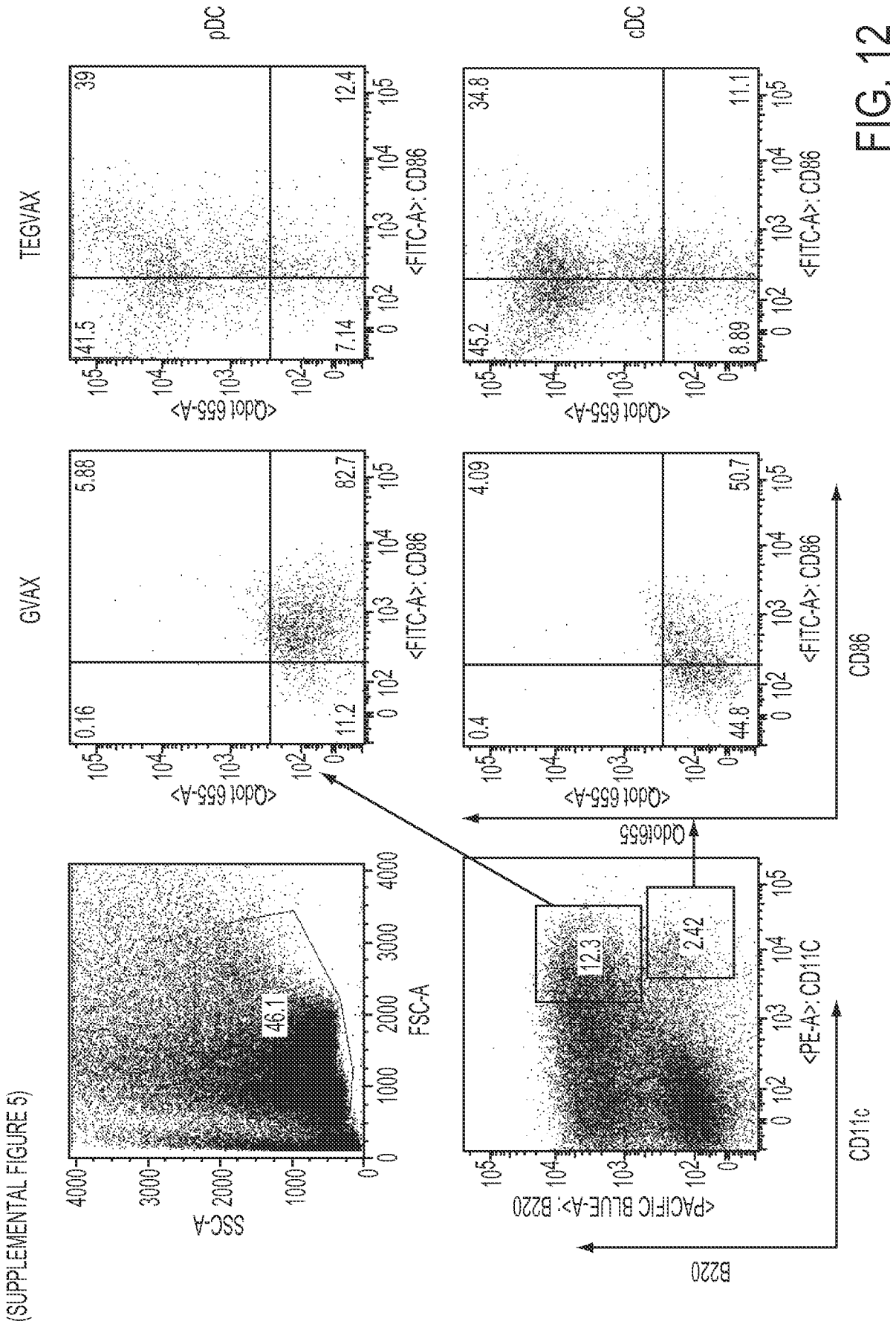

(SUPPLEMENTAL FIGURE 5)
SSC-A
FSC-A
46.1
B220
CD11c
<PACIFIC BLUE-A>: B220
<PE-A>: CD11C
12.3
2.42
QdotI655
CD86
<FITC-A>: CD86
<QdotI 655-A>
GVAX
TEGVAX
pDC
cDC
0.16
11.2
5.88
82.7
0.4
44.8
4.09
50.7
41.5
7.14
39
12.4
45.2
8.89
34.8
11.1

FIG. 12

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2012/055992

A. CLASSIFICATION OF SUBJECT MATTER

A61K 39/395(2006.01)i, A61K 38/19(2006.01)i, A61K 38/18(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 39/395; A61K 39/39; A61P 35/00; A61K 39/00; A61P 37/04; A61K 38/20; C07K 14/535

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

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

eKOMPASS(KIPO internal) & Keywords: cancer, anti-PD-1 antibody, TLR agonist, GM-CSF, GVAX, TEGVAX

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DAVIS et al. "Intratumoral administration of TLR4 agonist absorbed into a cellular vector improves anti-tumor responses" Clin. Cancer Res., 15 June 2011, Vol. 17, No. 12, pp. 3984-3992. See abstract; p. 3985; and results.	1-11,23-27
Y	US 2010-0086550 A1 (KANG et al.) 08 April 2010 See abstract and claim 1.	1-11,23-27
A	US 2008-0145375 A1 (BEMBRIDGE et al.) 19 June 2008 See abstract; and claims 1 and 49.	1-11,23-27
A	CHIANG et al. "Whole tumor antigen vaccines" Seminars in Immunology, 2010, Vol. 22, No. 3, pp. 132-143. See abstract; and p. 134 - p. 135, left-column.	1-11,23-27
A	US 2009-0081157 A1 (KORNBLUTH et al.) 26 March 2009 See abstract and claim 1.	1-11,23-27
A	WO 2010-024897 A2 (CELL GENESYS, INC. et al.) 04 March 2010 See abstract; and claims 1, 2, 14-20, and 51.	1-11,23-27

☐ 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	Date of mailing of the international search report
25 FEBRUARY 2013 (25.02.2013)	**25 FEBRUARY 2013 (25.02.2013)**
Name and mailing address of the ISA/KR Facsimile No. 82-42-472-7140	Authorized officer CHOI, Sung Hee Telephone No. 82-42-481-8740

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2012/055992

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.: 12-22
because they relate to subject matter not required to be searched by this Authority, namely:
Claims 12-22 are directed to a treatment method 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.:
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:

3. ☐ Claims Nos.:
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 addtional 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.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/US2012/055992

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2010-0086550 A1	08.04.2010	EP 2172219 A1	07.04.2010
		JP 2010-083863 A	15.04.2010
		JP 5054057 B2	24.10.2012
		KR 10-1050829 B1	20.07.2011
		KR 10-2010-0037899 A	12.04.2010
		US 2012-0237522 A1	20.09.2012
US 2008-0145375 A1	19.06.2008	AU 2004-271726 A1	24.03.2005
		BR PI0414381 A	21.11.2006
		CA 2538197 A1	24.03.2005
		CN 1878567 A	13.12.2006
		EP 1682175 A2	26.07.2006
		GB 0321615 D0	15.10.2003
		IL 174131 D0	01.08.2006
		IS 8363 A	20.03.2006
		JP 2007-505827 A	15.03.2007
		KR 10-2007-0029111 A	13.03.2007
		MA 28323 A1	01.12.2006
		MX PA06002969 A	26.03.2007
		NO 20061242 A	01.06.2006
		NZ 545948 A	31.07.2009
		RU 2006106848 A	27.10.2007
		RU 2370537 C2	20.10.2009
		SG 145767 A1	29.09.2008
		WO 2005-025614 A2	24.03.2005
		ZA 200602156 A	25.07.2007
US 2009-0081157 A1	26.03.2009	AU 2007-239095 A1	25.10.2007
		CA 2636424 A1	25.10.2007
		EP 1979488 A2	15.10.2008
		JP 2009-522372 A	11.06.2009
		WO 2007-120368 A2	25.10.2007
WO 2010-024897 A2	04.03.2010	CA 2735502 A1	04.03.2010
		EP 2328921 A2	08.06.2011
		US 2010-0150946 A1	17.06.2010