Title: SECONDARY SITE OF ANTIGEN STIMULATION FOR THERAPEUTIC VACCINATION

Abstract: A method for eliciting and sustaining an anti-tumor immune response is carried out by administering a device containing an amount of antigen sufficient to stimulate an innate immune response and an adaptive immune response. A method for eliciting and sustaining an anti-tumor immune response is also carried out by administering a plurality of devices, e.g., a first antigen-loaded acellular biomaterial device (PLG vaccine) at a first time point to stimulate an innate immune response and an adaptive immune response and administering a second device to a second anatomical site at a second time point to prolong the adaptive immune response.

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SECON ARY SITE OF ANTIGEN STIMULATION FOR THERAPEUTIC VACCINATION

RELATED APPLICATIONS
This application claims priority to U.S.S.N. 61/281,663, filed November 20, 2009, the contents of which are incorporated herein by reference in their entirety.

STATEMENT AS TO FEDERALLY SPONSORED RESEARCH
This invention was made with Government support under the National Institutes of Health award UL1 RR 025758-01, the National Institute of Dental and Craniofacial Research award R01-DE019917, and the Department of Defense (BC084682 Idea Award). The Government has certain rights in the invention.

FIELD OF THE INVENTION
This invention relates generally to the field of immunology.

BACKGROUND OF THE INVENTION
Vaccines are largely ineffective for patients with established disease, as advanced pathology requires potent and sustained activation of CD8(+), cytotoxic T lymphocyte (CTL) mediated responses. Established diseases, such as cancer, normally suppress responses by the immune system leading to immune tolerance to foreign antigen and the disease. As such, there is a pressing need for new strategies to activate the immune system in patients with established disease.

SUMMARY OF THE INVENTION
The methods described herein provide a solution to the problems associated with previous tumor vaccines. Accordingly, a method for eliciting and sustaining an anti-tumor immune response is carried out by administering a device containing an amount of antigen sufficient to stimulate an innate immune response and an adaptive immune response. In another approach, a method for eliciting and sustaining an anti-tumor immune response is carried out by administering to a first anatomical site a first antigen-loaded acellular biomaterial device (polyactide-co-glycolide (PLG) vaccine) at a first time point to elicit an innate immune response and administering to a second anatomical site at a second time point a second antigen loaded acellular biomaterial device to sustain an adaptive immune response.
Preferably, the antigen comprises a tumor cell lysate, granulocyte-macrophage colony-stimulating factor (GM-CSF), and/or cytosine-guanosine (CpG)-oligodeoxynucleotides (ODN). Suitable tumor cells include those from, e.g., central nervous system (CNS) cancers, lung cancer, breast cancer, Leukemia, Multiple Myeloma, Renal Cancer, Malignant Glioma, Medulloblastoma, and Melanoma. In some embodiments, purified tumor antigen (e.g., those listed below) is used instead of or together with tumor lysate as the antigen. The methods described herein are suitable for any mammal in need of treatment to elicit or boost an immune response. The mammal can be, e.g., any mammal, e.g., a human, a primate, a mouse, a rat, a dog, a cat, a cow, a horse, or a pig. In a preferred embodiment, the mammal is a human.

Each implant stimulates both innate and adaptive immune responses. After implantation, the device first recruits antigen presenting cells (dendritic cells (DCs) and macrophages), and then at a later time (at the same implant) cells of the adaptive immune system (T cells) are stimulated. Stimulation of innate immunity followed by adaptive immunity occurs after administration of a single device, provided that antigen is present in the device longer than the onset of innate immunity, i.e., antigen is not depleted from the device during the first 3 days after implantation. For example, an improved device contains enough antigen to be make the antigen available for continued presentation to endogenous cells for at least 3 days, 7 days, 12 days, 16 days, 30 days, 45 days, 90 days, 120 days, or more to prolong the adaptive immune response. In another approach, a second (and subsequent administrations/implantations of the device) are made to prolong the stimulation of the immune response to generate adaptive immune cells. Multiple rounds of vaccinations increase and extend an anti-tumor response.

Vaccination requires stimulation for at least 3 days, e.g., at least 4 days, 5 days, 6 days, or 7 days) to induce the onset of innate immunity (i.e., DC responses) and effector cytotoxic T lymphocyte (CTL) responses are maintained until antigen is depleted from the vaccine site. The innate immune response, which is initially induced by the first device during the first time period (days following the first time point) is characterized by an infiltration of dendritic cells and macrophages, i.e., antigen-presenting cells. For example, the innate immune response is characterized by an increase in plasmacytoid dendritic cells (pDCs). Preferably, the vaccine induces persistent interleukin-12 (IL-12) and interferon-γ (IFN-γ) production consistent with DC and T cell infiltration into the vaccine site. The adaptive immune response is characterized by cytotoxic T cells (CTLs). The second time
point occurs after onset of the innate immune response and before depletion of antigen in the biomaterial device. A plurality of devices is administered to elicit and sustain an anti-tumor immune response for an extended period of time. The first and second time points are sequential or simultaneous (or closely spaced, e.g., within minutes or hours of one another).

For example, the second time point is between 1-120 days, e.g., 1-5 days, 1-10 days, 1-15 days, 1-20 days, 1-30 days, 1-60 days, or 3-38 days, after the first time point. An exemplary vaccination protocol is characterized by a first vaccination followed by a second vaccination that is 10 days after said first time point (first vaccination).

Alternatively, implantation of a plurality of devices (scaffolds), e.g., 2-10 or more devices, occurs at or about the same time. The secondary antigen site is provided using a single device containing an ample amount of antigen to span the time during which both innate and adaptive immunity is developed or by a plurality of devices that are temporally spaced and/or geographically spaced such that multiple devices provide a continuing source of antigen to sustain the adaptive immune response to reduce and eliminate tumors.

The vaccine devices and methods described herein are useful to reduce tumor burden, e.g., tumor mass of solid tumors, and eradicate such tumors. Types of cancers to be treated include central nervous system (CNS) cancers, CNS germ cell tumors, lung cancer, breast cancer, prostate cancer, liver cancer, Leukemia, Multiple Myeloma, renal cancer, Malignant Glioma, Medulloblastoma, and Melanoma. The methods and devices are also useful to treat infections, e.g., at discrete anatomical sites caused by microbial pathogens such as bacteria, viruses, fungi.

A primary antigen (device) site is at or near a target tumor site, and a secondary antigen site or sites (device(s)) are implanted at, near, or at a distant site relative to the location of the tumor. For example, device(s) are implanted at a distance of 1, 3, 5, 10, 15, 20, 25, 40 mm from a tumor site or site of excision, e.g., 16-21 mm away from a tumor mass. The second or subsequent implant(s) are optionally placed anywhere in the body. For microbial infections, a similar device location strategy is employed.

An adjuvant is optionally included in the secondary antigen site to boost adaptive responses. Exemplary adjuvants are listed as follows: mineral salts, e.g., aluminium hydroxide and aluminium or calcium phosphate gels; oil emulsions and surfactant based formulations, e.g., MF59 (microfluidised detergent stabilised oil-in-water emulsion), QS21 (purified saponin), AS02 [SBAS2] (oil-in-water emulsion + MPL + QS-21), Montanide ISA-51 and ISA-720 (stabilised water-in-oil emulsion); particulate adjuvants, e.g., virosomes (unilamellar liposomal vehicles incorporating influenza haemagglutinin), AS04 ([SBAS4] Al
salt with MPL), ISCOMS (structured complex of saponins and lipids), polylactide co-glycolide (PLG); microbial derivatives (natural and synthetic), e.g., monophosphoryl lipid A (MPL), Detox (MPL + M. Phlei cell wall skeleton), AGP [RC-529] (synthetic acylated monosaccharide), DC_Chol (lipoidal immunostimulators able to self organize into liposomes), OM-174 (lipid A derivative), modified LT and CT (genetically modified bacterial toxins to provide non-toxic adjuvant effects); endogenous human immunomodulators, e.g., hGM-CSF or hIL-12 (cytokines that can be administered either as protein or plasmid encoded), Immudaptin (C3d tandem array); inert vehicles, such as gold particles.

Thus, the invention includes a method for progressively reducing tumor burden by successively administering an antigen-loaded acellular biomaterial device. For example, functionalized biomaterials incorporating various combinations of an inflammatory cytokine, immune danger signal, and tumor lysates are administered to control the activation and localization of host dendritic populations in situ. The methods include the steps of identifying a subject with an established disease (at a defined location) and implementing a successive administration schedule until the disease, e.g., tumor or infection, is substantially reduced or eradicated. The schedule comprises a first implantation and a second implantation of an antigen-loaded acellular biomaterial device (e.g., PLG vaccine device) and a second implantation occurring at least 24 hours prior to antigen depletion of first device. This schedule, first and second implantations, are repeated successively for as many cycles as required to progressively reduce (or eliminate) tumor burden.

The invention therefore features methods for treating established pathologies using vaccines that contain secondary immunostimulatory sites of sustained antigen presentation. Effective vaccination against established tumors was dramatically enhanced with sustained presentation of tumor associated antigens at a secondary site during the clearance of the disease. Secondary sites include immunostimulatory signals to amplify immune responses. Vaccination is enhanced by sustained antigen stimulation to immune cells that are maintained longer than the onset of innate immunity (i.e., infiltration of macrophages, DCs - typically 24 hours). Multiple vaccinations (at least two rounds of administration) are better than one vaccination to sustain antigen presentation. Appropriate timing between multiple vaccinations is between 3-28 days to effectively sustain antigen presentation, i.e., vaccines are administered between days 3-28 after administration of the previous vaccine.

The device recruit cells in vivo, modifies these cells, and then promotes their migration to another site in the body. This approach is exemplified herein in the context of
immune cells and cancer vaccine development, but is also useful to other vaccines such as those against microbial pathogens as well as cell therapies in general.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims. Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. All published foreign patents and patent applications cited herein are incorporated herein by reference. Genbank and NCBI submissions indicated by accession number cited herein are incorporated herein by reference. All other published references, documents, manuscripts and scientific literature cited herein are incorporated herein by reference. In the case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

**BRIEF DESCRIPTION OF THE DRAWINGS**

Figure 1 is a series of panels illustrating that granulocyte-macrophage colony-stimulating factor (GM-CSF) delivery from polyactide-co-glycolide (PLG) matrices promotes CD1 lb+ DC recruitment and activation. (A) H&E staining of sectioned PLG scaffolds explanted from subcutaneous pockets in the backs of C57BL/6j mice after 14 days: Blank scaffolds (BLANK), and GM-CSF (3000 ng) loaded scaffolds (GM-CSF). (B) The number of CD1 lc(+) DCs isolated from PLG scaffolds at day 14 after implantation in response to doses of 0, 1000, 3000 and 7000 ng of GM-CSF. (C) Fluorescence-activated cell sorting (FACS) plots of cells isolated from explanted scaffolds and stained for CD1 lc and CD1 lb. Cells were isolated from PLG matrices incorporating 3,000 ng of GM-CSF at day 10 post-implantation. Numbers in FACS plots indicate the percentage of the cell population positive for CD1 lc and CD1 lb, or for both markers. (D) The number of CD1 lc(+)CD86(+), CD1 lc(+) CCPv7(+), and Cdl lc(+) major histocompatibility complex II (MHCII)(+) DCs isolated from PLG scaffolds at day 14 after implantation in response to doses of 0, 400, 3000 and 7000 ng of GM-CSF. Values in B and D represent mean and standard deviation (n=4 or 5). * P<0.05 ** P<0.01 as compared to blank matrices unless otherwise noted.

Figure 2 is a series of panels demonstrating that cytosine-guanosine (CpG)-oligodeoxynucleotides (ODN) and GM-CSF delivery from PLG matrices promotes plasmacytoid DC generation and the production of anti-tumor cytokines. (A) FACS plots of
cells isolated from explanted scaffolds and stained for the plasmacytoid DC markers, CD1c and PDCA-1. Cells were isolated from PLG matrices incorporating 0, 1, 10, and 100 µg of CpG-ODN at day 10 post-implantation. Numbers in FACS plots indicate the percentage of the cell population positive for CD1c only or for both markers. The number of (B) plasmacytoid DCs, and (C) CD1 lc(+)CD1 lb(+) cDCs at day 10 post-implantation in blank scaffolds (Blank) or in response to doses of 100 μg (100) of CpG-ODN or 3000 ng GM-CSF alone (GM), or GM-CSF in combination with 1 (1+GM), 10 (10+GM), or 100 μg (100+GM) of CpG-ODN. The in vivo concentrations of (D) IFN-a, and (E) IFN-γ at Day 10 post implantation at the implant site of blank PLG matrices (Blank), or matrices loaded with 3000 ng GM-CSF alone (GM) or 1(γ) or 100μg (100) of CpG-ODN alone or GM-CSF in combination with 10 (10+GM), or 100 μg (100+GM) of CpG-ODN. Values in B-E represent mean and standard deviation (n=4 or 5). * P<0.05 ** P<0.01 as compared to blank matrices unless otherwise noted.

Figure 3 is a series of panels showing that tumor lysate, CpG-ODN, and GM-CSF co-delivery from PLG matrices stimulates CD8+ DC generation and IL-12 production. (A) FACS density plots of CD1 lc and CD8 staining of cells infiltrating Blank PLG matrices (blank) or matrices loaded with 3000 ng GM-CSF and 100 µg CpG-ODN without (CpG+GM) or with tumor lysates (CpG+GM+Ant) at day 10. Numbers in FACS plots indicate the percentage of the cell population positive for CD1 lc and CD8 or for both markers. (B-D) The number of (B) CD1 lc(+)CD8(+) cDCs, (C) plasmacytoid DCs, and (D) CD1 lc(+)CD1 lb(+)cDCs at day 10 after implantation in blank matrices (Blank) and in response to 3000ng GMCSF (GM) or 100 µg CpG-ODN (CpG) alone or in combination (CpG+GM) or co-presented with tumor lysates (GM+Ant, CpG+Ant and CpG+GM+Ant). (E-G) The in vivo concentration of (E) IL-12, (F) IFN-a, and (G) IFN-γ at day 10 after implantation in blank matrices (Blank) and in response to doses of 3000 ng GM-CSF (GM) or 100 µg CpG-ODN (CpG) alone or in combination (CpG+GM) or co-presented with tumor lysates (GM+Ant, CpG+Ant and CpG+GM+Ant). Values in B-G represent mean and SD (n=4 or 5). * P<0.05 ** P<0.01, as compared to blank matrices, unless otherwise noted.

Figure 4 is a series of panels illustrating that tumor lysate, CpG-ODN, and GM-CSF co-delivery in PLG matrices stimulates potent local and systemic CD8+ cytotoxic T cells. (A) FACS plots of cells isolated from explanted matrices and stained for the cytotoxic T cell markers, CD3 and CD8a. Cells were isolated from PLG matrices with 3000 ng GM-CSF, 100 µg CpG-ODN and tumor lysates at days 1, 5, 12 and 21 after implantation. Numbers in FACS plots indicate the percentage of the cell population that was either single positive for
CD3 (upper left quadrant of each plot) or CD8 (lower right), or double positive for both markers (upper right). (B) The total number of CD3(+)CD8(+) cytotoxic T cells isolated from PLG matrices loaded with GM-CSF, CpG-ODN and tumor lysates as a function of time after implantation. (C) The number of CD8 T cells at day 12 after implantation in blank scaffolds (Blank) or in response to lysate alone (Lys) or in combination with CpG-ODN (CpG+Lys) or GM-CSF (GM+Lys) or both factors (GM+Lys+CpG). (D) FACS plots of splenocytes of naïve mice and mice vaccinated with PLG vaccines containing 3000 ng GM-CSF, 100 µgCpG-ODN, and tumor lysates at day 16 post-implantation. Cells were stained with anti-CD8-PE Ab, and Kb/TRP2 pentamers. The gates represent the TRP2-specific, CD8(+) T cells and numbers provide the percentage of gated cells. (E) The total number of TRP2-specific, CD8(+) T cells in the spleens of mice vaccinated with PLG matrices loaded with GM-CSF, CpG-ODN and tumor lysates as a function of time after implantation. Values in B, C and E represent mean and standard deviation (n=4 or 5). * P<0.05 as compared to all other experimental conditions.

Figure 5 is a series of graphs demonstrating that tumor protection stimulated by engineered PLG matrices is correlated with DC subsets and IL-12 production. Survival times of mice vaccinated with PLG vaccines 14 days prior to B16-F10 melanoma tumor challenge (10^5 cells). (A) A comparison of survival times in mice treated with blank PLG matrices or with PLG matrices loaded with tumor lysates and 1, 10, 50 or 100 µg of CpG-ODN. (B) A comparison of survival times in mice vaccinated with PLG matrices loaded with tumor lysates, 3000 ng GM-CSF and either 1, 10, 50 or 100 µg of CpG-ODN. (C) The fraction of the total CD1 lc(+) DC population consisting of CD1 lb(+) cDCs (white bar), PDCA-1(+) pDCs (black bar), and CD8(+) cDCs (striped bar) generated at the PLG vaccine site at day 10. Vaccines were loaded with either 3000 ng GM-CSF, or 100 µg of CpG-ODN alone or in combination. Survival percentages recorded at day 100 after tumor challenge. Plots of the numbers of (D) CD1 lc(+)CD8(+) cDCs (E) CD1 lc(+)PDCA-1(+) pDCs, and (F) CD1 lc(+) CD1 lb(+) cDCs, and (G) the concentration of IL-12 at the PLG vaccine site at day 10 versus the percent of animals surviving B16-F10 melanoma tumor challenge at Day 100 (survival data taken from experimental conditions in A and B). Values in D-G represent mean and SD (n=4 or 5). r values in D-F represent the linear correlation coefficient between DC numbers or IL-12 concentration and survival percentage.

Figure 6 is a series of charts showing that engineered PLG matrices attenuate FoxP3+ Tregs and immunosuppressive cytokines. (A) The total number of CD3(+)CD4(+) T cells isolated from PLG matrices loaded with GMCSF, CpG-ODN and tumor lysates as a function...
of time after implantation. (B) The number of CD4 T cells at day 12 after implantation in blank scaffolds (Blank) or in response to lysate alone (Lys) or in combination with CpG-ODN (CpG+Lys) or GM-CSF (GM+Lys) or both factors (GM+Lys+CpG). The in vivo concentrations of (C) TGF-β and (D) IL-10 at Day 12 post implantation at the implant site of blank scaffolds (Blank) or scaffolds presenting lysate alone (Lys) or in combination with CpG-ODN (CpG+Lys) or GM-CSF (GM+Lys) or both factors (GM+Lys+CpG). (E) FACS plots of cells isolated from explanted scaffolds and stained for the T regulatory cell markers, CD3 and FoxP3. Cells were isolated from PLG matrices incorporating GM-CSF and lysates without (GM+Lys) or with GM-CSF, lysates and CpGODN (GM+Lys+CpG) at day 12 after implantation. Numbers in FACS plots indicate the percentage of the cell population positive for both markers. (F) The number of FoxP3(+) Tregs at day 12 post-implantation in blank scaffolds (Blank) or in response to lysate alone (Lys) or in combination with CpG-ODN (CpG+Lys) or GM-CSF (GM+Lys) or both factors (GM+Lys+CpG). (G) The ratio of CD8α T cells versus FoxP3(+) Tregs residing within PLG scaffolds loaded with GM-CSF and lysates (GM+Lys) alone or in combination with CpG-ODN (GM +Lys+CpG) at day 12 post-implantation. Values in A-D, F and G represent mean and SD (n=4 or 5). * P<0.05 ** P<0.01 as compared to all other experimental conditions unless otherwise noted.

Figure 7 is a series of graphs demonstrating that Engineered PLG matrices stimulate the regression of established melanomas A comparison of the (A) tumor growth and (B) survival of mice bearing established melanoma tumors (inoculated with $5 \times 10^5$ B16-F10 cells and allowed to develop for 9 days) and treated with either blank PLG matrices (Blank), or matrices loaded with 3000 ng GM-CSF and 100 µg CpG-ODN (GM+CPG). Mice were also treated once (Vax, 1x; at day 9) or twice (Vax, 2x; at days 9 and 19) with PLG matrices incorporating GM-CSF, CpG-ODN and tumor lysates (Vax). Mice were also vaccinated with $5 \times 10^5$ irradiated, GM-CSF transduced B16-F10 cells. (C) The individual tumor growth curves for each mouse surviving tumor challenge ($5 \times 10^5$ cells) after a two-time treatment with PLG vaccines at days 9 and 19. A comparison of the (D) tumor growth and (E) survival of mice bearing established melanoma tumors (inoculated with $5 \times 10^5$ B16-F10 cells and allowed to develop for 13 days) and treated with either blank PLG matrices (Blank), or once with PLG vaccines (Vax, 1x; at day 13) or twice (Vax, 2x; at days 13 and 23). (F) The individual tumor growth curves for each mouse surviving tumor challenge ($5 \times 10^5$ cells) after a two-time treatment with PLG vaccines at days 13 and 23. Values in A & D (A-F; n=15 per condition) represent mean and standard error of the mean.
Figure 8 is a series of FACS plots of cells isolated from explanted scaffolds and stained for the DC marker, CD1lc, and for activation markers MHCII and CCR7. Cells were isolated from blank PLG matrices or matrices incorporating either 3 or 7 µg of GM-CSF at day 28 post-implantation. Numbers in FACS plots indicate the percentage of the cell population positive for CD1lc only or positive for both CD1lc and either MHCII or CCR7.

Figure 9 shows the effect of PLG vaccine duration on efficacy in B16 melanoma tumor models. (A) Photomicrograph of surface of macroporous, PLG-based vaccine loaded with GM-CSF, tumor lysates and CpG-ODN. (B) SEM micrograph of cross section of macroporous, PLG vaccine. Scale bar - 200 µm. (C) Schematic of PLG vaccine regimen for both prophylactic and therapeutic B16 models in mice. [Prophylactic] Vaccine duration is varied from 0, 1, 3, 7, 12, and 16 days. (D) A comparison of the survival time and (E) the survival percentage at day 100 in mice treated with blank PLG scaffolds, and PLG vaccines for a duration of 1, 3, 7, 12, 16 days or indefinitely (>16). Mice were challenged with 10^4 B16-F10 melanoma tumor cells at day 14 after the start of vaccination. (F) Effect of vaccine duration on therapeutic efficacy [Therapeutic]. Tumor growth curves for mice vaccinated at day 9 after tumor challenge (10^5 B16 melanoma cells), with PLG vaccines for a duration of 1, 3, 7, 12, 16 days or indefinitely (>16). The total GM-CSF dose was 3000 ng, and CpG-ODN dose was 100 µg. Values in E represent mean and standard error.

Figure 10 demonstrates the kinetics of DC and T cell infiltration into PLG vaccine site. (A) Hematoxylin & Eosin staining of sectioned PLG vaccines explanted from subcutaneous pockets in the backs of C57BL/6J mice after 3 and 14 days. (B) FACS histograms of CD1lc(+) DCs and CD3(+) T cells infiltrating PLG matrices loaded with 3000 ng GM-CSF, 100 µg CpG-ODN and tumor antigens at days 5 and 12. Histograms of isotype control (tinted line) are also included. (C) The total number of CD1lc(+) DCs and CD3(+) T cells isolated from PLG vaccines as a function of time post implantation. (D) The ratio of CD8a Tcells vs CD4 Tcells residing within PLG vaccines at day 12 post-implantation. Values in B & C represent mean and standard deviation (n=4 or 5). ** P<0.01 as compared to controls.

Figure 11 illustrates the kinetics of IL-12 and IFN-γ production at vaccine site. (A) The concentrations of IL-12 at the implant site of PLG vaccines as a function of time post-implantation. (B) The in vivo concentration of IL-12 at the implant site of blank matrices (Blanks), PLG vaccines (GM+CpG+LYS) or matrices loaded with either Lysate alone (Lys) or Lysate with 3000 ng of GM-CSF (GM+Lys) or Lysate with 100 µg PEI-CpG-ODN
(CpG+Lys) or the combination of GM-CSF and PEI-CpG-ODN (GM+CpG) at Day 12 after implantation into the backs of C57BL/6J mice. (C) The concentration of IFN-γ at the implant site of PLG vaccines as a function of time post-implantation. (D) The in vivo concentration of IFN-γ at the implant site of blank matrices (Blanks), PLG vaccines (GM+CpG+LYS) or matrices loaded with either Lysate alone (Lys) or Lysate with 3000 ng of GM-CSF (GM+Lys) or Lysate with 100 μg PEI-CpG-ODN (CpG+Lys) or the combination of GM-CSF and PEI-CpG-ODN (GM+CpG) at Day 12 after implantation into the backs of C57BL/6J mice. Values in A, B, C & D represent mean and standard deviation (n=4 or 5). *P<0.05** P<0.01 as compared to controls.

Figure 12 demonstrates the effects of PLG vaccination on cells in draining lymph nodes (A & B) The number of phagocytic cells: CD1 lb(+)/CD1 lc(-) macrophages, CD1 lc(+) DCs, and PDCA-1(+)/CD1 lc(+) plasmacytoid DCs, in the draining inguinal lymph nodes of mice at (A) day 3 and at (B) day 10 after implantation of blank matrices (Blank) or PLG matrices containing GM-CSF and Lysate (GM-CSF+LYS) only or PLG vaccines (GM-CSF+Lys+CpG). (C) The number of T cells: CD3(+) T cells, CD3(+)CD8(+) T cells, and CD3(+)CD4(+) T cells, in the draining inguinal lymph nodes of mice at day 10 after implantation of blank matrices (Blank) or PLG matrices containing GM-CSF and Lysate (GM-CSF+LYS) only or with CpG-ODN (GM-CSF+Lys+CpG). (D) FACS plots of cells in the draining inguinal lymph nodes of mice stained for CD3(+)FoxP3(+) cells at day 16 after implantation of PLG matrices containing GM-CSF and Lysate (GM-CSF+LYS) only or with CpG-ODN (GM-CSF+LYS+CpG). Numbers represent percentage of cells positive for both FoxP3 and CD3 (upper left quadrant). (E) The number of CD3(+)FoxP3(+) T regs in the draining inguinal lymph nodes of mice at day 16 after implantation of blank matrices (Blank) or PLG matrices containing GM-CSF and Lysate (GM-CSF+LYS) only or with CpG-ODN (GM-CSF+Lys+CpG). Values in A, B, C and E represent mean and standard deviation (n=4 or 5). *P<0.05** P<0.01 as compared to controls.

Figure 13 shows the sustained tumor antigen presentation by PLG vaccines. Cumulative release of tumor lysates, as measured by mass of released protein, delivered from PLG vaccines total lysate dose ~680μg. Values represent mean and standard deviation.

**DETAILED DESCRIPTION**

The invention described herein is based on the discovery that recruitment and activation of multiple dendritic cell (DC) and T cell subsets provide therapeutic vaccination
against established tumors. Specifically, the methods described herein are utilized to treat established pathologies using vaccines containing secondary and immunostimulatory sites of antigen presentation during immune clearance of disease. As described in detail below, secondary and stimulatory sites of antigen presentation are required to maintain effector T cell responses until the primary site of established disease has cleared. In some cases, one implant (single administration of vaccine) is sufficient to both stimulate innate immunity and provide the secondary site of antigen stimulation required to further stimulate an adaptive response. As long as the vaccine is fabricated to persist for longer than the onset of innate immunity, it also provides a secondary site of antigen presentation. Thus, as described herein, vaccination utilizing PLG vaccines resulted in two distinct phases of immunity \textit{in situ}. The methods described herein are suitable for any mammal in need of treatment. The mammal can be, \textit{e.g.}, any mammal, \textit{e.g.}, a human, a primate, a mouse, a rat, a dog, a cat, a cow, a horse, or a pig. In a preferred embodiment, the mammal is a human.

Dendritic cells (DCs) are promising effectors of immunotherapy as they are essential for initiating and regulating T cell immunity. As components of the innate immune response, dendritic cells (DCs) are involved in the initial reactions to infection, as they detect foreign antigens and are activated by stimuli, such as pathogen associated molecular patterns (PAMPs) unique to invading pathogens (J. Banchereau, R.M. Steinman. Taking dendritic cells into medicine. Nature. 49, 419-426 (2007); Gilboa, E. Dendritic cell based vaccines. J Clin Invest. 117, 1195-1203 (2007); Schuler G., Schuler-Thurner B., Steinman R.M. The use of dendritic cells in cancer immunotherapy. Curr Opin Immunol. 15, 138-147 (2003)). PAMPs, including lipopolysacharides and cytosine-guanosine (CpG) sequences in bacterial deoxyribonucleic acid (DNA), trigger a particular toll-like receptor (TLR), that allows DCs to classify the pathogen and induce their expression of T cell-activating molecules (Agrawal S. \textit{et al.} Cutting edge: different Toll-like receptor agonists instruct dendritic cells to induce distinct T\textsubscript{H} responses via differential modulation of extracellular signal-regulated kinase-mitogen-activated protein kinase and c-Fos. J. Immunol. 171, 4984-4989 (2003)). Activated DCs then migrate to draining lymph nodes (LN) where they prime the appropriate T cell response, via the presentation of antigen, costimulatory molecules and the appropriate cytokines.

While innate immunity encompasses many rapid reactions to infection, adaptive immunity, in contrast, is learned or acquired more slowly via cellular messengers (\textit{e.g.}, DCs), over time frames of days to weeks (Pope C, Kim A, Marzo D, Masopust K, Williams J, Jiang H, Lefrancois L. Organ-specific regulation of the CD8 T cell response to \textit{Listeria}...

Chronic exposure to tumor antigens with inappropriate co-stimulation and immunomodulation by T regulatory (Treg) cells allows solid tumors to develop by dysregulating DC activity and the cytotoxic T lymphocyte (CTL) responses required to kill tumor cells (S. A. Rosenberg, J. C. Yang, N. P. Restifo, Cancer immunotherapy: Moving beyond current vaccines. Nat. Med. 10,909-915 (2004); C. A. Klebanoff, L. Gattinoni, N. P. Restifo, CD8+ T-cell memory in tumor immunology and immunotherapy. Immunol. Rev. 211, 214-224 (2006)). Cancer vaccines are frequently developed with easily accessible, patient-derived blood monocytes that are transformed into DCs ex vivo with cytokine mixtures and pulsed with tumor antigens to promote antigen presentation (R. M. Steinman, J. Banchereau, Taking dendritic cells into medicine. Nature 449, 419-426 (2007); E. Gilboa, DC-based cancer vaccines. J. Clin. Invest. 111, 1195-1203 (2007); G. Schuler, B. Schuler-Thurner, R. M. Steinman, The use of dendritic cells in cancer immunotherapy. Cuff. Opin. Immunol. 15, 138-147 (2003)). These antigen-loaded DCs are then infused back into cancer patients with the goal of inducing antitumor immune responses mediated primarily by T helper 1 (TH1) cells and CTLs. Although clinical trials utilizing such ex vivo DC vaccines in patients with advanced cancer have resulted in antigen-specific T cell expansion and the production of protective cytokines in vivo (T. J. Curiel, D. T. Curiel, Tumor immunotherapy: Inching toward the finish line. J. Clin. Invest. 109,3 11-3 12(2002)), many vaccines do not increase patients' survival over traditional treatments (for example, chemotherapy) and have failed to consistently cause the regression of solid tumors. In both murine models and humans, these strategies are likely unable to generate the necessary numbers of functional CD8+ CTLs for the duration required to induce regression of solid invasive tumors in both mice and humans.
Instead, they may amplify defective CTLs that never become fully functional effectors at the tumor site because of high local concentrations of immunosuppressive cytokines (for example, transforming growth factor-b (TGF-b) and interleukin-10 (IL-10)) and Treg cells, which dampen immune responses.

Hematopoietic precursor cells of both the myeloid and lymphoid lineage have the capacity to differentiate into two main categories of DCs: conventional DCs (cDCs) and plasmacytoid DCs (pDCs) (S. H. Naik, P. Sathe, H. Y. Park, D. Metcalf, A. I. Proietto, A. Dakic, S. Carotta, M. O’Keeffe, M. Bahlo, A. Papenfuss, J. Y. Kwak, L. Wu, K. Shortman, Development of plasmacytoid and conventional dendritic cell subtypes from single precursor cells derived in vitro and in vivo. Nat. Immunol. 8, 1217-1226 (2007); A. O’Garra, G. Trinchieri, Are dendritic cells afraid of commitment? Nat. Immunol. 5, 1206-1208 (2004); A. D’Amico, L. Wu, The early progenitors of mouse dendritic cells and plasmacytoid predendritic cells are within the bone marrow hemopoietic precursors expressing Flt3. J. Exp. Med. 198, 293-303 (2003)). Effective cancer vaccines may require both types of DCs, as each is equipped with a specific defense mechanism in response to invading pathogens.

cDCs include CD1 lc+CD1 lb+ and CD1 lc+CD8+ cells and exhibit classic DC morphology, protruding dendrites that make these cells especially adept at antigen processing and antigen presentation to T cells (S. H. Naik, P. Sathe, H. Y. Park, D. Metcalf, A. I. Proietto, A. Dakic, S. Carotta, M. O’Keeffe, M. Bahlo, A. Papenfuss, J. Y. Kwak, L. Wu, K. Shortman, Development of plasmacytoid and conventional dendritic cell subtypes from single precursor cells derived in vitro and in vivo. Nat. Immunol. 8, 1217-1226 (2007); A. O’Garra, G. Trinchieri, Are dendritic cells afraid of commitment? Nat. Immunol. 5, 1206-1208 (2004); A. D’Amico, L. Wu, The early progenitors of mouse dendritic cells and plasmacytoid predendritic cells are within the bone marrow hemopoietic precursors expressing Flt3. J. Exp. Med. 198, 293-303 (2003)). Plasmacytoid DCs exhibit a spherical morphology and can produce large amounts of type 1 interferons (IFNs) in response to "danger signals," such as unmethylated CpG dinucleotide sequences found in bacterial or viral DNA (A. M. Krieg, Development of TLR9 agonists for cancer therapy. J. Clin. Invest. 112, 1184-1194 (2007); T. Kawai, S. Akira, Innate immune recognition of viral infection. Nat. Immunol. 7, 131-137 (2006)). Plasmacytoid DC-derived type 1 IFNs link innate and adaptive immunity to viral infection by directly inducing naïve T cell differentiation to TH1 cells (J. J. O’Shea, R. Visconti, Type 1 IFNs and regulation of TH1 responses: Enigmas both resolved and emerge. Nat. Immunol. 1, 17-19 (2000)) and by triggering antigen cross-presentation to CD8+ T cells and IL production (e.g., IL-12) by cDCs that facilitate the clonal expansion of CTLs. The
plasticity of hematopoietic precursors likely allows for the recruitment and generation of the DC population most proficient at eliciting the appropriate immune response in a particular situation (J. A. Villadangos, P. Schnorrer, Intrinsic and cooperative antigen-presenting functions of dendritic-cell subsets in vivo. Nat. Rev. Immunol. 7, 543-555 (2007)). Prior to the invention described herein, vaccines were unable to recapitulate ex vivo the development of this broad DC response, which is critical to the development of potent CTL immune responses.

Prior to the invention described herein, vaccine methods produced short-lived bioactivity, and cancer vaccines have failed to reproducibly cause the regression of solid tumors (Rosenberg SA, Yang JC and Restifo, NP. Cancer immunotherapy: moving beyond current vaccines. Nat Med. 10, 909-915 (2004); Klebanoff CA, Gattinoni, L and Restifo NP. CD8+ T-cell memory in tumor immunology and immunotherapy. Immunol Rev. 2006 June ; 211: 214-224). Prior to the invention described herein, delivery methods for experimental vaccines often included bolus injections/effusions of antigen and adjuvants with short half-lives, or activated cell products that lose significant viability upon in vivo transplantation (Ali, O.A. and Mooney, D.J. "Converging Cell Therapy with Biomaterials." Cell Transplantation from Laboratory to Clinic. Elsevier Inc. Burlington, MA. 591-609 (2006)). As described below, biomaterials are utilized to extend the duration of immunostimulation, and act as adjuvants and to enable controlled presentation of antigens to the immune system (Hubbell JA, Thomas SN, Swartz MA. Materials engineering for immunomodulation. Nature. 462, 449-60. (2009); Uchida M, Natsume H, Kishino T, Seki T, Ogihara M, Juni K, Kimura M, Morimoto Y. Immunization by particle bombardment of antigen-loaded poly-(DL-lactide-co-glycolide) microspheres in mice. Vaccine. 12, 2120-30. (2006)). Material-based particulate systems control antigen localization in tissue or to target host DCs specifically via antibody or ligand conjugation (Reddy, S. T. et al. Exploiting lymphatic transport and complement activation in nanoparticle vaccines. Nature Biotechnol. 25, 1159-1164 (2007); Kwon YJ, et al. In vivo targeting of dendritic cells for activation of cellular immunity using vaccine carriers based on pH-responsive microparticles, Proc. Natl. Acad. Sci. U. S. A. 102 18264-18268. (2005)). Although biomaterial-based vaccines prior to the invention described herein could enhance antigen loading to DCs, leading to antigen specific T cell activation, they lacked concurrent regulation of effector T cell activity. As described below, therapeutic vaccination against an established disease, including solid tumors, requires methods that stimulate not only innate immunity and DCs, but also persistent cytotoxic T lymphocyte (CTL) responses, which kill tumor cells, until the disease has cleared. Therefore, the natural
kinetics of innate and adaptive immune responses and the coordination of these responses by vaccines impact vaccine design and application.

Described herein is the in situ generation of a heterogeneous DC network capable of CTL induction to activate robust CD8+ T cell effector responses to established tumors, by providing a secondary immunostimulatory site of tumor antigen presentation. Specifically, the results presented herein describe the kinetics of innate DC responses and adaptive T cell responses and illustrate their fundamental relationship to potent tumor rejection when implanted subcutaneously in a mouse B16 model of late-stage melanoma. Also described in detail below is the relationship between vaccine duration and efficacy in melanoma models and its correlation to local kinetics of DC and T-cell responses to infection-mimicking polymers. Implantable, synthetic polylactide-co-glycolide (PLG) matrices that spatially and temporally control the in vivo presentation of cytokines, tumor antigens and TLR-activating, danger signals have been described (Ali OA, Huebsch N, Cao L, Dranoff G, Mooney DJ. Infection-mimicking materials to program dendritic cells in situ. Nat Mater 2, 151-158 (2009); Ali OA, Emerich D, Dranoff G, and Mooney DJ. In situ Regulation of DC Subsets and T Cells Mediates Tumor Regression in Mice Sci Transl Med. 1, 8-19. (2009)). As described below, granulocyte-macrophage colony-stimulating factor (GM-CSF) was released from these PLG matrices to recruit DC precursors and DCs. CpG-rich oligonucleotides (CpG-ODN) were immobilized on the matrices as danger signals, and antigen (tumor lysates) was released to matrix resident DCs to program DC development and maturation. These PLG vaccine systems (PLG vaccines) were utilized to manipulate the in situ generation of DC networks programmed to induce and activate CD8(+) CTL responses that are antigen specific, while down regulating negative feedback mechanisms (i.e., immunosuppressive pathways). Importantly, the vaccination systems described herein cause immune-mediated eradication of distant and established B16 melanoma tumors in mice. As described below, the duration of vaccination was varied to determine the impact on vaccine efficacy. Also described herein is the kinetics of innate (DCs) and adaptive cellular responses (cytotoxic T cells) to these vaccines, and the inflection point when innate responses transition to effective anti-tumor immune responses after vaccination. Because the cytokines interleukin (IL)-12 and interferon (IFN)-y are important mediators of cytotoxic T cell responses to viruses and tumors (O'Shea JJ, and Visconti R. Type 1 IFNs and regulation of TH1 responses: enigmas both resolved and emerge. Nat Immunol 1, 17 - 19 (2000); Magram, J., et al. IL-12-deficient mice are defective but not devoid of type 1 cytokine responses. Ann NY Acad Sci 795, 60-
70. (1996)), their concentrations were measured at the PLG vaccine site over time. Additionally, the vaccine's induction of systemic cellular responses were monitored at the inguinal LN draining the site of vaccination, as these are points where naïve T cells interact with antigen presenting cells (APCs; DCs and macrophages) and are important locations where CD8+ CTL cells are primed.

Prior to the invention described herein, vaccines were unable to monitor and trigger the T effector profile described here, but instead induced at least partially dysfunctional T cells that are more likely to undergo exhaustion within the immunosuppressive microenvironment of many viral infections and tumors. The results described herein show that secondary and stimulatory sites of antigen presentation are required to maintain effector T cell responses until the primary site of established disease has cleared. This methodology provides a new platform to utilize vaccines not only for disease prevention but also as effective therapy.

**Scaffold compositions and architecture**

Antigen-laden, macroporous poly (lactide-co-glycolide) (PLG) scaffolds induce potent dendritic cell (DC) and cytotoxic T-lymphocyte (CTL) responses that maintain a "danger" microenvironment via the controlled signaling of inflammatory cytokines and Toll-like receptor agonists (PLG vaccines). Scaffolds for cell transplantation are generally described in US 2008-0044900 A1 and PCT Publication WO 2009/102465, both of which are incorporated herein by reference in their entirety. Components of the scaffolds are organized in a variety of geometric shapes (e.g., beads, pellets), niches, planar layers (e.g., thin sheets). For example, multicomponent scaffolds are constructed in concentric layers each of which is characterized by different physical qualities (% polymer, % crosslinking of polymer, chemical composition of scaffold, pore size, porosity, and pore architecture, stiffness, toughness, ductility, viscoelasticity, and or composition of bioactive substances such as growth factors, homing/migration factors, differentiation factors. Each niche has a specific effect on a cell population, e.g., promoting or inhibiting a specific cellular function, proliferation, differentiation, elaboration of secreted factors or enzymes, or migration. Cells incubated in the scaffold are educated and induced to migrate out of the scaffold to directly affect a target tissue, e.g., and injured tissue site. For example, stromal vascular cells and smooth muscle cells are useful in sheetlike structures are used for repair of vessel-like structures such as blood vessels or layers of the body cavity. For example, such structures are used to repair abdominal wall injuries or defects such as gastroschisis. Similarly, sheetlike scaffolds seeded with dermal stem cells and/or keratinocytes are used in bandages or wound
dressings for regeneration of dermal tissue. The device is placed or transplanted on or next to a target tissue, in a protected location in the body, next to blood vessels, or outside the body as in the case of an external wound dressing. Devices are introduced into or onto a bodily tissue using a variety of known methods and tools, e.g., spoon, tweezers or graspers, hypodermic needle, endoscopic manipulator, endo- or trans-vascular- catheter, stereotaxic needle, snake device, organ-surface-crawling robot (United States Patent Application 20050154376; Ota et al, 2006, Innovations 1:227-231), minimally invasive surgical devices, surgical implantation tools, and transdermal patches. Devices can also be assembled in place, for example by sequentially injecting or inserting matrix materials. Scaffold devices are optionally recharged with cells or with bioactive compounds, e.g., by sequential injection or spraying of substances such as growth factors or differentiation factors.

A scaffold or scaffold device is the physical structure upon which or into which cells associate or attach, and a scaffold composition is the material from which the structure is made. For example, scaffold compositions include biodegradable or permanent materials such as those listed below. The mechanical characteristics of the scaffold vary according to the application or tissue type for which regeneration is sought. It is biodegradable (e.g., collagen, alginates, polysaccharides, polyethylene glycol (PEG), poly(glycolide) (PGA), poly(L-lactide) (PLA), or poly(lactide-co-glycolide) (PLGA) or permanent (e.g., silk). In the case of biodegradable structures, the composition is degraded by physical or chemical action, e.g., level of hydration, heat or ion exchange or by cellular action, e.g., elaboration of enzyme, peptides, or other compounds by nearby or resident cells. The consistency varies from a soft/pliant (e.g., a gel) to glassy, rubbery, brittle, tough, elastic, stiff. The structures contain pores, which are nanoporous, microporous, or macroporous, and the pattern of the pores is optionally homogeneous, heterogenous, aligned, repeating, or random.

Alginate are versatile polysaccharide based polymers that may be formulated for specific applications by controlling the molecular weight, rate of degradation and method of scaffold formation. Coupling reactions can be used to covalently attach bioactive epitopes, such as the cell adhesion sequence RGD to the polymer backbone. Alginate polymers are formed into a variety of scaffold types. Injectable hydrogels can be formed from low MW alginate solutions upon addition of a cross-linking agents, such as calcium ions, while macroporous scaffolds are formed by lyophilization of high MW alginate discs. Differences in scaffold formulation control the kinetics of scaffold degradation. Release rates of morphogens or other bioactive substances from alginate scaffolds is controlled by scaffold formulation to present morphogens in a spatially and temporally controlled manner.
controlled release not only eliminates systemic side effects and the need for multiple injections, but can be used to create a microenvironment that activates host cells at the implant site and transplanted cells seeded onto a scaffold.

The scaffold comprises a biocompatible polymer matrix that is optionally biodegradable in whole or in part. A hydrogel is one example of a suitable polymer matrix material. Examples of materials which can form hydrogels include polylactic acid, polyglycolic acid, PLGA polymers, alginites and alginate derivatives, gelatin, collagen, agarose, natural and synthetic polysaccharides, polyamino acids such as polypeptides particularly poly(lysine), polyesters such as polyhydroxybutyrate and poly-epsilon-caprolactone, polyanhydrides; polyphosphazenes, poly(vinyl alcohols), poly(alkylene oxides) particularly poly(ethylene oxides), poly(allylamines)(PAM), poly(acrylates), modified...
styrene polymers such as poly(4-aminomethylstyrene), pluronic polyls, polyoxamers, poly(uronie acids), poly(vinylpyrrolidone) and copolymers of the above, including graft copolymers.

The scaffolds are fabricated from a variety of synthetic polymers and naturally-occurring polymers such as, but not limited to, collagen, fibrin, hyaluronic acid, agarose, and laminin-rich gels. One preferred material for the hydrogel is alginate or modified alginate material. Alginate molecules are comprised of (1-4)-linked β-D-mannuronic acid (M units) and a L-guluronic acid (G units) monomers, which can vary in proportion and sequential distribution along the polymer chain. Alginate polysaccharides are polyelectrolyte systems which have a strong affinity for divalent cations (e.g. Ca²⁺, Mg²⁺, Ba²⁺) and form stable hydrogels when exposed to these molecules. See, Martinsen A., et al., Biotech. & Bioeng., 33 (1989) 79-89. For example, calcium cross-linked alginate hydrogels are useful for dental applications, wound dressings chondrocyte transplantation and as a matrix for other cell types.

An exemplary device utilizes an alginate or other polysaccharide of a relatively low molecular weight, preferably of size which, after dissolution, is at the renal threshold for clearance by humans, e.g., the alginate or polysaccharide is reduced to a molecular weight of 1000 to 80,000 daltons. Preferably, the molecular mass is 1000 to 60,000 daltons, particularly preferably 1000 to 50,000 daltons. It is also useful to use an alginate material of high guluronate content since the guluronate units, as opposed to the mannuronate units, provide sites for ionic crosslinking through divalent cations to gel the polymer. U.S. Patent Number 6,642,363, incorporated herein by reference discloses methods for making and using polymers containing polysaccharides such as alginates or modified alginates that are particularly useful for cell transplantation and tissue engineering applications.

Useful polysaccharides other than alginates include agarose and microbial polysaccharides such as those listed in the table below.

**Polysaccharide scaffold compositions**

<table>
<thead>
<tr>
<th>Polymers a</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fungal</td>
<td></td>
</tr>
<tr>
<td>Pullulan (N)</td>
<td>1,4-; 1,6-a-D-Glucan</td>
</tr>
<tr>
<td>Scleroglucan (N)</td>
<td>1,3; 1,6- a -D-Glucan</td>
</tr>
<tr>
<td>Chitin (N)</td>
<td>1,4-β-D-Acetyl Glucosamine</td>
</tr>
<tr>
<td>Chitosan (C)</td>
<td>1,4- β.-D-N-Glucosamine</td>
</tr>
</tbody>
</table>
Elsinan (N) 1,4-; 1,3- a -D-Glucan
Bacterial
Xanthan gum (A) 1,4- β .-D-Glucan with D-mannose;
D-glucuronic Acid as side groups
Curdlan (N) 1,3- β.-D-Glucan (with branching)
Dextran (N) 1,6- a -D-Glucan with some 1,2; 1,3-;
1,4- a - linkages
Gellan (A) 1,4- β.-D-Glucan with rhamose,
D-glucuronic acid
Levan (N) 2,6- β.-D-Fructan with some
β.-2,1 -branching
Emulsan (A) Lipoheteropolysaccharide
Cellulose (N) 1,4- β-D-Glucan

a N-neutral, A = anionic and C=cationic.

The scaffolds of the invention are porous or non-porous. For example, the scaffolds are nanoporous having a diameter of less than about 10 nm; microporous wherein the diameter of the pores are preferably in the range of about 100 nm-20 μm; or macroporous wherein the diameter of the pores are greater than about 20 μm, more preferably greater than about 100 μm and even more preferably greater than about 400μm. In one example, the scaffold is macroporous with aligned pores of about 400-500μm in diameter. The preparation of polymer matrices having the desired pore sizes and pore alignments are described in the Examples. Other methods of preparing porous hydrogel products are known in the art. (U.S. Pat. No. 6,5 11,650 incorporated herein by reference).

**Bioactive compositions**

The device includes one or more bioactive compositions. Bioactive compositions are purified naturally-occurring, synthetically produced, or recombinant compounds, e.g., polypeptides, nucleic acids, small molecules, or other agents. The compositions described herein are purified. Purified compounds are at least 60% by weight (dry weight) the compound of interest. Preferably, the preparation is at least 75%, more preferably at least 90%, and most preferably at least 99%, by weight the compound of interest. Purity is measured by any appropriate standard method, for example, by column chromatography,
polyacrylamide gel electrophoresis, or HPLC analysis. Exemplary bioactive composition includes tumor antigens, tumor cell lysates, or tumor cells. Preferably, the bioactive composition includes tumor cell lysate, GM-CSF, and CpG-ODN.

**Vaccine device**

The biocompatible scaffolds are useful as delivery vehicles for cancer vaccines. The cancer vaccine stimulates an endogenous immune response against cancer cells. Prior to the invention described herein, vaccines predominantly activated the humoral immune system (*i.e.*, the antibody dependent immune response). Other vaccines focused on activating the cell-mediated immune system including cytotoxic T lymphocytes which are capable of killing tumor cells. Cancer vaccines generally enhance the presentation of cancer antigens to both antigen presenting cells (*e.g.*, macrophages and dendritic cells) and/or to other immune cells such as T cells, B cells, and NK cells. Although cancer vaccines may take one of several forms, their purpose is to deliver cancer antigens and/or cancer associated antigens to antigen presenting cells (APC) in order to facilitate the endogenous processing of such antigens by APC and the ultimate presentation of antigen presentation on the cell surface in the context of MHC class I molecules. One form of cancer vaccine is a whole cell vaccine which is a preparation of cancer cells which have been removed from a subject, treated *ex vivo* and then reintroduced as whole cells in the subject. These treatments optionally involve cytokine exposure to activate the cells, genetic manipulation to overexpress cytokines from the cells, or priming with tumor specific antigens or cocktails of antigens, and expansion in culture. Dendritic cell vaccines activate antigen presenting cells directly, and their proliferation, activation and migration to lymph nodes is regulated by scaffold compositions to enhance their ability to elicit an immune response. Types of cancers to be treated include central nervous system (CNS) cancers, CNS Germ Cell tumor, lung cancer, Leukemia, Multiple Myeloma, Renal Cancer, Malignant Glioma, Medulloblastoma, and Melanoma.

For the purpose of eliciting an antigen-specific immune response, a scaffold device is implanted into a mammal. The device is tailored to activate immune cells and prime the cells with a specific antigen thereby enhancing immune defenses and destruction of undesired tissues and targeted microorganisms such as bacterial or viral pathogens. The device attracts appropriate immune cells, such as macrophages, T cells, B cells, NK cells, and dendritic cells, by containing and/or releasing signaling substances such as GM-CSF. These signaling substances are incorporated in the scaffold composition in such a way as to control their release spatially and temporally using the same techniques used to integrate other bioactive compounds in the scaffold composition.
Once the immune cells are inside the device, the device programs the immune cells to attack or cause other aspects of the immune system to attack undesired tissues (e.g., cancer, adipose deposits, or virus-infected or otherwise diseased cells) or microorganisms. Immune cell activation is accomplished by exposing the resident immune cells to preparations of target-specific compositions, e.g., ligands found on the surface of the undesired tissues or organisms, such as cancer cell surface markers, viral proteins, oligonucleotides, peptide sequences or other specific antigens. For example, useful cancer cell-specific antigens and other tissue or organism-specific proteins are listed in the table below.

The device optionally contains multiple ligands or antigens in order to create a multivalent vaccine. The compositions are embedded in or coated on the surface of one or more compartments of the scaffold composition such that immune cells migrating through the device are exposed to the compositions in their traverse through the device. Antigens or other immune stimulatory molecules are exposed or become exposed to the cells as the scaffold composition degrades. The device may also contain vaccine adjuvants that program the immune cells to recognize ligands and enhance antigen presentation. Exemplary vaccine adjuvants include chemokines/cytokines, CpG rich oligonucleotides, or antibodies that are exposed concurrently with target cell-specific antigens or ligands.

The device attracts immune cells to migrate into a scaffold where they are educated in an antigen-specific manner and activated. The programmed immune cells are then induced to egress towards lymph nodes in a number of ways. The recruitment composition and deployment signal/composition, e.g., a lymph node migration inducing substance, is released in one or more bursts, programmed by the method of incorporation and/or release from the scaffold material, or controlled by the sequential degradation of scaffold compartments which contain the attractant. When a burst dissipates, the cells migrate away. Compartments containing repulsive substances are designed to degrade and release the repulsive substance in one or more bursts or steadily over time. Relative concentration of the repulsive substances cause the immune cells to migrate out of the device. Alternatively, cells which have been placed in or have migrated into the device are programmed to release repulsive substances or to change their own behavior. For example, localized gene therapy is carried out by cell exposure to plasmid DNA attached to the scaffold. Useful repulsive substances include chemokines and cytokines. Alternatively, the device may cause immune cells to egress by degrading and releasing them.

Target disease states, stimulatory molecules and antigens useful in vaccine device construction are listed below.
Bioactive factors to promote immune responses

a. Interleukins: IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-15, IL-18 etc.
b. TNF-α
c. IFN-γ
d. IFN-α
e. GM-CSF
f. G-CSF
g. Flt-3 ligand
h. MIP-3 β (CCL19)
i. CCL21
j. M-CSF
k. MIF
l. CD40L
m. CD3

o. Anti CTLA-4 antibodies
p. TGF-β
q. CPG rich DNA or oligonucleotides
r. Sugar moieties associated with Bacteria: Lipopolysacharides (LPS) is an example

s. Fas ligand
t. Trail
u. Lymphotactin
v. Mannan (M-FP)
w. Heat Shock Proteins (apg-2, Hsp70 and Hsp 90 are examples)

iv. Breast cancer

1. MUC-1
2. Sosl
3. Protein kinase C-binding protein
4. Reverse trascriptase protein
5. AKAP protein
6. VRK1
7. KIAA1735
8. T7-1, T11-3, T11-9
v. Other general and specific purified cancer antigens

1. Homo Sapiens telomerase ferment (hTRT)
2. Cytokeratin-19 (CYFRA21-1)
3. SQUAMOUS CELL CARCINOMA ANTIGEN 1 (SCCA-1), (PROTEIN T4-A)
4. SQUAMOUS CELL CARCINOMA ANTIGEN 2 (SCCA-2)
5. Ovarian carcinoma antigen CA125 (1A1-3B) (KIAA0049)
6. MUCIN 1 (TUMOR-ASSOCIATED MUCIN), (CARCINOMA-ASSOCIATED MUCIN), (POLYMORPHIC EPITHELIAL MUCIN), (PEANUT-REACTIVE URINARY MUCIN), (PUM), (BREAST CARCINOMA-ASSOCIATED ANTIGEN DF3)
7. CTCL tumor antigen sel-1
8. CTCL tumor antigen sel4-3
9. CTCL tumor antigen se20-4
10. CTCL tumor antigen se20-9
11. CTCL tumor antigen se33-l
12. CTCL tumor antigen se37-2
13. CTCL tumor antigen se57-l
14. CTCL tumor antigen se89-l
15. Prostate-specific membrane antigen
16. 5T4 oncofetal trophoblast glycoprotein
17. Orf73 Kaposi's sarcoma-associated herpesvirus
18. MAGE-C1 (cancer/testis antigen CT7)
19. MAGE-B1 ANTIGEN (MAGE-XP ANTIGEN) (DAM10)
20. MAGE-B2 ANTIGEN (DAM6)
21. MAGE-2 ANTIGEN
22. MAGE-4a antigen
23. MAGE-4b antigen
24. Colon cancer antigen NY-CO-45
25. Lung cancer antigen NY-LU-12 variant A
26. Cancer associated surface antigen
27. Adenocarcinoma antigen ART1
28. Paraneoplastic associated brain-testis-cancer antigen (onconeuronal antigen MA2; paraneoplastic neuronal antigen)
29. Neuro-oncological ventral antigen 2 (NOVA2)
30. Hepatocellular carcinoma antigen gene 520
31. TUMOR-ASSOCIATED ANTIGEN CO-029
32. Tumor-associated antigen MAGE-X2
33. Synovial sarcoma, X breakpoint 2
34. Squamous cell carcinoma antigen recognized by T cell
35. Serologically defined colon cancer antigen 1
36. Serologically defined breast cancer antigen NY-BR-15
37. Serologically defined breast cancer antigen NY-BR-16
38. Chromogranin A; parathyroid secretory protein 1
39. DUPAN-2
40. CA 19-9
41. CA 72-4
42. CA 195
43. Carcinoembryonic antigen (CEA)

**Example 1: In situ Regulation of DC Subsets and T Cells Mediates Tumor Regression in Mice**

Vaccines are largely ineffective for patients with established cancer, as advanced disease requires potent and sustained activation of CD8+ cytotoxic T lymphocytes (CTLs) to kill tumor cells and clear the disease. Subsets of dendritic cells (DCs) specialize in antigen cross-presentation and in the production of cytokines, which regulate both CTLs and T regulatory (Treg) cells that shut down effector T cell responses. Coordinated regulation of a DC network, and plasmacytoid DCs (pDCs) and CD8+ DCs in particular, enhances host immunity in mice. Functionalized biomaterials incorporating various combinations of an inflammatory cytokine, immune danger signal, and tumor lysates were used to control the activation and localization of host DC populations in situ. The numbers of pDCs and CD8+ DCs, and the endogenous production of interleukin-12, all correlated strongly with the magnitude of protective antitumor immunity and the generation of potent CD8+ CTLs. Vaccination by this method maintained local and systemic CTL responses for extended periods while inhibiting FoxP3 Treg activity during antigen clearance, resulting in complete regression of distant and established melanoma tumors. The efficacy of this vaccine as a
monotherapy against large invasive tumors is a result of the local activity of pDCs and CD8+ DCs induced by persistent danger and antigen signaling at the vaccine site. These results indicate that a critical pattern of DC subsets correlates with the evolution of therapeutic antitumor responses. Provision of secondary immunostimulatory site of tumor antigen presentation allows one to manipulate the in situ generation of a heterogeneous DC network capable of CTL induction, and activate robust CD8 T cell effector responses to established tumors.


Implantable synthetic polymer matrices (antigen-loaded acellular biomaterial device) that spatially and temporally control the in vivo presentation of cytokines, tumor antigens, and danger signals were utilized herein. GM-CSF is released from these polylactide-co-glycolide (PLG) [a Food and Drug Administration (FDA)-approved biomaterial] matrices into the surrounding tissue to recruit DC precursors and DCs. CpG-rich oligonucleotides are immobilized on the matrices as danger signals, and antigen (tumor lysates) is released to
matrix-resident DCs to program DC development and maturation. These matrices quantitatively regulate DC activation and trafficking in situ and induce prophylactic immunity against inoculations of murine B16-F10 melanoma cells (P. Schnorrer, G. M. Behrens, N. S. Wilson, J. L. Pooley, C. M. Smith, D. El-Sukkari, G. Davey, F. Kupresanin, M. Li, E. Maraskovsky, G. T. Belz, F. R. Carbone, K. Shortman, W. R. Heath, J. A. Villadangos, The dominant role of CD8+ dendritic cells in cross-presentation is not dictated by antigen capture. Proc. Natl. Acad. Sci. U.S.A. 103, 10729-10734 (2006)). As described herein, this system administered repeatedly over time to controls the recruitment and activation of multiple DC and T cell subsets and is effective as a therapeutic vaccine against established tumors.

The following materials and methods were used to generate the data described herein.

**Matrix fabrication**

An 85:15, 120-kD copolymer of ε,ε-L-lactide and glycolide (PLG) (Alkermes) was utilized in a gas-foaming process to form porous PLG matrices (L. D. Harris, B. S. Kim, D. J. Mooney, Open pore biodegradable matrices formed with gas foaming. J. Biomed. Mater. Res. 42, 396-402 (1998)). In brief, PLG microspheres encapsulating GM-CSF were first made with standard double emulsion (S. Cohen, T. Yoshioka, M. Lucarelli, L. H. Hwang, R. Langer, Controlled delivery systems for proteins based on poly(lactic/glycolic acid) microspheres. Pharm. Res. 8, 713-720 (1991)). PLG micro-spheres were then mixed with 150 mg of the porogen, sucrose (sieved to a particle size between 250 and 425 mm), and compression molded. The resulting disc was allowed to equilibrate within a high-pressure CO₂ environment, and a rapid reduction in pressure causes the polymer particles to expand and fuse into an interconnected structure. The sucrose was leached from the scaffolds by immersion in water, yielding scaffolds that were 90% porous. To incorporate tumor lysates into PLG scaffolds, the biopsies of B16-F10 tumors that had grown subcutaneous ly in the backs of C57BL/6J mice (Jackson Laboratory) were digested in collagenase (250 U/ml) (Worthington) and suspended at a concentration equivalent to 10⁷ cells per milliliter after filtration through 40-μm cell strainers. The tumor cell suspension was subjected to four cycles of rapid freeze in liquid nitrogen and thaw (37°C) and then centrifuged at 400 rpm for 10 min. The supernatant (1 ml) containing tumor lysates was collected, incubated with the PLG microspheres, and lyophilized, and the resulting mixture was utilized in the high-pressure CO₂ process to foam macroporous PLG matrices incorporating tumor lysates. To incorporate CpG-ODNs into PLG scaffolds, CpG-ODN 1826, 5’-tcctacagttcctgaagg-3’ (Invivogen) was condensed with PEI (Mₙ -60,000) molecules by dropping ODN 1826
solutions into PEI solution while vortexing the mixture (L. D. Harris, B. S. Kim, D. J. Mooney, Open pore biodegradable matrices formed with gas foaming. J. Biomed. Mater. Res. 42, 396-402 (1998); S. Cohen, T. Yoshioka, M. Lucarelli, L. H. Hwang, R. Langer, Controlled delivery systems for proteins based on poly(lactic/glycolic acid) microspheres. Pharm. Res. 8, 713-720 (1991); Y. C. Huang, M. Connell, Y. Park, D. J. Mooney, K. G. Rice, Fabrication and in vitro testing of polymeric delivery system for condensed DNA. J. Biomed. Mater. Res. A 67, 1384-1392(2003)). The charge ratio between PEI and CpG-ODN (NH$_3$\textsuperscript{+}:PO$_4$\textsuperscript{2-}) was kept constant at 7 during condensation. PEI-CpG-ODN condensate solutions were then vortexed with 60 μl of 50% (w/v) sucrose solution, lyophilized, and mixed with dry sucrose to a final weight of 150 mg. The sucrose containing PEI-CpG-ODN condensate was then mixed with blank, GM-CSF, and/or tumor lysate-loaded PLG microspheres to make PLG cancer vaccines.

**In situ identification of DC subsets and T cells**

Blank PLG matrices and matrices containing 3000 ng of GM-CSF alone or in combination with either 1, 10, 50, or 100 μg of CpG-ODN (studies were also performed with tumor lysates copresented with either 3000 ng of GM-CSF or 100 μg of CpG-ODN alone or in combination) were implanted into subcutaneous pockets on the back of 7- to 9-week-old male C57BL/6J mice. For histological examination, scaffolds were excised and fixed in Z-fix solution (Anatech), embedded in paraffin, and stained with hematoxylin and eosin (H&E). To analyze DC recruitment, scaffolds were excised at various time points and digested the ingrown tissue into single-cell suspensions with a collagenase solution (250 U/ml; Worthington) that was agitated at 37°C for 45 min. The cell suspensions were then poured through a 40-μm cell strainer to isolate cells from scaffold particles, and the cells were pelleted and washed with cold PBS and counted with a Z2 coulter counter (Beckman Coulter). To assess DC infiltration and activation, subsets of the total cell population isolated from PLG matrices were stained with primary antibodies (BD Pharmingen) conjugated to fluorescent markers to allow for analysis by flow cytometry. Allophycocyanin (APC)-conjugated CD11c (DC marker) and phycoerythrin (PE)-conjugated CD86 (B7, costimulatory molecule) stains were conducted for DC recruitment analysis, and APC-conjugated CD11c, fluorescein isothiocyanate (FITC)-conjugated CCR7, and PE-conjugated MHCII stains were conducted for DC programming analysis. To further delineate the presence of specific DC subsets, cells were stained with APC-conjugated CD11c and PE-conjugated PDCA-1 (pDC marker), APC-conjugated CD11c and PE-conjugated CD8 (CD8 DCs), or APC-conjugated CD11c and FITC-conjugated CD11b (CD11b DCs). To assess T
cell infiltration, PE-Cy7-conjugated CD3 stains were performed in conjunction with APC-conjugated CD8a (CD8 T cells), FITC-conjugated CD4 (CD4 T cells), and PE-conjugated FoxP3 (Treg) and analyzed with flow cytometry. Cells were gated according to positive FITC, APC, and PE with isotype controls, and the percentage of cells staining positive for each surface antigen was recorded.

**Tumor growth assays, protective cytokines, and TRP2 pentamer analysis**

PLG scaffolds with melanoma tumor lysates and various dosages of GM-CSF and/or various quantities of PEI-CpG-ODN condensates were implanted subcutaneously into the lower left flank of C57BL/6J mice. For prophylactic vaccinations, animals were challenged 14 days later with a subcutaneous injection of 10⁵ B16-F10 melanoma cells [American Type Culture Collection (ATCC)] in the back of the neck. Animals were monitored for the onset of tumor growth (~1 mm³) and killed for humane reasons when tumors grew to 20 to 25 mm (longest diameter).

To assess PLG vaccine efficacy in the therapeutic setting, C57BL/6J mice were challenged with a subcutaneous injection of 5 × 10⁵ B16-F10 melanoma cells (ATCC) in the back of the neck. At either day 9 or day 13 after tumor challenge, PLG vaccines loaded with 3000 ng of GM-CSF, 100 μg of CpG-ODN, and tumor lysates were implanted subcutaneously into the lower left flank of C57BL/6J mice. A subset of mice was vaccinated again at 10 days after the initial vaccination (days 19 and 23).

To determine *in vivo* IL-12p70, IFN-a, IFN-γ, and TGF-β concentrations at the matrix implant site, the adjacent tissue was excised and digested with tissue protein extraction reagent (Pierce). After centrifugation, the concentrations of IL-12, IFN-α, IFN-γ, and TGF-β in the supernatant were then analyzed with enzyme-linked immunosorbent assay (R&D Systems) according to the manufacturer’s instructions.

To determine the generation of TRP2-specific CTLs, single-cell suspensions were prepared from the spleens of mice immunized with PLG vaccines (lysat e + 3000 ng of GM-CSF + 100 μg of CpG) at various time points. These cells were initially stained with APC-H-2Kb-TRP2 pentamers (Proimmune) and subsequently stained with PE-conjugated monoclonal antibody to CD8 (BD Pharmingen) before being analyzed by flow cytometry.

The data indicate that an implanted copolymer matrix (antigen-loaded acellular biomaterial device) that incorporates inflammatory cytokines, immune danger signal, and tumor antigens elicits an immune response network that eradicates established tumors *in vivo.*
**Statistical analysis**

All values in the present study were expressed as mean ± SD. The significant differences between the groups were analyzed by a Student's t test and a P value of <0.05 was considered significant.

**Local GM-CSF delivery promotes recruitment of CD11b+ DCs**

Macroporous PLG matrices were fabricated for GM-CSF release to recruit DCs and with an interconnected porous structure facilitates cell infiltration. Matrices were loaded with 0, 3000, and 7000 ng of GM-CSF and implanted into the subcutaneous pockets of C57BL/6J mice. Histological analysis at day 14 after implantation of PLG matrices loaded with 3000 ng of GM-CSF revealed enhanced cellular infiltration when compared to blank controls (Figure 1A). Fluorescence-activated cell sorting (FACS) analysis for CD11c DCs showed that GM-CSF delivery recruited significantly more DCs (a factor of ~8 increase) than blank PLG matrices (Figure 1B). The matrix-resident DCs were almost exclusively CD11b+ (~87%), in accordance with other studies of GM-CSF effects on DC recruitment in vivo (N. Mach, S. Gillessen, S. B. Wilson, C. Sheehan, M. Mihm, G. Dranoff, Differences in dendritic cells stimulated in vivo by tumors engineered to secrete granulocyte-macrophage colonystimulating factor or Flt3-ligand. Cancer Res. 60, 3239-3246 (2000); E. Daro, B. Pulendran, K. Brasel, M. Teepe, D. Pettit, D. H. Lynch, D. Vremec, L. Robb, K. Shortman, H. J. McKenna, C. R. Maliszewski, E. Maraskovsky, Polyethylene glycolmodified GM-CSF expands CD11bhighCD11chigh but not CD11biowCD11chigh murine dendritic cells in vivo: A comparative analysis with Flt3 ligand. J. Immunol. 165, 49-58 (2000)). The total number of DCs recruited and their expression of the costimulatory molecule CD86 increased with GM-CSF delivery in a dose-dependent manner (Figure 1D). However, the highest dose (7000 ng) of GM-CSF reduced the number of activated DCs at the implant site, as indicated by diminished major histocompatibility complex class II (MHCII) and CCR7 expression at day 14 after implantation (Figure 1D and Figure 8). Because total DC recruitment and activation both peaked at 3000 ng of GM-CSF, this dose was utilized to recruit and generate DCs. GM-CSF delivery promoted greater cellular penetration into and association with the PLG material, as indicated by histological analysis (Figure 8) and measurement of DC numbers (Figure 1, B and D), allowing for the subsequent programming of resident DC precursors and DCs.
**In situ delivery of CpG-oligodeoxynucleotide promotes pDC recruitment and IFN production**

The ability of local presentation of danger signals to regulate the ratio of distinct DC subtypes was next examined by immobilizing TLR-activating, polyethylenimine (PEI)-condensed CpG-oligodeoxynucleotide (ODN) molecules into the matrices. Condensation of oligonucleotides with the polycationic polymer PEI results in positively charged particles that bind electrostatically to the anionic PLG matrix. PLG matrices incorporating CpG-ODN alone recruited CD11c⁺-PDCA-1⁺-pDCs to the PLG matrix (Figure 2A). This effect was enhanced with coadministration of GM-CSF (Figure 2B). The dose of CpG-ODN presented in combination with 3000 ng of GM-CSF was altered to regulate the numbers of resident pDCs, resulting in 190,000, 520,000, and 1,200,000 cells at doses of 0, 10, and 100 µg of CpG-ODN, respectively (Figure 2B). Copresentation of CpG-ODN had little effect on the ability of GM-CSF to enhance CD11c⁺-CD11b⁺ cDCs (Figure 2C). High doses of CpG-ODN promoted the local production of IFN-α (-10¹⁰ pg/ml) and IFN-γ (-600 pg/ml) independently of the presence of GM-CSF (Figure 2, E and F). These results indicate that controlled GM-CSF and CpG-ODN danger signaling from synthetic extracellular matrices cooperates to regulate resident pDC and CD11c⁺CD11b⁺ cDC numbers, along with the production of protective cytokines commonly linked to TH1 and CTL immunity.

**Tumor lysate co-delivery with CpG-ODN and GM-CSF stimulates CD8⁺ generation and IL-12 production**

Experiments were carried out to determine whether co-presenting cancer antigens with CpG-ODNs to matrix-resident DCs would promote further DC development, activation, and CTL antigen sensitization. In this context, necrotic tumor cells may be particularly immunostimulatory, as they release a variety of endogenous mediators (for example, heat shock proteins and damaged nucleic acids) that trigger innate immune recognition (C. Fonseca, G. Dranoff, Capitalizing on the immunogenicity of dying tumor cells. *Clin. Cancer Res.* 14, 1603-1608 (2008)). Thus, freeze-thaw lysates of B16 melanomas were prepared, and antigen-presenting matrices were fabricated by encapsulating these lysates into the PLG material, resulting in localized and sustained antigen presentation to the infiltrating cell population (O. A. Ali, N. Huebsch, L. Cao, G. Dranoff, D. J. Mooney, Infection-mimicking materials to program dendritic cells in situ. *Nat. Mater.* 8, 151-158 (2009)). These antigen-presenting matrices unexpectedly stimulated CD8⁺ DC generation in situ (Figure 3A). On viral invasion, CD8⁺CD11c⁺ cDCs are especially efficient at cross-presenting exogenous antigen on MHCII molecules (J. D. Farrar, H. Asnagli, K. M. Murphy, T helper subset
development: Roles of instruction, selection, and transcription. *J. Clin. Invest.* 109, 431-435 (2002); D. Skokos, M. C. Nussenzweig, CD8 DCs induce IL-12-independent Th1
differentiation through Delta 4 Notch-like ligand in response to bacterial LPS. *J. Exp. Med.*
204, 1525-1531 (2007); J. M. den Haan, S. M. Lehar, M. J. Bevan, CD8+ but not CD8-
and at producing the T_{H1}-promoting cytokine IL-12 (M. Moser, K. M. Murphy, Dendritic cell
regulation of TH1-TH2 development. *Nat. Immunol.* 1, 199-205 (2000); D. Jankovic, M. C.
Kullberg, S. Hieny, P. Caspar, C. M. Collazo, A. Sher, In the absence of IL-12, CD4+ T cell
responses to intracellular pathogens fail to default to a Th2 pattern and are host protective in
an IL-10+ setting. *Immunity* 16, 429-439 (2002); V. E. Schijns, B. L. Haagmans, C. M.
Wierda, B. Kruithof, I. A. Heijnen, G. Alber, M. C. Horzinek, Mice lacking IL-1 2 develop
polarized Thl cells during viral infection. *J. Immunol.* 160, 3958-3964 (1998); J. Magram, J.
Sfarra, S. Connaughton, D. Faherty, R. Warrier, D. Carvajal, C. Y. Wu, C. Stewart, U.
Sarmiento, M. K. Gately, IL-12-deficient mice are defective but not devoid of type 1 cytokine
responses. *Ann. N.Y. Acad. Sci.* 795, 60-70 (1996), which are two mechanisms that aid in
priming CTL immunity to viruses and tumors. This activity, however, is normally associated
with lymphoid tissues. Co-presentation of tumor lysates with CpG-ODN led to the presence
of 200,000 CD8+ DCs, which increased to ~670,000 (a factor of 9 increase over blank
matrices) when GM-CSF was added to stimulate recruitment (Figure 3B). Additionally,
tumor lysate in combination with GM-CSF and CpG enhanced the numbers of recruited
pDCs at day 10 after implantation by a factor of 2 over matrices without lysate and by a
factor of 10 over blank controls (Figure 3C). No significant difference in pDC numbers was
observed with tumor lysate in combination with only GM-CSF or CpG signaling. The
CD1 c+CD1 b+DC population at the vaccine site depended on GM-CSF alone (Figure 3D),
as tumor lysate or CpG signaling alone or in combination had no significant effect on the
recruitment and expansion of these DCs (Figure 3D).

*In situ* production of the T cell growth factor IL-12 at matrices that deliver both tumor
lysate and CpG-ODN to cell populations recruited by GM-CSF was about four times at blank
matrices and at least twice at all other matrix formulations (Figure 3E). However, tumor
lysates in the matrix did not increase the high concentrations of IFN-a and IFN-γ induced by
CpG-ODN alone (Figure 3, F and G). These results indicate that the engineered matrices
manipulated both the number and the function of specific DC subsets, as well as the
accompanying CTL-polarizing activity.
PLG matrices co-delivering GM-CSF, CpG-ODN, and tumor lysates stimulate potent local and systemic CD8+ cytotoxic T cells

To elucidate the adaptive immune mechanisms induced by PLG vaccines that deliver tumor lysate, GM-CSF, and CpG-ODN, the activity of both local and systemic CTLs was examined. Flow cytometric analysis of cells infiltrating the vaccine site revealed a significant CD3+CD8+ T cell response by day 5 (representative sample: -1.9 × 10^5 cells), which peaked at day 12 when a relatively large proportion of the matrix-resident cells were CTLs (representative sample: 8.5% of cells; -8.5 × 10^5 cells) (Figure 4A). Local CD8+ T cell numbers dropped sharply by day 16 and were negligible at day 21 (Figure 4B) likely because of antigen clearance. PLG vaccines containing tumor lysates, GM-CSF, and CpG-ODN preferentially tuned and promoted CD8+ cytotoxic immune responses relative to other matrix formulations devoid of CpG (Figure 4C). Further, the activation and persistence of systemic CTL responses was monitored by staining splenocytes with MHCII-tyrosinase-related protein 2 (TRP2) peptide pentamers to identify CTLs with specificity to TRP2, which is a major antigenic target of melanoma vaccines in mice and humans. A significant expansion of TRP2-specific CTLs was observed in the spleens of vaccinated mice by day5, which continued and peaked between days 7 and 16 before falling at days 21 to 28 (Figure 4, D and E), indicating that systemic anti-melanoma responses were being generated and sustained for extended periods.

Tumor protection induced by PLG matrices correlated with DC subsets and IL-12 production

This system is capable of generating prophylactic immunity against poorly immunogenic B16-F10 melanoma (O. A. Ali, N. Huebsch, L. Cao, G. Dranoff, D. J. Mooney, Infection-mimicking materials to program dendritic cells in situ. Nat. Mater. 8, 151-158 (2009)). The relation of this antitumor efficacy to the specific DC networks invoked by various vaccine formulations was investigated. C57BL/6J mice were vaccinated with PLG-based matrices incorporating B16 tumor lysates, GM-CSF, and CpG-ODN in varying combinations and then challenged with live B16-F10 melanoma tumor cells at day 14 after vaccination. PLG vaccines with both B16- F10 tumor lysates and either 1, 10, 50, or 100 mg doses of CpG-ODN danger signaling allowed 10 to 30% of the vaccinated mice to survive, tumor-free (Figure 5A), after an otherwise lethal cell challenge, whereas 100% of unvaccinated mice were killed by day 23 due to tumor burden. When GM-CSF-mediated DC recruitment was combined with lysate and CpG-ODN delivery, the mice showed...
significant protection from tumor-induced lethality. CpG-ODN doses of 10, 50, and 100 µg resulted in 50%, 60%, and 90% survival rates, respectively (Figure 5B).

The ability of vaccine systems to create a heterogeneous DC population correlated with the marked increase in antitumor efficacy. In comparison to antigen matrices delivering GM-CSF alone, the antigen-loaded matrices delivering CpG and GM-CSF together resulted in a higher proportion of pDCs (~31% versus 7%) and CD8+ cDCs (~14% versus 5.5%) (Figure 5C), which correlated with a significant enhancement in mouse survival (90% versus 20%), although total DC numbers in situ were statistically similar (3.0 ± 0.6 versus 4.2 ± 0.9 million DCs; two-tailed Student's t test, n = 5). Survival rates were proportional to the number of pDCs and CD8+ cDCs, but not CD11b+ DCs, generated at the PLG vaccine site at day 10 (Figure 5, D to F). Additionally, the endogenous production of IL-12 was correlated with animal survival (Figure 5G), indicating the importance of cross-presentation and T<sub>H1</sub>-promoting cytokines to vaccine efficacy.

**Engineered PLG matrices incorporating CpG-ODN attenuate immune regulation by FoxP3 Treg number and immunosuppressive cytokines**

with GM-CSF and CpG revealed peak activity at days 5 and 7, which decreased to negligible concentrations by day 12 after implantation (Figure 6A). By contrast, matrices containing GM-CSF and tumor lysate led to a significant enhancement of CD4 T cell infiltration at day 12 (Figure 6B), and these cells likely contribute to regulation of CTL responses.

Incorporation of GM-CSF and tumor lysate into the vaccine matrix led to a factor of 10 increase in TGFP concentrations (Figure 6C) and a significant increase in IL-10 (Figure 6D) at the vaccine site; these are cytokines commonly associated with Treg activity and immunosuppression. Further, as observed previously in GM-CSF-based vaccines, GM-CSF cosignaling with tumor antigens resulted in a significant CD3+FoxP3+ response at the vaccine site (Figure 6, E and F) when compared to all other matrix formulations, resulting in an almost even ratio of CD8+ effectors and FoxP3 Treg cells (Figure 6G). CpG-ODN presentation in concert with both tumor lysate and GM-CSF counteracted these immunosuppressive mechanisms, as TGFP and IL-10 concentrations and Treg activity were not enhanced over the control matrices, and CD8 CTLs outnumbered FoxP3 T cells by a factor of ~25 at day 12 after implantation (Figure 6, C to G). These findings indicate that the vaccine system promotes and extends CTL responses through naïve T cell differentiation induced by pDCs and CD8+ DCs, the corresponding production of type 1 IFNs and IL-12, and inhibition of negative feedback mechanisms.

**Engineered PLG matrices cause regression of established melanoma**

Because a high ratio of CD8+ T cell effectors to FoxP3+ Treg cells has been linked to therapeutic tumor immunity in murine and human systems (F. S. Hodi, M. Butler, D. A. Oble, M. V. Seiden, F. G. Haluska, A. Kruse, S. Macrae, M. Nelson, Canning, I. Lowy, A. Korman, D. Lautz, S. Russell, M. T. Jaklitsch, N. Ramaiya, T. C. Chen, D. Neuberger, J. P. Allison, M. C. Mihm, G. Dranoff, Immunologic and clinical effects of antibody blockade of cytotoxic T lymphocyte-associated antigen 4 in previously vaccinated cancer patients. *Proc. Natl. Acad. Sci. U.S.A.* 105, 3005-3010 (2008)), the activity of PLG vaccines against B16-F10 tumors that had been established for 9 days (inoculation of 5 × 10^5 cells at day 0) was tested. Tumor-bearing mice implanted with blank PLG matrices displayed rapid tumor growth and were killed by day 24 (Figure 7A). Vaccination of mice once with the PLG vaccine significantly decreased the rate of tumor progression (Figure 7A), and an increase in mean survival time over controls was observed, but all animals required euthanization by day 58, depending on the tumor size at the time of vaccination (Figure 7B).

Vaccination of mice twice (days 9 and 19) with PLG vaccines had a more dramatic effect on tumor progression and caused complete regression of tumors in a subset (7 of 15) of
the animals (Figure 7C). By contrast, a single treatment with irradiated, GM-CSF-secreting
B16-F10 cells, a widely used cell therapy now in clinical trials, attenuated tumor progression
modestly, and all animals had to be killed by day 36 (Figure 7A). Tumor antigen
presentation from PLG matrices enhanced protection and was required to induce tumor
regression, as matrices with GM-CSF and CpG did not enhance survival times significantly
(Figure 7, A and B). Strikingly, 47% of the mice (animals bearing day 9 tumors) vaccinated
twice with PLG vaccines survived long-term and free of detectable tumors; this treatment
regimen was able to completely eradicate tumors of up to 25 mm² in size (Figure 7C).

To test whether PLG vaccines could be effective against an even greater tumor
burden, melanoma tumors were established for 13 days and then mice were vaccinated. One-
time (day 13) and two-time (days 13 and 23) vaccination decreased tumor progression
(Figure 7D). Two-time vaccination doubled the mean survival time and led to complete
tumor regression in 20% of the animals with advanced solid tumors (day 13 tumors; n = 15)
(Figure 7, D to F). Because vaccinations were initiated at days 9 and 13 of tumor growth,
and required 5 days for CTL generation, the effector window for immune responses was
small (about 6 to 10 days) before untreated animals succumbed to tumor burden. Variations
in tumor size at the time of vaccination likely accounted for the lack of complete regression
in all animals; vaccination may not have resulted in generation of sufficient numbers of killer
T cell in time to control and clear larger tumors. Slight hair loss and depigmentation was
observed at the vaccine site, in agreement with the past study using this vaccination system,
but no significant toxicities were observed with vaccination.

Taken together, by implanting two vaccines, 10 days apart, this formulation was able
to completely clear large and established melanoma tumors in the aggressive murine model
(up to 25 mm²). This vaccine efficacy, as a monotherapy against invasive and large tumors,
is unprecedented. Sustained antigen presentation at an immunostimulatory site was required
during the clearance of the tumor by the immune system, as single vaccinations were unable
to maintain tumor regression for long enough periods (complete regressions for 2
vaccinations occurred over 34 days for 25 mm² tumors). The engineered matrices program T
cell responses efficiently by providing a site of sustained, immunostimulatory tumor antigen
presentation after the primary induction of innate immunity and stimulation has occurred.
Only vaccination times (greater than 3 days) that included the maintenance of effector CTL
responses resulted in the regression of solid tumors and high survival rates (>80%). Other
experimental vaccine formulations achieve only short-lived stimulation with infusions of
protein capable of stimulating only the components of innate immunity (i.e. DCs).
Cancer vaccination to decrease established tumors


As described herein, engineered PLG vaccines evoke a coordinated response of multiple DC subtypes, which together trigger sustained and potent antitumor CD8+ CTLs while inhibiting immunoregulatory pathways. The combination of tumor cell lysates, GM-CSF, and CpG-ODN in the vaccine matrix was required for optimal tumor protection, which was strongly associated with the recruitment of pDCs and CD8+ DCs and the local production of IL-12. The accumulation of CD8+ DCs at the vaccine site is a notable feature of this vaccination strategy because this DC subset is typically localized to secondary lymphoid structures. Plasmacytoid DC numbers were closely linked with the generation of type I IFNs (H. Kanzler, F. J. Barrat, E. M. Hessel, R. L. Coffman, Therapeutic targeting of innate immunity with Toll-like receptor agonists and antagonists. Nat. Med. 13, 552-559 (2007)), and these cells helped to support the activation of CD8 DCs and their cross-presentation of tumor antigens to TH1 cells and CTLs. The data demonstrate that the presence of a broader set of DC subtypes evokes more potent antitumor responses.

The findings also suggest that a minimum number of DCs are required to induce high concentrations of protective immunity. Vaccines that generated about 1,200,000 pDCs and 600,000 CD8+ DCs (-43% of total DCs) in a total population of ~4.2 million DCs resulted in 90% survival in a subsequent tumor challenge. The engineered matrices appear to program T
cell responses efficiently by providing a site of sustained immunostimulatory tumor antigen presentation, which evokes robust CTLs, both locally and systemically, and attenuates immune regulation mediated through TGF-β, IL-10, and FoxP3+ Treg cells. The kinetics of the adaptive immune response to this system suggest that CTLs manifested potent effector function, as vaccination resulted in a prototypical activation phase that gradually plateaued, followed by a contraction phase as antigen was cleared. Other vaccine formulations achieve only short-lived stimulation with infusions of protein or manipulated cells and may not trigger this T effector profile, but instead induce at least partially dysfunctional T cells that are more likely to undergo exhaustion within the immunosuppressive tumor microenvironment.

The results highlight a critical array of DC subtypes that are generated during the evolution of therapeutic antitumor responses in mice, which provide a template for rational vaccine design. The vaccine system described herein is useful to modulate DC and CTL responses for the control of other solid cancers and perhaps chronic infections. The approach also facilitates the study of DC subset development and the mechanisms through which these subsets are coordinated in vivo for the eradication of established diseases. It is striking that tumor regression induced by these PLG vaccines outperformed gene-modified tumor cell vaccines in direct comparison and outperformed ex vivo DC vaccines reported in literature. This acellular biomaterial system was designed with components that either are FDA approved (PLG and GM-CSF) or have been utilized clinically (CpG-ODN) and do not require the maintenance and modification of live cell cultures. This PLG system has considerable advantages in terms of clinical application as compared to other approaches. Scaling to humans does not require significant modification of the size or structure of the material but simply require utilizing effective human analogs (for example, human GM-CSF and CpG-ODN sequences) that evoke human DC and CTL responses.

Example 2: Relationship of Vaccine Efficacy to the Kinetics of DC and T Cell Responses

Induced by PLG-Based Cancer Vaccines

Cancer vaccines are typically formulated for bolus injection and often produce short-lived immunostimulation resulting in poor temporal control over immune cell activation, and weak oncolytic activity. One means of overcoming these limitations utilizes immunologically active biomaterial constructs. Antigen-laden, macroporous PLG scaffolds induce potent dendritic cell (DC) and cytotoxic T-lymphocyte (CTL) responses via the controlled signaling of inflammatory cytokines, antigen and toll-like receptor agonists. Described herein are the kinetics of these responses and their fundamental relationship to
potent tumor rejection when implanted subcutaneously in a mouse B16 model of melanoma. By explanting scaffolds from mice at times ranging from 1-7 days a seamless relationship was observed between the production of controlled CTL responses, tumor growth and long-term survival in both prophylactic and therapeutic models. Scaffolds must be implanted for >7 days to augment CTL responses via the prolonged presentation of tumor antigen, and the benefits included a notable regression of established tumors. Host DC infiltration into the porous material persisted for 12 days (peaking at day 5 ~1.4x10^6 cells), and a sharp attenuation in DC numbers coincided with the peak of the CD8+ CTL response at day 12 (~8x10^5 cells). Importantly, these PLG systems enhanced DC numbers in the draining lymph node resulting in increased CD8(+) CTL subsets at days 10-16 of vaccination. These results indicate that material systems finely control innate and adaptive immune cell responses to kill typically untreated melanoma tumors and provide critical kinetic data for the design of vaccine carriers.

The following materials and methods were used to generate the data described herein.

Matrix fabrication

A 85:15, 120 kD copolymer of D.L-lactide and glycolide (PLG) (Lakeshore Biomaterials, Birmingham, Al) was utilized in a gas-foaming process to form porous PLG matrices (Harris, L.D., Kim, B.S., and Mooney, D.J. Open pore biodegradable matrices formed with gas foaming. J. Biomed.Mater. Res. 42,396-402 (1998)). In brief, PLG microspheres encapsulating GM-CSF were first made using a standard double emulsion process (Cohen S., Yoshioka T., Lucarelli, M., Hwang L.H., and Langer R. Controlled delivery systems for proteins based on poly(lactic/glycolic acid) microspheres. Pharm. Res. 8,713-720 (1991)). PLG microspheres were then mixed with 150 mg of the porogen, sucrose (sieved to a particle size between 250 µm and 425 µm), and compression molded. The resulting disc was allowed to equilibrate within a high-pressure C0₂ environment, and a rapid reduction in pressure causes the polymer particles to expand and fuse into an interconnected structure. The sucrose was leached from the scaffolds by immersion in water yielding scaffolds that were 90% porous. To incorporate tumor lysates into PLG scaffolds, biopsies of B16-F10 tumors that had grown subcutaneously in the backs of C57BL/6J mice (Jackson Laboratory, Bar Harbor Maine), were digested in collagenase (250 U/ml) (Worthington, Lakewood, NJ) and suspended at a concentration equivalent to 10^7 cells per ml after filtration through 40 µm cell strainers. The tumor cell suspension was subjected to 4 cycles of rapid freeze in liquid nitrogen and thaw (37°C) and then centrifuged at 400 rpm for 10 min. The
supernatant (1ml) containing tumor lysates was collected, incubated with the PLG microspheres and lyophilized and the resulting mixture was used to make PLG scaffold-based cancer vaccines. To incorporate CpG-ODNs into PLG scaffolds, CpG-ODN 1826, 5'-tcg atg acg ttc ctg acg tt-3', (Oligofactory, Holliston, MA) was first condensed with poly(ethylenimine) (PEI, M₁ -60,000, Sigma Aldrich) molecules by dropping ODN-1826 solutions into PEI solution, while vortexing the mixture. The charge ratio between PEI and CpG-ODN (NH₃+:P04-) was kept constant at 7 during condensation. PEI-CpG-ODN condensate solutions were then vortexed with 60 μl of 50% (wt/vol) sucrose solution, lyophilized and mixed with dry sucrose to a final weight of 150 mg. The sucrose containing PEI-CpG-ODN condensate was then mixed with blank, GM-CSF and/or tumor lysate loaded PLG microspheres to make PLG cancer vaccines.

**In situ identification of DC and T cell infiltration**

Blank PLG matrices and matrices containing 3000ng GM-CSF alone or in combination with either 1, 10, 50, or 100 μg CpG-ODN (studies were also performed with tumor lysates co-presented with either 3000ng GM-CSF or 100 μg CpG-ODN alone or in combination) were implanted into subcutaneous pockets on the back of 7-9 week old male C57BL/6J mice. For histological examination scaffolds were excised and fixed in Z-fix solution, embedded in paraffin, and stained with hematoxylin and eosin. For SEM analysis, matrices were sputter coated with gold prior to visualization.

To analyze DC and T cell recruitment, scaffolds were excised at various time-points and the ingrown tissue was digested into single cell suspensions using a collagenase solution (Worthington, 250 U/ml) that was agitated at 37°C for 45 minutes. The cell suspensions were then poured through a 40μm cell strainer to isolate cells from scaffold particles and the cells were pelleted and washed with cold PBS and counted using a Z2 coulter counter (Beckman Coulter). To assess DC infiltration, subsets of the total cell population isolated from PLG matrices were then stained with primary antibodies (BD Pharmingen, San Diego, CA) conjugated to fluorescent markers to allow for analysis by flow cytometry. APC-conjugated CD11c (dendritic cell marker) stains were conducted for DC recruitment analysis. To assess T cell infiltration, PE-Cy7 conjugated CD3 stains were performed in conjunction with APC-conjugated CD8a (CD8 T cells), FITC-conjugated CD4 (CD4 T cells) and analyzed with flow cytometry. Cells were gated according to positive FITC, APC and PE using isotype controls, and the percentage of cells staining positive for each surface antigen was recorded.
**Tumor growth assays, and in situ cytokine concentrations**

PLG-based vaccines were developed utilizing matrices incorporating melanoma tumor lysates, 3,000ng GM-CSF and 100 µg PEI-CpG-ODN condensates and were implanted subcutaneously into the lower left flank of C57BL/6J mice. For prophylactic vaccinations, animals were challenged 14 days later with a subcutaneous injection of $10^5$ B16-F10 melanoma cells (ATCC, Manassas, NJ) in the back of the neck. To assess the effect of the duration of vaccination, PLG vaccines were explanted at days, 1, 3, 7, 12, and 16 days after implantation. Animals were monitored for the onset of tumor growth (approximately $1mm^3$) and sacrificed for humane reasons when tumors grew to 20 - 25 mm (longest diameter).

To determine in vivo GM-CSF concentrations in adjacent tissue and IL-12p70, and IFN-γ, concentrations at the matrix implant site excised tissue was digested with tissue protein extraction reagent (Pierce). After centrifugation, the concentration of GM-CSF, IL-12p70, and IFN-γ in the supernatant was then analyzed with ELISA (R&D systems), according to the manufacturers' instructions.

**Statistical analysis**

All values in the present study were expressed as mean ± S.D. The significant differences between the groups were analyzed by a Student's t test and a P value of less than 0.05 was considered significant.

**Effect of vaccine duration on vaccine efficacy**

Macroporous PLG scaffolds were fabricated to control the presentation of GM-CSF, tumor lysate (B16 melanoma), and CpG-ODN to serve as vaccines (PLG vaccines) (Figure 9A &B). The duration of vaccination using PLG vaccines was controlled by explanting the material system at various timepoints. The relationship between vaccine duration and efficacy was examined in prophylactic and therapeutic B16-F10 melanoma models. PLG vaccines were implanted subcutaneously into the backs of C57BL/6J mice and removed at days 1, 3, 7, 12 and 16 after implantation and these mice were challenged at day 14 with an otherwise lethal dose of B16-F10 cells (Figure 9C).

By explanting scaffolds from mice at times ranging from 1-7 days a seamless relationship was observed between the production of controlled CTL responses and tumor growth and long-term survival in both prophylactic and therapeutic melanoma models (Figure 9D-9F). When the scaffold was implanted for > 7 days these benefits included a notable regression of large, established tumors and survival rates of up to 80% (Figure 9D-9F). Prophylactic vaccination times of less than 7 days resulted in little survival benefit.
(Figure 9D). However, vaccination times of 7, 12 and 16 days resulted in significant long-term survival, as 60, and 80 percent of animals survived tumor challenge (Figure 9D & 9E). Continuous vaccination (matrix not removed) conferred a small increase in immune protection (90% survival) over vaccination times of 16 days (80% survival). These results clearly indicate that longer term antigen/immune cell stimulation was required to induce significant protection.

The relationship between vaccine duration and tumor rejection was also determined by varying vaccination times in therapeutic treatment of established B16 melanomas. Mice were inoculated with $10^5$ B16 tumor cells and melanomas were allowed to develop for 9 days, at which point animals received PLG vaccines. Vaccination was then arrested by the removal of the implant at days 1, 3, 7, 12 and 16 after implantation (Figure 9C). Therapeutic vaccination times of less than 7 days had no impact on tumor progression (Figure 9D). Vaccination times of greater than 12 days significantly impacted tumor progression and extended the lifespan of mice (Figure 12F). Importantly, continuous vaccination (>16 days) prolonged anti-tumor attack, the suppression of tumor growth, and conferred a 3-fold increase in median survival (Figure 9F).

**Kinetics of DC and T cell responses at PLG vaccine site**

To further delineate the relation between the duration of vaccination and the associated immunologic response, the kinetics of cell recruitment by the PLG vaccines were quantified. The engineered PLG matrices were designed to release a pulse of GM-CSF to recruit host DCs. Histological analysis at day 14 post-implantation of PLG matrices loaded with 3000 ng of GM-CSF revealed enhanced cellular infiltration and penetration into the void volume of the material, relative to cell infiltration at 3 days after implantation (Figure 10A). As described previously, these PLG vaccines were embedded with B16-melanoma tumor lysates and CpG-ODN molecules to serve as tumor antigen and danger signals to infiltrating immune cells (Ali OA, Huebsch N, Cao L, Dranoff G, Mooney DJ. Infection-mimicking materials to program dendritic cells *in situ*. Nat Mater 2, 151-158 (2009)). Incorporation of approximately 680 µg of protein derived from B16-F10 melanoma tumor lysate resulted in sustained release of tumor-associated proteins for at least 40 days, *in vitro*, with approximately 80 µg being released between days 5 and 40 (Figure 13). At day 40, the percent of tumor protein retained within the matrix remained high (77%) indicating that the vaccine serves as a site of sustained antigen presentation that may program immune cells for extended periods.
To assess the kinetics of DC and T cell responses to PLG vaccines, matrices were explanted at various times and total cell infiltrates were isolated and analyzed using FACs analysis to determine CD11c(+) DC and the CD3(+) T cell subpopulations. DC numbers (innate response) were detectable at day 3 post-implantation, peaked at days 5 and 7 (Figure 10 B&C), and dropped sharply at Day 12 post-implantation. The T cell response to PLG vaccines is predominantly comprised of CD8(+) cytotoxic T cells (Figure 10D). Local cytotoxic T cell responses persisted at significant levels between days 7 and 28 after implantation. Specifically, CD8(+) cytotoxic T-lymphocytes (CTLs) were detectable at day 5, peaked at day 12 and subsided at day 28. These data indicate that the vaccine site transitions from primarily activating innate immune responses and DCs to a T cell effector site between 7 to 12 days after implantation, and this CD8(+) T cell responses is maintained for at least 28 days.

Therefore, CTLs manifested potent effector function, as vaccination resulted in a prototypical activation phase that gradually plateaus, followed by a contraction phase as antigen is cleared. The data indicates that the CTL response also effectively terminated the innate response by day 12 as indicated by the sharp decline of antigen presenting DCs, likely via effector, CTL killing. These results demonstrate that vaccination requires stimulation for at least 3 days to induce the onset of innate immunity (i.e., dendritic cell responses) and that antigen clearance by CTLs can be maintained as long as antigens are presented within an immunostimulatory site.

**Kinetics of IL-12 and IFN-γ production at vaccine site**

IL-12, which is a T cell growth and stimulating factor and an activating factor for DCs, is produced by DCs and macrophages in response to intercellular pathogens and tumor cells. Local IL-12 concentrations peaked at 800 pg/ml after one day of vaccination, and then subsided to approximately 300 pg/ml between days 5-16 of vaccination (Figure 11A). All three components of the vaccine, GM-CSF, CpG-ODN and tumor lysates were required to promote and maintain high IL-12 concentrations, as blank controls and all other combinations of the vaccine's bioactive factors produced significantly lower IL-12 levels (Figure 11B). Interestingly, the IL-12 concentration subsided to undetectable levels after day 21 of vaccination, and the time over which IL-12 was detected coincided with the time course of DC infiltration (Figure 10B & C) at the vaccine site. IFN-γ levels at the vaccine sites were first detected at day 3 after vaccination, peaked at day 12, and subsided at days 16-21; these kinetics mirror the time-course of DC infiltration (Figure 11C & 13B). Taken together, this
data demonstrate that DCs are exposed to high IL-12 concentrations while the CpG-ODN danger signals and tumor lysates are presented from the vaccine. Provision of GM-CSF and CpG-ODN signaling into the vaccine dramatically increased IFN-γ production in situ (Figure 1ID), likely due to their role in promoting DC recruitment and activation (including ligation of TLR-9) (Krieg AM. Development of TLR9 agonists for cancer therapy. J Clin Invest. 17,1 184-94 (2007)). Importantly, PLG vaccines sustained the induction of IL-12 and IFN-γ from infiltrating immune cells for 16 days, which is likely to be important for Th1 polarization and prolonged CD8(+) CTL responses to the tumor antigens embedded within the vaccine's matrix.

10 **Effects of PLG vaccination on cell populations in draining LN**

To determine the systemic effects of PLG vaccines, cellular populations in the draining LNs were monitored over time. Blank matrices and PLG matrices containing GM-CSF, lysate and CpG-ODN (PLG vaccines) were implanted subcutaneously into the backs of mice at a distance of approximately 9 mm from the inguinal LN. PLG vaccines resulted in a rapid enhancement in the numbers of phagocytic cells in the proximal inguinal LN, as 4 fold increases in resident DCs and plasmacytoid DCs numbers were observed at day 3 (Figure 12A). Vaccination induced persistent APC activity in LNs, and at day 10 PLG vaccines maintained approximately 3-fold increases in macrophage, DC and plasmacytoid DC numbers in LNs (in comparison to blank PLG matrices) (Figure 12B). Consequently, significant T cell expansion was observed at the draining inguinal LNs at day 10 of vaccination, as a 2.5-fold increase in CD3(+) T cell numbers (~1.3x10^6 T cells) was generated in comparison to blank matrices (5x10^5 T cells). Importantly, the T cell expansion induced by PLG vaccines was mostly due to the expansion of the CD8(+) CTL subset, as their numbers in LNs increased similarly in scale from 2.8x10^5 CTLs (blank matrices) to 8.2x10^5 CTLs (PLG vaccines) at day 10 of vaccination (Figure 12C). Provision of persistent CpG-ODN signaling alongside antigenic signals (tumor lysate) was required of the vaccines ability to induce polarization of LN T cells toward CD8(+) CTL expansion (Figure 12C), and to down-regulate FoxP3(+) T regulatory cell activity in LNs (Figure 12D and E). FoxP3 T cells may suppress the cytotoxicity of CD8(+) T cells and extinguish vaccine activity.

Many infections provide antigenic and stimulatory signals to the immune system that activate DCs, which then process and present instructive signals to prime effector T cell responses. Optimally, these effector T cells mediate the killing of infectious agents and persist until antigens and pathogens are cleared, but chronic infections and tumors have
evolved mechanisms to evade and subdue these destructive responses. Thus, established
tumors require not only the stimulation of innate DC responses, but also the maintenance of
effector immune responses until tolerance to the disease has been broken, and all tumor cells
have been killed by effectors. Described herein are the kinetics of both innate DC responses
and effector T cell responses to a biomaterial based vaccine, and its relationship to vaccine
efficacy. The immune responses to PLG vaccines as described herein propagate with kinetics
similar to viral and bacterial infections. These results also demonstrate that the vaccine's
ability to coordinate persistent DC activation and CD8(+) CTL responses \textit{in situ} was
manifested both locally and systemically, resulting in enhanced APC recruitment to LNs
followed by CTL expansion with similar kinetics (Figure 10 & 12). A unique feature of
these PLG vaccines is their prolonged maintenance of local CTL responses (detectable at 28
days) by sustained antigen presentation after the conclusion of DC recruitment and activation,
which likely underlies the therapeutic efficacy of the vaccine.

Vaccination utilizing melanoma vaccines fabricated into PLG matrices resulted in two
distinct phases of immune responses \textit{in situ}. First, innate responses and DC activation began
at day 3, peaked at day 5 and subsided at day 12 post-vaccination (Figure 10A & B). The
second phase consisted of adaptive T cell responses consisting mostly of CD8(+) cytotoxic T-
lymphocytes (CTLs) (Figure IOC), which began at day 5, peaked at day 12 and subsided at
day 28 (Figure 10A & B). Indeed, CTL responses to the PLG matrix manifested potent
effector function, with vaccination resulting in a prototypical activation phase that gradually
plateaued, and was followed by a contraction phase as the antigen was cleared. The time lag
associated with the onset of the effector response (detected at day 5 after implantation) is
likely the time required to induce sufficient DC recruitment and priming.

The vaccine also induced persistent IL-12 and IFN-\(\gamma\) production consistent with DC
and T cell infiltration into the vaccine site; this is a hallmark of Th1 and cytotoxic T cell
responses against tumors. PLG vaccines induce CTLs expressing TCRs specific for the
melanoma antigen, Trp-2, and the kinetics of cytotoxic T cell homing to the vaccine site is
likely in response to prolonged antigen presentation (tumor lysates) by the PLG matrix. The
data described herein demonstrates that the CTL response terminated the local innate
responses as the significant drop in local DC numbers after day 7 was likely due to CD8(+)T-
cell cytotoxicity against these antigen-presenting cells. Importantly, similar kinetics of APC
recruitment and CTL expansion were observed systemically in the draining, inguinal LNs.
These results demonstrate that vaccination requires stimulation for at least 3 days to induce
the onset of innate immunity (i.e. DC responses) and that effector CTL responses can be maintained until antigen is depleted from the vaccine site.

The relationship between vaccine duration and vaccine efficacy suggests the importance of prolonged maintenance of effector responses against established tumors. By explanting the PLG vaccines between 1 and 7 days - before the onset of significant effector CTL responses in situ - little to no benefit was observed in regards to tumor progression or survival. When the scaffolds were implanted for greater than 7 days, the engineered matrices efficiently maintained potent T cell responses by providing a secondary site of tumor antigen presentation, after the primary induction of innate DC activation had occurred. Vaccination times that included the maintenance of effector CTL responses greater than 7 days, resulted in enhanced prophylactic protection and the significant regression of solid tumors. In tumor-bearing animals, prolonged durations of vaccination (>16 days) significantly augmented persistent CTL responses locally and slowed tumor progression, resulting in an almost 3-fold increase in mouse survival.

Current vaccination methods include protein fusions, antibody conjugation to antigen and viral gene therapy to enhance the half-life, targeting and immunogenecity of antigen. Biomaterials have also been utilized to sustain or target antigen delivery to immune cells in animal models, predominantly as particulate systems. Although current biomaterials are capable of extending the in vivo half-life of antigen delivery from hours to days, allowing for prolonged DC stimulation, this lifespan is shortened by biodegradation, lymphatic drainage, and renal clearance (Pooyan S, Qiu B, Chan MM, Fong D, Sinko PJ, Leibowitz MJ, et al. Conjugates bearing multiple formyl-methionyl peptides display enhanced binding to, but not activation of phagocytic cells. Bioconjugate Chem 2002;13(2):216-23; Wan L, Pooyan S, Hu P, Leibowitz MJ, Stein S, Sinko PJ. Peritoneal macrophage uptake, pharmacokinetics and biodistribution of macrophage-targeted PEG-fMLF (N-formyl-methionyl-leucyl-phenylalanine) nanocarriers for improving HIV drug delivery. Pharm Res. 24, 2 110-21 19. (2007); Bachelder EM, BeaudetteTT, Broaders KE, Paramonov SE, Dashe J, Frochet JM. Acid-Degradable Polyurethane Particles for Protein-Based Vaccines: Biological Evaluation and in vitro Analysis of Particle Degradation Products. Mol. Pharm. 5 876-884 (2008)).

Thus, the ability of prior methods to effectively coordinate adaptive responses (for example, T cells) is inadequate, as exemplified by their inability to stimulate reproducible tumor regression against solid and invasive tumors in mice and humans. This may be attributed to a rapid loss of vaccine activity (days to a week) in vivo.
In summary, this study highlights the benefit of augmenting effector T cell responses in therapeutic vaccine formulations and suggests specific temporal requirements for the bioactivity of vaccine systems. As described herein, approximately 7 days of immunostimulation by vaccines is required to stimulate effector T cell responses. Importantly, long-term antigen stimulation (>12 days; after DC activation) provided a distinct benefit in the maintenance of CTL responses and the efficacy of therapeutic vaccines to established tumors. This duration of stimulation is absent in prior vaccine approaches.
OTHER EMBODIMENTS

While the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

The patent and scientific literature referred to herein establishes the knowledge that is available to those with skill in the art. All United States patents and published or unpublished United States patent applications cited herein are incorporated by reference. All published foreign patents and patent applications cited herein are hereby incorporated by reference. Genbank and NCBI submissions indicated by accession number cited herein are hereby incorporated by reference. All other published references, documents, manuscripts and scientific literature cited herein are hereby incorporated by reference.

While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the scope of the invention encompassed by the appended claims.
CLAIMS

1. A method for eliciting and sustaining an anti-tumor immune response, comprising
   administering to a first anatomical site an antigen-loaded acellular biomaterial
device at a first time point to elicit an innate immune response and an adaptive immune
response; and
   administering to a second anatomical site a second antigen-loaded acellular
biomaterial device at a second time period to sustain said adaptive immune response.

2. The method of claim 1, wherein said antigen comprises a tumor cell lysate,
granulocyte-macrophage colony-stimulating factor (GM-CSF), or cytosine-guanosine
(CpG)-oligodeoxynucleotides (ODN).

3. The method of claim 1, wherein said anti-tumor immune response is characterized
   by the production of interleukin-12 (IL-12) or interferon-γ (IFN-γ) at said anatomical
site.

4. The method of claim 1, wherein said innate immune response is characterized by
   an infiltration of dendritic cells and macrophages.

5. The method of claim 1, wherein said innate immune response is characterized by
   an increase in plasmacytoid dendritic cells (pDCs).

6. The method of claim 1, wherein said adaptive immune response is characterized
   by cytotoxic T cells (CTLs).

7. The method of claim 1, wherein said second time period occurs after onset of said
   innate immune response and before depletion of antigen in said biomaterial device.

8. The method of claim 1, wherein said second time period is between 1-120 days
   after said first time point.
9. The method of claim 1, wherein said second time period is between 3-38 days after said first time point.

10. The method of claim 1, wherein said second time period is 10 days after said first time point.

11. The method of claim 1, wherein said tumor is a solid tumor.

12. The method of claim 1, wherein said tumor is a melanoma.

13. The method of claim 1, wherein said tumor is selected from the group consisting of central nervous system (CNS) cancers, lung cancer, breast cancer, Leukemia, Multiple Myeloma, Renal Cancer, Malignant Glioma, Medulloblastoma, and Melanoma.

14. A method for eliciting and sustaining an anti-tumor immune response, comprising implanting an antigen-loaded acellular biomaterial device at or near a target tumor site, wherein said device presents antigen longer than the time of onset of innate immunity.

15. The method of claim 14, wherein said device presents antigen for greater than 3 days after implantation.

16. The method of claim 14, wherein said device presents antigen for greater than 7 days after implantation.

17. The method of claim 14, wherein said device presents antigen for greater than 12 days after implantation.

18. The method of claim 14, wherein said device presents antigen for greater than 30 days after implantation.
19. The method of claim 14, wherein said device presents antigen for greater than 90 days after implantation.

20. The method of claim 14, wherein said device presents antigen for greater than 120 days after implantation.

21. An acellular antigen-loaded biomaterial device comprising a tumor antigen, a danger signal, and a recruitment composition, wherein the amount of said antigen is sufficient for presentation to endogenous cells for greater than 30 days.

22. The device of claim 21, further comprising an adjuvant.

23. The device of claim 21, wherein the amount of said antigen is sufficient for presentation to endogenous cells for greater than 60 days.

24. A method for progressively reducing tumor burden, comprising successively administering an antigen-loaded acellular biomaterial device, wherein said administering comprises a first implantation and a second implantation, said second implantation occurring at least 24 hours prior to antigen depletion of said device, wherein said first and said second implantations are repeated to progressively reduce tumor burden.

25. The method of claim 24, wherein said antigen comprises a tumor cell lysate, granulocyte-macrophage colony-stimulating factor (GM-CSF), or cytosine-guanosine (CpG)-oligodeoxynucleotides (ODN).
FIG. 4E

Twelve 28

CD8^+ TRP2 Pentamer Splenocytes (cell no.)

Time (days)

FIG. 5A

% Survival

Time (days)

100 μg

100 μg

50 μg

BLANK 1 μg