

HYPERACTIVE DENDRITIC CELLS ENABLE DURABLE ADOPTIVE CELL TRANSFER-BASED ANTI-TUMOR IMMUNITY

CLAIM OF PRIORITY

This application claims the benefit of U.S. Provisional Application Serial No.
5 62/937,075, filed on November 18, 2019. The entire contents of the foregoing are
incorporated herein by reference.

FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

This invention was made with government support under Grant No. AI116550
awarded by the National Institutes of Health. The Government has certain rights in the
10 invention.

TECHNICAL FIELD

The present application is related to cancer immunotherapy, e.g. stimulation of T cell
mediated anti-tumor therapy.

BACKGROUND

15 Central to the understanding of protective immunity to infection and cancer are
dendritic cells (DCs), which are migratory phagocytes that patrol the tissues of the body (D.
Alvarez, E. H. *et al.*, *Immunity*, vol. 29, no. 3, pp. 325–42, Sep. 2008). DCs survey the
environment for threats to the host, most commonly infection or evidence of tissue damage.
This surveillance is achieved through the actions of a superfamily of threat assessment
20 receptors (classically known as pattern recognition receptors (PRRs), which recognize
microbial products or host-encoded molecules indicative of tissue injury (S. W. Brubaker, *et al.* *Annu. Rev. Immunol.*, vol. 33, pp. 257–90, 2015; C. A. Janeway and R. Medzhitov, *Annu.*
Rev. Immunol., vol. 20, pp. 197–216, Jan. 2002). Microbial ligands for PRRs are classified as
pathogen associated molecular patterns (PAMPs) whereas host-derived PRR ligands are
25 damage associated molecular patterns (DAMPs) (P. Matzinger, *Science*, vol. 296, no. 5566,
pp. 301–5, Apr. 2002).

Upon detection of PAMPs, PRRs unleash signaling pathways that fundamentally alter
the physiology of the DCs that express these receptors (A. Iwasaki and R. Medzhitov, *Nat.*
Immunol., vol. 16, no. 4, pp. 343–53, Apr. 2015; O. Joffre, *et al. Immunol. Rev.*, vol. 227, no.
30 1, pp. 234–247, Jan. 2009). For example, prior to PRR activation, DCs are typically viewed
as non-inflammatory cells. Upon encountering extracellular PAMPs, PRRs stimulate the

rapid and robust upregulation of numerous inflammatory mediators, including cytokines, chemokines and interferons. Co-incident with the expression of these genes is the migration of DCs to draining lymph nodes (dLN) and the upregulation of factors important for T cell activation, such as MHC and co-stimulatory molecules. Thus, the process of PRR signaling leads to a shift in DC activities from a non-stimulatory (naïve) state to an “activated” state (K. Inaba, *et al. J. Exp. Med.*, vol. 191, no. 6, pp. 927–36, Mar. 2000 I. Mellman and R. M. Steinman, *Cell*, vol. 106, no. 3, pp. 255–8, Aug. 2001).

SUMMARY

There is a need to diversify current approaches to cancer immunotherapy. Accordingly, the present application is directed to methods of generating a population of therapeutic dendritic cells, methods of inducing an immune response in a subject, methods for treating cancer, methods of hyperactivating dendritic cells (DCs) which induce T helper type I (TH1) and cytotoxic T lymphocyte (CTL) responses in the absence of TH2 immunity. Hyperactivating stimuli drive T cell responses that protect against tumors that are sensitive or resistant to PD-1 inhibition. These protective responses depend on inflammasomes in DCs and can be generated using tumor lysates as immunogens.

In certain embodiments, a method of inducing a protective immune response to an immunogen in a subject, comprises obtaining dendritic cells, culturing the dendritic cells with an effective amount of a non-canonical inflammasome-activating lipid *ex vivo* and administering to the subject the living dendritic cells in an effective amount to enhance a protective immune response, thereby inducing a protective immune response. In some embodiments, the therapeutically effective amount of the non-canonical inflammasome-activating lipid hyperactivates the dendritic cells.

In certain embodiments, the dendritic cells are optionally cultured with an immunogen *ex vivo*. In certain embodiments, the dendritic cells are optionally cultured with a cytokine *ex vivo*.

In certain embodiments, the non-canonical inflammasome-activating lipid comprises: 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine (PAPC), oxidized 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine (oxPAPC), species of oxPAPC, components thereof or combinations thereof.

In certain embodiments, the method further comprises administering a chemotherapeutic agent.

In certain embodiments, the treatment approaches disclosed herein could also be combined with any of the following therapies: radiation, chemotherapy, surgery, therapeutic antibodies, immunomodulatory agents, proteasome inhibitors, pan-deacetylase (DAC) inhibitors, histone deacetylase (HDAC) inhibitors, checkpoint inhibitors, adoptive cell therapies include CAR T and NK cell therapy and vaccines.

Preferably, the methods described herein inhibit the growth or progression of cancer, e.g., a tumor, or a viral infection in a subject. For example, the methods described herein inhibit the growth of a tumor by at least 1%, e.g., by at least 2%, at least 3%, at least 4%, at least 5%, at least 6%, at least 7%, at least 8%, at least 9%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90% or 100%. In other cases, the methods described herein reduce the size of a tumor by at least 1 mm in diameter, e.g., by at least 2 mm in diameter, by at least 3 mm in diameter, by at least 4 mm in diameter, by at least 5 mm in diameter, by at least 6 mm in diameter, by at least 7 mm in diameter, by at least 8 mm in diameter, by at least 9 mm in diameter, by at least 10 mm in diameter, by at least 11 mm in diameter, by at least 12 mm in diameter, by a least 13 mm in diameter, by at least 14 mm in diameter, by at least 15 mm in diameter, by at least 20 mm in diameter, by at least 25 mm in diameter, by at least 30 mm in diameter, by at least 40 mm in diameter, by at least 50 mm in diameter or more. In some cases, the subject has had the bulk of the tumor resected.

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. Methods and materials are described herein for use in the present invention; other, suitable methods and materials known in the art can also be used. The materials, methods, and examples are illustrative only and not intended to be limiting. All publications, patent applications, patents, sequences, database entries, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control.

Other features and advantages of the invention will be apparent from the following detailed description and figures, and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1A-1H are a series of graphs demonstrating that hyperactive DCs are superior antigen-presenting cells and drive TH1-skewed immune responses, with no evidence of TH2

immunity. FIGS. 1A-1F: WT BMDCs were either left untreated (none) or treated with LPS alone, or Alum alone, or oxPAPC or PGPC alone for 24h, or BMDCs were primed for 3h with LPS, then treated with indicated stimuli for 21h. FIG. 1A: IL-1 β , and TNF α cytokine release was monitored by ELISA. FIG. 1B: Percentage of cell death was measured by LDH release in the cell supernatants. FIG. 1C: BMDCs treated with indicated stimuli as in FIG. 1A, were stained with live-dead violet kit, CD11c and CD40. The Mean fluorescence intensity (MFI) of surface CD40 (among CD11c⁺ live cells) is measured by flow cytometry. FIG. 1D: BMDCs pretreated with indicated stimuli were transferred onto CD40-coated plates and cultured for 24h. IL-12p70 cytokine release was measured by ELISA. FIG. 1E: BMDCs pretreated with indicated stimuli as in FIG. 1A, were incubated with OVA protein for 2h or FITC labeled-OVA for 45 minutes. OVA-FITC uptake (left panel) was assessed by flow cytometry. Data are represented as the percentage of OVA-associated CD11c⁺ BMDCs at 37°C and normalized to OVA-associated CD11c⁺ BMDCs at 4°C. OVA peptide presentation on MHC-I (right panel) was monitored using PE-conjugated antibody to H-2Kb bound to the OVA peptide SIINFEKL (SEQ ID NO: 1). Data are represented as the frequency of SIINFEKL (SEQ ID NO: 1)-associated DCs among CD11c⁺ live cells. Means and SDs from three replicates are shown and data are representative of at least three independent experiments. FIG. 1F: BMDCs treated with indicated stimuli as in FIG. 1A, were loaded (or not) with OVA protein or the OVA peptide SIINFEKL for 1h, then incubated for 4 days with splenic OT-II naïve CD4⁺ T cells or OT-I naïve CD8⁺ T cells. FIG. 1F: Supernatants were collected at day 4 and IFN γ , IL-2, IL-10, TNF α and IL-13 cytokine release was measured by ELISA. FIG. 1G: BMDCs were either left untreated (none), or treated with LPS for 24h, or BMDCs were primed with LPS for 3h, then treated with PGPC or Alum for 21h. Treated BMDCs were then cultured with splenic OT-I or OT-II T cells as in FIG. 1F. 4 days post-culture, CD4⁺ and CD8⁺ T cells were stimulated for 5h with PMA plus ionomycin in the presence of brefeldin-A and monensin. The frequency of TH1 cells as TNF α ⁺ IFN γ ⁺, and TH2 cells as Gata3⁺ IL-4⁺ IL-10⁺ among CD4⁺ T cells was measured by intracellular staining. Data are represented as the ratio of TH1/TH2 cells (left panel). The frequency of IFN γ ⁺ among CD8⁺ T cells is represented in the right panel. Means and SDs from three replicates are shown and each panel is representative of at least two independent experiments. FIG. 1H: C57BL/6 mice were injected subcutaneously on the right flank with endofit-OVA protein either alone or with LPS that were emulsified in either incomplete Freund's adjuvant (IFA) or in Alum as indicated. Alternatively, mice were injected with endofit-OVA protein and LPS plus OxPAPC or PGPC all emulsified in IFA. 40 days post immunization, CD4⁺ and CD8⁺ T

cells were isolated from the skin draining lymph nodes (dLN). T cells were then cultured with naïve BMDC loaded (or not) with OVA or with SIINFEKL peptide for 5 days. IFN γ , IL-10, and IL-13 secretion was measured by ELISA. Means and SDs of four mice are shown and each panel is representative of two independent experiments. *P < 0.05; **P < 0.01; ***P < 0.005.

FIG. 2: C57BL/6 WT mice were inoculated with 5×10^5 live B16OVA cells s.c. on the left upper back. 7, 14 and 21 days post tumor challenge, mice were injected s.c. on the right flank with 5×10^6 of either untreated WT BMDCs (DC^{naïve}), or active WT BMDCs treated with LPS for 23h then pulsed with B16OVA WTL for 1h (DC^{LPS}), or WT, or NLRP3^{-/-}, or casp1/11^{-/-} BMDCs that were primed with LPS for 3h, treated with PGPC for 20h then pulsed with B16OVA WTL for 1h (DC^{LPS+PGPC}). Survival was monitored every day (n=5 mice per group).

FIGS. 3A-3E are a series of graphs demonstrating that hyperactive DCs are superior antigen-presenting cells and drive TH1-skewed immune responses, with no evidence of TH2 immunity. FIGS. 3A, 3B: BMDC generated with GMCSF were left untreated (None), or were treated with MPLA alone, Alum alone, or OxPAPC or PGPC alone or BMDCs were primed for 3h with MPLA, then treated with indicated stimuli for 21h. FIG. 3A: IL-1 β , and TNF α cytokine release was monitored by ELISA. FIG. 3B: Percentage of cell death was measured by LDH release in the cell supernatants. FIGS. 3C, 3D: Splenic CD11c⁺ were sorted and either left untreated (None), or were treated either with LPS alone, or Alum alone, or PGPC alone or DCs were primed for 3h with LPS, then treated with indicated stimuli for 21h. FIG. 3C: IL-1 β , and TNF α cytokine release was monitored by ELISA. FIG. 3D: Percentage of cell death was measured by LDH release in the cell supernatants. FIGS. 3E-3F: BMDCs generated with GMCSF treated with indicated stimuli as in A, were stained with live-dead violet kit, anti-CD11c, anti-CD80, anti-CD69, and anti-H2kb antibodies. FIG. 3E: The Mean fluorescence intensity (MFI) of surface CD80 (left panel) and CD69 (middle panel) and H2Kb (right panel) among CD11c⁺ live cells was measured by flow cytometry. Means and SDs of three replicates are shown and all panels are representative of at least three independent experiments. *P < 0.05.

FIGS. 4A-4C are a graph and a series of plots demonstrating that hyperactive DCs are superior antigen-presenting cells and drive TH1-skewed immune responses, with no evidence of TH2 immunity. FIGS. 4A-4C: WT BMDCs were either left untreated (none) or treated with LPS alone, or Alum alone, or OxPAPC or PGPC alone for 24h, or BMDCs were primed

for 3h with LPS, then treated with indicated stimuli for 21h. FIG. 4A: BMDCs were incubated with fixable FITC labeled-OVA at 37°C or at 4°C for 45 minutes. BMDCs were then stained with live-dead violet kit. Gating strategy to determine the frequency of OVA-FITC associated BMDCs at 37°C as compared to OVA-associated BMDCs at 4°C by flow cytometry. FIG. 4B: BMDCs were incubated with endofit-OVA protein for 2 hours. Gating strategy to determine the frequency of SIINFEKL (SEQ ID NO: 1) peptide bound to H2kb on the surface of live BMDCs as measured by flow cytometry using PE-conjugated antibody to H-2Kb bound to the OVA peptide SIINFEKL. Each panel is representative of three replicates of one out of three experiments. FIG. 4C: C57BL/6 mice were injected subcutaneously on the right flank with endofit-OVA protein either alone or with LPS that were emulsified in either incomplete Freund's adjuvant (IFA) or in Alum as indicated. Alternatively, mice were injected with endofit-OVA protein and LPS plus OxPAPC or PGPC all emulsified in IFA. 40 days post immunization, CD4⁺ T cells were isolated from the skin draining lymph nodes (dLN). T cells were then cultured with naïve BMDC loaded (or not) with OVA for 5 days. IL-4 secretion was measured by ELISA. Means and SDs of four mice are shown and each panel is representative of two independent experiments ***P < 0.005.

FIG. 5 is a series of plots demonstrating that hyperactive DCs are superior antigen-presenting cells and drive TH1-skewed immune responses, with no evidence of TH2 immunity. BMDCs were either left untreated (none), or treated with LPS for 24h, or BMDCs were primed with LPS for 3h, then treated with PGPC or Alum for 21h. Treated BMDCs were then cultured with splenic OT-II T cells at a ratio of 1:5 (BMDC: T cell). 4 days post-co-culture, CD4⁺ T cells were stimulated for 5h with PMA plus ionomycin in the presence of brefeldin-A and monensin. Gating strategy to determine the frequency of TH2 cells as IL-4⁺ IL-10⁺ among live CD4⁺ T cells as measured by intracellular staining. Each panel is representative of three replicates of one out of three experiments.

FIGS. 6A-6D are a series of graphs and an immunostain demonstrating that cDC1 and cDC2 cells achieve a state of hyperactivation *in vitro*. (FIGS. 6A-6B) Splenic DCs (left panels) or FLT3 generated DCs (right panels) were sorted as cDC1 (CD11c⁺CD24⁺) or cDC2 (CD11c⁺Sirpa⁺). DCs were either left untreated (none) or treated with LPS alone, or Alum alone, or OxPAPC or PGPC alone for 24h, or DCs were primed for 3h with LPS, then treated with indicated stimuli for 21h. (FIG. 6A) Cell death was measured by LDH release in the supernatants. (FIG. 6B) IL-1β and TNFα cytokine release was monitored by ELISA. Means and SDs of 3 independent experiments are shown. (FIG. 6C) FLT3-DCs treated with

indicated stimuli were stained with Phalloidin-FITC and DAPI. Images were obtained with a 40X oil immersion lens on a Zeiss confocal microscope. (FIG. 6D) FLT3-DCs treated with indicated stimuli were stained with Live-dead violet kit, CCR7 PE, and CD11c-APC. The Mean fluorescence intensity (MFI) of CCR7 (gated on CD11c⁺ Live cells) was measured by flow cytometry.

FIG. 7 is a schematic and a plot demonstrating that hyperactive cDC1 control tumor rejection induced by hyperactivation-based immunotherapy. WT mice were inoculated with 3×10^5 live B16OVA cells subcutaneously (s.c.) on the back. 7, 14 and 21 days post tumor challenge, mice were either left untreated, or were injected s.c. on the right flank with 1×10^6 of untreated WT cDC1 or WT cDC1 treated with LPS for 23h (cDC1^{active}), or WT cDC1 that were primed with LPS for 3h then treated with PGPC for 20h (cDC1^{hyperactive}). All DCs were pulsed with tumor lysate for 1h prior to their injection. Survival was monitored every day (n=5 mice/group).

FIGS. 8A-8C is a series of graphs, plots and a schematic demonstrating that hyperactive cDC1 control tumor rejection and enhance tumor infiltration of anti-tumor specific T cells. FIGS. 8A-8B: *Batf3*^{-/-} mice were inoculated with 3×10^5 live B16OVA cells s.c. on the back. 7, 14 and 21 days post tumor challenge, mice were injected s.c. on the right flank with 1×10^6 of either untreated WT cDC1 or WT cDC1 treated with LPS for 23h then pulsed with B16OVA tumor lysate for 1h (cDC1^{active}), or WT cDC1 that were primed with LPS for 3h then treated with PGPC for 20h then pulsed with tumor lysate for 1h (cDC1^{hyperactive}). (FIG. 8C) Survival was monitored every day (n=5 mice/group). (FIG. 8B) skin draining lymph node (dLN), tumor, and spleen tissues were dissected from immunized mice 15 days post tumor inoculation. The percentage of antigen specific CD8⁺ and CD4⁺ T cells was measured using SIINFEKL and AAHAIEINEA tetramer staining respectively (n=5 mice/group). (FIG. 8C) Representative plot of SIINFEKL⁺ CD8⁺ T cells in the tumors and dLN of treated mice.

FIGS. 9A and 9B are a series of schematics and plots demonstrating that hyperactive cDC1 control tumor rejection in an inflammasome-dependent manner. (FIG. 9A) Casp1/11^{-/-} mice, (FIG. 9B) NLRP3^{-/-} mice were inoculated with 3.105 live B16OVA cells s.c. on the back. 7, 14 and 21 days post tumor challenge, mice were injected s.c. on the right flank with 1.106 of either untreated WT cDC1 (cDC1^{naive}) or WT cDC1 treated with LPS for 23h then pulsed with B16OVA tumor lysate for 1h (cDC1^{active}), or with WT or Casp1/11^{-/-} cDC1 that were primed with LPS for 3h then treated with PGPC for 20h (cDC1^{hyperactive}). All DCs

were pulsed with tumor lysate for 1h prior to their injection. Survival was monitored every day (n=5 mice/group) in (FIG. 9A) Casp1/11^{-/-} mice and (FIG. 9B) NLRP3 mice.

FIGS. 10A and 10B show the mass spectrometry of synthesized lipids. Mass spectrometry analysis of non-oxidized PAPC (FIG. 10A), oxPAPC (FIG. 10B), PEIPC-enriched oxPAPC (FIG. 10C) and biotin-labeled oxPAPC (FIG. 10D).

FIGS. 11A-B. Oxidized phospholipids induce hyperactive cDC1 and cDC2 cells that display a hypermigratory phenotype. (A) Wild-type or NLRP3^{-/-} or Casp1/11^{-/-} BMDCs generated using FLT3L were either left untreated (none) or treated with LPS alone, or Alum alone, or PGPC alone for 24h, or BMDCs were primed for 3h with LPS, then treated with indicated stimuli for 21h. IL-1 β and TNF α release was monitored by ELISA. The percentage of cell death was measured by LDH release in the cell supernatants. Means and SDs from three replicates are shown and data are representative of at least three independent experiments. (B) Wild-type BMDCs generated using FLT3L were sorted as cDC1 or cDC2 cells then treated with indicated stimuli as in A. IL-1 β and TNF α release was monitored by ELISA. The percentage of cell death was measured by LDH release in the cell supernatants. Means and SDs from three independent experiments done in two different laboratories.

FIGS. 12A-B. Hyperactive DCs induce strong CTL responses and a long lived anti-tumor immunity that is dependent on CCR7 expression and on inflammasome activation. (A-B) Wild-type BMDCs generated using FLT3L were either left untreated (DC^{naive}) or treated with LPS alone (DC^{active}) for 18 hours, or BMDCs were primed with LPS for 3h then treated with PGPC (DC^{hyperactive}) or Alum (DC^{pyroptotic}) for 15 hours. Alternatively, BMDCs from NLRP3^{-/-} or CCR7^{-/-} mice were primed with LPS for 3 hours then treated with PGPC for 15 hours. 1.10e6 BMDCs were incubated with OVA protein for 1 hour, then injected subcutaneously into wild-type mice. The injection of BMDCs that were not loaded with OVA protein served as a control group. 7 days post BMDCs injection, skin draining lymph nodes were dissected and stained with live-dead violet kit, OVA peptides tetramer antibodies, anti-CD45, anti-CD3, anti-CD8a, anti-CD4. (A) The percentage of SIINFEKL⁺ CD8⁺ T cells (upper panel), and AAHAEINEA⁺ CD4⁺ live T cells were measured by flow cytometry. (B) The absolute number of SIINFEKL⁺ CD8⁺ T cells (upper panel) and AAHAEINEA⁺ CD4⁺ live T cells were measured by flow cytometry using CounterBright beads.

FIGS. 13A-E. Hyperactive stimuli induce strong CTL response in an inflammasome dependent manner. (A) C57BL/6 mice were injected subcutaneously on the right flank with

OVA either alone or with LPS or with PGPC, or with LPS plus oxPAPC or PGPC all emulsified in incomplete Freud's adjuvant (IFA). 7 or 40 days post immunization, T cells were isolated from the skin draining lymph nodes (dLN) by magnetic enrichment using anti-CD8 beads. (A) The percentage of T effector cells (Teff) as CD44^{low}CD62L^{low}, T effector memory cells (TEM) as CD44^{hi}CD62L^{low}, and T central memory cells (TCM) as CD44^{hi}CD62L^{hi} are represented among CD3+CD8+ live cells. (B) CD8+ T cells were sorted from the dLN 7 days post immunization, then treated either with PMA plus ionomycin, or co-cultured with B16OVA cells (target cells) at ratio of 1:3 (effector: target) for 5h. The degranulation of CD8+ T cells was assessed by monitoring the percentage of CD107a+ among live CD8+T cells using flow cytometry. Means and SDs of five-ten mice are shown. (C) Mice were injected s.c. on the right flank with OVA either alone or with LPS, or with LPS plus oxPAPC or PGPC all emulsified in IFA, or with LPS plus Alum. Alternatively, NLRP3^{-/-} mice were injected with OVA with LPS plus PGPC emulsified in IFA. 7 days post-immunization, CD8+ T cells were sorted from the skin dLN of immunized mice, and co-cultured with BMDCs loaded (or not) with OVA for 7 days at a ratio of 1:10 (DC: T cell). The percentage of SIINFEKL+ IFN γ + among CD8+ live T cells was measured using OVA peptide tetramer staining followed by an intracellular IFN γ staining. (D-E) CD45.1 Mice were irradiated then reconstituted with Bone marrow ZBTB46DTR mice plus either WT or NLRP3^{-/-} or Casp1/11^{-/-} or CCR7^{-/-} (ratio 5:1), all on a CD45.2 C57BL/6 background. 6 weeks post-reconstitution, mouse chimeras were injected with tamoxifen every other day for 7 days. Chimeric mice were then immunized s.c. on the right flank with OVA with LPS plus PGPC emulsified in IFA. 7 days post-immunization, CD8+ T cells were isolated from the skin draining lymph nodes (dLN) or from the spleen by magnetic enrichment using anti-CD8 beads. (D) The percentage of Teff, TEM, TCM and T naïve cells in the skin dLN was measured by flow cytometry. (E) The percentage of SIINFEKL+ among CD8+ live T cells in the dLN (left panel) or in the spleen (right panel) was measured using OVA peptide tetramer staining by flow cytometry. Total CD8+ T cells were sorted from the dLN and co-cultured with untreated BMDCs loaded (or not) with OVA for 7 days at a ratio of 1:10 (DC: T cell).

FIGs. 14A-D. The Immunization with hyperactivating stimuli eradicate tumors with immunogenicity ranging from hot to icy tumors. (A) C57BL/6 mice were inoculated subcutaneously with 5×10^5 of live MC38OVA cells on the left upper back. 14 days later, mice were either left untreated (unimmunized) or were injected subcutaneously on the right flank with syngeneic MC38OVA whole tumor lysate (WTL), plus LPS and PGPC with or

without injection of neutralizing anti IL-1 β intravenously (i.v.), or anti-CD4, or anti-CD8a intraperitoneally. Mice received 2 boost injections with WTL and LPS plus PGPC on day 37 and on day 55 post tumor inoculation. Tumors were allowed to reach 20mm of diameter. The percentage of survival is indicated (n=10 mice per group). (B) C57BL/6 mice were inoculated s.c. with 3X10⁵ live B16OVA cells on the left upper back. 10 days later, mice were either left untreated (unimmunized), or injected intraperitoneally with anti-PD1 antibody. Alternatively, mice were injected s.c. on the right flank with syngeneic B16OVA WTL, plus LPS and PGPC with or without neutralizing antibodies anti IL-1 β intravenously (i.v.), or anti-CD4, or anti-CD8a intraperitoneally. Mice received 2 boost injections with B16OVA WTL, plus LPS and PGPC on day 17 and on day 24 post tumor inoculation. The percentage of survival is indicated (n=10 mice/group). (C) C57BL/6 mice were inoculated s.c. on the left upper back with 3x10⁵ live B16-F10 cells. 7 days later, mice were either left untreated (unimmunized), or injected intraperitoneally with anti-PD1 antibody. Alternatively, mice were immunized s.c. on the right flank with syngeneic B16-F10 WTL, plus LPS and PGPC with or without the neutralizing antibodies anti IL-1 β intravenously (i.v.), or anti-CD4, or anti-CD8a intraperitoneally. Mice received 2 boost injections on day 14 and day 21 post tumor inoculation. The percentage of survival is indicated (n=10 mice per group). (D) BALB/c WT mice were inoculated s.c. with 3x10⁵ live CT26 cells on the left back. 7 days later, mice were either left untreated (unimmunized), or injected intraperitoneally with anti- PD1 antibody. Alternatively, mice were injected s.c. on the right flank with syngeneic CT26 WTL, plus LPS and PGPC with or without the neutralizing antibodies anti IL-1 β intravenously (i.v.), or anti-CD4, or anti-CD8a intraperitoneally. Mice received 2 boost injections on day 14 and day 21 post tumor inoculation. The percentage of survival is indicated (n=10 mice/group).

FIG. 15A-F. Hyperactive cDC1s can use complex antigen sources to stimulate T cell mediated anti-tumor immunity. (A) Zbtb46DTR mice were s.c. injected with B16OVA cells. Mice were either injected with diptheria toxin (DTx) every other day for 4 consecutive injections, or mice were injected with PBS. 7 days post tumor injection, all mice were immunized with B16OVA WTL plus LPS and PGPC, followed by two boosts injections. The percentage of mice survival is indicated (n=10 mice per group). (B) CD45.1 mice were irradiated then reconstituted with mixed BM from Zbtb46DTR mice plus either WT or Nlrp3^{-/-} or Casp1/11^{-/-} or Ccr7^{-/-} mice. 6 weeks post-reconstitution, mouse chimeras were injected s.c. with B61OVA cells, then all mice received DTx 3 times a week for a total of 12 consecutive injections. 7 days post tumor inoculation, chimeric mice were immunized with

B16OVA WTL and LPS plus PGPC and received two boosts injections. The percentage of mice survival is indicated (n=5 mice per group). (C-D) WT or Batf3^{-/-} mice were injected s.c with B16OVA cells. 7 days post-tumor inoculation, mice were either left untreated or WT and Batf3^{-/-} mice were immunized with B16OVA WTL and LPS plus PGPC followed by two
 5 boosts injections. (C) The percentage of mice survival is indicated (n=10 mice per group). (D) 21 days post tumor inoculation, the percentage of OVA specific CD8⁺ T cells and CD4⁺ T cells were assessed using tetramer staining (n=5 mice per group). (E-F) Batf3^{-/-} mice were injected s.c on the right flank with B16OVA cells. 7 days post tumor inoculation, mice were either left untreated (no cDC1 injection), or mice were injected s.c. on the left flank with
 10 FLT3-derived naïve cDC1s or active cDC1s treated with LPS or with hyperactive cDC1s pretreated with LPS plus PGPC. All cDC1s were loaded with B16OVA WTL for 1 hour prior to their injection. (E) The percentage of mice survival is indicated (n=5 mice per group). (F) 21 days post tumor inoculation, OVA specific CD8⁺ T cells and CD4⁺ T cells were assessed using tetramer staining (n=5 mice per group).

15 FIGs. 16A-C. Oxidized phospholipids induce inflammasome dependent IL-1 β secretion by cDC1 and cDC2 cells, and promote a hypermigratory DC phenotype. (A) Wild-type BMDCs generated using FLT3L were either left untreated (none) or treated with CpG 1806 alone, or PGPC alone for 24h, or BMDCs were primed for 3h with CpG 1806, then
 20 treated with indicated stimuli for 21h. IL-1 β and TNF α release was monitored by ELISA. The percentage of cell death was measured by LDH release in the cell supernatants. Means and SDs from three replicates are shown and data are representative of at least three independent experiments. (B) Gating strategy to isolate cDC1 or cDC2 from FLT3L-generated BMDCs or from the spleen of wild type mice. Purity post-sorting is indicated for splenic cDCs or FLT3L DCs. (C) splenic cDC1 or cDC2 were either left untreated (none) or treated with LPS alone,
 25 or Alum alone, or oxPAPC or PGPC alone for 18h, or BMDCs were primed for 3h with LPS, then treated with indicated stimuli for 15h. IL-1 β and TNF α release was monitored by ELISA. The percentage of cell death was measured by LDH release in the cell supernatants. Means and SDs from three independent experiments done in two different laboratories.

FIGs. 17A-C. Hyperactive DCs induce strong CTL responses and a long lived anti-
 30 tumor immunity that is dependent on CCR7 expression and on inflammasome activation. Wild-type BMDCs generated using FLT3L were either left untreated (DC^{naïve}) or treated with LPS alone (DC^{active}) for 18 hours, or BMDCs were primed with LPS for 3h then PGPC (DC^{hyperactive}) or Alum (DC^{pyroptotic}) were added to the culture media for 15 hours.

Alternatively, BMDCs from NLRP3^{-/-} or CCR7^{-/-} mice were primed with LPS for 3 hours then PGPC was added to the culture media for 15 hours. BMDCs were washed then incubated with FITC labeled-OVA for 45 minutes, or with non-fluorescent OVA protein for 2h. (A) OVA peptide presentation on MHC-I was monitored using PE-conjugated antibody to H-2Kb bound to the OVA peptide SIINFEKL. Data are represented as the frequency of SIINFEKL-associated DCs among CD11c⁺ live cells. Means and SDs from three replicates are shown and data are representative of three independent experiments. (B) Wild-type or NLRP3^{-/-} or CCR7^{-/-} BMDCs generated using FLT3L were stimulated as in A. BMDCs were washed then stained with live-dead violet kit, CD11c and CD40. The mean fluorescence intensity (MFI) of surface CD40 (among CD11c⁺ live cells) was measured by flow cytometry. (C) CCR7^{-/-} BMDCs generated using FLT3L were either left untreated (none) or treated with LPS alone, or Alum alone, or PGPC alone for 24h. Alternatively, BMDCs were primed for 3h with LPS, then treated with indicated stimuli for 21h. IL-1 β and TNF α release was monitored by ELISA. The percentage of cell death was measured by LDH release in the cell supernatants. Means and SDs from three replicates are shown and data are representative of at least three independent experiments.

FIGs. 18A-D. Hyperactivating stimuli enhance memory T cell generation and potentiate antigen-specific IFN γ effector responses in an inflammasome-dependent manner. C57BL/6 mice were injected subcutaneously on the right flank with OVA either alone or with LPS or with PGPC, or with LPS plus oxPAPC or PGPC all emulsified in incomplete Freud's adjuvant (IFA). 7 days post immunization, T cells were isolated from the skin draining lymph nodes (dLN) by magnetic enrichment using anti-CD8 beads. (A) Gating strategy to determine the percentage of T effector cells (Teff) as CD44^{low}CD62L^{low}, T effector memory cells (TEM) as CD44^{high}CD62L^{low}, and T central memory cells (TCM) as CD44^{high}CD62L^{high}. (B) Absolute number of Teff or TEM cells in the skin dLN per mouse was assessed by flow cytometry among total CD3⁺ live cells. (C) CD8⁺ T cells were sorted from the dLN 7 days post immunization, then cultured with untreated BMDC loaded (or not) with a serial dilution of OVA protein starting from 1000 ug/ml. IFN γ cytokine secretion was measured by ELISA. Means and SDs of five mice are shown. (D) CD8⁺ T cells were sorted from the dLN 7 days post immunization, then treated either with PMA plus ionomycin, or co-cultured with B16OVA cells (target cells) at ratio of 1:3 (effector: target) for 5h. Gating strategy to determine the percentage of CD107a⁺ among live CD8⁺T cells by flow cytometry. Each panel is representative of five mice. *P < 0.05; **P < 0.01.

FIGs. 19A-B. Hyperactivating stimuli enhance memory T cell generation and potentiate antigen-specific IFN γ effector responses in an inflammasome-dependent manner. (A-B) CD45.1 Mice were irradiated then reconstituted with Bone marrow ZBTB46DTR mice plus either WT or NLRP3^{-/-} or Casp1/11^{-/-} or CCR7^{-/-} (ratio 5:1), all on a CD45.2 C57BL/6 background. 6 weeks post-reconstitution, mouse chimeras were injected with tamoxifen every other day for 7 days. Chimeric mice were then immunized subcutaneously on the right flank with OVA with LPS plus PGPC emulsified in IFA. 7 days post-immunization, CD8⁺ T cells were isolated from the skin draining lymph nodes (dLN) or from the spleen by magnetic enrichment using anti-CD8 beads. (A) The percentage of Teff, TEM, TCM and T naïve cells in the skin dLN was measured by flow cytometry. Each panel is representative of five mice. (B) The percentage of SIINFEKL⁺ among CD8⁺ live T cells in the dLN (upper panel) or in the spleen (lower panel) was measured using OVA peptide tetramer staining by flow cytometry.

FIGs. 20A-C. (A-B) Mice were injected subcutaneously (s.c.) on the right flank with PBS (unimmunized), B16OVA cell lysate alone (none) or with LPS, or B16OVA lysate plus LPS and oxPAPC or PGPC all emulsified in incomplete Freud's adjuvant (IFA). 15 days post immunization, mice were challenged s.c. on the left upper back with 3x10⁵ of viable B16OVA cells. 150 days later, tumor-free mice were re-challenged s.c. with 5x10⁵ of viable B16OVA cells on the back. (A) Tumor growth was monitored every 2 days (upper panel). For survival experiments (lower panel), mice were allowed to reach 20mm of diameter (n=8-15 mice per group). (B-C) Tumors were harvested at the endpoint of tumor growth and dissociated to obtain single-cell tumor suspension. (B) The percentage of tumor infiltrating CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells among enriched CD45⁺ live cells was assessed by flow cytometry. (C) Tumor infiltrating CD3⁺ T cells were sorted then stimulated for 24h in the presence of anti-CD3 and anti-CD28 dynabeads. IFN γ release was measured by ELISA (lower panel) (n=4 mice per group).

FIGs. 21A-D. (A-B) Absolute number of CD8⁺ T cells and CD69⁺ CD103⁺ T resident memory CD8⁺ T cells was assessed at the immunization or tumor injection site of survivor mice and measured by flow cytometry (n=4 mice). (C-D) Circulating memory CD8⁺ T cells (TCM) were isolated from the spleen, and T resident memory CD8⁺ cells (TRM) were isolated from the skin inguinal adipose tissue of survivor mice or age-matched unimmunized tumor-bearing mice. (C) TCM and TRM from survivor mice were co-cultured with B16OVA or B16-F10 or CT26 tumor cells for 5h at a ratio of 1:5 (tumor cell: T cell).

Cell death by cytolytic CD8+T cells was measured by LDH release in the supernatants. (D) Mice were either left untreated (no Tx), or were inoculated intravenously (i.v.) with 5×10^5 of CD8+ TCM cells and/or intradermally (i.d.) with 5×10^5 of CD8+ TRM cells isolated from survivor mice or age-matched unimmunized tumor-bearing mice. 7 days later, all mice were
5 challenged with 3×10^5 of viable B16OVA cells. Percentage of survival was monitored every 2 days. Mice were allowed to reach 20mm of diameter (n=5 mice per group).

DETAILED DESCRIPTION

The innate immune system has classically been viewed to operate in an all-or-none fashion, with DCs operating either to mount inflammatory responses that promote adaptive
10 immunity, or not. Toll-like receptors (TLRs) expressed by DCs are therefore believed to be of central importance in determining immunogenic potential of these cells. The mammalian immune system is responsible for detecting microorganisms and activating protective responses that restrict infection. Central to this task are the dendritic cells, which sense microbes and subsequently promote T-cell activation. It has been suggested that dendritic
15 cells can gauge the threat of any infection and instruct a proportional response (Blander, J.M. (2014). *Nat Rev Immunol* 14, 601-618; Vance, R.E. et al., (2009) *Cell host & microbe* 6, 10-21), but the mechanisms by which these immuno-regulatory activities could occur are unclear.

PRRs act to either directly or indirectly detect molecules that are common to broad
20 classes of microbes. These molecules are classically referred to as pathogen associated molecular patterns (PAMPs), and include factors such as bacterial lipopolysaccharides (LPS), bacterial flagellin or viral double stranded RNA, among others.

An important attribute of PRRs as regulators of immunity is their ability to recognize specific microbial products. As such, PRR-mediated signaling events should provide a
25 definitive indication of infection. It was postulated that a “GO” signal is activated by PRRs expressed on DCs that promote inflammation and T-cell mediated immunity. Interestingly, several groups have recently proposed that DCs may not simply operate in this all-or-none fashion (Blander, J.M., and Sander, L.E. (2012). *Nat Rev Immunol* 12, 215-225; Vance, R.E. et al., (2009) *Cell host & microbe* 6, 10-21). Rather, DCs may have the ability to gauge the
30 threat (or virulence) that any possible infection poses and mount a proportional response. The most commonly discussed means by which virulence can be gauged is based on the ability of virulent pathogens to activate a greater diversity of PRRs than non-pathogens. However, not

- all microbes have a common set of PRR activators, and not all PRR activators are of comparable potency. The number of PRRs activated during an infection may therefore not be an ideal gauge of virulence. Moreover, increasing the number of PRRs activated during an infection will lead to a greater inflammatory response in general, which may indirectly
- 5 promote greater T-cell responses. Conditions previously suggested to heighten the state of DC activation (*e.g.* through the use of virulent pathogens as stimuli) are also expected to heighten the state of MΦ activation (Vance, R.E. et al., (2009) *Cell host & microbe* 6, 10-21). Thus, it remains unclear if mechanisms are truly in place for the immune system (*i.e.* DCs) to gauge the threat of an infection specifically.
- 10 One possible means by which the threat of infection could be assessed would be through the well-recognized process of coincidence detection, where independent inputs result in a response that differs from the one elicited by any single input. In the context of PRRs, one such input must be a microbial product as an indicator of infection, regardless of the threat of virulence. In order to gauge the virulence threat, a second input must exist.
- 15 Without wishing to be bound by theory, it is now thought that this putative second input is a molecule produced at the site of tissue injury, as cellular damage is often a feature associated with highly pathogenic microbes. Candidate molecules that may provide a second stimulus to DCs are the diverse family of molecules called danger associated molecules patterns (DAMPs), which are also known as alarmins (Kono, H., and Rock, K.L. (2008) *Nat Rev*
- 20 *Immunol* 8, 279-289; Pradeu, T., and Cooper, E.L. (2012) *Front Immunol* 3, 287). DAMPs have been found at sites of infectious and non-infectious tissue injury, and have been proposed to modulate inflammatory responses, although their mechanisms of action remain unclear. One such class of DAMPs is represented by oxidized phospholipids derived from 1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphorylcholine (PAPC), which are collectively
- 25 known as oxPAPC. These lipids are produced at the sites of both infectious and non-infectious tissue injury (Berliner, J.A., and Watson, A.D. (2005). *N Engl J Med* 353, 9-11; Imai, Y. *et al.* (2008) *Cell* 133, 235-249; Shirey, K.A. *et al.* (2013) *Nature* 497, 498-502) and are found at very high levels in the membranes of dying cells (Chang, M.K. et al., (2004) *J Exp Med* 200, 1359-1370). oxPAPC is also an active component of oxidized low density lipoprotein
- 30 (oxLDL) aggregates that promote inflammation in atherosclerotic tissues (Leitinger, N. (2003) *Curr Opin Lipidol* 14, 421-430), where local concentrations can be as high as 10-100μM (Oskolkova, O.V. *et al.* (2010) *J Immunol* 185, 7706-7712). The association between oxPAPC and dying cells raised the possibility that these lipids could serve as a generic

indicator of tissue health. In the presence of microbial product(s), oxPAPC may therefore indicate an increased infectious threat.

Due to the aforementioned features of activated DCs, these cells are well-equipped to stimulate antigen-specific T cell responses and numerous strategies have been undertaken to promote DC activation to drive protective immunity. These strategies commonly involve the use of synthetic or natural microbial products that stimulate PRRs of the Toll-like Receptor (TLR) family, with a notable example being the molecule monophosphoryl lipid A (MPLA), an FDA-approved TLR4 ligand that is used to adjuvant an increasing number of vaccines (J. Paavonen, *Lancet*, vol. 374, no. 9686, pp. 301–314, Jul. 2009; M. Kundi, *Expert Rev. Vaccines*, vol. 6, no. 2, pp. 133–140, Apr. 2007; A. M. Didierlaurent, *et al. J. Immunol.*, vol. 183, no. 10, pp. 6186–6197, Nov. 2009). Notably, TLRs alone do not upregulate all the molecular signals needed to promote T cell mediated immunity. Members of the interleukin-1 (IL-1) family of cytokines are critical regulators of many aspects of T cell differentiation, long-lived memory T cell generation and effector function (S. Z. Ben-Sasson, *et al. Proc. Natl. Acad. Sci. U. S. A.*, vol. 106, no. 17, pp. 7119–24, Apr. 2009; S. Z. Ben-Sasson, *et al. J. Exp. Med.*, vol. 210, no. 3, pp. 491–502, Mar. 2013; A. Jain, *et al. Nat. Commun.*, vol. 9, no. 1, pp. 1–13, 2018). The expression of IL-1 β , a well-characterized family member, is highly induced by TLR signals, but this cytokine lacks an N-terminal secretion signal and is therefore not released from cells via the conventional biosynthetic pathway. Rather, IL-1 β accumulates in an inactive state in the cytosol of DCs that have been activated by TLR ligands (C. Garlanda, *et al. Immunity*, vol. 39, no. 6, pp. 1003–1018, Dec. 2013). The lack of IL-1 β release from activated DCs raises the possibility that TLR signals alone are not sufficient to maximally stimulate T cell responses and protective immunity.

The DC activation state is not the only cell fate DCs can achieve upon PRR signaling. Indeed, different PRRs stimulate distinct fates of these cells. One such fate is a commitment to an inflammatory form of cell death known as pyroptosis. Pyroptosis is a regulated process that results from the actions of inflammasomes, which are supramolecular organizing centers (SMOCs) that assemble in the cytosol of DCs and other cells (A. Lu, *et al. Cell*, vol. 156, no. 6, pp. 1193–1206, Mar. 2014; J. C. Kagan, *et al. Nat. Rev. Immunol.*, vol. 14, no. 12, pp. 821–826, Dec. 2014). Inflammasome assembly is commonly stimulated upon detection of PAMPs or DAMPs in the cytosol of the host cell and as such, cytosolic PRRs are responsible for linking threat assessment in the cytosol to inflammasome-dependent pyroptosis (K. J. Kieser and J. C. Kagan, *Nat. Rev. Immunol.*, vol. 17, no. 6, pp. 376–390, May 2017; M.

Lamkanfi and V. M. Dixit, *Cell*, vol. 157, no. 5, pp. 1013–22, May 2014). The process of pyroptosis leads to the release of IL-1 β and other IL-1 family members from the cell, therefore providing the signal to T cells that TLRs cannot offer. Despite this gain in activity, in terms of promoting IL-1 β release, pyroptotic cells are dead and have therefore lost the ability to participate in the days-long process needed to stimulate and differentiate naïve T cells in dLN (T. R. Mempel, *et al. Nature*, vol. 427, no. 6970, pp. 154–159, Jan. 2004). Indeed, stimuli that promote pyroptosis, such as the commonly used vaccine adjuvant alum (S. C. Eisenbarth, *et al. Nature*, vol. 453, no. 7198, pp. 1122–1126, Jun. 2008; M. Kool, *et al. J. Immunol.*, vol. 181, no. 6, pp. 3755–3759, Sep. 2008), are best-appreciated for their ability to stimulate type 2 immune responses (P. Marrack, *et al. Nat. Rev. Immunol.*, vol. 9, no. 4, pp. 287–293, Apr. 2009), which are not suited for the elimination of many microbial infections or cancers.

Adoptive cell therapy (ACT) (including allogeneic and autologous hematopoietic stem cell transplantation (HSCT) and recombinant cell (i.e., CAR T) therapies) is the treatment of choice for many malignant disorders (for reviews of HSCT and adoptive cell therapy approaches, see, Rager & Porter, *Ther Adv Hematol* (2011) 2(6) 409–428; Roddie & Peggs, *Expert Opin. Biol. Ther.* (2011) 11(4):473-487; Wang *et al. Int. J. Cancer.* (2015)136, 1751–1768; and Chang, Y.J. and X.J. Huang, *Blood Rev*, 2013. 27(1): 55-62). Such adoptive cell therapies include, but are not limited to, allogeneic and autologous hematopoietic stem cell transplantation, donor leukocyte (or lymphocyte) infusion (DLI), adoptive transfer of tumor infiltrating lymphocytes, or adoptive transfer of T cells or NK cells (including recombinant cells, i.e., CAR T, CAR NK, gene-edited T cells or NK cells, see Hu *et al. Acta Pharmacologica Sinica* (2018) 39: 167–176, Irving *et al. Front Immunol.* (2017) 8: 267). Beyond the necessity for donor-derived cells to reconstitute hematopoiesis after radiation and chemotherapy, immunologic reconstitution from transferred cells is important for the elimination of residual tumor cells. The efficacy of ACT as a curative option for malignancies is influenced by a number of factors including the origin, composition and phenotype (lymphocyte subset, activation status) of the donor cells, the underlying disease, the pre-transplant conditioning regimen and post-transplant immune support (i.e., IL-2 therapy) and the graft-versus-tumor (GVT) effect mediated by donor cells within the graft. Additionally, these factors must be balanced against transplant-related mortality, typically arising from the conditioning regimen and/or excessive immune activity of donor cells within the host (i.e., graft-versus-host disease, cytokine release syndrome, etc.).

The present application is based, in part, on the finding that stimuli which activate dendritic cells (DCs), or promote DC pyroptosis, induce a mixed T cell response consisting of type I and type 2 T helper (Th) cells. Stimuli that hyperactivate DCs, in contrast, selectively stimulate TH1 and cytotoxic T lymphocyte (CTL) immune responses, with no evidence of TH2-induced immunity. The TH1-biased immunity generated by hyperactive DCs endows these cells with the unique ability to mediate long-term protective anti-tumor immunity, even when a complex antigen source is used (e.g. tumor cell lysates). As demonstrated herein, hyperactive DCs can be generated *ex vivo*, and used, e.g., for adoptive cell therapy.

Thus, provided herein is a method of generating a population of therapeutic dendritic cells that comprises obtaining living dendritic cells from a cell donor, priming the dendritic cells with a TLR ligand *ex vivo*, culturing the primed dendritic cells with a non-canonical inflammasome-activating lipid *ex vivo*, and loading the dendritic cells with an immunogen, thereby generating a population of therapeutic dendritic cells.

Also provided herein is a method of inducing an immune response in a subject that comprises obtaining living dendritic cells from a cell donor, priming the dendritic cells with a TLR ligand *ex vivo*, culturing the primed dendritic cells with a non-canonical inflammasome-activating lipid *ex vivo*, loading the dendritic cells with an immunogen, thereby generating a population of therapeutic dendritic cells, and administering the population of therapeutic dendritic cells to the subject, thereby inducing an immune response in the subject.

Also provided herein is a method of treating cancer, comprising obtaining living dendritic cells from a cell donor, priming the dendritic cells with a TLR ligand *ex vivo*, culturing the primed dendritic cells with a non-canonical inflammasome-activating lipid *ex vivo*, loading the dendritic cells with an immunogen, thereby generating a population of therapeutic dendritic cells, and administering the population of therapeutic dendritic cells to the subject, thereby treating cancer in the subject.

Dendritic Cells

The methods disclosed herein involve obtaining living dendritic cells from a cell donor. The dendritic cells obtained from the cell donor may be immature or mature. The dendritic cells can be differentiated *in vivo* or *in vitro*.

In some embodiments, obtaining living dendritic cells from a cell donor comprises harvesting progenitor cells from the cell donor and culturing the progenitor cells *ex vivo* under conditions effective to induce differentiation, thereby obtaining dendritic cells from a

cell donor. Methods for in vitro differentiation of progenitor cells into dendritic cells are known in the art. *See, e.g.,* Ardavin et al., “Origin and Differentiation of Dendritic Cells,” *TRENDS in Immunol.* 22(12):691–700 (2001).

5 In some embodiments, the progenitor cells are lymphoid progenitor cells. In some embodiments, the progenitor cells are myeloid progenitor cells. In some embodiments, the progenitor cells are blood monocytes.

In some embodiments, the progenitor cells are derived from bone marrow. In some embodiments, the progenitor cells are derived from blood. In some embodiments, the progenitor cells are derived from peripheral blood mononuclear cells. In some embodiments,
10 the progenitor cells are derived from umbilical cord blood.

In some embodiments, culturing the progenitor cells *ex vivo* under conditions effective to induce differentiation comprises culturing the progenitor cells are cultured in the presence of one or more cytokines.

In some embodiments, culturing the progenitor cells *ex vivo* under conditions
15 effective to induce differentiation comprises culturing the progenitor cells are cultured in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-4 (IL-4), tumor necrosis factor α (TNF- α), transforming growth factor β (TGF- β), interleukin 7 (IL-7), stem cell factor (SCF), fms-like tyrosine kinase 3 ligand (FLT3-L), interleukin 1 (IL-1), or combinations thereof.

20 In some embodiments, the progenitor cells are cultured *ex vivo* for between about 1 to about 48 hours. In some embodiments, the progenitor cells are cultured for about 6 to about 48, about 12 to about 48, about 18 to about 48, about 24 to about 48, about 30 to about 48, about 36 to about 48, about 42 to about 48, about 1 to about 42, about 6 to about 42, about 12 to about 42, about 18 to about 42, about 24 to about 42, about 30 to about 42, about 36 to
25 about 42, about 1 to about 36, about 6 to about 36, about 12 to about 36, about 18 to about 36, about 24 to about 36, about 30 to about 36, about 1 to about 30, about 6 to about 30, about 12 to about 30, about 18 to about 30, about 24 to about 30, about 1 to about 24, about 6 to about 24, about 12 to about 24, about 18 to about 24, about 1 to about 18, about 6 to about 18, about 12 to about 18, about 1 to about 12, about 6 to about 12, or about 1 to about 6 hours.

30 In some embodiments, the progenitor cells are blood monocytes. In some embodiments, the blood monocytes are cultured in the presence of GM-CSF and/or IL-4.

In some embodiments, obtaining dendritic cells from a cell donor comprises harvesting *in vivo* differentiated dendritic cells from the cell donor. In some embodiments, the *in vivo* differentiated dendritic cells are immature dendritic cells. In some embodiments, the *in vivo* differentiated dendritic cells are mature dendritic cells.

5 In some embodiments, *in vivo* differentiated dendritic cells are harvested from the cell donor's spleen. In some embodiments, *in vivo* differentiated dendritic cells are harvested from the cell donor's lymph nodes. In some embodiments, *in vivo* differentiated dendritic cells are harvested from the cell donor's thymus. In some embodiments, *in vivo* differentiated dendritic cells are harvested from the cell donor's blood. In some
10 embodiments, *in vivo* differentiated dendritic cells are harvested from the cell donor's skin.

In some embodiments, obtaining dendritic cells from a subject comprises freezing the progenitor cells and/or *in vivo* differentiated dendritic cells.

Methods disclosed herein comprise priming dendritic cells with a TLR ligand *ex vivo*. Suitable TLR ligands are described herein. Priming dendritic cells can comprise incubating
15 progenitor cells, *ex vivo* differentiated dendritic cells, and/or *in vivo* differentiated dendritic cells in the presence of a TLR ligand.

In some embodiments, the dendritic cells are primed with a TLR ligand *ex vivo* for about 1 to about 24 hours. In some embodiments, the dendritic cells are primed with a TLR ligand *ex vivo* for about 3 to about 24, about 6 to about 24, about 9 to about 24, about 12 to
20 about 24, about 15 to about 24, about 18 to about 24, about 21 to about 24, about 1 to about 21, about 3 to about 21, about 6 to about 21, about 9 to about 21, about 12 to about 21, about 15 to about 21, about 18 to about 21, about 1 to about 18, about 3 to about 18, about 6 to about 18, about 9 to about 18, about 12 to about 18, about 15 to about 18, about 1 to about 15, about 3 to about 15, about 6 to about 15, about 9 to about 15, about 12 to about 15, about 1 to
25 about 12, about 3 to about 12, about 6 to about 12, about 9 to about 12, about 1 to about 9, about 3 to about 9, about 6 to about 9, about 1 to about 6, about 3 to about 6, or about 1 to about 3 hours.

In embodiments where progenitor cells are differentiated *ex vivo*, priming the dendritic cells can occur before culturing the progenitor cells *ex vivo* under conditions
30 effective to induce differentiation. In some embodiments, priming the dendritic cells can occur after culturing the progenitor cells *ex vivo* under conditions effective to induce

differentiation. In some embodiments, priming the dendritic cells can occur simultaneously with culturing the progenitor cells *ex vivo* under conditions effective to induce differentiation.

Methods disclosed herein comprise culturing primed dendritic cells with a non-canonical inflammasome-activating lipid *ex vivo*. Suitable non-canonical inflammasome-activating lipids are described herein. Culturing primed dendritic cells can comprise incubating progenitor cells, *ex vivo* differentiated dendritic cells, and/or *in vivo* differentiated dendritic cells in the presence of a non-canonical inflammasome-activating lipid.

In some embodiments, culturing primed dendritic cells with a non-canonical inflammasome-activating lipid *ex vivo* is carried out for between about 1 to about 48 hours. In some embodiments, the progenitor cells are cultured for about 6 to about 48, about 12 to about 48, about 18 to about 48, about 24 to about 48, about 30 to about 48, about 36 to about 48, about 42 to about 48, about 1 to about 42, about 6 to about 42, about 12 to about 42, about 18 to about 42, about 24 to about 42, about 30 to about 42, about 36 to about 42, about 1 to about 36, about 6 to about 36, about 12 to about 36, about 18 to about 36, about 24 to about 36, about 30 to about 36, about 1 to about 30, about 6 to about 30, about 12 to about 30, about 18 to about 30, about 24 to about 30, about 1 to about 24, about 6 to about 24, about 12 to about 24, about 18 to about 24, about 1 to about 18, about 6 to about 18, about 12 to about 18, about 1 to about 12, about 6 to about 12, or about 1 to about 6 hours.

In some embodiments, culturing primed dendritic cells with a non-canonical inflammasome-activating lipid *ex vivo* is carried out simultaneously with priming the dendritic cells with a TLR ligand *ex vivo*. In some embodiments, culturing primed dendritic cells with a non-canonical inflammasome-activating lipid *ex vivo* is carried out after priming the dendritic cells with a TLR ligand *ex vivo*.

Methods disclosed herein comprise loading the dendritic cells with an immunogen. Suitable immunogens are disclosed herein. Loading the dendritic cells with an immunogen can comprise culturing the dendritic cells with an immunogen.

In some embodiments, loading the dendritic cells with an immunogen can be carried out for about 1 to about 24 hours. In some embodiments, loading the dendritic cells with an immunogen can be carried out for about 3 to about 24, about 6 to about 24, about 9 to about 24, about 12 to about 24, about 15 to about 24, about 18 to about 24, about 21 to about 24, about 1 to about 21, about 3 to about 21, about 6 to about 21, about 9 to about 21, about 12 to about 21, about 15 to about 21, about 18 to about 21, about 1 to about 18, about 3 to about 18,

about 6 to about 18, about 9 to about 18, about 12 to about 18, about 15 to about 18, about 1 to about 15, about 3 to about 15, about 6 to about 15, about 9 to about 15, about 12 to about 15, about 1 to about 12, about 3 to about 12, about 6 to about 12, about 9 to about 12, about 1 to about 9, about 3 to about 9, about 6 to about 9, about 1 to about 6, about 3 to about 6, or
 5 about 1 to about 3 hours.

In some embodiments, loading the dendritic cells with an immunogen is carried out simultaneously with culturing dendritic cells with a non-canonical inflammasome-activating lipid *ex vivo*. In some embodiments, loading the dendritic cells with an immunogen is carried out after culturing dendritic cells with a non-canonical inflammasome-activating lipid *ex vivo*.

10 In some embodiments of methods of generating a population of therapeutic dendritic cells and/or methods of inducing an adaptive immune response, the dendritic cells and/or progenitor cells are frozen. In some embodiments, the dendritic cells and/or progenitor cells are frozen prior to loading with an immunogen. In some embodiments, the dendritic cells are frozen after loading with an immunogen.

15 Methods for obtaining and loading dendritic cells, *e.g.*, with a cancer immunogen, are described in the art, *e.g.*, in US20060134067A1, US9694059B2, US9962433B2, US20080254537A1, US9701942B2, US20060057129A1, US20160263206A1, US20150352200A1, US20070292448A1, US6251665B1, WO2003010292A3, US2017036325A1, US20040197903A1, and US10731130B2.

20 **TLR Ligands**

As used herein, the term “pattern recognition receptor ligand” refers to molecular compounds that activate one or more members of the Toll-like Receptor (TLR) family, RIG-I like Receptor (RLR) family, Nucleotide binding leucine rich repeat containing (NLR) family, cGAS, STING or AIM2-like Receptors (ALRs). Specific examples of pattern recognition
 25 receptor ligands include natural or synthetic bacterial lipopolysaccharides (LPS), natural or synthetic bacterial lipoproteins, natural or synthetic DNA or RNA sequences, natural or synthetic cyclic dinucleotides, and natural or synthetic carbohydrates. Cyclic dinucleotides include cyclic GMP-AMP (cGAMP), cyclic di-AMP, cyclic di-GMP.

In some embodiments, the TLR ligand is selected from a TLR1 ligand, a TLR2
 30 ligand, a TLR3 ligand, a TLR4 ligand, a TLR5 ligand, a TLR6 ligand, a TLR7 ligand, a TLR8 ligand, a TLR9 ligand, a TLR10 ligand, a TLR11 ligand, a TLR12 ligand, a TLR13 ligand, and combinations thereof.

In some embodiments, the TLR ligand is a TLR4 ligand. In some embodiments, the TLR4 ligand is LPS. In some embodiments, the TLR4 ligand is MPLA.

Oxidized Phospholipids

The term “non-canonical inflammasome-activating lipid”, as used herein, refers to a lipid capable of eliciting an inflammatory response in a caspase 11-dependent inflammasome of a cell. Exemplary “non-canonical inflammasome-activating lipids” include PAPC, oxPAPC and species of oxPAPC (e.g., HOdiA-PC, KOdiA-PC, HOOA-PC, KOOA-PC, POVPC, PGPG), as well as Rhodo LPS (LPS-RS or LPS from *Rhodobacter sphaeroides*).

The term “oxPAPC” or “oxidized PAPC”, as used herein, refers to lipids generated by the oxidation of 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine (PAPC), which results in a mixture of oxidized phospholipids containing either fragmented or full length oxygenated sn-2 residues. Well-characterized oxidatively fragmented species contain a five-carbon sn-2 residue bearing omega-aldehyde or omega-carboxyl groups. Oxidation of arachidonic acid residue also produces phospholipids containing esterified isoprostanes. oxPAPC includes HOdiA-PC, KOdiA-PC, HOOA-PC and KOOA-PC species, among other oxidized products present in oxPAPC

In some embodiments, the non-canonical inflammasome-activating lipid comprises a species of oxidized 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine (oxPAPC).

Species of oxPAPC are known and described in the art. *See, e.g.,* Ni et al., “Evaluation of Air Oxidized PAPC: A Multi Laboratory Study by LC-MS/MS,” *Free Radical Biology and Medicine* 144:156–66 (2019); Table 1.

In some embodiments, the non-canonical inflammasome-activating lipid comprises 2-[[[(2R)-2-[(E)-7-carboxy-5-hydroxyhept-6-enoyl]oxy-3-hexadecanoyloxypropoxy]-hydroxyphosphoryl]oxyethyl-trimethylazanium (HOdiA-PC), [(2R)-2-[(E)-7-carboxy-5-oxohept-6-enoyl]oxy-3-hexadecanoyloxypropyl] 2-(trimethylazaniumyl)ethyl phosphate (KOdiA-PC), 1-palmitoyl-2-(5-hydroxy-8-oxo-octenoyl)-*sn*-glycero-3-phosphorylcholine (HOOA-PC), 2-[[[(2R)-2-[(E)-5,8-dioxooct-6-enoyl]oxy-3-hexadecanoyloxypropoxy]-hydroxyphosphoryl]oxyethyl-trimethylazanium (KOOA-PC), [(2R)-3-hexadecanoyloxy-2-(5-oxopentanoyloxy)propyl] 2-(trimethylazaniumyl)ethyl phosphate (POVPC), [(2R)-2-(4-carboxybutanoyloxy)-3-hexadecanoyloxypropyl] 2-(trimethylazaniumyl)ethyl phosphate (PGPC), [(2R)-3-hexadecanoyloxy-2-[4-[3-[(E)-[2-[(Z)-oct-2-enyl]-5-oxocyclopent-3-en-1-ylidene]methyl]oxiran-2-yl]butanoyloxy]propyl] 2-(trimethylazaniumyl)ethyl phosphate

(PEPC), [(2R)-3-hexadecanoyloxy-2-[4-[3-[(E)-[3-hydroxy-2-[(Z)-oct-2-enyl]-5-oxocyclopentylidene]methyl]oxiran-2-yl]butanoyloxy]propyl] 2-(trimethylazaniumyl)ethyl phosphate (PEIPC), or a combination thereof.

In some embodiments, the non-canonical inflammasome-activating lipid comprises
 5 [(2R)-2-(4-carboxybutanoyloxy)-3-hexadecanoyloxypropyl] 2-(trimethylazaniumyl)ethyl phosphate (**PGPC**).

In some embodiments, the oxPAPC species is an oxPAPC species set forth in Table 1, or combinations thereof.

Table 1. Oxidized PAPC molecular species identified in Ni et al., with corresponding
 10 elemental composition (neutral), exact mass, adduct, m/z, ID and proposed structures.
 Nomenclature: “Lipid nomenclature is based on the LIPID MAPS consortium recommendations [31]. For instance, the shorthand notation PC 36:4 represents a phosphatidylcholine lipid containing 36 carbons and four double bonds. When the fatty acid identities and sn-position are known, as in our case, the slash separator is used (e.g., PC
 15 16:0/20:4). Since no unified nomenclature is available for oxidized lipids, the short hand notations provided by LPptiger tool were used [28]. Short chain oxidized lipids were indicated by the corresponding terminal enclosed in angular brackets (e.g., “<” and “>”), with the truncation site indicated by the carbon atom number (e.g., <COOH@C9> and
 20 <CHO@C12>). For long chain products our recommendation is to indicate the number of oxygen addition after the fully identified parent lipid (e.g. PC 16:0/20:4 + 1O) when the type of addition is not known, or in parenthesis for known functional groups (e.g. PC 16:0/20:4[1xOH@C11]).” Ni et al., “Evaluation of Air Oxidized PAPC: A Multi Laboratory Study by LC-MS/MS,” *Free Radical Biology and Medicine* 144:156–66 (2019) at 2.7.

Elemental composition (neutral)	Exact mass	Adduct	m/z	ID	L1	L2	L3	L4	Proposed structures
C24H50NO7P	495.3325	[M+HCOO] ⁻	540.3301	PC(16:0/0:0)	x		x	x	PC(16:0/0:0)
C28H50NO7P	543.3325	[M+HCOO] ⁻	588.3301	PC(0:0/20:4)				x	PC(0:0/20:4)
C28H54NO10P	595.3485	[M-H] ⁻	594.3407	PC(16:0/4:0<COOH@C4>)			x		PC(16:0/4:0<COOH@C4>)
C29H56NO10P	609.3642	[M-H] ⁻	608.3564	PC(16:0/5:0<COOH@C5>)	x		x	x	PC(16:0/5:0<COOH@C5>)
C28H54NO9P	579.3536	[M+HCOO] ⁻	624.3513	PC(16:0/4:0<CHO@C4>)	x		x	x	PC(16:0/4:0<CHO@C4>)

Elemental composition (neutral)	Exact mass	Adduct	m/z	ID	L1	L2	L3	L4	Proposed structures
C31H58NO10P	635.3798	[M-H]-	634.3720	PC(16:0/7:1[1xDB]<COOH@C7>)			x	x	PC(16:0/7:1[1xDB]<COOH@C7>)
C29H56NO9P	593.3693	[M+HCOO]-	638.3669	PC(16:0/5:0<CHO@C5>)	x		x	x	PC(16:0/5:0<CHO@C5>)
C32H60NO10P	649.3955	[M-H]-	648.3877	PC(16:0/8:1[1xDB]<COOH@C8>)			x	x	PC(16:0/8:1[1xDB]<COOH@C8>)
C31H58NO11P	651.3748	[M-H]-	650.3669	PC(16:0/7:1[1xDB]<COOH@C7>)+O			x		PC(16:0/7:1[1xDB,1xOH]<COOH@C7>)
C32H58NO11P	663.3748	[M-H]-	662.3669	PC(16:0/8:1[1xDB]<COOH@C8>)+O-2H	x		x	x	PC(16:0/8:1[1xDB,1xKETO]<COOH@C8>)
C31H58NO9P	619.3849	[M+HCOO]-	664.3826	PC(16:0/7:1[1xDB]<CHO@C7>)	x	x	x	x	PC(16:0/7:1[1xDB]<CHO@C7>)
C31H56NO10P	633.3642	[M+HCOO]-	678.3618	PC(16:0/7:1[1xDB]<CHO@C7>)+O-2H			x		PC(16:0/7:1[1xDB,1xKETO]<CHO@C7>)
C31H58NO10P	635.3798	[M+HCOO]-	680.3775	PC(16:0/7:1[1xDB]<CHO@C7>)+O			x	x	PC(16:0/7:1[1xDB,1xOH]<CHO@C7>)
C32H62NO12P	683.4010	[M-H]-	682.3931	PC(16:0/8:0<COOH@C8>)+2O			x		PC(16:0/8:0[2xOH]<COOH@C8>)
C34H60NO11P	689.3904	[M-H]-	688.3826	PC(16:0/10:2[2xDB]<COOH@C10>)+O-2H			x	x	PC(16:0/10:2[2xDB,1xKETO]<COOH@C10>)
C35H64NO10P	689.4268	[M-H]-	688.4190	PC(16:0/11:2[2xDB]<COOH@C11>)			x		PC(16:0/11:2[2xDB]<COOH@C11>)
C32H58NO10P	647.3798	[M+HCOO]-	692.3775	PC(16:0/8:1[1xDB]<CHO@C8>)+O-2H	x			x	PC(16:0/8:1[1xDB,1xKETO]<CHO@C8>)
C32H60NO10P	649.3955	[M+HCOO]-	694.3931	PC(16:0/8:1[1xDB]<CHO@C8>)+O	x		x	x	PC(16:0/8:1[1xDB,1xOH]<CHO@C8>) PC(16:0/8:0[1xEPOXY]<CHO@C8>)
C31H60NO11P	653.3904	[M+HCOO]-	698.3881	PC(16:0/7:1[1xDB]<CHO@C7>)+2O	x		x	x	PC(16:0/7:0[2xOH]<CHO@C7>) PC(16:0/7:0[1xOOH]<CHO@C7>)
C34H62NO9P	659.4162	[M+HCOO]-	704.4139	PC(16:0/10:2[2xDB]<CHO@C10>)	x	x	x	x	PC(16:0/10:2[2xDB]<CHO@C10>)

Elemental composition (neutral)	Exact mass	Adduct	m/z	ID	L1	L2	L3	L4	Proposed structures
C32H60NO11P	665.3904	[M+HCOO] ⁻	710.3881	PC(16:0/8:1[1xDB]<CHO@C8>) +2O	x		x	x	PC(16:0/8:1[1xDB,1xOOH]<CHO@C8>)
									PC(16:0/8:1[1xDB,2xOH]<CHO@C8>)
									PC(16:0/8:0[1xOH,1xEPOXY]<CHO@C8>)
C32H62NO11P	667.4061	[M+HCOO] ⁻	712.4037	PC(16:0/8:0<CHO@C8>) +2O	x		x	x	PC(16:0/8:0[2xOH]<CHO@C8>)
									PC(16:0/8:0[1xOOH]<CHO@C8>)
C35H62NO12P	719.4010	[M-H] ⁻	718.3931	PC(16:0/11:2[2xDB]<COOH@C11>) +2O-2H			x	x	PC(16:0/11:3[3xDB,2xOH]<COOH@C11>)
									PC(16:0/11:2[2xDB,1xOH,1xKETO]<COOH@C11>)
C35H64NO9P	673.4319	[M+HCOO] ⁻	718.4295	PC(16:0/11:2[2xDB]<CHO@C11>)			x	x	PC(16:0/11:2[2xDB]<CHO@C11>)
C34H62NO10P	675.4111	[M+HCOO] ⁻	720.4088	PC(16:0/10:2[2xDB]<CHO@C10>) +O			x	x	PC(16:0/10:2[2xDB,1xOH]<CHO@C10>)
C34H58NO11P	687.3748	[M+HCOO] ⁻	732.3724	PC(16:0/10:2[2xDB]<CHO@C10>) +2O-4H			x	x	PC(16:0/10:2[2xDB,2xKETO]<CHO@C10>)
C35H62NO10P	687.4111	[M+HCOO] ⁻	732.4088	PC(16:0/11:2[2xDB]<CHO@C11>) +O-2H			x	x	PC(16:0/11:2[2xDB,1xKETO]<CHO@C11>)
C35H64NO10P	689.4268	[M+HCOO] ⁻	734.4244	PC(16:0/11:2[2xDB]<CHO@C11>) +O	x			x	PC(16:0/11:2[2xDB,1xOH]<CHO@C11>)
									PC(16:0/11:1[1xDB,1xEPOXY]<CHO@C11>)
C34H62NO11P	691.4061	[M+HCOO] ⁻	736.4037	PC(16:0/10:2[2xDB]<CHO@C10>) +2O			x	x	PC(16:0/10:2[2xDB,1xOOH]<CHO@C10>)
									PC(16:0/10:2[2xDB,2xOH]<CHO@C10>)
									PC(16:0/10:1[1xDB,1xOH,1xEPOXY]<CHO@C10>)
									PC(16:0/10:0[2xEPOXY]<CHO@C10>)
C37H66NO9P	699.4475	[M+HCOO] ⁻	744.4452	PC(16:0/13:3[3xDB]<CHO@C13>)	x		x	x	PC(16:0/13:3[3xDB]<CHO@C13>)

Elemental composition (neutral)	Exact mass	Adduct	m/z	ID	L1	L2	L3	L4	Proposed structures
C35H62NO11P	703.4061	[M+HCOO] ⁻	748.4037	PC(16:0/11:2[2xDB]<CHO@C11>)+2O-2H				x	PC(16:0/11:2[2xDB, 1xOH, 1xKETO]<CHO@C11>)
C35H64NO11P	705.4217	[M+HCOO] ⁻	750.4194	PC(16:0/11:2[2xDB]<CHO@C11>)+2O			x	x	PC(16:0/11:2[2xDB, 1xOOH]<CHO@C11>)
									PC(16:0/11:2[2xDB, 2xOH]<CHO@C11>)
									PC(16:0/11:1[1xDB, 1xOH, 1xEPOXY]<CHO@C11>)
									PC(16:0/11:0[2xEPOXY]<CHO@C11>)
C37H64NO10P	713.4268	[M+HCOO] ⁻	758.4244	PC(16:0/13:3[3xDB]<CHO@C13>)+O-2H			x	x	PC(16:0/13:3[3xDB, 1xKETO]<CHO@C13>)
C37H66NO10P	715.4424	[M+HCOO] ⁻	760.4401	PC(16:0/13:3[3xDB]<CHO@C13>)+O				x	PC(16:0/13:3[3xDB, 1xOH]<CHO@C13>)
C35H62NO12P	719.4010	[M+HCOO] ⁻	764.3986	PC(16:0/11:2[2xDB]<CHO@C11>)+3O-2H				x	PC(16:0/11:2[2xDB, 1xKETO, 1xOOH]<CHO@C11>)
									PC(16:0/11:2[2xDB, 2xOH, 1xKETO]<CHO@C11>)
C35H64NO12P	721.4166	[M+HCOO] ⁻	766.4143	PC(16:0/11:2[2xDB]<CHO@C11>)+3O			x	x	PC(16:0/11:2[2xDB, 1xOH, 1xOOH]<CHO@C11>)
									PC(16:0/11:2[2xDB, 3xOH]<CHO@C11>)
									PC(16:0/11:1[1xDB, 2xOH, 1xEPOXY]<CHO@C11>)
									PC(16:0/11:0[1xOH, 2xEPOXY]<CHO@C11>)
C35H66NO12P	723.4323	[M+HCOO] ⁻	768.4299	PC(16:0/11:1[1xDB]<CHO@C11>)+3O			x	x	PC(16:0/11:1[1xDB, 3xOH]<CHO@C11>)
									PC(16:0/11:1[1xDB, 1xOH, 1xOOH]<CHO@C11>)
									PC(16:0/11:0[1xOOH, 1xEPOXY]<CHO@C11>)
C37H66NO11P	731.4374	[M+HCOO] ⁻	776.4350	PC(16:0/13:3[3xDB]<CHO@C13>)+2O	x		x	x	PC(16:0/13:3[3xDB, 1xOOH]<CHO@C13>)

Elemental composition (neutral)	Exact mass	Adduct	m/z	ID	L1	L2	L3	L4	Proposed structures
									PC(16:0/13:3[3xDB, 2xOH]<CHO@C13>)
									PC(16:0/13:2[2xDB, 1xOH, 1xEPOXY]<CHO@C13>)
									PC(16:0/13:1[1xDB, 2xEPOXY]<CHO@C13>)
C35H64NO13P	737.4115	[M+HCOO] ⁻	782.4092	PC(16:0/11:2[2xDB]<CHO@C11>) +4O			x	x	PC(16:0/11:2[2xDB, 2xOOH]<CHO@C11>)
									PC(16:0/11:2[2xDB, 2xOH, 1xOOH]<CHO@C11>)
									PC(16:0/11:1[1xDB, 1xOH, 1xOOH, 1xEPOXY]<CHO@C11>)
									PC(16:0/11:0[1xOOH, 2xEPOXY]<CHO@C11>)
C35H66NO13P	739.4272	[M+HCOO] ⁻	784.4248	PC(16:0/11:1[1xDB]<CHO@C11>) +4O			x	x	PC(16:0/11:1[1xDB, 2xOOH]<CHO@C11>)
C44H78NO9P	795.5414	[M+HCOO] ⁻	840.5391	PC(16:0/20:4[4xDB]) +O-2H	x	x	x	x	PC(16:0/20:4[4xDB, 1xKETO])
C44H80NO9P	797.5571	[M+HCOO] ⁻	842.5547	PC(16:0/20:4[4xDB]) +O	x	x	x	x	PC(16:0/20:4[4xDB, 1xOH])
									PC(16:0/20:3[3xDB, 1xEPOXY])
C44H76NO10P	809.5207	[M+HCOO] ⁻	854.5183	PC(16:0/20:4[4xDB]) +2O-4H	x		x	x	PC(16:0/20:4[4xDB, 2xKETO])
									PC(16:0/[epoxyisoprostane D2 -H2O])
									PC(16:0/[epoxyisoprostane E2 -H2O])
C44H80NO10P	813.5520	[M+HCOO] ⁻	858.5496	PC(16:0/20:4[4xDB]) +2O	x	x	x	x	PC(16:0/20:4[4xDB, 1xOOH])
									PC(16:0/20:4[4xDB, 2xOH])
									PC(16:0/20:3[3xDB, 1xOH, 1xEPOXY])
									PC(16:0/20:2[2xDB, 2xEPOXY])
C44H78NO11P	827.5313	[M+HCOO] ⁻	872.5289	PC(16:0/20:4[4xDB]) +3O-2H	x	x	x	x	PC(16:0/20:4[4xDB, 1xKETO, 1xOOH])
									PC(16:0/20:4[4xDB, 2xOH, 1xKETO])

Elemental composition (neutral)	Exact mass	Adduct	m/z	ID	L1	L2	L3	L4	Proposed structures
									PC(16:0/20:3[3xDB, 1xOH, 1xKETO, 1xEPOXY])
									PC(16:0/20:2[2xDB, 1xKETO, 2xEPOXY])
									PC(16:0/[PGK2])
									PC(16:0/[epoxyisoprostane D2])
									PC(16:0/[epoxyisoprostane E2])
									PC(16:0/[epoxyisoprostane H2])
									PC(16:0/[epoxyisoprostane I2])
									PC(16:0/[epoxy TXA])
C44H80NO11P	829.5469	[M+HCOO] ⁻	874.5446	PC(16:0/20:4[4xDB]) +3O	x	x	x	x	PC(16:0/20:4[4xDB, 1xOH, 1xOOH])
									PC(16:0/20:4[4xDB, 1xOOH, 1xEPOXY])
									PC(16:0/20:4[4xDB, 3xOH])
									PC(16:0/20:3[3xDB, 2xOH, 1xEPOXY])
									PC(16:0/20:2[2xDB, 1xOH, 2xEPOXY])
									PC(16:0/20:1[1xDB, 3xEPOXY])
									PC(16:0/[PGD2])
									PC(16:0/[PGE2])
									PC(16:0/[PGH2])
									PC(16:0/[PGI2])
C44H76NO12P	841.5105	[M+HCOO] ⁻	886.5082	PC(16:0/20:4[4xDB]) +4O-4H				x	PC(16:0/20:4[4xDB, 2xKETO, 1xOOH])
									PC(16:0/20:4[4xDB, 2xOH, 2xKETO])
									PC(16:0/20:3[3xDB, 1xOH, 2xKETO, 1xEPOXY])
									PC(16:0/20:2[2xDB, 2xKETO, 2xEPOXY])
C44H78NO12P	843.5262	[M+HCOO] ⁻	888.5238	PC(16:0/20:4[4xDB]) +4O-2H		x		x	PC(16:0/20:4[4xDB, 1xOH, 1xKETO, 1xOOH])
									PC(16:0/20:4[4xDB, 3xOH, 1xKETO])

Elemental composition (neutral)	Exact mass	Adduct	m/z	ID	L1	L2	L3	L4	Proposed structures
									PC(16:0/20:3[4xDB, 1xKETO, 1xOOH, 1xEPOXY])
									PC(16:0/20:3[3xDB, 2xOH, 1xKETO, 1xEPOXY])
									PC(16:0/20:2[2xDB, 1xOH, 1xKETO, 2xEPOXY])
									PC(16:0/20:1[1xDB, 1xKETO, 3xEPOXY])
C44H80NO12P	845.5418	[M+HCOO] ⁻	890.5395	PC(16:0/20:4[4xDB]) +4O	x	x	x	x	PC(16:0/20:4[4xDB, 2xOOH])
									PC(16:0/20:4[4xDB, 2xOH, 1xOOH])
									PC(16:0/20:4[4xDB, 4xOH])
									PC(16:0/20:3[3xDB, 1xOH, 1xOOH, 1xEPOXY])
									PC(16:0/20:2[2xDB, 1xOOH, 2xEPOXY])
									PC(16:0/[PGG2])
C44H78NO13P	859.5211	[M+HCOO] ⁻	904.5187	PC(16:0/20:4[4xDB]) +5O-2H	x	x	x	x	PC(16:0/20:4[4xDB, 1xKETO, 2xOOH])
									PC(16:0/20:4[4xDB, 2xOH, 1xKETO, 1xOOH])
									PC(16:0/20:4[4xDB, 1xOH, 1xKETO, 1xOOH, 1xEPOXY])
									PC(16:0/20:4[4xDB, 1xKETO, 1xOOH, 2xEPOXY])
									PC(16:0/20:4[4xDB, 4xOH, 1xKETO])
									PC(16:0/20:3[3xDB, 3xOH, 1xKETO, 1xEPOXY])
									PC(16:0/20:2[2xDB, 2xOH, 1xKETO, 2xEPOXY])
									PC(16:0/20:1[1xDB, 1xOH, 1xKETO, 3xEPOXY])
									PC(16:0/20:0[1xOH, 1xKETO, 4xEPOXY])

Elemental composition (neutral)	Exact mass	Adduct	m/z	ID	L1	L2	L3	L4	Proposed structures
C44H80NO13P	861.5367	[M+HCOO] ⁻	906.5344	PC(16:0/20:4[4xDB]) +5O	x		x	x	PC(16:0/20:4[4xDB, 1xOH, 2xOOH])
									PC(16:0/20:3[4xDB, 2xOOH, 1xEPOXY])
									PC(16:0/20:4[4xDB, 3xOH, 1xOOH])
									PC(16:0/20:3[3xDB, 2xOH, 1xOOH, 1xEPOXY])
									PC(16:0/20:2[2xDB, 1xOH, 1xOOH, 2xEPOXY])
									PC(16:0/20:1[1xDB, 1xOOH, 3xEPOXY])
C44H82NO13P	863.5524	[M+HCOO] ⁻	908.5500	PC(16:0/20:3[3xDB]) +5O			x	x	PC(16:0/20:3[3xDB, 1xOH, 2xOOH])
									PC(16:0/20:2[2xDB, 2xOOH, 1xEPOXY])
									PC(16:0/20:3[3xDB, 3xOH, 1xOOH])
									PC(16:0/20:2[2xDB, 2xOH, 1xOOH, 1xEPOXY])
									PC(16:0/20:1[1xDB, 1xOH, 1xOOH, 2xEPOXY])
									PC(16:0/20:0[1xOH, 3xEPOXY])
C44H80NO14P	877.5316	[M+HCOO] ⁻	922.5293	PC(16:0/20:4[4xDB]) +6O	x	x	x	x	PC(16:0/20:4[4xDB, 3xOOH])
									PC(16:0/20:4[4xDB, 2xOH, 2xOOH])
									PC(16:0/20:3[3xDB, 1xOH, 2xOOH, 1xEPOXY])
									PC(16:0/20:2[2xDB, 2xOOH, 2xEPOXY])
C44H80NO15P	893.5266	[M+HCOO] ⁻	938.5242	PC(16:0/20:4[4xDB]) +7O		x	x	x	PC(16:0/20:4[4xDB, 1xOH, 3xOOH])
									PC(16:0/20:3[3xDB, 3xOOH, 1xEPOXY])
C44H80NO16P	909.5215	[M+HCOO] ⁻	954.5191	PC(16:0/20:4[4xDB]) +8O			x	x	PC(16:0/20:4[4xDB, 4xOOH])
									PC(16:0/20:4[4xDB, 2xOH, 3xOOH])
									PC(16:0/20:3[3xDB, 1xOH, 3xOOH, 1xEPOXY])

Elemental composition (neutral)	Exact mass	Adduct	m/z	ID	L1	L2	L3	L4	Proposed structures
									PC(16:0/20:2[2xDB, 3xOOH, 2xEPOXY])

Immunogens

- “Immunogen” and “antigen” are used interchangeably and mean any compound to which a cellular or humoral immune response is to be directed against. Non-living immunogens include, e.g., killed immunogens, subunit vaccines, recombinant proteins or peptides or the like. The adjuvants disclosed herein can be used with any suitable immunogen. Exemplary immunogens of interest include those constituting or derived from a virus, a mycoplasma, a parasite, a protozoan, a prion or the like. Accordingly, an immunogen of interest can be from, without limitation, a human papilloma virus, a herpes virus such as herpes simplex or herpes zoster, a retrovirus such as human immunodeficiency virus 1 or 2, a hepatitis virus, an influenza virus, a rhinovirus, respiratory syncytial virus, cytomegalovirus, adenovirus, *Mycoplasma pneumoniae*, a bacterium of the genus *Salmonella*, *Staphylococcus*, *Streptococcus*, *Enterococcus*, *Clostridium*, *Escherichia*, *Klebsiella*, *Vibrio*, *Mycobacterium*, amoeba, a malarial parasite, and/or *Trypanosoma cruzi*.
- An immunogen of interest is expressed by diseased target cells (e.g., neoplastic cell, infected cells), and expressed in lower amounts or not at all in other tissue. Examples of target cells include cells from a neoplastic disease, including but not limited to sarcoma, lymphoma, leukemia, a carcinoma, melanoma, carcinoma of the breast, carcinoma of the prostate, ovarian carcinoma, carcinoma of the cervix, colon carcinoma, carcinoma of the lung, glioblastoma, and astrocytoma. Alternatively, the target cell can be infected by, for example, a virus, a mycoplasma, a bacterium, a parasite, a protozoan, a prion and the like. Accordingly, an immunogen of interest can be from, without limitation, a human papilloma virus (see below), a herpes virus such as herpes simplex or herpes zoster, a retrovirus such as human immunodeficiency virus 1 or 2, a hepatitis virus, an influenza virus, a rhinovirus, respiratory syncytial virus, cytomegalovirus, adenovirus, *Mycoplasma pneumoniae*, a bacterium of the genus *Salmonella*, *Staphylococcus*, *Streptococcus*, *Enterococcus*, *Clostridium*, *Escherichia*, *Klebsiella*, *Vibrio*, *Mycobacterium*, amoeba, a malarial parasite, and *Trypanosoma cruzi*.

In some embodiments, an infection with the infectious agent is associated with the development of cancer. *See, e.g.,* Kuper et al., “Infections as a Major Preventable Cause of Human Cancer,” *Journal of International Medicine* 249(S741):61–74 (2001).

In addition to tumor antigens and antigens of infectious agents, mutants of tumor suppressor gene products including, but not limited to, p53, BRCA1, BRCA2, retinoblastoma, and TSG101, or oncogene products such as, without limitation, RAS, W T, MYC, ERK, and TRK, can also provide target antigens to be used according to the present disclosure. The target antigen can be a self-antigen, for example one associated with a cancer or neoplastic disease. In an embodiment, the immunogen is a peptide from a heat shock protein (hsp)-peptide complex of a diseased cell, or the hsp-peptide complex itself.

By “cancer” as used herein is meant, a disease, condition, trait, genotype or phenotype characterized by unregulated cell growth or replication as is known in the art; including colorectal cancer, as well as, for example, leukemias, e.g., acute myelogenous leukemia (AML), chronic myelogenous leukemia (CML), acute lymphocytic leukemia (ALL), and chronic lymphocytic leukemia, AIDS related cancers such as Kaposi's sarcoma; breast cancers; bone cancers such as Osteosarcoma, Chondrosarcomas, Ewing's sarcoma, Fibrosarcomas, Giant cell tumors, Adamantinomas, and Chordomas; Brain cancers such as Meningiomas, Glioblastomas, Lower-Grade Astrocytomas, Oligodendrocytomas, Pituitary Tumors, Schwannomas, and Metastatic brain cancers; cancers of the head and neck including various lymphomas such as mantle cell lymphoma, non-Hodgkins lymphoma, adenoma, squamous cell carcinoma, laryngeal carcinoma, gallbladder and bile duct cancers, cancers of the retina such as retinoblastoma, cancers of the esophagus, gastric cancers, multiple myeloma, ovarian cancer, uterine cancer, thyroid cancer, testicular cancer, endometrial cancer, melanoma, lung cancer, bladder cancer, prostate cancer, lung cancer (including non-small cell lung carcinoma), pancreatic cancer, sarcomas, Wilms' tumor, cervical cancer, head and neck cancer, skin cancers, nasopharyngeal carcinoma, liposarcoma, epithelial carcinoma, renal cell carcinoma, gallbladder adeno carcinoma, parotid adenocarcinoma, endometrial sarcoma, multidrug resistant cancers; and proliferative diseases and conditions, such as neovascularization associated with tumor angiogenesis, macular degeneration (e.g., wet/dry AMD), corneal neovascularization, diabetic retinopathy, neovascular glaucoma, myopic degeneration and other proliferative diseases and conditions.

Immunogens, e.g., cancer immunogens, and their use, e.g., in loading dendritic cells are known and described in the art. *See, e.g.,* Michael J.P. Lawman and Patricia D. Lawman

(eds.) "Cancer Vaccines, Methods and Protocols" *Methods in Molecular Biol.* 1136 (2014); Chiang et al., "Whole Tumor Antigen Vaccines: Where Are We?" *Vaccines* (Basel) 3(2):344–72 (2015); Thumann et al., "Antigen Loading of Dendritic Cells with Whole Tumor Cell Preparations," *J. Immunol. Methods* 277:1–16 (2003); Kamigaki et al., "Immunotherapy of Autologous Tumor Lysate-Loaded Dendritic Cell Vaccines by a Closed-Flow Electroporation System for Solid Tumors," *Anticancer Res.* 33:2971–6 (2013); US3823126A; US3960827A; and US4160018A.

In some embodiments, the immunogen is a cancer antigen. In some embodiments, the cancer antigen is selected from a tumor lysate, an apoptotic body, a peptide, a tumor RNA, a tumor derived exosome, a tumor-DC fusion, or combinations thereof.

In some embodiments, the immunogen is a whole tumor lysate.

In some embodiments, the whole tumor lysate is prepared by irradiating, boiling, and or freeze-thaw lysis.

In some embodiments, the immunogen is autologous. In some embodiments, the immunogen is allogenic.

In some embodiments of methods of inducing an immune response in a subject, the immunogen is a tumor lysate derived from the cell donor.

Cell Donors and Subjects

The terms "patient" or "individual" or "subject" are used interchangeably herein, and refers to a mammalian subject to be treated, with human patients being preferred. In some cases, the methods disclosed herein find use in experimental animals, in veterinary application, and in the development of animal models for disease, including, but not limited to, rodents including mice, rats, and hamsters, and primates.

In some embodiments, the cell donor and/or subject is a mammalian subject. The term "mammal" as used herein is intended to include, but is not limited to, humans, laboratory animals, domestic pets and farm animals.

In some embodiments, the cell donor and/or subject is a human subject.

In some embodiments of methods of inducing an immune response in a subject, the cell donor is the subject. In some embodiments the cell donor is not the subject. In some embodiments, the progenitor cells and/or in vivo differentiated dendritic cells are autologous.

In some embodiments, the progenitor cells and/or in vivo differentiated dendritic cells are allogenic.

Administration

In methods disclosed herein, a population of therapeutic dendritic cells is administered to a subject. In some embodiments, a therapeutically effective amount of living dendritic cells are administered to a subject.

The dosage of the population of therapeutic dendritic cells disclosed herein will vary depending on the nature of the immunogen and the condition of the dendritic cells, but should be sufficient to enhance the efficacy of the living dendritic cells in evoking an immunogenic response. For therapeutic or prophylactic treatment, the amount of living dendritic cells administered can range from 1×10^3 , 1×10^4 , 1×10^5 , 1×10^6 , 1×10^7 , 1×10^8 , 1×10^9 , 1×10^{10} or 1×10^{11} cells per dose or more. The dendritic cells of the present disclosure are generally non-toxic, and generally can be administered as living cells in relatively large amount without causing life-threatening side effects.

Among the methods include off-the-shelf methods. In some embodiments, the methods include isolating cells from the subject, preparing, processing, culturing, as described herein, and re-introducing them into the same patient, before or after cryopreservation.

The administration of population of therapeutic dendritic cells disclosed herein is by any suitable means that results in a concentration of cells that is effective in ameliorating, reducing, or stabilizing cancer. The population of therapeutic dendritic cells can be provided in a dosage form that is suitable for parenteral (e.g., subcutaneous, intravenous, intramuscular, intravesicular, intratumoral or intraperitoneal) administration route.

Human dosage amounts are initially determined by extrapolating from the amount of the population of therapeutic dendritic cells disclosed herein used in mice or non-human primates, as a skilled artisan recognizes it is routine in the art to modify the dosage for humans compared to animal models. For example, the dosage can vary from between about 1×10^3 , 1×10^4 , 1×10^5 , 1×10^6 , 1×10^7 , 1×10^8 , 1×10^9 to about 1×10^{11} cells per dose or more.

A “suitable dosage level” refers to a dosage level that provides a therapeutically reasonable balance between pharmacological effectiveness and deleterious effects (e.g., sufficiently immunostimulatory activity imparted by an administered dendritic cells disclosed

herein, with sufficiently low macrophage stimulation levels). For example, this dosage level can be related to the peak or average serum levels in a subject of, *e.g.*, an anti-immunogen antibody produced following administration of an immunogenic composition (comprising dendritic cells disclosed herein) at the particular dosage level.

5 As defined herein, a “therapeutically effective” amount of a compound or agent (*i.e.*, an effective dosage) means an amount sufficient to produce a therapeutically (*e.g.*, clinically) desirable result. The compositions can be administered from one or more times per day to one or more times per week; including once every other day. The skilled artisan will appreciate that certain factors can influence the dosage and timing required to effectively
10 treat a subject, including but not limited to the severity of the disease or disorder, previous treatments, the general health and/or age of the subject, and other diseases present. Moreover, treatment of a subject with a therapeutically effective amount of living dendritic cells disclosed herein can include a single treatment or a series of treatments.

 The population of therapeutic dendritic cells disclosed herein are administered
15 parenterally by injection, infusion or implantation (subcutaneous, intravenous, intramuscular, intratumoral, intravesicular, intraperitoneal) in dosage forms, formulations, or via suitable delivery devices or implants containing conventional, non-toxic pharmaceutically acceptable carriers. The formulation and preparation of such carriers are well known to those skilled in the art of pharmaceutical formulation. Formulations can be found in Remington: The Science
20 and Practice of Pharmacy, *supra*.

 As used herein, a “pharmaceutically acceptable” component/carrier etc. is one that is suitable for use with humans and/or animals without undue adverse side effects (such as toxicity, irritation, and allergic response) commensurate with a reasonable benefit/risk ratio.

 Provided herein are methods of treating cancer or symptoms thereof which comprise
25 administering a population of therapeutic dendritic cells. Thus, one embodiment is a method of treating a subject suffering from or susceptible to a cancer. The method includes the step of administering to the subject a therapeutic amount of the population of therapeutic dendritic cells disclosed herein, in a dose sufficient to treat the disease or disorder or symptom thereof, under conditions such that the disease or disorder is treated.

30 An “effective amount” as used herein, means an amount which provides a therapeutic or prophylactic benefit.

To “treat” a disease as the term is used herein, means to reduce the frequency or severity of at least one sign or symptom of a disease or disorder, e.g. cancer, experienced by a subject.

“Treatment” is an intervention performed with the intention of preventing the development or altering the pathology or symptoms of a disorder. Accordingly, “treatment” refers to both therapeutic treatment and prophylactic or preventative measures. “Treatment” can also be specified as palliative care. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented. Accordingly, “treating” or “treatment” of a state, disorder or condition can include: (1) preventing or delaying the appearance of clinical symptoms of the state, disorder or condition developing in a human or other mammal that can be afflicted with or predisposed to the state, disorder or condition but does not yet experience or display clinical or subclinical symptoms of the state, disorder or condition; (2) inhibiting the state, disorder or condition, *i.e.*, arresting, reducing or delaying the development of the disease or a relapse thereof (in case of maintenance treatment) or at least one clinical or subclinical symptom thereof; or (3) relieving the disease, *i.e.*, causing regression of the state, disorder or condition or at least one of its clinical or subclinical symptoms. The benefit to an individual to be treated is either statistically significant or at least perceptible to the patient or to the physician.

Thus, in the case of cancer, “treatment” can include: (1) reducing tumor size and/or number; (2) reducing the number of circulating tumor cells; (3) reducing risk of metastasis; (4) reducing risk of cancer occurrence and/or recurrence.

The “modulation” of, *e.g.*, a symptom, level or biological activity of a molecule, or the like, refers, for example, to the symptom or activity, or the like that is detectably increased or decreased. Such increase or decrease can be observed in treated subjects as compared to subjects not treated with hyperactive DCs, where the untreated subjects (*e.g.*, subjects administered immunogen in the absence of adjuvant lipid) have, or are subject to developing, the same or similar disease or infection as treated subjects. Such increases or decreases can be at least about 2%, 5%, 10%, 15%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, 100%, 150%, 200%, 250%, 300%, 400%, 500%, 1000% or more or within any range between any two of these values. Modulation can be determined subjectively or objectively, *e.g.*, by the subject's self-assessment, by a clinician's assessment or by conducting an appropriate assay or measurement, including, *e.g.*, assessment of the extent and/or quality of immunostimulation in a subject achieved by an administered

dendritic cell disclosed herein. Modulation can be transient, prolonged or permanent or it can be variable at relevant times during or after dendritic cells disclosed herein are administered to a subject or is used in an assay or other method described herein or a cited reference, *e.g.*, within times described *infra*, or about 12 hours to 24 or 48 hours after the administration or use of an adjuvant lipid disclosed herein to about 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 21, 28 days, or 1, 3, 6, 9 months or more after a subject(s) has received such an immunostimulatory composition/treatment.

The present disclosure includes methods of inducing an immune response. In some embodiments, the immune response is an adaptive immune response.

10 In some embodiments, the immune response is a therapeutic immune response. The term “therapeutic immune response”, as used herein, refers to an increase in humoral and/or cellular immunity, as measured by standard techniques, which is directed toward the target antigen. Preferably, the induced level of immunity directed toward the target antigen is at least four times, and preferably at least 5 times the level prior to the administration of the immunogen. The immune response can also be measured qualitatively, wherein by means of
15 a suitable in vitro or in vivo assay, an arrest in progression or a remission of a neoplastic or infectious disease in the subject is considered to indicate the induction of a therapeutic immune response.

The methods herein include administering to the subject (including a subject
20 identified as in need of such treatment) an effective amount of a population of therapeutic dendritic cells disclosed herein, to produce such effect. Identifying a subject in need of such treatment can be in the judgment of a subject or a health care professional and can be subjective (*e.g.* opinion) or objective (*e.g.* measurable by a test or diagnostic method).

The therapeutic methods disclosed herein (which include prophylactic treatment) in
25 general comprise administration of a therapeutically effective amount of the population of therapeutic dendritic cells disclosed herein, to a subject (*e.g.*, animal, human) in need thereof, including a mammal, particularly a human. Such treatment will be suitably administered to subjects, particularly humans, suffering from, having, susceptible to, or at risk for cancer or symptom thereof. Determination of those subjects “at risk” can be made by any objective or
30 subjective determination by a diagnostic test or opinion of a subject or health care provider (*e.g.*, genetic test, enzyme or protein marker, Marker (as defined herein), family history, and the like).

The present disclosure also provides a method of monitoring treatment progress. The method includes the step of determining a level of diagnostic marker (Marker) (e.g., any target delineated herein modulated by a compound herein, a protein or indicator thereof, etc.) or diagnostic measurement (e.g., screen, assay) in a subject suffering from or susceptible to a disorder or symptoms thereof associated with cancer in which the subject has been administered a therapeutic amount of a compound herein sufficient to treat the disease or symptoms thereof. The level of Marker determined in the method can be compared to known levels of Marker in either healthy normal controls or in other afflicted patients to establish the subject's disease status. In some cases, a second level of Marker in the subject is determined at a time point later than the determination of the first level, and the two levels are compared to monitor the course of disease or the efficacy of the therapy. In certain aspects, a pre-treatment level of Marker in the subject is determined prior to beginning treatment according to the methods disclosed herein; this pre-treatment level of Marker can then be compared to the level of Marker in the subject after the treatment commences, to determine the efficacy of the treatment.

In some embodiments, the population of therapeutic dendritic cells disclosed herein are administered as part of a pharmaceutical composition.

In some embodiments, the pharmaceutical compositions are administered systemically, for example, formulated in a pharmaceutically-acceptable buffer such as physiological saline. Preferable routes of administration include, for example, instillation into the bladder, subcutaneous, intravenous, intraperitoneal, intramuscular, intratumoral or intradermal injections that provide continuous, sustained or effective levels of the composition in the patient. Treatment of human patients or other animals is carried out using a therapeutically effective amount of a therapeutic identified herein in a physiologically-acceptable carrier. Suitable carriers and their formulation are described, for example, in Remington's Pharmaceutical Sciences by E. W. Martin. The amount of the therapeutic agent to be administered varies depending upon the manner of administration, the age and body weight of the patient, and with the clinical symptoms of the cancer. Generally, amounts will be in the range of those used for other agents used in the treatment of other diseases associated with cancer, although in certain instances lower amounts will be needed because of the increased specificity of the compound. Living Dendritic cells are administered at a dosage that enhances an immune response of a subject, or that reduces the proliferation,

survival, or invasiveness of a neoplastic or, infected cell as determined by a method known to one skilled in the art.

Compositions comprising populations of therapeutic dendritic cells disclosed herein can be administered cutaneously, subcutaneously, intravenously, intramuscularly, parenterally, intrapulmonarily, intravaginally, intrarectally, nasally or topically. The composition can be delivered by injection, orally, by aerosol, or particle bombardment.

The pharmaceutical compositions of population of therapeutic dendritic cells disclosed herein can be included in a kit, container, pack, or dispenser together with instructions for administration.

10 **Combination Therapy**

As used herein, the term “in combination” in the context of the administration of a therapy to a subject refers to the use of more than one therapy for therapeutic benefit. The term “in combination” in the context of the administration can also refer to the prophylactic use of a therapy to a subject when used with at least one additional therapy. The use of the term “in combination” does not restrict the order in which the therapies (e.g., a first and second therapy) are administered to a subject. A therapy can be administered prior to (e.g., 1 minute, 5 minutes, 15 minutes, 30 minutes, 45 minutes, 1 hour, 2 hours, 4 hours, 6 hours, 12 hours, 24 hours, 48 hours, 72 hours, 96 hours, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 8 weeks, or 12 weeks before), concomitantly with, or subsequent to (e.g., 1 minute, 5 minutes, 15 minutes, 30 minutes, 45 minutes, 1 hour, 2 hours, 4 hours, 6 hours, 12 hours, 24 hours, 48 hours, 72 hours, 96 hours, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 8 weeks, or 12 weeks after) the administration of a second therapy to a subject which had, has, or is susceptible to cancer. The therapies are administered to a subject in a sequence and within a time interval such that the therapies can act together. In a particular embodiment, the therapies are administered to a subject in a sequence and within a time interval such that they provide an increased benefit than if they were administered otherwise. Any additional therapy can be administered in any order with the other additional therapy.

As used herein, the term “cancer therapy” refers to a therapy useful in treating cancer. Examples of anti-cancer therapeutic agents include, but are not limited to, e.g., surgery, chemotherapeutic agents, immunotherapy, growth inhibitory agents, cytotoxic agents, agents used in radiation therapy, anti-angiogenesis agents, apoptotic agents, anti-tubulin agents, and other agents to treat cancer, such as anti-HER-2 antibodies (e.g., HERCEPTINTM), anti-CD20

antibodies, an epidermal growth factor receptor (EGFR) antagonist (e.g., a tyrosine kinase inhibitor), HER1/EGFR inhibitor (e.g., erlotinib (TARCEVATM)), platelet derived growth factor inhibitors (e.g., GLEEVECTM (Imatinib Mesylate)), a COX-2 inhibitor (e.g., celecoxib), interferons, cytokines, antagonists (e.g., neutralizing antibodies) that bind to one or more of the following targets ErbB2, ErbB3, ErbB4, PDGFR-beta, BlyS, APRIL, BCMA or VEGF receptor(s), TRAIL/Apo2, and other bioactive and organic chemical agents, etc. Combinations thereof are also contemplated for use with the methods described herein.

Some embodiments of methods of inducing an immune response in a subject include administering an anti-cancer agent to the subject. In some embodiments, the anti-cancer agent is a chemotherapeutic agent. In some embodiments, the anti-cancer agent is an immune checkpoint modulator.

Anti-Cancer Agents: In certain embodiments, the method further comprises administering an anti-cancer agent. In some embodiments, the anti-cancer agent is a chemotherapeutic or growth inhibitory agent, a T cell expressing a chimeric antigen receptor, an antibody or antigen-binding fragment thereof, an antibody-drug conjugate, an angiogenesis inhibitor, and combinations thereof.

In some embodiments, the anti-cancer agent is a chemotherapeutic or growth inhibitory agent. For example, a chemotherapeutic or growth inhibitory agent can include an alkylating agent, an anthracycline, an anti-hormonal agent, an aromatase inhibitor, an anti-androgen, a protein kinase inhibitor, a lipid kinase inhibitor, an antisense oligonucleotide, a ribozyme, an antimetabolite, a topoisomerase inhibitor, a cytotoxic agent or antitumor antibiotic, a proteasome inhibitor, an anti-microtubule agent, an EGFR antagonist, a retinoid, a tyrosine kinase inhibitor, a histone deacetylase inhibitor, and combinations thereof.

A “chemotherapeutic agent” is a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents can include erlotinib (TARCEVATM, Genentech/OSI Pharm.), bortezomib (VELCADETM, Millennium Pharm.), disulfiram, epigallocatechin gallate, salinosporamide A, carfilzomib, 17-AAG (geldanamycin), radicicol, lactate dehydrogenase A (LDH-A), fulvestrant (FASLODEXTM, AstraZeneca), sunitib (SUTENTTM, Pfizer/Sugen), letrozole (FEMARATTM, Novartis), imatinib mesylate (GLEEVECTM, Novartis), finasunate (VATALANIBTM, Novartis), oxaliplatin (ELOXATINTM, Sanofi), 5-FU (5-fluorouracil), leucovorin, Rapamycin (Sirolimus, RAPAMUNETTM, Wyeth), Lapatinib (TYKERBTM, GSK572016, Glaxo Smith Kline), Lonafamib (SCH 66336), sorafenib (NEXAVARTTM, Bayer Labs), gefitinib (IRESSATM,

AstraZeneca), AG1478, alkyl sulfonates such as busulfan, improsulfan and piposulfan;
 aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and
 methylamelamines including altretamine, triethylenemelamine, triethylenephosphoramidate,
 triethylenethiophosphoramidate and trimethylmelamine; acetogenins (especially bullatacin
 5 and bullatacinone); a camptothecin (including topotecan and irinotecan); bryostatin;
 callystatin; CC-1065 (including its adozelesin, carzelesin and bizelesin synthetic analogs);
 cryptophycins (particularly cryptophycin 1 and cryptophycin 8); adrenocorticosteroids
 (including prednisone and prednisolone); cyproterone acetate; 5 α -reductases including
 finasteride and dutasteride); vorinostat, romidepsin, panobinostat, valproic acid, mocetinostat
 10 dolastatin; aldesleukin, talc duocarmycin (including the synthetic analogs, KW-2189 and
 CB1-TM1); eleutherobin; pancratistatin; a sarcodictyin; spongistatin; antibiotics such as the
 enediyne antibiotics (e.g., calicheamicin, especially calicheamicin γ II and calicheamicin
 .omega.II (Angew Chem. Intl. Ed. Engl. 1994 33:183-186); dynemicin, including dynemicin
 A; bisphosphonates, such as clodronate; an esperamicin; as well as neocarzinostatin
 15 chromophore and related chromoprotein enediyne antibiotic chromophores), aclacinomysins,
 actinomycin, anthramycin, azaserine, bleomycins, cactinomycin, carabacin, caminomycin,
 carzinophilin, chromomycinis, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-
 norleucine, ADRIAMYCINTM (doxorubicin), morpholino-doxorubicin, cyanomorpholino-
 doxorubicin, 2-pyrrolino-doxorubicin and deoxydoxorubicin), epirubicin, esorubicin,
 20 idarubicin, marcellomycin, mitomycins such as mitomycin C, mycophenolic acid,
 nogalamycin, olivomycins, peplomycin, porfiromycin, puromycin, quelamycin, rodorubicin,
 streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such
 as methotrexate and 5-fluorouracil (5-FU); folic acid analogs such as denopterin,
 methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-
 25 mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine,
 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine;
 androgens such as calusterone, dromostanolone propionate, epitostanol, mepitostane,
 testolactone; anti-adrenals such as aminogluthetamide, mitotane, trilostane; folic acid
 replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic
 30 acid; eniluracil; amsacrine; bestabucil; bisantrene; edatraxate; defofamine; demecolcine;
 diaziquone; elfomithine; elliptinium acetate; an epothilone; etoglucid; gallium nitrate;
 hydroxyurea; lentinan; lonidainine; maytansinoids such as maytansine and ansamitocins;
 mitoguazone; mitoxantrone; mopidamnol; nitraerine; pentostatin; phenamet; pirarubicin;
 losoxantrone; podophyllinic acid; 2-ethylhydrazide; procarbazine; PSKTM polysaccharide

complex (JHS Natural Products, Eugene, Oreg.); razoxane; rhizoxin; sizofuran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2''-trichlorotriethylamine; trichothecenes (especially T-2 toxin, verracurin A, roridin A and anguidine); urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside
 5 (“Ara-C”); cyclophosphamide; thiotepa; taxoids, e.g., TAXOL (paclitaxel; Bristol-Myers Squibb Oncology, Princeton, N.J.), ABRAXANETM (Cremophor-free), albumin-engineered nanoparticle formulations of paclitaxel (American Pharmaceutical Partners, Schaumburg, Ill.), and TAXOTERETM (docetaxel, doxetaxel; Sanofi-Aventis); chlorambucil; GEMZARTM (gemcitabine); 6-thioguanine; mercaptopurine; methotrexate; platinum analogs
 10 such as cisplatin and carboplatin; vinblastine; etoposide (VP-16); ifosfamide; mitoxantrone; vincristine; NAVELBINETM (vinorelbine); novantrone; teniposide; edatrexate; daunomycin; aminopterin; capecitabine (XELODATM); ibandronate; CPT-11; topoisomerase inhibitor RFS 2000; difluoromethylornithine (DMFO); retinoids such as retinoic acid; and pharmaceutically acceptable salts, acids and derivatives of any of the above.

15 In some embodiments, a chemotherapeutic agent can include alkylating agents (including monofunctional and bifunctional alkylators) such as thiotepa, CYTOXANTM cyclophosphamide, nitrogen mustards such as chlorambucil, chlormaphazine, chlorophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil
 20 mustard; nitrosoureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, and ranimustine; temozolomide; and pharmaceutically acceptable salts, acids and derivatives of any of the above.

In some embodiments, a chemotherapeutic agent can include anthracyclines such as daunorubicin, doxorubicin, epirubicin, idarubicin, mitoxantrone, valrubicin, and
 25 pharmaceutically acceptable salts, acids and derivatives of any of the above.

In some embodiments, a chemotherapeutic agent can include an anti-hormonal agent such as anti-estrogens and selective estrogen receptor modulators (SERMs), including, for example, tamoxifen (including NOLVADEXTM; tamoxifen citrate), raloxifene, droloxifene, iodoxyfene, 4-hydroxytamoxifen, trioxifene, keoxifene, LY117018, onapristone, and
 30 FARESTONTM (toremifine citrate); and pharmaceutically acceptable salts, acids and derivatives of any of the above.

In some embodiments, a chemotherapeutic agent can include an aromatase inhibitor that inhibit the enzyme aromatase, which regulates estrogen production in the adrenal glands, such as, for example, 4(5)-imidazoles, aminoglutethimide, MEGASETM (megestrol acetate), AROMASINTM (exemestane; Pfizer), formestane, fadrozole, RIVISORTM (vorozole),
5 FEMARATM (letrozole; Novartis), and ARIMIDEXTM (anastrozole; AstraZeneca); and pharmaceutically acceptable salts, acids and derivatives of any of the above.

In some embodiments, a chemotherapeutic agent can include an anti-androgen such as flutamide, nilutamide, bicalutamide, leuprolide and goserelin; buserelin, triptorelin, medroxyprogesterone acetate, diethylstilbestrol, premarin, fluoxymesterone, all transretinoic
10 acid, fenretinide, as well as troxacitabine (a 1,3-dioxolane nucleoside cytosine analog); and pharmaceutically acceptable salts, acids and derivatives of any of the above.

In some embodiments, a chemotherapeutic agent can include a protein kinase inhibitors, lipid kinase inhibitor, or an antisense oligonucleotide, particularly those which inhibit expression of genes in signaling pathways implicated in aberrant cell proliferation,
15 such as, for example, PKC- α , Ral and H-Ras.

In some embodiments, a chemotherapeutic agent can include a ribozyme such as VEGF expression inhibitors (e.g., ANGIOZYMETM) and HER2 expression inhibitors.

In some embodiments, a chemotherapeutic agent can include a cytotoxic agent or antitumor antibiotic, such as dactinomycin, actinomycin, bleomycins, plicamycin,
20 mitomycins such as mitomycin C, and pharmaceutically acceptable salts, acids and derivatives of any of the above.

In some embodiments, a chemotherapeutic agent can include a proteasome inhibitor such as bortezomib (VELCADETM, Millennium Pharm.), epoxomicins such as carfilzomib (KYPROLISTM, Onyx Pharm.), marizomib (NPI-0052), MLN2238, CEP-18770, oprozomib,
25 and pharmaceutically acceptable salts, acids and derivatives of any of the above.

In some embodiments, a chemotherapeutic agent can include an anti-microtubule agent such as Vinca alkaloids, including vincristine, vinblastine, vindesine, and vinorelbine; taxanes, including paclitaxel and docetaxel; podophyllotoxin; and pharmaceutically acceptable salts, acids and derivatives of any of the above.

30 In some embodiments, a chemotherapeutic agent can include an "EGFR antagonist," which refers to a compound that binds to or otherwise interacts directly with EGFR and prevents or reduces its signaling activity, and is alternatively referred to as an "EGFR i."

Examples of such agents include antibodies and small molecules that bind to EGFR.

Examples of antibodies which bind to EGFR include MAb 579 (ATCC CRL HB 8506), MAb 455 (ATCC CRL HB8507), MAb 225 (ATCC CRL 8508), MAb 528 (ATCC CRL 8509) (see, U.S. Pat. No. 4,943,533, Mendelsohn et al.) and variants thereof, such as chimerized 225 (C225 or Cetuximab; ERBUTIX) and reshaped human 225 (H225) (see, WO 96/40210, Imclone Systems Inc.); IMC-11F8, a fully human, EGFR-targeted antibody (Imclone); antibodies that bind type II mutant EGFR (U.S. Pat. No. 5,212,290); humanized and chimeric antibodies that bind EGFR as described in U.S. Pat. No. 5,891,996; and human antibodies that bind EGFR, such as ABX-EGF or Panitumumab (see WO98/50433, Abgenix/Amgen); EMD 55900 (Stragliotto et al. Eur. J. Cancer 32A:636-640 (1996)); EMD7200 (matuzumab) a humanized EGFR antibody directed against EGFR that competes with both EGF and TGF-alpha for EGFR binding (EMD/Merck); human EGFR antibody, HuMax-EGFR (GenMab); fully human antibodies known as E1.1, E2.4, E2.5, E6.2, E6.4, E2.11, E6.3 and E7.6.3 and described in U.S. Pat. No. 6,235,883; MDX-447 (Medarex Inc); and mAb 806 or humanized mAb 806 (Johns et al., J. Biol. Chem. 279(29):30375-30384 (2004)). The anti-EGFR antibody can be conjugated with a cytotoxic agent, thus generating an immunoconjugate (see, e.g., EP659439A2, Merck Patent GmbH). EGFR antagonists include small molecules such as compounds described in U.S. Pat. Nos. 5,616,582, 5,457,105, 5,475,001, 5,654,307, 5,679,683, 6,084,095, 6,265,410, 6,455,534, 6,521,620, 6,596,726, 6,713,484, 5,770,599, 6,140,332, 5,866,572, 6,399,602, 6,344,459, 6,602,863, 6,391,874, 6,344,455, 5,760,041, 6,002,008, and 5,747,498, as well as the following PCT publications: WO98/14451, WO98/50038, WO99/09016, and WO99/24037. Particular small molecule EGFR antagonists include OSI-774 (CP-358774, erlotinib, TARCEVATM Genentech/OSI Pharmaceuticals); PD 183805 (CI 1033, 2-propenamide, N-[4-[(3-chloro-4-fluorophenyl)amino]-7-[3-(4-morpholinyl)propoxy]-6-quin- azolonyl]-, dihydrochloride, Pfizer Inc.); ZD1839, gefitinib (IRESSATM) 4-(3'-Chloro-4'-fluoroanilino)-7-methoxy-6-(3-morpholinopropoxy)quinazoline, AstraZeneca); ZM 105180 ((6-amino-4-(3-methylphenyl-amino)-quinazoline, Zeneca); BIBX-1382 (N8-(3-chloro-4-fluoro-phenyl)-N2-(1-methyl-piperidin-4-yl)-pyrimido[5,4--d]pyrimidine-2,8-diamine, Boehringer Ingelheim); PKI-166 ((R)-4-[4-(1-phenylethyl)amino]-1H-pyrrolo[2,3-d]pyrimidin-6-yl]-phenol)- ; (R)-6-(4-hydroxyphenyl)-4-[(1-phenylethyl)amino]-7H-pyrrolo[2,3-d]pyrimidine); CL-387785 (N-[4-[(3-bromophenyl)amino]-6-quinazolonyl]-2-butyramide); EKB-569 (N-[4-[(3-chloro-4-fluorophenyl)amino]-3-cyano-7-ethoxy-6-quinolonyl]-4-(- dimethylamino)-2-butenamide) (Wyeth); AG1478 (Pfizer); AG1571 (SU 5271; Pfizer); dual EGFR/HER2 tyrosine kinase

inhibitors such as lapatinib (TYKERBTM, GSK572016 or N-[3-chloro-4-[(3-fluorophenyl)methoxy]phenyl]-6[[[2-methylsulfonyl]ethyl]amino]methyl]-2--furan-2-yl]-4-quinazolinamine).

In some embodiments, a chemotherapeutic agent can include a tyrosine kinase inhibitor, including the EGFR-targeted drugs noted in the preceding paragraph; small molecule HER2 tyrosine kinase inhibitor such as TAK165 available from Takeda; CP-724,714, an oral selective inhibitor of the ErbB2 receptor tyrosine kinase (Pfizer and OSI); dual-HER inhibitors such as EKB-569 (available from Wyeth) which preferentially binds EGFR but inhibits both HER2 and EGFR-overexpressing cells; lapatinib (GSK572016; available from Glaxo-SmithKline), an oral HER2 and EGFR tyrosine kinase inhibitor; PKI-166 (available from Novartis); pan-HER inhibitors such as canertinib (CI-1033; Pharmacia); Raf-1 inhibitors such as antisense agent ISIS-5132 available from ISIS Pharmaceuticals which inhibit Raf-1 signaling; non-HER targeted TK inhibitors such as imatinib mesylate (GLEEVECTM, available from Glaxo SmithKline); multi-targeted tyrosine kinase inhibitors such as sunitinib (SUTENTTM, available from Pfizer); VEGF receptor tyrosine kinase inhibitors such as vatalanib (PTK787/ZK222584, available from Novartis/Schering AG); MAPK extracellular regulated kinase I inhibitor CI-1040 (available from Pharmacia); quinazolines, such as PD 153035, 4-(3-chloroanilino)quinazoline; pyridopyrimidines; pyrimidopyrimidines; pyrrolopyrimidines, such as CGP 59326, CGP 60261 and CGP 62706; pyrazolopyrimidines, 4-(phenylamino)-7H-pyrrolo[2,3-d]pyrimidines; curcumin (diferuloyl methane, 4,5-bis (4-fluoroanilino)phthalimide); tyrphostins containing nitrothiophene moieties; PD-0183805 (Warner-Lambert); antisense molecules (e.g. those that bind to HER-encoding nucleic acid); quinoxalines (U.S. Pat. No. 5,804,396); tyrphostins (U.S. Pat. No. 5,804,396); ZD6474 (AstraZeneca); PTK-787 (Novartis/Schering AG); pan-HER inhibitors such as CI-1033 (Pfizer); Affinitac (ISIS 3521; Isis/Lilly); imatinib mesylate (GLEEVECTM); PKI 166 (Novartis); GW2016 (Glaxo SmithKline); CI-1033 (Pfizer); EKB-569 (Wyeth); Semaxinib (Pfizer); ZD6474 (AstraZeneca); PTK-787 (Novartis/Schering AG); INC-1C11 (Imclone), rapamycin (sirolimus, RAPAMUNETM); or as described in any of the following patent publications: U.S. Pat. No. 5,804,396; WO 1999/09016 (American Cyanamid); WO 1998/43960 (American Cyanamid); WO 1997/38983 (Warner Lambert); WO 1999/06378 (Warner Lambert); WO 1999/06396 (Warner Lambert); WO 1996/30347 (Pfizer, Inc); WO 1996/33978 (Zeneca); WO 1996/3397 (Zeneca) and WO 1996/33980 (Zeneca).

In some embodiments, a chemotherapeutic agent can include a retinoid such as retinoic acid and pharmaceutically acceptable salts, acids and derivatives of any of the above.

In some embodiments, a chemotherapeutic agent can include an anti-metabolite.

Examples of anti-metabolites can include folic acid analogs and antifolates such as

- 5 denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as 5-fluorouracil (5-FU), ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine; nucleoside analogs; and nucleotide analogs.

- 10 In some embodiments, a chemotherapeutic agent can include a topoisomerase inhibitor. Examples of topoisomerase inhibitors can include a topoisomerase I inhibitor such as LURTOTECANTM and ABARELIXTM rmRH; a topoisomerase II inhibitor such as doxorubicin, epirubicin, etoposide, and bleomycin; and topoisomerase inhibitor RFS 2000.

- 15 In some embodiments, a chemotherapeutic agent can include a histone deacetylase (HDAC) inhibitor such as vorinostat, romidepsin, belinostat, mocetinostat, valproic acid, panobinostat, and pharmaceutically acceptable salts, acids and derivatives of any of the above.

- Chemotherapeutic agents can also include hydrocortisone, hydrocortisone acetate, cortisone acetate, tixocortol pivalate, triamcinolone acetonide, triamcinolone alcohol, mometasone, amcinonide, budesonide, desonide, fluocinonide, fluocinolone acetonide,
- 20 betamethasone, betamethasone sodium phosphate, dexamethasone, dexamethasone sodium phosphate, fluocortolone, hydrocortisone-17-butyrate, hydrocortisone-17-valerate, aclometasone dipropionate, betamethasone valerate, betamethasone dipropionate, prednicarbate, clobetasone-17-butyrate, clobetasol-17-propionate, fluocortolone caproate, fluocortolone pivalate and fluprednidene acetate; immune selective anti-inflammatory
- 25 peptides (ImSAIDs) such as phenylalanine-glutamine-glycine (FEG) and its D-isomeric form (feG) (IMULAN BioTherapeutics, LLC); anti-rheumatic drugs such as azathioprine, ciclosporin (cyclosporine A), D-penicillamine, gold salts, hydroxychloroquine, leflunomide, minocycline, sulfasalazine, tumor necrosis factor alpha (TNF α) blockers such as etanercept (Enbrel), infliximab (Remicade), adalimumab (Humira), certolizumab pegol
- 30 (Cimzia), golimumab (Simponi), Interleukin 1 (IL-1) blockers such as anakinra (Kineret), T cell costimulation blockers such as abatacept (Orencia), Interleukin 6 (IL-6) blockers such as tocilizumab (ACTEMERATM); Interleukin 13 (IL-13) blockers such as lebrikizumab;

- Interferon alpha (IFN) blockers such as Rontalizumab; Beta 7 integrin blockers such as rhuMAb Beta7; IgE pathway blockers such as Anti-M1 prime; Secreted homotrimeric LTa3 and membrane bound heterotrimer LTa1/β2 blockers such as Anti-lymphotoxin alpha (LTa); radioactive isotopes (e.g., 211At, 131I, 125I, 90Y, 186Re, 188Re, 212Bi, 32P, 212Pb and radioactive isotopes of Lu); miscellaneous investigational agents such as thioplatin, PS-341, phenylbutyrate, ET-18-OCH3, or farnesyl transferase inhibitors (L-739749, L-744832); polyphenols such as quercetin, resveratrol, piceatannol, epigallocatechine gallate, theaflavins, flavanols, procyanidins, betulinic acid and derivatives thereof; autophagy inhibitors such as chloroquine; delta-9-tetrahydrocannabinol (dronabinol, MARINOL™); beta-lapachone; lapachol; colchicines; betulinic acid; acetylcamptothecin, scoplectin, and 9-aminocamptothecin; podophyllotoxin; tegafur (UFTORAL™); bexarotene (TARGRETIN™); bisphosphonates such as clodronate (for example, BONEFOST™ or OSTACTM), etidronate (DIDROCAL™), NE-58095, zoledronic acid/zoledronate (ZOMETATM), alendronate (FOSAMAX™), pamidronate (AREDIA™), tiludronate (SKELID™), or risedronate (ACTONEL™); and epidermal growth factor receptor (EGF-R); vaccines such as THERATOPETM vaccine; perifosine, COX-2 inhibitor (e.g. celecoxib or etoricoxib), proteasome inhibitor (e.g. PS341); CCI-779; tipifarnib (R11577); orafenib, ABT510; Bcl-2 inhibitor such as oblimersen sodium (GENASENSE™); pixantrone; farnesyltransferase inhibitors such as lonafarnib (SCH 6636, SARASAR™); and pharmaceutically acceptable salts, acids or derivatives of any of the above; as well as combinations of two or more of the above such as CHOP, an abbreviation for a combined therapy of cyclophosphamide, doxorubicin, vincristine, and prednisolone; and FOLFOX, an abbreviation for a treatment regimen with oxaliplatin (ELOXATIN™) combined with 5-FU and leucovorin.
- Chemotherapeutic agents can also include non-steroidal anti-inflammatory drugs with analgesic, antipyretic and anti-inflammatory effects. NSAIDs include non-selective inhibitors of the enzyme cyclooxygenase. Specific examples of NSAIDs include aspirin, propionic acid derivatives such as ibuprofen, fenoprofen, ketoprofen, flurbiprofen, oxaprozin and naproxen, acetic acid derivatives such as indomethacin, sulindac, etodolac, diclofenac, enolic acid derivatives such as piroxicam, meloxicam, tenoxicam, droxicam, lomoxicam and isoxicam, fenamic acid derivatives such as mefenamic acid, meclofenamic acid, flufenamic acid, tolfenamic acid, and COX-2 inhibitors such as celecoxib, etoricoxib, lumiracoxib, parecoxib, rofecoxib, rofecoxib, and valdecoxib. NSAIDs can be indicated for the symptomatic relief of

conditions such as rheumatoid arthritis, osteoarthritis, inflammatory arthropathies, ankylosing spondylitis, psoriatic arthritis, Reiter's syndrome, acute gout, dysmenorrhoea, metastatic bone pain, headache and migraine, postoperative pain, mild-to-moderate pain due to inflammation and tissue injury, pyrexia, ileus, and renal colic.

5 *Immune Checkpoint Modulation:* In certain embodiments, immune checkpoint modulators are co-administered with the hyperactivated dendritic cells. Immune checkpoints refer to inhibitory pathways of the immune system that are responsible for maintaining self-tolerance and modulating the duration and amplitude of physiological immune responses.

10 Certain cancer cells thrive by taking advantage of immune checkpoint pathways as a major mechanism of immune resistance, particularly with respect to T cells that are specific for tumor antigens. For example, certain cancer cells may overexpress one or more immune checkpoint proteins responsible for inhibiting a cytotoxic T cell response. Thus, immune checkpoint modulators can be administered to overcome the inhibitory signals and permit and/or augment an immune attack against cancer cells. Immune checkpoint modulators may
15 facilitate immune cell responses against cancer cells by decreasing, inhibiting, or abrogating signaling by negative immune response regulators (e.g. CTLA4), or may stimulate or enhance signaling of positive regulators of immune response (e.g. CD28).

20 Immunotherapy agents targeted to immune checkpoint modulators can be administered to encourage immune attack targeting cancer cells. Immunotherapy agents can be or include antibody agents that target (e.g., are specific for) immune checkpoint modulators. Examples of immunotherapy agents include antibody agents targeting one or more of CTLA-4, PD-1, PD-L1, GITR, OX40, LAG-3, KIR, TIM-3, CD28, CD40; and CD137. Specific examples of antibody agents can include monoclonal antibodies. Certain monoclonal antibodies targeting immune checkpoint modulators are available. For instance,
25 ipilimumab targets CTLA-4; tremelimumab targets CTLA-4; pembrolizumab targets PD-1, etc.

30 The Programmed Death 1 (PD-1) protein is an inhibitory member of the extended CD28/CTLA-4 family of T cell regulators (Okazaki *et al.* (2002) *Curr Opin Immunol* 14: 391779-82; Bennett *et al.* (2003) *J. Immunol.* 170:711-8). Other members of the CD28 family include CD28, CTLA-4, ICOS and BTLA. Two cell surface glycoprotein ligands for PD-1 have been identified, Program Death Ligand 1 (PD-L1) and Program Death Ligand 2 (PD-L2). PD-L1 and PD-L2 have been shown to downregulate T cell activation and cytokine

secretion upon binding to PD-1 (Freeman *et al.* (2000) *J Exp Med* 192:1027-34; Latchman *et al.* (2001) *Nat Immunol* 2:261-8; Carter *et al.* (2002) *Eur J Immunol* 32:634-43; Ohigashi *et al.* (2005) *Clin Cancer Res* 11:2947-53).

PD-L1 (also known as cluster of differentiation 274 (CD274) or B7 homolog 1 (B7-H1)) is a 40 kDa type 1 transmembrane protein. PD-L1 binds to its receptor, PD-1, found on activated T cells, B cells, and myeloid cells, to modulate activation or inhibition. Both PD-L1 and PD-L2 are B7 homologs that bind to PD-1, but do not bind to CD28 or CTLA-4 (Blank *et al.* (2005) *Cancer Immunol Immunother.* 54:307-14). Binding of PD-L1 with its receptor PD-1 on T cells delivers a signal that inhibits TCR-mediated activation of IL-2 production and T cell proliferation. The mechanism involves inhibition of ZAP70 phosphorylation and its association with CD3.zeta. (Sheppard *et al.* (2004) *FEBS Lett.* 574:37-41). PD-1 signaling attenuates PKC- θ activation loop phosphorylation resulting from TCR signaling, necessary for the activation of transcription factors NF- κ B and AP-1, and for production of IL-2. PD-L1 also binds to the costimulatory molecule CD80 (B7-1), but not CD86 (B7-2) (Butte *et al.* (2008) *Mol Immunol.* 45:3567-72).

Expression of PD-L1 on the cell surface has been shown to be upregulated through IFN- γ stimulation. PD-L1 expression has been found in many cancers, including human lung, ovarian and colon carcinoma and various myelomas, and is often associated with poor prognosis (Iwai *et al.* (2002) *PNAS* 99:12293-7; Ohigashi *et al.* (2005) *Clin Cancer Res* 11:2947-53; Okazaki *et al.* (2007) *Intern. Immun.* 19:813-24; Thompson *et al.* (2006) *Cancer Res.* 66:3381-5). PD-L1 has been suggested to play a role in tumor immunity by increasing apoptosis of antigen-specific T-cell clones (Dong *et al.* (2002) *Nat Med* 8:793-800). It has also been suggested that PD-L1 might be involved in intestinal mucosal inflammation and inhibition of PD-L1 suppresses wasting disease associated with colitis (Kanai *et al.* (2003) *J Immunol* 171:4156-63).

Exemplary anti-PD1 antibodies include pembrolizumab (MK-3475, Merck), nivolumab (BMS-936558, Bristol-Myers Squibb), and pidilizumab (CT-011, Curetech LTD.). Anti-PD1 antibodies are commercially available, for example from ABCAMTM (AB137132), BIOLEGENDTM (EH12.2H7, RMP1-14) and Affymetrix Ebioscience (J105, J116, MIH4).

The practice of the present disclosure employs, unless otherwise indicated, conventional techniques of chemistry, molecular biology, microbiology, recombinant DNA,

- genetics, immunology, cell biology, cell culture and transgenic biology, which are within the skill of the art. See, e.g., Maniatis et al., 1982, *Molecular Cloning* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.); Sambrook et al., 1989, *Molecular Cloning*, 2nd Ed. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.); Sambrook and Russell, 2001, *Molecular Cloning*, 3rd Ed. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.); Ausubel et al., 1992, *Current Protocols in Molecular Biology* (John Wiley & Sons, including periodic updates); Glover, 1985, *DNA Cloning* (IRL Press, Oxford); Anand, 1992; Guthrie and Fink, 1991; Harlow and Lane, 1988, *Antibodies*, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.); Jakoby and Pastan, 1979; *Nucleic Acid Hybridization* (B. D. Hames & S. J. Higgins eds. 1984); *Transcription And Translation* (B. D. Hames & S. J. Higgins eds. 1984); *Culture Of Animal Cells* (R. I. Freshney, Alan R. Liss, Inc., 1987); *Immobilized Cells And Enzymes* (IRL Press, 1986); B. Perbal, *A Practical Guide To Molecular Cloning* (1984); the treatise, *Methods In Enzymology* (Academic Press, Inc., N.Y.); *Gene Transfer Vectors For Mammalian Cells* (J. H. Miller and M. P. Calos eds., 1987, Cold Spring Harbor Laboratory); *Methods In Enzymology*, Vols. 154 and 155 (Wu et al. eds.), *Immunochemical Methods In Cell And Molecular Biology* (Mayer and Walker, eds., Academic Press, London, 1987); *Handbook Of Experimental Immunology*, Volumes I-IV (D. M. Weir and C. C. Blackwell, eds., 1986); Riott, *Essential Immunology*, 6th Edition, Blackwell Scientific Publications, Oxford, 1988; Hogan et al., *Manipulating the Mouse Embryo*, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986); Westerfield, M., *The zebrafish book. A guide for the laboratory use of zebrafish (Danio rerio)*, (4th Ed., Univ. of Oregon Press, Eugene, 2000).

Genes: All genes, gene names, and gene products disclosed herein are intended to correspond to homologs from any species for which the compositions and methods disclosed herein are applicable. It is understood that when a gene or gene product from a particular species is disclosed, this disclosure is intended to be exemplary only, and is not to be interpreted as a limitation unless the context in which it appears clearly indicates. Thus, for example, for the genes or gene products disclosed herein, are intended to encompass homologous and/or orthologous genes and gene products from other species.

Ranges: throughout this disclosure, various aspects of the disclosure can be presented in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the invention. Accordingly, the description of a range should be considered to have

specifically disclosed all the possible subranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed subranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 2.7, 3, 4, 5, 5.3, and 6. This applies regardless of the breadth of the range.

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

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. It is to be understood and expected that variations in the principles of invention herein disclosed may be made by one skilled in the art and it is intended that such modifications are to be included within the scope of the present invention.

EXAMPLES

The invention is further described in the following examples, which do not limit the scope of the invention described in the claims.

Example 1: Hyperactive dendritic cells stimulate durable anti-tumor immunity to complex antigen mixtures

The ideal strategy of stimulating protective immunity would be to combine the benefits of activated and pyroptotic DCs, whereby activated cells would have the ability to release IL-1 β while maintaining viability. The inventors have recently identified a new activation state of DCs which display these attributes. When DCs are exposed to PAMPs (*e.g.* TLR ligands) and a collection of oxidized phospholipids released from dying cells (DAMPs), the cells achieve a long-lived state of “hyperactivation” (I. Zanoni, *et al. Science*, vol. 352, no. 6290, pp. 1232–1236, 2016; I. Zanoni, *et al. Immunity*, vol. 47, no. 4, p. 697–709.e3, 2017). The collection of oxidized lipids are known as oxPAPC (oxidized 1-palmitoyl-2-arachidonyl-*sn*-glycero-3-phosphorylcholine). Hyperactive DCs display the activities of activated DCs, in terms of cytokine release (*e.g.* TNF α), but they have gained the ability to also release IL-1 β over the course of several days. Consistent with their assignment as “hyperactive” DCs, these cells are superior to their activated counterparts, in terms of their ability to stimulate T cell responses to model antigens.

Mechanisms underlying the hyperactive state of DCs have been defined, as the DAMPs in question (oxPAPC) are able to bind and stimulate the cytosolic PRR caspase-11 (I. Zanoni, *et al.* 2016). Caspase-11 stimulation results in the activation of NLRP3 and the assembly of an inflammasome that does not lead to pyroptosis, but rather leads to the release of IL-1 β from living cells. IL-1 β release from hyperactive cells is mediated by the pore forming protein gasdermin D, which serves as a conduit for the secretion of these cytokines (C. L. Evavold, *et al. Immunity*, 2018 Jan 16;48(1):35-44.e6.; X. Liu, *et al. Nature*, vol. 535, no. 7610, pp. 153–158, 2016; R. A. Aglietti, *et al. Proc. Natl. Acad. Sci. U. S. A.*, vol. 113, no. 28, pp. 7858–63, Jul. 2016; N. Kayagaki, *et al. Nature*, vol. 526, no. 7575, pp. 666–671, Sep. 2015). It is thought that the plasma membrane is repaired to remove gasdermin D pores in a fashion that ensures cell viability (S. Rühl, *et al. Science*, vol. 362, no. 6417, pp. 956–960, Nov. 2018), whereas in other instances (alum stimulation) membrane repair pathways may be overwhelmed and pyroptosis ensues. Despite this insight into the mechanisms of how IL-1 β can be released from living cells, the physiological benefits of the hyperactive cell state in terms of instruction of adaptive immunity have been poorly defined.

Materials and Methods

Mouse strains, and Tumor cell lines: C57BL/6J (Jax 000664), caspase-1/-11 dKO mice (Jax 016621), NLRP3KO (Jax 021302), Casp11KO (Jax 024698), OT-I (Jax 003831) and OT-II (Jax 004194) and BALB/c (Jax 000651) mice were purchased from Jackson Labs. For syngeneic tumor models in C57BL/6J, two melanoma cell lines were used. The parental cell line: B16.F10 and an OVA expressing cell line: B16.F10OVA. For a syngeneic colorectal model, MC-38 cell line expressing OVA derived from C57BL/6 murine colon adenocarcinoma cells was used. These cell lines were a gift from Arlene Sharpe Laboratory. For a syngeneic colon cancer model in BALB/c mice, CT26 cell line was used (a gift from Jeff Karp laboratory).

Reagents: *E. coli* LPS (Serotype O55:B5- TLRGRADE™) was purchased from Enzo and used at 1 μ g/ml in cell culture or 10 μ g/mice for *in vivo* use. Monophosphoryl Lipid A from *S. minnesota* R595 (MPLA) was purchased from Invivogen and used at 1 μ g/ml in cell culture or 20 μ g/mice for *in vivo* use. OxPAPC was purchased from Invivogen, resuspended in pre-warmed serum-free media and was used as 100 μ g/ml for cell stimulation, or 65 μ g/mice for *in vivo* use. POVPC and PGPC were purchased from Cayman Chemical. Reconstitution of commercially available POVPC and PGPC was performed as previously described (C. L. Evavold *et al., Immunity*, 2018 Jan 16;48(1):35-44). Briefly, ethanol solvent

is evaporated using a gentle nitrogen gas stream. Pre-warmed serum-free media was then immediately added to the dried lipids to a final concentrations of 1 mg/ml. Reconstituted lipids were incubated at 37°C for 5-10 mins and were sonicated for 20 s before adding to cells. POVPC or PGPC were used at 100 µg/ml for cell stimulation or 65µg/mice for *in vivo* use. EndoFit chicken egg ovalbumin protein with endotoxin levels <1 EU/mg and OVA 257-264 peptide were purchased from Invivogen for *in vivo* use at a concentration of 200µg/mice or *in vitro* use at a concentration of 500 or 100µg/ml. Incomplete Freund's Adjuvant (F5506) was purchased from Sigma and used for *in vivo* immunizations at a working concentration of 1:4 (IFA : antigen emulsion). Alhydrogel referred to as alum was purchased from Accurate Chemical, and used for *in vivo* immunization at a working concentration of 2mg/mouse. In some experiments, Addavax which is a Squalene-oil-in-water adjuvant was used instead of IFA at a working concentration of 1:2 (AddaVax: antigen).

Cell culture: BMDCs were generated by differentiating bone marrow in IMDM (Gibco), 10% B16-GM-CSF derived supernatant, 2µM 2-mercaptoethanol, 100 U/ml penicillin, 100µg/ml streptomycin (Sigma-Aldrich) and 10% FBS. 6 day after culture, BMDCs were washed with PBS and re-plated in IMDM with 10% FBS at a concentration of 1×10^6 cells/ml in a final volume of 100µl. CD11c⁺ DC purity was assessed by flow cytometry using BD Fortessa and was routinely above 80%. Splenic DCs from mice injected with B16-FLT3 for 15 days, were purified as CD11c⁺MHC⁺ live cells, then plated at a concentration of 1×10^6 cells/ml in a final volume of 100µl in complete IMDM. To induce hyperactive or pyroptotic BMDCs, DCs were primed with LPS (1µg/ml) for 3 hours, then stimulated with OxPAPC or PGPC (100µg/ml) or alum (100µg/ml) for 21h in complete IMDM. In some cases, activated BMDCs were re-stimulated for additional 24h onto plate-bound agonistic anti-CD40, using Ultra-LEAF anti-mouse CD40 (clone 1C10; BioLegend). T cells were cultured in RPMI-1640 (Gibco) supplemented with 10% FBS, 100 U/ml penicillin, 100µg/ml streptomycin (Sigma-Aldrich), and 50 µM β-mercaptoethanol (Sigma-Aldrich). Tumor cell lines were all cultured in DMEM supplemented with 10% FBS. For OVA expressing cell lines, puromycin (2µg/ml) was added to the media.

LDH Assay and ELISAs: Fresh supernatants were clarified by centrifugation after BMDC stimulation, then assayed for LDH release assay using the Pierce LDH cytotoxicity colorimetric assay kit (Life Technologies) following the manufacturer's protocol. Measurements for absorbance readings were performed on a Tecan plate reader at wavelengths of 490 nm and 680 nm. To measure secreted cytokines, supernatants were

collected, clarified by centrifugation and stored at -20°C. ELISA for IL-1 β , TNF α , IL-10, IL-12p70, IFN γ , IL-2, IL-13, IL-4 and IL-17 were performed using eBioscience Ready-SET-Go! (now ThermoFisher) ELISA kits according to the manufacturer's protocol.

Flow cytometry: After FcR blockade, 7 days BMDCs were resuspended in MACS
 5 buffer (PBS with 1% FCS and 2 mM EDTA), and stained with the following fluorescently conjugated antibodies (BioLegend): anti-CD11c (clone N418), anti-I-A/I-E (clone M5/114.15.2), anti-CD40 (clone 3/23), anti-CD80(16-10A1), anti-CD69 clone (H1.2F3), anti-H-2Kb (clone AF6-88.5). Single cell suspension from the tumor or draining inguinal lymph nodes, or skin inguinal adipose tissue were resuspended in MACS buffer (PBS with
 10 1% FCS and 2 mM EDTA), and stained with the following fluorescently conjugated antibodies (BioLegend): anti-CD8 α (clone 53-6.7), anti-CD4 (clone RM4-5), anti-CD44 (clone IM7), anti-CD62L (MEL-14), anti-CD3 (17A2), anti-CD103 (2E7), anti-CD69 clone (H1.2F3), anti-CD45 (A20 or 30F11). LIVE/DEAD™ Fixable Violet Dead Cell Stain Kit (Molecular probes) was used to determine the viability of cells, and cells were stained for
 15 20minutes in PBS at 4°C. Draining inguinal lymph nodes T cells were stained with OVA-peptide tetramers at room temperature for 1h. PE-conjugated H2K(b) SIINFEKL (OVA 257-264; SEQ ID NO: 1) and APC conjugated I-A(b) AAHAEINEA (OVA 329-337; SEQ ID NO: 2) were used. I-A(b) and H2K(b) associated with CLIP peptides were used as isotype controls. Tetramers were purchased for NIH tetramer core facility. In some experiments,
 20 FITC anti-CD8.1 (clone Lyt-2.1 CD8-E1) purchased from accurate chemical was used with tetramers. To determine absolute number of cells, countBright counting beads (Molecular probes) were used, following the manufacturer's protocol. Appropriate isotype controls were used as a staining control. Data were acquired on a BD FACS ARIA or BD Fortessa. Data were analyzed using FlowJo software.

Antigen uptake assay: To examine antigen uptake and the endocytic ability of BMDC during different activation states (active, hyperactive or pyroptotic states), FITC labeled-chicken OVA (FITC-OVA) was used (Invitrogen-Molecular Probes). Briefly, pretreated BMDCs were incubated with either FITC-OVA or AF488-dextran (0.5 mg/ml) during 45 minutes at 37°C, or 4°C (as a control for surface binding of the antigen). BMDCs were then
 30 washed, and stained with Live/Dead Fixable Violet Dead Cell Stain Kit (Molecular probes) to distinguish living cells from dead cells. Cells were then fixed with BD fixation solution and resuspended in MACS buffer (PBS with 1% FCS and 2 mM EDTA). FITC fluorescence of live cells was measured every 15 minutes using Fortessa flow cytometer (Becton-Dickenson).

Fluorescence values of BMDCs incubated at 37°C were reported as percentage of OVA-FITC or Dextran-AF488 associated cells and data were normalized to the percentage of OVA-FITC associated cells incubated at 4°C.

- OVA antigen presentation assay: To measure the efficiency of OVA antigen presentation on MHC-I, (0.5×10⁶) BMDCs treated with an activation (LPS), a hyperactivation (LPS+PGPC or LPS+OxPAPC) or a pyroptotic stimuli (LPS+Alum) were incubated with Endofit-OVA protein (0.5 mg/ml) for 2 hours at 37°C. Cells were then washed with MACS buffer and stained on ice for 20 to 30 min with APC anti-mouse H-2K^b antibody (Clone AF6-88.5, BioLegend), and PE-conjugated antibody that binds to H-2K^b bound to the OVA peptide SIINFEKL (SEQ ID NO: 1; Clone 25-D1.16, BioLegend). Appropriate isotype controls were used as a staining control. The percentage of total surface H-2K^b, and the percentage of cells associated with the OVA peptide on MHC-I was calculated. Data were acquired on a Fortessa flow cytometer (Becton-Dickenson) and analyzed with FlowJo software (Tree Star).
- OT-I and OT-II in vitro T-cell stimulation:* Splenic CD8⁺ and CD4⁺ T cells were sorted from OT-I and OT-II mice by magnetic cell sorting with anti-CD8 beads or anti-CD4 beads respectively (Miltenyi Biotech). Sorted T cells were then seeded in 96-well plates at a concentration of 100,000 cells per well in the presence of either 20,000 or 10,000 DCs (5:1 or 10:1 ratio) that were pretreated with either LPS (activation stimuli) or LPS+PGPC (hyperactivation stimuli) or LPS+Alum (pyroptotic stimuli) and pulsed (or not) with OVA protein or SIINFEKL (SEQ ID NO: 1) peptide at 100 µg/ml for 2 hours. 5 days post culture, supernatants were collected and clarified by centrifugation for short-term storage at -20°C and cytokine measurement by ELISA.

- Intracellular staining:* For intracellular cytokine staining, cells were stimulated with 50 ng/ml phorbol 12-myristate 13-acetate (PMA) and 500 ng/ml ionomycin (Sigma-Aldrich) in the presence of GolgiStop (BD) and brefeldin A for 4-5 h. Cells were then washed twice with PBS, and stained with LIVE/DEAD™ Fixable violet or green Dead Cell Stain Kit (Molecular probes) in PBS for 20 min at 4°C. Cells were washed with MACS buffer, and stained for appropriate surface markers for 20 min at 4°C. After two washes, cells were fixed and permeabilized using BD Cytofix/Cytoperm kit for 20 min at 4°C, then washed with 1X perm wash buffer (BD) per manufacturer's protocol. Intracellular cytokine staining was performed in 1X perm buffer for 20-30 min at 4°C with the following conjugated antibodies all purchased from BioLegend: anti-Ki67(clone 16A8), anti-IFN-γ (clone XMG1.2), anti

TNF α (clone MP6-XT22), anti-Gata3 (16E10A23), anti-IL4(11B11), anti-IL10 (clone JES5-16E3). Data were acquired on a BD FACS ARIA or BD Fortessa. Data were analyzed using FlowJo software.

CD107a Degranulation Assay: To evaluate the effector antitumor activity of CD8⁺ T cells, surface exposure of the lysosomal-associated protein CD107a was assessed by flow cytometry. Briefly, CD8⁺ T cells from the skin draining lymph nodes of immunized mice were isolated by magnetic cell enrichment with anti-CD8 beads and columns (Miltenyi Biotec), then sorted as CD3⁺CD8⁺Live cells on FACS ARIA(BD). Freshly sorted CD8⁺ T cells were resuspended in complete RPMI at a concentration of 1×10^6 cells/ml. PerCP/Cy5.5 anti-mouse CD107a (LAMP-1) antibody (Clone1D4B, BioLegend) was added at a concentration of 1 μ g/ml to this media, in the presence of GolgiStop (BD). T cells were then immediately seeded as 100,000 cells onto 10,000 MC38OVA or B16OVA tumor cells/well in 96 wells plates. Alternatively, CD8⁺ T cells were seeded alone and stimulated with 50 ng/ml phorbol 12-myristate 13-acetate (PMA) and 500 ng/ml ionomycin (Sigma-Aldrich). 5 hours post-culture, cells were washed with MACS buffer, stained with LIVE/DEADTM Fixable Violet Dead Cell Stain Kit (Molecular probes), and APC anti-CD8 (Clone 53-6.7, BioLegend). Cells were then Fixed with BD fixation solution for 20 min at 4°C and resuspended in MACS buffer. The percentage of CD107a⁺ cells was determined by flow cytometry on the Fortessa flow cytometer (BD).

In vitro cytotoxicity assay: CD8⁺ T cells from the spleen, or the skin inguinal adipose tissue of survivor mice were isolated using anti-CD8 MACS beads and columns (Miltenyi Biotec). Enriched T cells were then sorted as live CD45⁺CD3⁺CD8⁺ cells using FACS ARIA. Purity post-sorting was >97%. Tumor cell lines such as B16OVA, B16F-10 or CT26 cells were seeded onto 96-well plates (2×10^4 cells/well) in complete DMEM at least 5 hours prior their co-culture with T cells. 10^5 CD8⁺ T cells were seeded onto tumor cells for 12h, then cytotoxicity was assessed by LDH release assay using the Pierce LDH cytotoxicity colorimetric assay kit (Life Technologies) following the manufacturer's protocol.

Whole tumor cell lysates preparation: To prepare whole tumor cell lysates (WTL) for immunization, tumor cell lines were cultured for 4-5 days in complete DMEM. When cells became confluent, supernatants were collected, and the cells were washed and dissociated using trypsin-EDTA (Gibco). Tumor cell lines were then resuspended at 5×10^6 cells/ml in their collected culture supernatant, then lysed by 3 cycles of freeze-thawing.

Tumor Infiltration: To assess the frequency of tumor-infiltrating lymphocytes (TIL) in immunized mice, tumors were harvested when their size reached 1.8-2 cm. Tumors were dissociated using the tumor Dissociation Kit (Milteny Biotec) and the gentleMACS dissociator following the manufacturer's protocol. After digestion, tumors were washed with PBS and passed through 70- μ m and 30- μ m filters. CD45⁺ cells were positively selected using CD45 microbeads (Milteny Biotec), and T cell infiltration was assessed by flow cytometry. Tumor infiltrating T cells were cultured with dynabeads mouse T-Activator CD3/CD28 (Gibco) for T cell activation and expansion.

Adoptive cell transfer: For T cell transfer, CD8⁺ T cells from the spleen, or the skin inguinal adipose tissue of survivor mice were isolated using anti-CD8 MACS beads and columns (Milteny Biotec). Enriched cells were then sorted as live CD45⁺CD3⁺CD8⁺ cells using FACS ARIA. Purity post-sorting was >97%. Sorted T cells were then stimulated for 24 h in 24-well plates ($\sim 2 \times 10^6$ cells/well) coated with anti-CD3 (4 μ g/ml) and anti-CD28 (4 μ g/ml) in the presence of IL-2 (50ng/ml). 5×10^5 of activated circulating splenic or skin inguinal adipose resident CD8⁺T cells were transferred by i.v. or intra dermal (i.d.) injection respectively into naïve recipient mice. Some mice received both T cell subsets.

For DCs transfer, BMDCs were harvested on day 6, and 5×10^6 cells were seeded in 6-well plates. DC activation was induced by incubation with hyperactive stimuli (LPS+PGPC) or activating stimuli (LPS). Tumor lysates were added to DC culture plates for 1 hour at the ratio of 1 DC to two tumor cell equivalents (i.e. 1:2). Non-loaded naïve DCs were used as negative controls.

Statistical analysis: Statistical significance for experiments with more than two groups was tested with two-way ANOVA with Tukey multiple comparison test correction. Adjusted p-values, calculated with Prism (Graphpad), are coded by asterisks: <0.05 (*); <0.0005 (***); ≤ 0.0001 (****).

Results

Hyperactivating stimuli upregulate several activities important for DCs to stimulate T cell immunity.

Virtually all studies of DC hyperactivation have focused on the ability of these cells to release IL-1 β while retaining viability. The spectrum of DC functions that are influenced by hyperactivating stimuli are undefined. To examine this spectrum, bone marrow derived DC (BMDCs) were primed with LPS and subsequently treated with oxPAPC or a specific and

pure lipid component of oxPAPC named PGPC (I. Zanoni, *et al. Science*, vol. 352, no. 6290, pp. 1232–1236, 2016). The resulting hyperactive cells were compared to traditionally activated BMDCs (treated with LPS) or pyroptotic BMDCs (primed with LPS and subsequently treated with alum). In contrast to activation stimuli, which expectedly did not induce the release of IL-1 β , pyroptotic or hyperactivating stimuli promoted IL-1 β release into the extracellular media (FIG. 1A). All stimuli examined promoted the secretion of the cytokine TNF α (FIG. 1A). These findings are in accordance with prior work, which established that LPS-activated BMDCs release TNF α but not IL-1 β (I. Zanoni *et al.*, 2016). IL-1 β secretion was co-incident with cell death in pyroptotic DCs, as assessed by the release of the cytosolic enzyme lactate dehydrogenase (LDH) (FIG. 1B). In contrast, IL-1 β secretion occurred in the absence of LDH release in hyperactive cells (FIG. 1B). Similar behaviors of BMDCs were observed when LPS was replaced with MPLA (FIGS. 3A, 3B), an FDA-approved TLR4 ligand that is used in vaccines against human papilloma virus (HPV) and hepatitis B virus (HBV). To determine if the behavior of hyperactive BMDCs extends to DCs that were differentiated *in vivo*, activities of CD11c⁺ DCs isolated from the spleen of mice that were injected with B16-FLT3, were examined. Similar to the behavior of GM-CSF-derived BMDCs, treatment with LPS and PGPC led to the release of TNF α and IL-1 β from splenic CD11c⁺ DCs in the absence of cell death, as assessed by LDH release (FIGS. 3C, 3D). These results indicate that the hyperactivating stimulus PGPC can be used to induce IL-1 β release from living DCs that have been differentiated *in vitro* or *in vivo*.

Several signals important for T cell differentiation, such as the expression of the co-stimulatory molecules CD80, CD69 and CD40, and the secretion of the p70 subunit of IL-12, were examined. CD80 surface expression was similar in DCs responding to all activation stimuli (FIG. 3E). In contrast, CD40 expression was highly influenced by activation stimulus. As compared to the activating stimulus LPS, hyperactivating stimuli induced greater expression of CD40 (FIG. 1C). Pyroptotic stimuli were very weak inducers of CD40 and CD69, even within the 20-30% of living cells that remained after LPS-alum treatments (FIGS. 1C and 3E). The differential expression of CD40 correlated with hyperactive DCs having the greatest ability to secrete IL-12p70 when cultured onto agonistic anti-CD40 coated plates (FIG. 1D).

Hyperactive BMDCs were no better than their activated counterparts at antigen capture, as assessed by the equivalent internalization of fluorescent ovalbumin (OVA-FITC) (FIGS. 4A, 6B), yet the former cell population displayed a greater abundance of OVA-

derived SIINFEKL peptide on MHC-I molecules at the cell surface (FIGS. 1E and 4C). Total surface MHC-I abundance did not differ between activated and hyperactivated cells (FIG. 3E). Taken together, as compared to other stimuli of DCs, hyperactivating stimuli exhibit an enhancement of several activities important for T cell differentiation.

5 *Hyperactive DCs stimulate a TH1-focused immune response, with no evidence of TH2 immunity.*

To assess the influence of DC activation states on T cell instruction, BMDCs were treated as described above, and were then loaded with OVA. These cells were exposed to naïve OT-II or OT-I T cells. OT-II cells express a T cell Receptor (TCR) specific for an MHC-II restricted OVA peptide (OVA 323-339), whereas OT-I cells express a TCR specific for an MHC-I restricted OVA peptide (OVA 257-264) (K. A. Hogquist, *et al. Cell*, vol. 76, no. 1, pp. 17–27, Jan. 1994; M. J. Barnden, *et al. Immunol. Cell Biol.*, vol. 76, no. 1, pp. 34–40, Feb. 1998). The activities of the responding T cells were assessed by ELISA, to determine T cell polarization towards TH1 responses (IFN γ production), or TH2 responses (IL-10, IL-4 or IL-13 production). Regardless of DC activation state, OVA treated BMDCs stimulated the production of IFN γ from OT-II T cells. The extent of IFN γ production varied modestly between activation stimuli examined (FIG. 1F). Similarly, TNF α production by responding OT-II cells was comparable when comparing all DC activation states (FIG. 1F). These results indicate that *in vitro*, TH1 responses are commonly induced, regardless of the activation state of the antigen presenting cell (APC). In contrast, TH2 responses were strikingly different when comparing DC activation states. Stimuli that induce BMDC activation (LPS) or pyroptosis (LPS+alum) promoted the release of large amounts of IL-10, and IL-13, whereas hyperactivating stimuli led to minimal production of these TH2-associated cytokines (FIG. 1F). Intracellular staining of single cells for TH1 (IFN γ and TNF α) and TH2 (IL-4 and IL-10) cytokines as well as the TH2-lineage defining transcription factor GATA3 permitted the calculation of the ratio of TH1 and TH2 cells generated by different DC activating stimuli. This analysis revealed that hyperactive BMDCs induce a strong skewing of individual T cells towards the IFN γ -producing TH1 lineage (FIGS. 1G and 5). The ratio of TH1 to TH2 cells under hyperactive conditions was greater than 100:1 (FIG. 1G). In contrast, all other activating stimuli induced a mixed T cell response, with pyroptotic stimuli leading to a nearly 1:1 ratio of TH1 to TH2 cells (FIG. 1G).

Similar studies were done with CD8⁺ OT-I T cells, which revealed a slight enhancement of IFN γ production by stimuli that hyperactivate BMDCs, as compared to

activating or pyroptotic stimuli (FIG. 1F). IL-2 production by responding OT-I cells was comparable when comparing all DC activation states (FIG. 1F). These collective results indicate that *in vitro*, the hyperactive BMDC state leads to a highly TH1-biased T cell response and a slightly enhanced CD8 T cell response. In contrast, activation or pyroptotic stimuli yielded a mixed TH1 and TH2 response.

Hyperactivation of inflammasome-competent DCs is sufficient to confer protective anti-tumor immunity. As DCs are the principal cells responsible for stimulating *de novo* T cell mediated immunity, it was sought to determine if conditions that specifically hyperactivate DCs is sufficient to confer anti-tumor immunity. This possibility was addressed by performing an adoptive transfer into mice of BMDCs that were stimulated *ex vivo* with different activation stimuli and WTL. BMDCs were chosen because these cells are 1) well-characterized to become hyperactivated and 2) are considered models for monocyte-derived DCs, which are the most common APCs used in DC-based immunotherapies in humans (R. L. Sabado *et al.*, *Cell Res.*, vol. 27, no. 1, pp. 74–95, Jan. 2017).

BMDCs were treated with various activation stimuli, along with WTL, and were then injected s.c. every 7 days for 3 consecutive weeks into B16OVA tumor-bearing mice. BMDCs that were activated with LPS and pulsed with B16OVA WTL provided a slight protection from B16OVA-induced lethality, as compared to mice injected with naïve BMDCs; 25–30% of mice that received DC transfer rejected tumors and remained tumor-free long after the last/third DC transfer procedure (FIG. 2). Notably, hyperactive BMDCs induced a complete rejection of B16OVA tumors in 100% of tumor-bearing mice (FIG. 2). The anti-tumor activity of hyperactive DCs was dependent on inflammasomes in these cells, as NLRP3^{-/-} and Casp1^{-/-}11^{-/-} BMDC transfers induced only a minor rejection that was comparable to active DCs (FIG. 2). These data therefore indicate that hyperactive DCs are sufficient to induce durable protective anti-tumor immunity, and that inflammasomes within DCs are essential for this process.

Discussion

In this study, the immunological activities that are upregulated upon treatment of DCs with hyperactivating stimuli were expanded. Not only are these stimuli capable of eliciting IL-1 β release from living cells, but hyperactivating stimuli also exceed other activation stimuli in their ability to induce CD40 expression and IL-12p70 secretion. Furthermore, cells exposed to hyperactivating stimuli exhibit enhanced surface expression of MHC-peptide

complexes. These collective findings underscore the hyperactive nature of the DCs that are exposed to oxPAPC or its pure component PGPC and provides evidence of an enhanced ability to stimulate adaptive immunity. While it was found that hyperactive DCs are indeed better stimulators of T cell responses than activated or pyroptotic cells, the most notable aspect of their activities may be their ability to stimulate a TH1- and CTL-focused response. Indeed, stimuli that hyperactivate DCs led to a 100:1 ratio of TH1:TH2 cells; no other strategy of DC activation induced such a biased T cell response.

It is noteworthy that alum, a well-defined inflammasome stimulus, does not exhibit the same activities as oxPAPC or PGPC. Indeed, it is well-recognized that alum induces TH2 immunity. These findings were verified in this study, as alum or alum+LPS treatments induced robust TH2 immunity. One possible reason for the lack of TH1-focused immunity of alum-treated cells is based on findings herein that alum is a poor inducer of several signals necessary for TH1 differentiation, such as CD40 expression and IL-12p70 secretion. Notably, even when the DCs that did not undergo pyroptosis in response to alum+LPS were examined, CD40 expression was strikingly low. The lack of high-level expression of these factors likely renders pyroptotic stimuli weak inducers of TH1 responses and consequently, anti-tumor immunity. Without wishing to be bound by theory, it was proposed that the TH1-focused immunity induced by hyperactive DCs result from the actions of inflammasomes, as well as several other features of these cells. These additional features include enhanced antigen presenting capacity, CD40 expression, IL-12p70 expression and increased viability. It is likely that each of these enhanced activities are important for DC functions as APCs and likely contribute to the strong TH1-focused immune responses observed under conditions of DC hyperactivation.

These results may help explain why certain chemotherapeutic agents (*e.g.* oxaliplatin) induce tumor cell death and inflammasome-dependent anti-tumor T cell immunity (F. Ghiringhelli *et al.*, *Nat. Med.*, vol. 15, no. 10, pp. 1170–1178, 2009). Oxaliplatin is a robust stimulator of reactive oxygen species (ROS) production, which can oxidize biological membranes and create a complex mixture of distinct oxidized phospholipid species including PGPC. It is therefore possible that the protective immunity induced by oxaliplatin results from the actions of hyperactive DCs that prime anti-tumor T cell responses.

It was found that hyperactive stimuli could be harnessed as an immunotherapy using complex mixtures of antigen. WTL is an attractive source of antigens for several reasons, not the least of which is from a practical perspective. A significant benefit of WTL-based

approaches is that it alleviates the need for neo-antigen identification. Despite the potential benefits offered by WTL-based immunotherapies, prior work in this area has yielded mixed results. The finding herein, is that hyperactivating stimuli are uniquely capable of adjuvanting WTL to elicit potent anti-tumor immunity may explain the lack of success in prior work, as the strategies of DC activation discovered herein, have not before been considered. On this latter point, it is noteworthy that DC hyperactivating strategies can protect mice from lethality associated with tumors that are sensitive to PD-1 blockade and those that are resistant to PD-1 blockade. The full spectrum of tumors amenable to treatment by hyperactivating stimuli is undefined, but these studies provide a mandate to further explore the value of DC-centric strategies of cancer immunotherapy.

Example 2: Hyperactive cDC1 control tumor rejection in an inflammasome-dependent manner.

Conventional dendritic cells (cDCs) are adept at presenting exogenous and endogenous antigens to T cells and regulating T cell proliferation, survival, and effector function. cDCs are divided into two major subsets named cDC1s and cDC2s. Resident cDC1s in the spleen and lymph nodes (LNs) express CD8 α , CD24, and XCR1, while cDC2s express CD4 and Sirp α . cDC1 are classical DCs that cross-present tumor-associated antigens and prime Th1 immunity and anti-tumor CD8⁺ T cells to efficiently reject tumors. On the other hand, cDC2s govern type 2 immune responses, against parasites in which they activate Th2 immunity.

To investigate whether resident cDCs can achieve a state of hyperactivation, cDC1 or cDC2 from the spleen of WT mice were sorted and were either left untreated, or treated with LPS for 24h, or they were primed with LPS for 3h then treated with the hyperactivating stimuli oxPAPC or PGPC, or with the pyroptotic stimuli Alum for 21h. In contrast to splenic cDC2, Splenic cDC1 died quickly after isolation, as measured by their LDH release (FIG. 6A left panel). Consequently, cDC1 cells were not primed with LPS, and were unable to produce TNFa cytokine in response to activation stimuli (LPS), hyperactivating stimuli (LPS+OxPAPC/PGPC) or pyroptotic stimuli (LPS+Alum) (FIG. 6B left panel). Scarce amount of IL-1 β release was observed in response to the hyperactivating stimuli (LPS+PGPC) (FIG. 6B left panel). In contrast, splenic cDC2 cells were efficiently primed with LPS and achieved a state of hyperactivation, which is identified by their ability to produce IL-1 β without undergoing cell death (FIGS. 6A-6B left panels). Since resident cDC1 were highly sensitive to ex vivo isolation and in-vitro stimulation, we alternatively generated cDCs from bone marrow (BM) progenitors using the cytokine FLT3 ligand (FLT3L). FLT3L generated cDC1 and cDC2

were sorted 9 days post culture, then treated as previously with activation stimuli, hyperactivating stimuli or pyroptotic stimuli. In contrast to resident cDC1 cells isolated from the spleen, FLT3L-generated cDC1 and cDC2 were efficiently primed with LPS and achieved a state of hyperactivation in response to their stimulation with LPS+PGPC but not with
 5 LPS+oxPAPC, as measured by their release of high amounts of IL-1 β and TNF α while maintaining their viability (FIG. 6A-6B right panels). These data indicate that the pure form of oxidized phospholipids PGPC can hyperactivate cDC1 and cDC2 subsets.

Hyperactive FLT3L-generated cDC1 displayed more stellate dendrites as compared to their naïve, active or pyroptotic counterparts implicating a higher migration potential. Indeed,
 10 hyperactive cDC1 and cDC2 upregulated the chemokine receptor CCR7 which guides the migratory DCs to the lymph nodes for T cells stimulation (FIGS. 6C-6D). In summary, these results indicate that cDC1 and cDC2 subsets achieve a hyperactivation state in vitro and display unique attributes as compared to their classically activated counterparts.

The unique function of cDC1 cells is crucial in the context of cancer, where cDC1 take
 15 up tumor antigens and cross-present them to T cells within the tumor microenvironment (TME) or after migration to draining lymph nodes. The fact that cDC1 get hyperactive in vitro provide a mandate to further explore the value of cDC hyperactivation state for cancer immunotherapy. Therefore, to investigate the role of the hyperactive state of cDC1 in the control of tumor rejection, mice were inoculated with B16OVA cells subcutaneously (s.c.) on the left back. 7,
 20 14 and 21 days post tumor challenge, mice were either left untreated, or were injected s.c. on the right flank with 1×10^6 of untreated WT cDC1 (cDC1 naïve), or WT cDC1 treated with LPS for 23h (cDC1 active), or WT cDC1 that were primed with LPS for 3h then treated with PGPC for 20h (cDC1 hyperactive). All cDCs were pulsed with B16OVA tumor lysates for 1h prior to their injection. Surprisingly, the adoptive transfer of hyperactive cDC1 conferred mice with a
 25 strong and long-lasting protection against tumor growth, while naïve or active cDC1 transfer induced only a minor tumor rejection (FIG. 7). These data represent the first evidence for the superior role of the hyperactive state of cDC1 in durable tumor rejection.

To further confirm the crucial role of hyperactive cDC1 in tumor control, we used Batf3^{-/-} mice which lack CD8⁺cDC1, and are defective in cross-presentation, consequently
 30 Batf3^{-/-} mice lack anti-tumor antigen-specific CD8⁺ T cell responses. Accordingly, Batf3^{-/-} mice inoculated with B16OVA cells failed to control tumor growth as compared to WT mice (FIG. 8A). However, when tumor-bearing Batf3^{-/-} mice were supplemented with WT cDC1 through their s.c. injection on day 7, 14 and 21 post tumor challenge, we found that in contrast

to naïve or activated cDC1, only hyperactive cDC1 were able to completely eradicate the tumors. This protection correlated with a higher frequency of tumor infiltrating OVA-specific CD8⁺ and CD4⁺ T cells that were restored in Batf3^{-/-} mice when injected with WT hyperactive cDC1 but not WT active or naïve cDC1 cells (FIGS. 8B-8C). Overall, these data provide a strong
 5 evidence that hyperactive cDC1 control tumor rejection by enhancing tumor infiltration of anti-tumor specific T cells.

The mechanisms underlying the hyperactive state of DCs have been well defined, as the oxidized phospholipids in question (oxPAPC/PGPC) are able to bind and stimulate the cytosolic pathogen recognition receptor (PRR) caspase-11. Caspase-1/11 stimulation results
 10 in the activation and the assembly of NLRP3 inflammasome that lead to the release of IL-1 β from living cells via gasdermin-D pores. To assess the role of IL-1 β in the anti-tumoral activity of hyperactive cDC1, Casp1/11^{-/-} mice or NLRP3^{-/-} mice, which are defective in IL-1 β secretion, were used. Casp1/11^{-/-} or NLRP3^{-/-} mice were inoculated with B16OVA cells on the left back. 7, 14 and 21 days post tumor challenge, mice were either left untreated (no DC
 15 injection), or they were injected s.c. on the right flank with 1.10⁶ of either untreated WT cDC1 (cDC1^{naïve}) or WT cDC1 treated with LPS for 23h (cDC1^{active}), or with WT or Casp1/11^{-/-} cDC1 that were primed with LPS for 3h then treated with PGPC for 20h (cDC1^{hyperactive}). All DCs were pulsed with B16OVA tumor lysate for 1h prior to their injection. Interestingly, we found that in contrast to WT naïve or active cDC1 which induced
 20 only a minor protection, the adoptive transfer of WT hyperactive cDC1 into Casp1/11^{-/-} and NLRP3^{-/-} recipient mice completely eradicated tumor growth in 100% of tumor-bearing. This protection was dependent on the inflammasome machinery, as cDC1 from casp1/11^{-/-} or NLRP3^{-/-} which cannot induce IL-1 β secretion in response to the hyperactivating stimuli (LPS+PGPC) failed to induce tumor rejection. In summary, the adoptive transfer of
 25 hyperactive DCs is sufficient to induce a durable anti-tumor response and recapitulate the protection observed by hyperactivating-based vaccines.

Overall, the herein data have the potential to shift the paradigm in DC based immunotherapy, regarding the activation state employed in adoptive cell transfer based immunotherapies, and thus may re-invigorate the attempts to “condition” DC in vitro to
 30 generate effective cancer immunotherapies.

Example 3: Oxidized phospholipids induce hyperactive cDC1 and cDC2 cells.

Virtually all studies assessing the state of cell-hyperactivation have focused on the ability of bone marrow derived DC (BMDCs), generated with the cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF), to release IL-1 β while retaining viability [24,31,32,33,34]. Recent report demonstrated that monocyte-derived macrophages, rather than DCs, are responsible for inflammasome activation and IL-1 β secretion [35]. To examine whether conventional DCs can achieve a hyperactivation state, we used BMDCs generated using the DC hematopoietin Fms-like tyrosine kinase 3 ligand (Flt3L). To assess hyperactivation, FLT3-DCs were primed with LPS and subsequently treated with the oxidized phospholipids oxPAPC or a pure lipid component of oxPAPC named PGPC [36]. Alternatively, FLT3-DCs were stimulated with traditional activation stimuli such as with LPS alone, or FLT3-DCs were primed with LPS then treated with pyroptotic stimuli such as alum. In contrast to traditional activation stimuli, which did not induce IL-1 β release from DCs, pyroptotic DCs promoted IL-1 β release into the extracellular media (FIG. 11A). IL-1 β secretion was co-incident with cell death in pyroptotic DCs, as assessed by the release of the cytosolic enzyme lactate dehydrogenase (LDH) (FIG. 11B). Interestingly, stimulation with the hyperactive stimuli LPS+PGPC, or to a lesser extent with LPS+oxPAPC induced IL-1 β secretion from DCs, which occurred in the absence of LDH release (FIG. 11A). All DCs primed or stimulated with LPS promoted the secretion of the cytokine TNF α (FIG. 11A). IL-1 β secretion in pyroptotic or hyperactive DCs was in both cases dependent on the inflammasome components NLRP3 and Caspase1/11 (FIG. 11A). These findings are in accordance with prior work, which defined the mechanisms underlying the hyperactive state of DCs, in which oxPAPC bind and stimulate the cytosolic PRR caspase-11, resulting in NLRP3 activation and the assembly of a non-pyroptotic inflammasome that lead to the release of IL-1 β from living cells [24]. Similar behaviors of DCs were observed when DCs were primed with other TLR agonists such as TLR9 agonist CpG (FIG. 16A). Thus, these data indicate that FLT3 DCs can achieve a state of hyperactivation. DCs are divided into two major subsets named cDC1s and cDC2s. cDC1 are classical DCs that can cross-present tumor-associated antigens and prime CD8 $^{+}$ T cells [37], [38]. On the other hand, cDC2s govern type 2 immune responses, against parasites in which they activate Th2 immunity. To determine if the behavior of hyperactive DCs extends to cDC1 or cDC2, we isolated cDC1 or cDC2 from FLT3-DCs or from the spleen of wild type naïve mice (FIG. 16B). Similar to the behavior of FLT3-derived DCs, treatment with LPS and PGPC and to a lesser extent with

LPS and oxPAPC, led to the release of TNF α and IL-1 β from FLT3-derived cDC1s and cDC2 in the absence of cell death, as assessed by LDH release (FIG. 11A). These data indicate that PGPC is the bioactive component of oxPAPC that induces cDC1 and cDC2 hyperactivation. We also observed a similar behavior of splenic cDC2, which produced IL-1 β in response to the pyroptotic stimuli LPS and alum concomitant with pyroptotic cell death, but also in response to the hyperactivating stimuli LPS and PGPC in the absence of cell death (FIG. 16C). On the contrary, splenic cDC1 produced minimal amount of IL-1 β in response to pyroptotic or hyperactivating stimuli, since these cells were very sensitive to cell death post-sorting, and were unable to get primed by LPS (FIG. 16C). Overall, these results indicate that hyperactivating stimulus can be used to induce IL-1 β release from living DCs that have been differentiated in vitro or in vivo. For practical reasons, we continued using in this paper FLT3-derived DCs as a source of DCs.

Example 4: Hyperactive DCs potentiate CTL responses in an inflammasome dependent manner.

IL-1 β is a critical regulator of T cell differentiation, long-lived memory T cell generation and effector function [12]–[14]. We wondered whether hyperactive DCs, which produce IL-1 β over the course of several days in the dLN, can enhance CD8 $^{+}$ T cell stimulation. To test this, we sought to adoptively transfer DCs loaded with OVA protein subcutaneously (s.c), then measure OVA- specific CD8 $^{+}$ T cells in the dLN. We first tested the ability of the disparate DCs states to uptake OVA protein and to cross-present the OVA peptide SIINFEKL on H2kb molecules. We found that all DCs at the disparate states uptake OVA to a similar extend as demonstrated by the equivalent internalization of fluorescent ovalbumin (OVA-FITC). However, we found that active DCs and hyperactive DCs that were primed with LPS or with CpG both exhibited an enhanced SIINFEKL cross presentation upon OVA protein loading as compared to their naïve counterparts. This is in accordance with previous work showing that DC maturation enhances their antigen presentation capacity. Surprisingly, the pyroptotic stimuli alum strongly reduced the cross-presentation capacity of DCs suggesting that pyroptotic DCs are unfit for optimal T cell stimulation. Accordingly, when we injected 1.106 DCs of OVA-loaded naïve, active, pyroptotic or hyperactive DCs into WT mice, we observed that hyperactive DCs induced the highest frequency and absolute number of SIINFEKL $^{+}$ CD8 $^{+}$ T cells in the dLN of recipient mice (FIG. 12A and FIG. 17B) as compared to naïve, active or pyroptotic DCs. The enhanced CD8 $^{+}$ T cell responses

mediated by hyperactive DCs was dependent on inflammasome activation since the injection of NLRP3^{-/-} DCs treated with LPS+PGPC induced weak OVA-specific T cell responses.

Example 5: Hyperactivating stimuli enhance memory T cell generation and potentiate antigen-specific IFN γ effector responses in an inflammasome-dependent manner.

We hypothesized that hyperactive stimuli may represent a strong adjuvant that could recapitulate the effect of hyperactive DC injection. To examine this possibility, mice were immunized s.c. with OVA alone, or OVA plus an activating stimulus (LPS), or OVA plus a hyperactivating stimulus (LPS+oxPAPC or PGPC). 7- and 40-days post-immunization, memory and effector T cell generation in the dLN was assessed by flow cytometry using CD44 and CD62L markers that distinguish T effector cells (Teff) as CD44^{low}CD62L^{low}, T effector memory cells (TEM) as CD44^{hi}CD62L^{low}, and T central memory cells (TCM) as CD44^{hi}CD62L^{hi} [47]. Seven days post-immunization, hyperactivating stimuli were superior than activating stimuli at inducing CD8⁺ Teff cells (FIG. 13A upper panels and FIGS. 18A–18B). Furthermore, at this early time point, hyperactivating stimuli induced the highest abundance of CD8⁺ TEM (FIG. 13A middle panels and FIGS. 18A–18B). Forty days post-immunization, ample TCM cells were observed in mice exposed to hyperactivating stimuli, whereas these cells were less abundant in mice immunized with OVA alone or with LPS (FIG. 13A lower panels). Teff and TEM cells were conversely more abundant in mice immunized with OVA alone or with LPS as compared to mice immunized with OVA plus 40 days post immunization. Thus, these data indicate that the hyperactivating stimuli oxPAPC and PGPC enhance the magnitude of effector and memory T cell generation. Furthermore, the increase in the frequency of Teff cells 7 days post-immunization correlated with the enhanced IFN γ responses of CD8⁺ T cells that were isolated from the dLN of mice immunized with OVA plus hyperactivating stimuli, upon their re-stimulated ex vivo in the presence of naïve BMDCs loaded with OVA (FIG. 18C). In addition, when total CD8⁺ T cells were isolated from mice immunized with hyperactive stimuli and co-cultured with the B16 tumor cell line expressing OVA (B16OVA), CD8⁺ T cells exhibited enhanced degranulation activity as compared with CD8⁺ T cells that were isolated from mice immunized with OVA alone or OVA plus LPS (FIG. 13B and FIG. 18D), indicating that hyperactive stimuli enhance CTLs function.

To assess the antigen-specificity of T cells that result from a s.c. immunization with the distinct activation stimuli, mice were injected with OVA, alone or with activating stimuli

(LPS), or with pyroptotic stimuli (LPS+alum) or with hyperactivating stimuli (LPS+oxPAPC or PGPC). Alternatively, mice were immunized s.c. with LPS+PGPC without the OVA antigen. 7 days post immunization, CD8⁺ T cells were isolated from the skin dLN of immunized mice and were re-stimulated ex vivo for 7 days with naïve BMDC loaded (or not) with OVA in order to enrich the OVA-specific T cell subset. T cell effector function of OVA-specific T cells was assessed by intracellular staining for IFN γ . TCR specificity was assessed by staining with MHC-restricted OVA peptide tetramers. H2kb restricted SIINFEKL (OVA 257-264) peptide tetramers were used. The frequency of tetramer⁺ IFN γ ⁺ double positive cells was measured for CD4⁺ and CD8⁺ T cell subsets. Strikingly, OVA with hyperactivating stimuli were superior at inducing antigen-specific T cells, as oxPAPC- or PGPC-based immunizations resulted in the generation of the highest frequency of tetramer⁺ IFN γ ⁺ responses upon CD8⁺ T cells re-stimulation with OVA antigen (FIG. 13C). In contrast, pyroptotic stimuli (LPS+alum) were the weakest inducers of antigen-specific IFN γ responses (FIG. 13C). These results are in accordance with previous studies which indicated that alum is an adjuvant that is effective for promoting humoral immunity and Th2 responses, but not Th1 or CTL responses [39,42,43].

Previous studies showed that antigen-specific T cell responses can be enhanced by co-immunization with recombinant IL-1 β [47,13], a cytokine whose bioactivity is naturally controlled by inflammasomes. However, despite the fact that both hyperactive and pyroptotic stimuli induce IL-1 β secretion, how hyperactive stimuli but not pyroptotic stimuli induce higher antigen-specific T cells? To determine if inflammasome-mediated events control the T cell responses generated with hyperactivating stimuli, side-by-side comparisons of T cell activity were performed in WT and NLRP3^{-/-} mice. Notably, we found that NLRP3 was required for the hyperactivation-induced enhancement of antigen-specific responses by CD8⁺ T cells (FIG. 13C). Thus these data indicate that non-pyroptotic vs pyroptotic inflammasome activation following immunization with hyperactivating stimuli or pyroptotic stimuli respectively, induce a striking differential adaptive immune T cell control.

Our previous results using DC injection strategies indicate that DCs stimulated with pyroptotic stimuli lose their ability to migrate to adjacent dLN and to stimulate T cell activation, whereas DCs exposed to hyperactive stimuli hypermigrate to dLN and potentiate CTL responses (FIGS. 12A–12B). However, It's unknown whether endogenous DCs can achieve hyperactivation in vivo following immunization with hyperactive stimuli. To assess this, we generated mouse chimeras using Zbtb46DTR and WT mice or Zbtb46DTR and

NLRP3^{-/-} mice or Zbtb46DTR and Casp1/11^{-/-} mice. To this end, 4 weeks old CD45.1 irradiated mice were reconstituted with mixed bone marrow of 80% of Zbtb46DTR and 20% of WT mice or 20% of NLRP3^{-/-} or 20% of Casp1/11^{-/-} mice on a CD45.2 background as previously described[51]. Six-weeks post-reconstitution, the efficacy of BM reconstitution in all mice was assessed by flow cytometry using cD45.1 vs CD45.2 markers. Chimera mice were treated every other day with diphtheria toxin (DT) to deplete Zbtb46⁺ conventional DCs, giving rise to mice that harbor either WT or inflammasome deficient (NLRP3^{-/-} or Casp1/11^{-/-}) DCs which can or cannot get hyperactive respectively. To test the effect of endogenous DC-hyperactivation on CD8⁺T cell responses, all chimera mice were s.c. immunized with OVA plus LPS+PGPC following 3 consecutive DT injections. 7 days post-immunization, CD8⁺ T cell response from the dLN was assessed. Interestingly, we found that the abundance of Teff CD8⁺ T cells was strongly reduced in the chimera mice harboring DCs that cannot get hyperactive such as in NLRP3^{-/-} and Casp1/11^{-/-} chimeras mice, as compared to the chimera mice harboring WT DCs that can get hyperactive (FIG. 13D, FIG. 19A). Furthermore, we discovered that the frequency of SIINFEKL⁺ CD8⁺ T cells in the dLN or the spleen was reduced in NLRP3^{-/-} and Casp1/11^{-/-} chimera mice harboring DCs that cannot get hyperactive, whereas high SIINFEKL⁺ CD8⁺ cells were observed in chimera harboring WT DCs (FIG. 13E, FIG. 19B). Thus, these data clearly show that: 1) endogenous DCs can achieve a state of hyperactivation in vivo, and potentiate CTLs responses following immunization with hyperactivating stimuli, and 2) inflammasome activation within endogenous DCs is crucial for hyperactivation-mediated protective CTL responses.

Example 6: Hyperactive DC entry to lymphoid tissues is essential for hyperactivation-mediated CTLs responses.

We previously showed that DCs stimulated with hyperactive stimuli hypermigrate to dLN and potentiate CTL responses (FIGS. 12A–12B). To assess if hyperactivation-mediated CTLs responses require the entry of endogenous hyperactive DCs to dLN, we generated mice chimeras as described above, using Zbtb46DTR and WT or Zbtb46DTR and CCR7^{-/-} BM (FIG. 13D). Overall, endogenous trafficking of hyperactive DCs to dLN is essential for hyperactivation-mediated CTLs function.

Example 7: Hyperactive stimuli can use complex antigen sources to stimulate prophylactic T cell mediated anti-tumor immunity.

Current efforts to stimulate anti-tumor immunity include strategies that invigorate resident T cell populations (e.g. PD-1 blockade) or personalized cancer vaccine strategies that stimulate the generation of de novo T cell responses to tumor-specific antigens (TSAs) [46]. This latter effort has been hampered by the inability to use tumor cell lysates as a source of TSAs, also known as neo-antigens. Consequently, efforts are underway to improve the identification of neo- antigens, which can be used in pure form to elicit T cell mediated anti-tumor immunity. While these efforts have yielded successes [50,49,51], the path to neo-antigen identification requires a pipeline for mutated, as well as aberrantly expressed TSAs discovery [54], which is laborious and not representative of the natural course of events. As previously discussed, WTL represent an attractive alternative source of antigens, as these lysates provide a large number of antigens that are required for the initiation of a personalized anti-tumor immune responses. However, fundamental questions such as what is the most effective type of adjuvant to be used in cancer vaccines (including the type of adjuvant to be associated with different type of antigens) still remain unanswered.

To address the possibility that hyperactivating stimuli can be an adjuvant to WTL, mice were immunized on the right flank with WTL alone, or WTL mixed with the activating stimulus LPS or the hyperactivating stimuli LPS+oxPAPC or LPS+PGPC. The source of the WTL was B16OVA cells. Fifteen days post-immunization, mice were challenged s.c on the left upper back with the parental B16OVA cells. Unimmunized mice or mice immunized with WTL alone did not exhibit any protection, and all mice harbored large tumors by day 24 after tumor inoculation and died (FIG. 20A). Similarly, WTL+LPS immunizations offered minimal protection. Two of eight mice immunized with WTL+LPS were tumor-free, but quickly relapsed after B16OVA re-challenge (FIG. 20A), indicating no protective immunity was conferred by stimuli that merely activate DCs. In contrast, WTL immunizations in the presence of LPS and oxPAPC induced a significant delay in the tumor growth and resulted in a strong protection against subsequent lethal re-challenge with parental B16OVA tumor cells; 50% of the immunized mice were fully protected (FIG. 20A). To determine if the protective responses induced by oxPAPC correlated with T cell responses, tumors were harvested from mice receiving each activation stimulus. Tumors from mice immunized with LPS+oxPAPC contained a substantial abundance of CD4⁺ and CD8⁺ T cells, as compared to LPS immunizations (Figure S7B). Moreover, when equal numbers of T cells from these tumors

were compared, oxPAPC-based immunizations resulted in intra-tumoral T cells that secreted the highest amounts of IFN γ upon anti-CD3 and anti-CD28 stimulation (FIG. 20C). Thus, the superior restriction of tumor growth induced by hyperactivating stimuli (LPS+oxPAPC) was coincident with inflammatory T cell infiltration into the tumor.

5 Notably, the protective phenotypes of oxPAPC were superseded by those elicited by the pure oxPAPC component PGPC. WTL immunizations in the presence of LPS+PGPC led to 100% of mice being tumor-free for 150 days post-tumor challenge. These mice completely rejected a lethal re-challenge with B16OVA cells and remained tumor-free 300 days post initial tumor challenge (FIG. 20A). Since these mice never relapsed, we wondered how tumor
10 cell growth is kept under control at the site of tumor injection in mice immunized with WTL plus LPS+PGPC?

 Among memory T cell subsets, T resident memory cells (TRM) are defined by the expression of CD103 integrin along with C-type lectin CD69, which contribute to their residency characteristic in the peripheral tissues [55]. CD8⁺ TRM cells have recently gained
15 much attention, as these cells accumulate at the tumor site in various human cancer tissues and correlate with the more favorable clinical outcome [54,55,56]. In an experimental cutaneous melanoma model, CD8⁺ TRM cells in the skin promoted durable protection against melanoma progression [58].

 We examined the presence of TRM cells at the tumor injection site, as well as the
20 immunization skin biopsies in the survivor mice that were previously immunized with the hyperactivating stimuli LPS+PGPC. Interestingly, 200 days post-tumor inoculation, CD8⁺ CD69⁺ CD103⁺ TRM cells were highly enriched at the site of tumor injection but were scarce at the immunization site in all survivor mice (FIGS. 21A–21B). These data are in accordance with clinical and experimental reports that correlate high levels of TRM with a
25 long-term tumor control, in which TRM are potentially maintained for long periods to survey tumor injection site [56,57]. Thus, it is likely that the immunization with WTL and the hyperactivating stimuli generate TRM that keeps tumor cells in check.

 To examine the functional specificity of these T cells, we monitored cytotoxic lymphocyte (CTL) activity ex vivo. Circulating memory CD8⁺ T cells and TRM cells were
30 isolated from the spleen or the skin adipose tissue of survivor mice that previously received hyperactivating stimuli. These cells were cultured with B16OVA cells, or B16 cells not expressing OVA or an unrelated cancer cell line CT26. CTL activity, as assessed by LDH

release, was only observed when CD8⁺ T cells were mixed with B16OVA or B16 cells (FIG. 21C). No killing of CT26 cells was observed (FIG. 21C), thus indicating the functional and antigen-specific nature of hyperactivation-induced T cell responses.

Based on the antigen-specific T cell responses induced by hyperactivating stimuli, we determined if T cells are sufficient to protect against tumor progression. CD8⁺ T cells were transferred from survivor mice into naïve mice and subsequently challenged with the parental tumor cell line used as the initial immunogen. Transfer of CD8⁺ TRM or circulating CD8⁺ T cells from survivor mice into naïve recipients conferred profound protection from a subsequent tumor challenge, with the TRM subset playing a dominant protective role (FIG. 21D). Transfer of both T cell subsets from survivor mice into naïve mice, one week before tumor inoculation, provided 100% protection of recipient mice from subsequent tumor challenges (FIG. 21D). These collective data indicate that PGPC-based hyperactivation stimuli confer optimal protection in a B16 melanoma model by inducing strong circulating and resident anti-tumor CD8⁺ T cell responses.

Example 8: Hyperactive stimuli protect against established anti-PD1 resistant tumors.

To determine if hyperactivating stimuli could be harnessed as a cancer immunotherapy, we examined anti-tumor responses in mice that harbored a growing tumor prior to any additional treatment. For these studies, rather than using cultured tumor cells as an antigen source, *ex vivo* WTL were generated using syngeneic tumors from unimmunized mice, in which 10mm harvested tumors were dissociated and then depleted of CD45⁺ cells. Mice were inoculated subcutaneously (s.c.) with tumor cells on the left upper back. When tumors reached a size of 3-4mm, tumor-bearing mice were either left untreated (unimmunized) or received a therapeutic injection on the right flank, which consisted of *ex vivo* WTL and LPS+PGPC. Two subsequent s.c. boosts of therapeutic injections were performed (FIG. 14A). Interestingly, hyperactivation-based therapeutic injections induced tumor eradication in a wide range of tumors such as B16OVA and B16F10 melanoma models, in MC38OVA and CT26 colon cancer tumor models (FIGS. 14B–14D). In all of these models, a high percentage of mice that received the immunotherapy regimen remained tumor-free long after the tumor inoculation (FIGS. 14B–14D). The efficacy of the immunotherapy was dependent on IL-1 β in all the tested tumor models, since the neutralization of IL-1 β abolished protection conferred by hyperactivating stimuli plus *ex vivo* WTL (FIGS. 14B–14D). In addition, CD8⁺ T cells were crucial for protection against

immunogenic tumor models such as B16OVA or MC38OVA tumors, whereas CD4⁺ and CD8⁺ T cells were both required for protection against less immunogenic tumors such as CT26, and B16F-10 (FIGS. 14B–14D) [63]. To determine how hyperactivation-based immunotherapy compare in efficacy to therapies based on PD-1 blockade, side-by-side assessments were performed. Hyperactivation-based immunotherapy was as efficient as anti-PD-1 therapy in the immunogenic B16OVA model, but more efficient in tumor models that are insensitive to anti-PD-1 treatment such as CT26, and B16F-10 (FIGS. 14B–14D).

Example 9: Endogenous hyperactive DCs stimulate T cell mediated durable anti-tumor immunity.

The adoptive transfer of hyperactive DCs into tumor-bearing mice induce strong anti-tumor immunity (FIGS. 12A–12B). To test whether endogenous DCs can initiate hyperactivation-mediated anti-tumor response, we used Zbtb46DTR mice in which conventional DCs are depleted by DT injection. Zbtb46DTR or WT mice were injected s.c. with B16OVA cells. DT was then injected every other day to fully deplete resident DCs in Zbtb46DTR mice prior to their immunization. When tumors reached 4mm of size, Zbtb46DTR or WT mice were immunized with B16OVA WTL plus the hyperactivating stimuli LPS+PGPC. We found that in contrast to WT mice, which rejected tumors in 90% of mice, Zbtb46DTR mice (which lack DCs) were unable to reject tumors. These data confirm that DCs are the initiator of hyperactivation-mediated protection (FIG. 15A).

Example 10: Hyperactive cDC1 can use complex antigen sources to stimulate T cell mediated anti-tumor immunity.

We demonstrated in vitro that cDC1 and cDC2 both can achieve a state of hyperactivation, as these cells produce IL-1 β in response to LPS+PGPC while maintaining their viability. These data provide the mandate to further define the specific DC subset that initiate hyperactivation-mediated anti-tumor response in vivo. Given the importance of cDC1 subset in tumor rejection, we hypothesized that cDC1 play a central role in inducing hyperactivation-mediated anti-tumor protection. To test this idea, we used Batf3^{-/-} mice (which lack cDC1, but harbor cDC2 cells) [65]. To this end, we immunized Batf3^{-/-} or WT tumor-bearing mice (harboring a B16OVA tumor of 3- 4mm) with LPS+PGPC and WTL. These mice received two s.c boost injections every 7 days. We observed that unimmunized Batf3^{-/-} mice displayed a more severe tumor growth as unimmunized WT mice, and all mice succumb to tumors as soon as 18 days post tumor inoculation. This data corroborate with

previous studies showing that the rejection of highly immunogenic tumors is strongly impaired in Batf3^{-/-} mice that lack cDC1 cells [65]. Interestingly, despite the fact that the immunization of Batf3^{-/-} mice improved their survival by few days as compared to unimmunized Batf3^{-/-} mice, all Batf3^{-/-} mice succumbed to tumor growth by 25 days post tumor inoculation. In contrast, WT mice rejected tumors in 100% of tumor-bearing mice (FIG. 15C). Thus, cDC1 play a critical role in hyperactivation-mediate anti-tumor immunity. In addition, while immunized WT mice induced high frequency of antigen-specific CD8⁺ and CD4⁺ T cells in the TME and in the skin dLN, immunized Batf3^{-/-} induced slightly reduced antigen CD4⁺ T cells, but strikingly no antigen-specific CD8⁺ T cells in the TME (FIG. 15D).

To further confirm the role of hyperactive cDC1 in inducing long-live anti-tumor protection, we sought to assess the ability of hyperactive cDC1 to restore anti-tumor protection in Batf3^{-/-}. We adoptively transferred naïve, active, or hyperactive cDC1 cells into Batf3^{-/-} mice. To this end, FLT3⁻ derived cDC1 were sorted from C57BL/6J mice as B220⁻ MHC-II⁺CD11c⁺CD24⁺ cells as previously described. cDC1 were treated as described above in vitro and loaded with B16OVA WTL, then 1.10e6 cells were injected s.c. into tumor-bearing Batf3^{-/-} mice. We observed that in contrast to naïve, or active cDC1 which provided only a slight improved mice survival as compared to uninjected mice, hyperactive cDC1 induced tumor rejection in 100% of tumor-bearing mice, which remained tumor-free for more than 60 days post tumor inoculation (FIG. 15E). Of note, hyperactive cDC1 injection restored CD8⁺ T cell responses in Batf3^{-/-} as measured by SIINFEKL tetramer staining in the tumor and in the skin dLN (FIG. 15F). In contrast, naïve or active cDC1 injection failed to restore antigen-specific CD8⁺ T cells. cDC1 mediated tumor rejection was dependent on inflammasome activation, since the injection of NLRP3^{-/-} cDC1 that were treated with LPS+PGPC did not provide any anti-tumor protection, and abrogated the ability of hyperactive cDC1 to restore CD8⁺ T cells responses (FIG. 15F).

In addition to their ability to produce IL-1 from living cells, hyperactive DCs highly migrate to adjacent dLN to potentiate CD8⁺ T cell responses (FIGS. 12A–12B).

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OTHER EMBODIMENTS

It is to be understood that 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
5 aspects, advantages, and modifications are within the scope of the following claims.