(54) Title: METHODS AND COMPOSITIONS FOR TREATING CANCER WITH DENDRITIC CELLS

(57) Abstract: This invention provides methods and compositions for the treatment and/or prevention of cancer, including ovarian cancer. In particular, the present invention relates to immunotherapy using antigen-presenting cells (e.g., dendritic cells) comprising at least one tumor-associated antigen whereas the antigen-presenting cells are also treated with at least one inhibitor of the mitogen-activated protein kinase (MAPK) signaling pathway. This immunotherapy enhances immune responses against cancer cells.
Methods and Compositions for Treating Cancer with Dendritic Cells

Cross Reference to Related Application
This application claims priority to U.S. Provisional Application No. 62/146,502 filed on April 13, 2015, which is incorporated herein by reference in its entirety.

Field of the Invention
The present invention relates to methods and compositions for the treatment and/or prevention of cancer. In particular, the present invention relates to immunotherapy using antigen-presenting cells loaded with tumor associated antigens.

Background of the Invention


Barber et al., Chimeric NKG2D receptor-bearing T cells as immunotherapy for ovarian cancer.


Cellular immunotherapy aims to induce tumor-specific helper and cytotoxic T-cells capable of efficiently targeting and eradicating ovarian cancer. This has been attempted by in vitro expansion of TIL, and engineered T-cells. Fujita et al., Prolonged disease-free period in patients with advanced epithelial ovarian cancer after adoptive transfer of tumor-infiltrating lymphocytes. Clin Cancer Res 1, 501-507 (1995). Nonetheless, the most efficient physiological process for T-cell priming requires dendritic cell (DC) activation and antigen presentation. One of the major obstacles to the development of effective DC-based immunotherapy in ovarian cancer is the circumvention of tumor-associated immunosuppressive mechanisms, the most notable being the accumulation of tumor-infiltrating regulatory T-cells (Tregs), which has been associated with increased mortality in patients. Curiel et al., Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. Nat Med 10,
Thus, it is important to "program" DCs towards activation of effector T-cell polarizing profiles and avoidance of Treg expansion.

Dendritic cells (DCs) are potent antigen-presenting cells (APCs) and are important for the efficient priming and activation of naive T-cells against specific antigens. DCs undergo maturation and activation in the presence of specific immunogenic antigens and serve as a critical link between innate and adaptive immunity resulting in antitumor responses [3]. DC vaccination studies have been conducted in several different neoplasms [4,5] with variable results [52].

Since the majority of patients with metastatic or progressive solid malignancies (SM) display a defective immune response to tumor antigens, the ex vivo activation of DCs, through their exposure to tumor associated antigens, is an attractive and active area of investigation [6,7]. A variety of early clinical studies have used antigen-pulsed DC therapy in patients with SM malignancies including metastatic prostate, hepatocellular, colorectal, renal, cervical, and breast carcinomas, as well as melanomas and glioblastomas [8-13]. The strategies tested have included pulsing DCs with whole tumor cell lysates or specific tumor-associated antigens [8-13]. Although safe, DC vaccination strategies have resulted in unsatisfactory clinical outcomes attributed to either the poor immunogenicity of the target antigens, suboptimal DC-dosing, generation of tolerogenic DC subtypes and activity of suppressor regulatory T-cells (Tregs).

DCs can be generated from blood-derived monocytes in the presence of GM-CSF and IL-4 and administered to cancer patients [14,15]. Moreover, DCs pulsed with specific tumor associated peptide antigens (TAPAs) are capable of eliciting immune and antitumor responses, without prohibitive toxicity [8-13,16].

Cancer/testis antigens (CTA) are a novel class of tumor-associated antigens displaying potent immunogenicity and high expression levels in tumor cells but negligible expression in normal tissues. We have successfully exploited sperm protein 17 (SP17), a cancer/testis antigen, for adoptive immunotherapy in and as a biomarker to track ovarian cancer progression in animal models. The suitability of SP17 as a target for immunotherapy is supported by the finding that it is expressed by primary and metastatic ovarian cancer lesions in up to 70% of patients. Nakazato et al., Sperm protein 17 influences the tissue-specific malignancy of clear cell adenocarcinoma in human epithelial ovarian cancer. Int J Gynecol Cancer 17, 426-432 (2007). Further, the relevance of SP17 expression in ovarian cancer has been confirmed by the demonstration that it


The MAPK signaling pathway is a main component in several steps of tumorigenesis including cancer cell proliferation, migration, invasion and survival. Overall, the activation of a MAPK employs a core three-kinase cascade. The extracellular mitogen binds to the membrane receptor (e.g., receptor tyrosine kinases, cytokine receptors, and some G protein-coupled receptors), which allows Ras (a GTPase) to swap its GDP for a GTP. It can now activate a MAPK kinase kinase (MAP3K or MAPKKK; e.g., Raf), which phosphorylates and activates a MAPK kinase (MAP2K, MEK, or MKK), which then phosphorylates and activates a MAPK (e.g., ERKs). Upon activation, MAPKs can phosphorylate and activate a variety of intracellular targets including transcription factors, nuclear pore proteins, membrane transporters, cytoskeletal elements, and other protein kinases. Seger et al., *FASEB J.*, 1995, 9: 726-735; Lewis et al., *Adv. Cancer Res.*., 1998, 74: 49-139; and Pearson et al., *Endocr. Rev.*, 2001, 22: 153-183. Hodis et al., 2012.

Regulation of the p38 and ERK signal transduction pathways in DC plays an important role in determining the expression of certain cytokines by DC. As an example, inhibition of MEK 1/2 and MAPK promotes IL-12 production and Thl responses, whereas inhibition of p38 MAPK blocks IL-12 production. Jackson *et al.*, Tumour-mediated disruption of dendritic cell function: inhibiting the MEK1/2-p44/42 axis restores IL-12 production and Thl-generation. *Int J Cancer* 123, 623-632 (2008). Because IL-12 facilitates the differentiation of type 1 T-helper cells, the inhibition of IL-12 secretion in DC is expected to negatively affect DC-driven anti-tumor T-cell responses. Jung *et al.*, *IL-32gamma Induces the Maturation of Dendritic Cells with Thl- and Thl7-Polarizing Ability through Enhanced IL-12 and IL-6 Production.* *J Immunol.* 6, 6 (2011). Interestingly, p38 inhibition promotes differentiation and survival of monocyte-derived DC, and p38 inhibition or ERK activation restores deficiencies in DC function in myeloma patients, suggesting that treatment of DC with pharmacological inhibitors of p38 signaling may confer some benefit. Xie *et al.*, Critical roles of Raf/MEK/ERK and PI3K/AKT signaling and inactivation of p38 MAP kinase in the differentiation and survival of monocyte-derived immature dendritic cells. *Exp Hematol* 33, 564-572 (2005). Wang *et al.*, Optimizing immunotherapy in multiple myeloma: Restoring the function of patients' monocyte-derived dendritic cells by inhibiting p38 or activating MEK/ERK MAPK and neutralizing interleukin-6.

There still remain a need to optimize DC-based immunotherapy to improve responses in cancer patients.
Summary

The present application provides for a method of treating and/or preventing cancer in a subject. The method may comprise the following steps: (a) introducing at least one tumor-associated antigen (or a fragment thereof) into antigen-presenting cells; (b) treating the antigen-presenting cells with at least one inhibitor of the mitogen-activated protein kinase (MAPK) signaling pathway; and (c) administering the antigen-presenting cells to the subject. Step (a) may be conducted before, after, simultaneously with or overlapping with step (b).

Also encompassed by the present application is a pharmaceutical composition comprising dendritic cells comprising at least one tumor-associated antigen or a fragment thereof, wherein the dendritic cells are treated with at least one MAPK signaling pathway inhibitor.

The present application provides for a pharmaceutical composition comprising dendritic cells comprising nucleic acids encoding at least one tumor-associated antigen, wherein the dendritic cells are treated with at least one MAPK signaling pathway inhibitor.

The tumor-associated antigen may be SP17, Ropporin, AKAP-4, PTTG1, Span-xb, Her-2/neu, HM1.24, NY-ESO-1, MAGE-1 or combinations thereof. In one embodiment, SP17 comprises the amino acid sequence of SEQ ID NO:6.

When introducing the tumor-associated antigen or a fragment thereof into antigen-presenting cells (e.g., in step (a) of the present method, or when preparing the present composition), the antigen-presenting cells may be infected with viral vectors (e.g., adeno-associated viral vectors) encoding the tumor-associated antigen.

The MAPK signaling pathway inhibitor may be an inhibitor of RAF, an inhibitor of MEK, an inhibitor of ERK, an inhibitor of RAS, an inhibitor of receptor tyrosine kinases (RTKs), or combinations thereof. In one embodiment, the MAPK signaling pathway inhibitor is an inhibitor of p38. The inhibitor may be a small molecule, a polynucleotide (e.g., a small interfering RNA (siRNA) or an antisense molecule), a polypeptide, or an antibody or antigen-binding portion thereof. Non-limiting examples of the MAPK signaling pathway inhibitors include ML3403, PLX4720, PD325901, GW5074, BAY 43-9006, ISIS 5132, PD98059, PD184352, U0126, Ro 09-2210, L-783,277, GSK-1 120212, trametinib, vemurafenib, purvalanol, and imidazolium trans-imidazoledimethyl sulfoxide-tetrachlororuthenate (NAMI-A).
Prior to administration of the antigen presenting cells to the subject (e.g., before step (c) the method) or administration of the present composition, at least one immunosuppressive agent may be administered to the subject. In certain embodiments, the immunosuppressive agent may be an alkylating agent. In one embodiment, the alkylating agent is cyclophosphamide.

After administration of the antigen presenting cells to the subject (e.g., after step (c) the method) or administration of the present composition, granulocyte-macrophage colony-stimulating factor (GM-CSF) may be administered to the subject.

The antigen-presenting cells may be dendritic cells, macrophages, B cells, etc. The dendritic cells may be derived from autologous monocytes. For example, the monocytes can be cultured in vitro to induce differentiation into dendritic cells. In one embodiment, the differentiation into dendritic cells is facilitated by at least one maturation factor, including, but not limited to, granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-4, IL-1β, TNFα, IFN-α, poly (I:C) and combinations thereof. The monocytes may be isolated from the subject's blood. Before the subject's blood is obtained, granulocyte-macrophage colony-stimulating factor (GM-CSF) may be administered to the subject.

The cancer may be solid malignancy or hematologic malignancy. In certain embodiments, the subject has ovarian cancer, melanoma, breast cancer, colon cancer, pancreatic cancer, cervical cancer, thyroid cancer, bladder cancer or combinations thereof. In one embodiment, the subject has ovarian cancer.
Brief Description of the Figures

Figure 1. (A) AAV-mSP17 vector map. (B) mSp17 expression in DCs. Total RNA was isolated 72 hours after infection. Histograms show the RT-qPCR analysis run in triplicate (error bars = standard deviations). (c) Virus stock titers. DNA extracted from the crude lysates of AAV-mSP17 was used as the template for PCR. The DNA from 1000 µl, 500 µl and 250 µl crude lysates was tested, respectively. EG = encapsulated genomes.

Figure 2. Characterization of DC. DC were analyzed by flow-cytometry. Four DC preparations were tested: infected with rAAV vector only (AAV), infected with rAAV expressing mSP-17 (AAV-mSP17), and infected with rAAV expressing mSP17 and treated with p38 MAPK inhibitor (AAV-mSP17 + p38i). Results represent analysis of DC obtained from pooled splenocytes (3 mice for each condition). Histograms display mean fluorescence intensity (MFI). Error bars, standard error of the mean. One-way ANOVA and Bonferroni’s post-test (a=0.05) were used to estimate significance of MFI differences between groups. n.s., not significant; **, Bonferroni’s post-test p<0.001.

Figure 3 (A,B,C). Survival rates and tumor dissemination in mice that received different DC vaccination protocols, a) Survival rates are presented as the percentage of live mice in each group per day. Experimental end-point was day 300. Statistically significant survival curves using a Log-rank (Mantel-Cox) Test (p< 0.0001) are shown. b) Influence of DC vaccination on tumor growth. Upper panel: macroscopic impact of AAV-mSP17+p38i DC vaccination on primary tumor mass and lymph node metastases. Lower panel: immunohistochemical detection of mSP17 expression in the peritoneum of ID8-injected, non-vaccinated mice but not of DC vaccinated and control mice. c) Prevention of tumor spread was confirmed by RT-qPCR, showing mSP17 mRNA expression in the peritoneum of untreated ID8-injected mice (no vaccine), but not of control (no tumor) or DC vaccinated (DC AAV-mSP17 + p38i) mice.

Figure 4 (A,B,C,D). Measurement of IgG and cytokines production following vaccinations, a) DC vaccine-induced production of anti-mSP17 IgGi. Humoral responses against mSP17 were evaluated through ELISA performed using pooled sera from each vaccination group (5 mice per group). b) ELISA assay for cytokine production. Sera from
vaccinated mice and controls (5 mice per group) were collected post-mortem and analyzed by ELISA assay. IFN-γ and TNF-a were highly expressed; bars, SD calculated in triplicates. c) ELISPOT assay for IFN-γ. d) ELISPOT assay for TNF-a. Splenocytes from vaccinated mice and controls (5 animals/group) were collected post-mortem and analyzed by ELISA assay. Data are presented as the frequency of IFN-γ and TNF-a spot-forming cells per 10^6 splenocytes. Spot numbers represent the mean of ten mice for each vaccination; bars, SD calculated in triplicates. Two-tailed t-test p value versus no treatment group < 0.05 (*) or < 0.01 (**).

**Figure 5** (A,B) Evaluation of lymphocyte response against ID8 cells *in vitro*, a) Cytotoxic lymphocyte responses against ID8 cells following DC vaccination. Anti-tumor cytotoxic activity of in vitro-stimulated splenic lymphocytes was evaluated through a EUROPIUM assay at the indicated effector (E): target (T) ratios. rAAV-mSP17 DC vaccination increased ID8 cell lysis in comparison with rAAV DC (*= two-tailed t-test p=0.03). Significantly higher rates of lysis were obtained following vaccination with rAAV-mSP17 DC + p38i (**= two-tailed t-test rAAV-mSP17 + p38i versus rAAV-mSP17 or rAAV p<0.01). b) DC vaccination promotes lymphocyte migration towards tumor cells. Splenocytes isolated from rAAV-mSP17 + p38i DC vaccinated (rAAV-mSP17 + p38i) or rAAV-DC vaccinated mice (rAAV-DC) were isolated and loaded in the upper chamber of a Transwell chemotaxis plate. The lower chambers were seeded with sub-confluent ID8 cells. After 4h-incubation at 37 °C and 5% C0₂, non-adherent cells in the lower chamber were counted and migration indexes were calculated. The histogram shows the migration indexes computed as the ratio between the number of migrated splenocytes in the presence of ID8 cells divided by the number of migrated splenocytes in the presence of culture medium alone (without tumor cells). The assay was run in triplicate and mean values ± SEM are shown (**= two-tailed t-test p<0.01).

**Figure 6. Flow cytometry estimation of splenic Thl7 and Treg frequencies following DC vaccination.** Splenocytes were harvested and duplicate samples from 5 mice per group were analyzed by flow cytometry (1. No tumor, 2. ID8 only, 3. rAAV only, 4. rAAV-mSp17, 5. rAAV-mSp17+p38i). Time points were selected according to the survival times shown in Figure 3a (no tumor: day 0; no vaccination, day 45; rAAV DC vaccination, day 45; rAAV-mSP17 DC vaccination, day 80; rAAV-mSP17 DC + p38 MAPK inhibition, day 300). CD4^+ Thl7 and Treg frequencies were based on intracellular staining for IL-17 expression and Foxp3 expression,
respectively, without any further manipulation in vitro. Plots display the percentage of positive events for splenocytes from individual mice treated with the indicated DC vaccine formulations.

**Figure 7.** Representative flow-cytometry analysis of human monocyte-derived DC. Histograms from one representative subject are shown. The cut-off was set at the maximum fluorescent intensity level given by the corresponding isotypic control.

**Figure 8.** Mean Fluorescence Intensity (MFI) analysis. The MFI of the indicated markers was recorded out of five experiments. Whiskers represent range of values, boxes represent first and third quartiles, while horizontal lines are the medians. Statistical analysis was performed by a two-tailed t-test (a=0.05; ***, p<0.001; NS= not significant).

**Figure 9 (A, B).** Flow cytometry analysis of DC-primed autologous PBMCs derived from human subjects. A shows the dot-plots of double staining analyses. B summarizes the percentage of positive events recorded out of five experiments and shows the statistical analyses (two-tailed t-test with a=0.05; ***, p<0.001; **, 0.001>p<0.01; NS= not significant).
Detailed Description

This invention provides methods and compositions for the treatment and/or prevention of cancer, including ovarian cancer. In particular, the present invention relates to immunotherapy using antigen-presenting cells (e.g., dendritic cells) loaded with tumor associated antigens whereas the antigen-presenting cells are also treated with at least one inhibitor of the mitogen-activated protein kinase (MAPK) signaling pathway. This immunotherapy enhances immune responses against cancer cells.

Encompassed by the present invention is a method of treating and/or preventing cancer in a subject. The method may contain the following steps: (a) introducing at least one tumor-associated antigen (or a fragment thereof) into antigen-presenting cells (or, loading antigen-presenting cells with at least one tumor-associated antigen or a fragment thereof); (b) treating the antigen-presenting cells with at least one inhibitor of the mitogen-activated protein kinase (MAPK) signaling pathway; and (c) administering the antigen-presenting cells to the subject.

The step of introducing at least one tumor-associated antigen (or a fragment thereof) into antigen-presenting cells may be conducted before, after, simultaneously with, or overlapping, the step of treating the antigen-presenting cells with at least one inhibitor of the MAPK signaling pathway.

Any component of the MAPK pathway may be inhibited by the present inhibitors. They include an inhibitor of RAF, an inhibitor of MEK, an inhibitor of MAPK (e.g., ERK), an inhibitor of RAS, an inhibitor of a receptor tyrosine kinase (RTK), or combinations thereof.

The present invention provides for a pharmaceutical composition comprising dendritic cells loaded with at least one tumor-associated antigen (or a fragment thereof), wherein the dendritic cells are also treated with at least one MAPK signaling pathway inhibitor.

Tumor-associated Antigens

The present methods and systems may target/use any tumor-associated antigen that is an antigenic substance produced in tumor cells. Non-limiting examples of tumor antigens include cancer-testis (CT) antigens, epidermal growth factor receptor (EGFR, such as Her-2/neu), Ropporin, PTTG1, Span-xb, HM1.24, mucins (e.g., mucin 16 or MUC16, also known as CA125), human epididymis protein 4 (HE4), Beta human chorionic gonadotropin (beta-hCG), urinary gonadotropin fragment, Alpha-fetoprotein (AFP), Inhibin, estradiol, carcinoembryonic
antigen (CEA), squamous cell carcinoma (SCC) antigen, Miüllerian inhibiting substance (MIS),
topoisomerase II, Carbohydrate antigen 19-9, Cancer antigen 27-29, human telomerase reverse
transcriptase (hTERT), ferritin, lysophosphatidic acid, MIBI -determined tumor growth fraction,
LI (CAM), Mesothelin, Osteopontin, Vascular endothelial growth factor (VEGF), Interleukin 8
(IL-8), Macrophage colony-stimulating factor (M-CSF), Insulinlike growth factor-binding

Cancer-testis (CT) antigens are proteins expressed in normal gametogenic tissues and in
different types of tumors (Scanlan et al., 2002, Immunol Rev. 188:22-32. Scanlan et al., 2004,
antigens are expressed exclusively in cells of the germ cell lineage, although there is a marked
variation in the protein expression pattern during different stages of sperm development.
Likewise, a heterogeneous expression is also observed in tumors. It has been proposed that the
aberrant expression of CT antigens in tumors recapitulates portions of the germline gene
expression program and is related to some characteristics of the neoplastic phenotype such as
immortality, invasiveness, immune evasion and metastatic capacity. Chiriva-Internati et al.,
Cancer Testis Antigens: A Novel Target in Lung Cancer, International Reviews of Immunology,
31:321-343, 2012. Pandey et al., Cancer Testes Antigens in Breast Cancer: Biological Role,
Regulation, and Therapeutic Applicability, International Reviews of Immunology, 31:302-320,
2012. Mirandola et al., Cancer Testis Antigens: Novel Biomarkers and Targetable Proteins for
Ovarian Cancer, International Reviews of Immunology, 30:127-137, 2011. Simpson et al.,
8,207,300.

Non-limiting examples of CT antigens include SP17, ASP, NY-ESO (e.g., NY-ESO-1,
etc.) CABYR, TSP50, BORIS, RQCD1, BAGE, SSX, SCP-1, Piwil2, OY-TES-1, LAGE-1,
AKAP (e.g., AKAP-4 etc.), SCP-I/HOM-TES-14, MAGE (e.g., MAGE-1, MAGE-A, MAGE-B,
MAGE-C, etc.), GAGE (GAGE-A, GAGE-B, etc.), PAGE, XAGE, CAGE, HOM-TES-85,
SAGE, BAGE, CT9/3BRDT, HAGE, SPOI1, and SPAG9.

In one embodiment, the present method comprises the step of introducing at least one
tumor-associated antigen, e.g., selected from Spl7, Ropporin, AKAP-4, PTTG1, Span-xb, Her-
2/neu, HM1.24, NY-ESO-1, MAGE-1 and combinations thereof, into an APC. A fragment of the
tumor-associated antigen (a tumor-associated peptide antigen) may be introduced into an APC. The APC is also treated with at least one inhibitor of the MAPK signaling pathway.

Sperm protein 17 (SP17) is a highly immunogenic spermatozoan protein which has been considered a potential therapeutic target for immuncontraception in the last few years [25]. We have identified SP17 as a novel cancer-testis antigen (CTA) in hematologic and SM. Using RT-PCR, SP17 transcripts were detected in more than 70% of tumor-cell enriched bone marrow from multiple myeloma (MM) patients. SP17 gene expression was associated with SP17 protein translation as demonstrated by Western blot analysis. Northern blot analysis and RT-PCR, demonstrated SP17 expression in normal testis and tumor cells, but not in normal tissues, suggesting SP17 could serve as an immunotherapeutic target in MM [26]. We have generated SP17-specific HLA-class I restricted cytotoxic T lymphocyte (CTL) after pulsing monocyte-derived DCs with recombinant SP17 protein [14]. These SP17-specific CTLs were able to lyse autologous target cells pulsed with recombinant SP17 protein as well as SP17-expressing fresh MM cells. Moreover, we have generated a synthetic SP17 peptide (103-111) (ILDSSEEDK; SEQ ID NO:7) capable of inducing HLA-AI restricted CTL recognition and activation [27]. We have also expanded our evaluation of SP17 potential as an immunotherapeutic target by recently demonstrating its strong immunogenicity in an ovarian cancer animal model [28]. Moreover, we have recently found SP17 is expressed in human lung cancer cell lines and tumor tissues, but not in normal lung [29]. More importantly, we have shown SP17-loaded DCs activate CTLs capable of eliciting antitumor responses, in vitro [29]. Dadabayev et al have reported the safety and clinical response of SP17-pulsed DCs in patients with MM and ovarian cancer, validating the clinical potential of this protein as an immunotherapeutic target [30]. Amino acid sequences of human SP17 may be found under the following NCBI Reference Sequence (RefSeq) accession number: NP_059121 (SEQ ID NO:6). Nucleic acid sequences encoding human SP17 may be found under the following NCBI RefSeq accession number: NM_017425, or GenBank accession number BC032457.

A-kinase anchoring protein 4 (AKAP-4) is a member of a family of scaffolding proteins involved in the control of signal transduction by targeting cyclic adenosine monophosphate-dependent protein kinase-A, and directing its actions [31, 32]. We have shown that AKAP-4 is expressed in lung cancer and MM (multiple myeloma) cells, at both the transcriptional and the
protein level, with no evidence of expression in human normal tissues, other than the testis [29, 33]. The lack of expression in normal tissues and the presence of AKAP-4 antibodies in lung cancer and MM patient sera supports the protein's immunogenicity and indicates AKAP-4 is a tumor-associated antigen. Additionally, we have also demonstrated AKAP-4 serves as a marker of disease status in a murine model of MM [34]. Thus, AKAP-4 is a potential target for developing specific immunotherapeutic strategies against MM, lung cancer and other SM [29, 35].

Ropporin is a rhophilin-associated protein normally expressed in the inner fibrous sheath of sperm flagella. Ropporin has previously been found to interact with other fibrous sheath proteins, including Spl7 and AKAP-1 10, suggesting a common or related biological function. A study by Li et al has demonstrated a very restricted RNA expression of ropporin in normal tissues, with the exception of testicular and fetal liver tissue [36]. Ropporin expression was also detected in tumor cells derived from the bone marrow in 6 of 16 (37.5%) patients with MM, 6 of 14 (43%) cases of CLL and 2 of 11 (18%) cases of acute myeloid leukemia. No ropporin transcripts were detected in the peripheral blood mononuclear cells of 17 healthy donors.

Importantly, these investigators detected high titers of antibodies against ropporin in 8 of 30 MM (26.7%), 7 of 24 AML (29.2%), 18 of 31 chronic lymphocytic leukemia (CLL) (58.1%), 9 of 27 chronic myelogenous leukemia (CML) (33.3%) and 1 of 3 acute lymphoblastic leukemia (ALL) (33.3%), compared to healthy donors. The presence of ropporin antibodies in patients with a variety of malignancies indicates this CTA is a highly immunogenic protein. Taken together, these findings support the notion that ropporin is a tumor-restricted CTA with a potential role in the biology of certain neoplasms. The restricted expression of ropporin in neoplastic cells, testes and fetal liver, makes it a suitable candidate as a target for immunotherapy.

PTTG-1 is a novel oncogene involved in transcriptional and cell cycle regulation with expression in the normal testis and thymus [37]. PTTG-1 has been shown to be highly expressed in different hematologic malignancies (HM) including promyelocytic leukemia (PML) cell line HL-60, CML cell line K-562, ALL cell line MOLT-4 and Burkitt's lymphoma cell line Raji [38]. PTTG-1 has also been shown to be associated with tumorigenesis, angiogenesis and cancer progression, making it a logical therapeutic target [37]. We and others have found PTTG-1 is expressed at the transcriptional level in MM, with PTTG-1 being expressed in 63% of MM patients and 66% of human MM cell lines studied, but not in normal tissues [39]. We also
documented cytoplasmic and surface PTTG-1 expression in plasma cells from MM patients supporting PTTG-1 potential role as a therapeutic target in patients with HM. More recently, we have also demonstrated PTTG-1 expression in lung cancer tissues and cell lines, and showed PTTG-1-loaded DCs can activate CTL-mediated lysis of human lung cancer cells, in vitro [29]. Therefore, our data indicates the suitability of using PTTG-1 as a potential target for immunotherapy in both HM and SD.

Span-xb is a novel CTA expressed in CML and other HM. Using RT-PCR, we have detected Span-xb transcripts in 20% of MM patients, 33% of patients with CLL, 29% of CML patients and 50% of patients with AML. In contrast, Span-xb expression was not detected in peripheral blood or bone marrow samples from healthy donors [40]. Importantly, span-xb gene expression has also been found in a variety of SM, including melanoma and carcinomas of the lung, colon and breast, making it a target for immunotherapeutic interventions [41].

HER-2/neu is a trans-membrane tyrosine-kinase involved in aberrant signal transduction in a variety of neoplasms [42,43]. HER-2/neu amplification has been demonstrated in certain HM and its functional inhibition, using anti-sense oligonucleotides, results in a reduced tumor cell proliferative rate. The observation that Her-2/neu is expressed at very low levels in normal tissues and preferentially expressed in neoplastic cells, suggest it could serve as an immunotherapeutic target in HER-2/neu-expressing SM. In fact, Scardino et al have demonstrated HLA-restricted CTL activation by high several high affinity HER-2/neu peptides capable of HLA-restricted killing of neoplastic cells of diverse origin [44]. Thus, specific Her-2/neu peptides may serve as good candidates for immunotherapy in HER-2-expressing SM.

HM1.24 is a 29-33 kDa membrane glycoprotein expressed in mature B-cells. We have proposed HM1.24 as a new antigen for CTLs activation against MM [45]. HM1.24 expression has been found in all five human MM cell lines assayed, as well as in mature, Ig-secreting B-cells (plasma cells and lymphoplasmacytoid cells), but not in non-B-Cells in the peripheral blood, bone marrow, liver, spleen, kidney, or heart of normal individuals or patients with non-plasma-cell-related malignancies. Thus, HM1.24 protein represents a specific marker of late-stage B-cell maturation and may potentially serve as a target antigen for the development of immunotherapeutic strategies specific against MM. HM1.24 is also expressed in SM including brain tumors, renal, hepatocellular, breast, ovarian, and breast carcinomas, with some expression in a few normal organs including liver and kidney [46]. Although HM1.24 function is unknown
at this time, its promise as a therapeutic target has been demonstrated using a specific HM1.24 monoclonal antibody (MoAb)[47].

NY-ESO-1 is one of the most immunogenic tumor antigens known to date. Spontaneous humoral and cellular immune responses against NY-ESO-1 are detected in a substantial proportion of patients with NY-ESO-1 expressing malignancies and NY-ESO-1 antibody titers correlate with clinical development of disease [48]. Moreover, the development of NY-ESO-1 serum antibody is associated with detectable NY-ESO-1-specific CD8+ T cell reactivity, suggesting this antigen is an excellent immunogen and potential therapeutic target, in vivo [49].

MAGE-1 is expressed in HM, including human MM cell lines and malignant plasma cells, as well as melanomas [50,51]. Both RNA and protein expression has been demonstrated in MM cells, but not in polyclonal, reactive plasma cells. Moreover, anti-MAGE-1 HLA-A1 cytotoxic T lymphocytes can efficiently kill MAGE-1 HLA-A1 expressing MM and melanoma cells, suggesting MAGE-1 represents a specific and potential immunotherapeutic target for patients with these malignancies.

**Inhibitors of MAPK Signaling Pathway**

Any component of the MAPK signaling pathway may be inhibited by the present inhibitors. They include an inhibitor of RAF, an inhibitor of MEK, an inhibitor of MAPK (ERK), an inhibitor of RAS, an inhibitor of a receptor tyrosine kinase (RTK), or combinations thereof.

Used in the present methods may be any MAPK signaling pathway inhibitor disclosed herein or any combination thereof.

Any isoform of any component the MAPK pathway may be inhibited by the present inhibitors. They include, but are not limited to: an inhibitor of BRAF, CRAF or ARAF; an inhibitor of MEK1, MEK2, MKK3, MKK4, MKK5, MKK6, or MKK7; an inhibitor of ERK1, ERK2, p38, INK or ERK5; an inhibitor of HRAS, KRAS or NRAS; an inhibitor of epidermal growth factor receptor (EGFR), ErbB-2, ErbB-3, ErbB-4, Trk A/B, Fibroblast growth factor receptor (FGFR) or PDGFR.

The present inhibitors may target the wild-type or mutant component of the MAPK pathway. For example, the inhibitors may target, inhibit or decrease activity of wild-type BRAF or a mutant BRAF (e.g., BRAF(V600); BRAF(G466); BRAF(G464); BRAF(G469);
BRAF(D594); BRAF(G596); BRAF(K601); BRAF(V600), etc.), wild-type MEK or a mutant MEK (e.g., MEK1/2(Q60), MEK1/2(P124), etc.), and wild-type RAS or a mutant RAS (e.g., N/K/H-RAS(Q61), N/K/H-RAS(G12), N/K/H-RAS(G13), etc.).

As used herein, the term "inhibitor" refers to agents capable of down-regulating or otherwise decreasing or suppressing the amount and/or activity of any component of the MAPK signaling pathway, including, but not limited to, the extracellular signal regulated mitogen-activated protein kinase (ERK-MAPK) signaling pathway.

The mechanism of inhibition may be at the genetic level (e.g., interference with or inhibit expression, transcription or translation, etc.) or at the protein level (e.g., binding, competition, etc.). The inhibitors may reduce MAPK signaling, reduce phosphorylation of components of the MAPK signaling pathways (e.g., MEK 1/2, ERK1/2), reduce levels of activated components of the MAPK signaling pathways (e.g., including but not limited to members of the Ras/Raf/MEK/ERK pathways), and/or sequester components of the MAPK signaling pathways and prevent signaling. For example, an inhibitor may be utilized that interferes with or inhibits expression of ERK1 and/or ERK2, or sequesters ERK 1 and/or ERK2 in the cytoplasm of the cell, preventing nuclear translocation and signaling (Brunet A. et al., EMBO J, 1999, 18: 664-674).

A wide variety of suitable inhibitors may be employed, guided by art-recognized criteria such as efficacy, toxicity, stability, specificity, half-life, etc.

Small Molecule Inhibitors

As used herein, the term "small molecules" encompasses molecules other than proteins or nucleic acids without strict regard to size. Non-limiting examples of small molecules that may be used according to the methods and compositions of the present invention include, small organic molecules, peptide-like molecules, peptidomimetics, carbohydrates, lipids or other organic (carbon containing) or inorganic molecules.


Non-limiting examples of RAF inhibitors include: PLX4720; PLX4032 (Vemurafenib; N-(3-[[5-(4-chlorophenyl)-1 H-pyrrolo[2,3-b]pyridin-3-yl]carbonyl]-2,4-difluorophenyl)propane-1 -sulfonamide); R7204; GSK21 18436; Sorafenib (BAY-43-9006); BMS-908662 (XL-281); RAF265 (Smalley and Flaherty (2009) Future Oncology, Volume 5, Number 6, pp. 775-778); RG-7256 (RO5212054, PLX3603); R05126766; ARQ-736; E-3810; DCC-2036; 4-(4-[[3-[4-chloro-3-[(trifluoromethyl)phenyl]ureido]phenoxy]-N2-methylpyridine-2-carboxamid e 4-methylbenzenesulfonylate (sorafenib); GW5074; BAY 43-9006; and ISIS 5132 (Lackey, K. et al., Bioorg. Med. Chem. Lett., 2000, 10: 223-226; Lyons, J. F. et al., Endocrine-related Cancer, 2001, 8: 219-225; and Monia, B. P. et al., Nat. Med., 1996, 2(6): 668-675).

Non-limiting examples of ERK inhibitors include: GW5074, BAY 43-9006, ISIS 5132, PD98059, PD184352, U0126, Ro 09-2210, L-783,277, purvalanol (Knockaert M. et al., Oncogene, 2002, 21: 6413-6424), imidazolium trans-imidazolediethyl sulfoxide-tetrachlororuthenate (NAMI-A), 3-cyano-4-(phenoxyanilino)quinolines (such as Wyeth-Ayerst Compound 14), resorcylic acid lactones (such as Ro 09-2210 and L-783,277), and purvalanol (Kohno M. et al., Progress in Cell Cycle Research, 2003, 5: 219-224). Information about ERK inhibitors and methods for their preparation are readily available in the art (see, for example, Kohno M. et al., Progress in Cell Cycle Research, 2003, 5: 219-224).

Non-limiting examples of p38 inhibitors include, RWJ 67657, SCIO 469, EO 1428, Org 48762-0, SD 169, SB 203580, SB 202190, SB 239063, SB 220025, VX-745, SB 242235, VX-
702, SD-282, PH-797804, pamapimod, BIRB 796, BMS 582949, PD 169316, PD 98059 (2'-Amino-3'-methoxyflavone, MEK Inhibitor V), U0126, SC-68376 (2-Methyl-4-phenyl-5-(4-pyridyl)oxazole), p38 MAP Kinase inhibitor (2-(4-Chlorophenyl)-4-(4-fluorophenyl)-5-pyridin-4-yl-1,2-dihydropyrazol-3-one), p38 MAP Kinase inhibitor III (ML3403, (RS)-{4-[5-(4-Fluorophenyl)-2-methylsulfanyl-3H-imidazol-4-yl]pyridin-2-yl-}(1-phenylethyl)amine), p38 MAP Kinase inhibitor IV (2,2′-Sulfonyl-bis-(3,4,6-trichlorophenol), MT4), p38 MAP Kinase inhibitor V (4-(3-(4-Chlorophenyl)-5-(1-methylpiperidin-4-yl)-1H-pyrazol-4-yl)pyrimidine, SC-409), p38 MAP Kinase inhibitor VI (N-(2-Methoxy-4-thiomethyl)benzoyl-4-benzylpiperidine), p38 MAP Kinase inhibitor VII (Indole-5-carboxamide), p38 MAP Kinase inhibitor VIII ((4-((2-Amino-4-bromophenyl)amino)-2-chlorophenyl)-(2-methylphenyl)methanone), p38 MAP Kinase inhibitor X (1-(5-tert-Butyl-2-p-tolyl-2H-pyrazol-3-yl)-3-[4-(2-morpholin-4-yl-ethoxy)naphthalen-1-yl]urea, JNK Inhibitor XVII, Doramapimod, BIRB796), p38 MAP Kinase inhibitor XX (Catalog No. 506385, EMD Millipore), and others. See, e.g., Kumar et al., "p38 MAP Kinases: Key Signaling Molecules as Therapeutic Targets for Inflammatory Diseases," Nature Reviews, 2:717-726 (2003); Brown et al., "p38 MAP kinase inhibitors as potential therapeutics for the treatment of joint degeneration and pain associated with osteoarthritis," J. Inflammation 5:22 (2008); Mayer et al., "p38 MAP kinase inhibitors: A future therapy for inflammatory diseases," Drug Discovery Today: Therapeutic Strategies 3(1): 49-54 (2006); and Regan et al., "Pyrazole Urea-Based Inhibitors of p38 MAP Kinase: from Lead Compound to Clinical Candidate," J. Med. Chem. 2002, 45, 2994-3008, the entirety of each of which is incorporated herein by reference.

Non-limiting examples of receptor tyrosine kinases (RTKs) include inhibitors to ErbB: HER1/EGFR (Erlotinib, Gefitinib, Lapatinib, Vandetanib, Sunitinib, Neratinib); HER2/neu (Lapatinib, Neratinib); RTK class III: C-kit (Axitinib, Sunitinib, Sorafenib), FLT3 (Lestaurtinib), PDGFR (Axitinib, Sunitinib, Sorafenib); and VEGFR (Vandetanib, Semaxanib, Cediranib, Axitinib, Sorafenib); bcr-abl (Imatinib, Nilotinib, Dasatinib); Src (Bosutinib) and Janus kinase 2 (Lestaurtinib). The inhibitors also include lapatinib (Tykerb®); Zactima (ZD6474), Iressa (gefitinib), imatinib mesylate (STI571; Gleevec), erlotinib (OSI-1774; Tarceva), canertinib (CI 1033), semaxinib (SU5416), vatalanib (PTK787/ZK222584), sorafenib (BAY 43-9006), sutent (SUI 1248) and lefltomomide (SU101). PTK/ZK is a tyrosine kinase inhibitor with broad specificity that targets all VEGF receptors (VEGFR), the platelet-derived growth factor (PDGF)
receptor, c-KIT and c-Fms. Drevs (2003) Idrugs 6(8):787-794. The chemical names of PTK/ZK are 1-[4-Chloroanilino]-4-[4-pyridylmethyl] phthalazine Succinate or 1-Phthalazinamine, N-(4-chlorophenyl)-4-(4-pyridinylmethyl)-butanedioate (1:1). Synonyms and analogs of PTK/TK are known as Vatalanib, CGP79787D, PTK787/ZK 222584, CGP-79787, DE-00268, PTK-787, PTK787A, VEGFR-TK inhibitor, ZK 222584 and ZK.

The MAP kinase signaling pathway inhibitor may be ERK Inhibitor II, FR1 80204; INK Inhibitor II; INK Inhibitor IX; MEK1/2 Inhibitor; MNK1 Inhibitor; MK2a Inhibitor; p38 MAP Kinase Inhibitor V; PD 98059; Raf Kinase Inhibitor IV; SB 203580; Tpl2 Kinase Inhibitor, ZM 336372, or combinations thereof.

Inhibitors of the MAPK signaling pathway are also disclosed in U.S. Patent Nos. 8,697,627 and 7,863,288; U.S. Patent Publication Nos. 2003/0060469; 2004/0048861; 2004/0082631; 2003-0232869; 20140275078, each of which is incorporated herein by reference in its entirety.

In certain embodiments, the MAPK pathway inhibitor used in the methods and compositions of the invention is a polynucleotide that reduces expression of one or more components of the MAPK pathway. Thus, the method involves administering an effective amount of a polynucleotide that specifically targets nucleotide sequence(s) within a target gene(s) of the MAPK pathways. The polynucleotides reduce expression of one or more genes within the MAPK pathways, to yield reduced levels of the gene product (the translated polypeptide).

The nucleic acid target of the polynucleotides (e.g., siRNA, antisense oligonucleotides, and ribozymes) of the invention may be any location within the gene or transcript of any component of the MAPK signaling pathway.

RNA Interference

SiRNAs (small interfering RNAs) or small-hairpin RNA (shRNA) may be used to reduce the level of any component of the MAPK signaling pathway.

SiRNAs may have 16-30 nucleotides, e.g., 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides. The siRNAs may have fewer than 16 or more than 30 nucleotides. The polynucleotides of the invention include both unmodified siRNAs and modified siRNAs such as siRNA derivatives etc.
SiRNAs can be delivered into cells in vitro or in vivo by methods known in the art, including cationic liposome transfection and electroporation. SiRNAs and shRNA molecules can be delivered to cells using viruses or DNA vectors.

**Antisense Polynucleotides**

In other embodiments, the polynucleotide of the invention is an antisense nucleic acid sequence that is complementary to a target region within the mRNA of any component of the MAPK signaling pathway. The antisense polynucleotide may bind to the target region and inhibit translation. The antisense oligonucleotide may be DNA or RNA, or comprise synthetic analogs of ribo-deoxynucleotides. Thus, the antisense oligonucleotide inhibits expression of any component of the MAPK signaling pathway.

An antisense oligonucleotide can be, for example, about 7, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, or more nucleotides in length.


The antisense nucleic acid molecules of the invention may be administered to a subject, or generated in situ such that they hybridize with or bind to the mRNA of a component of the MAPK signaling pathway. Alternatively, antisense nucleic acid molecules can be modified to target selected cells and then administered systemically. For systemic administration, antisense molecules can be modified such that they specifically bind to receptors or antigens expressed on a selected cell surface, e.g., by linking the antisense nucleic acid molecules to peptides or antibodies that bind to cell surface receptors or antigens. The antisense nucleic acid molecules can also be delivered to cells using viruses or DNA vectors.

**Ribozyme**

In other embodiments, the polynucleotide of the invention is a ribozyme that inhibits expression of the gene of any component of the MAPK signaling pathway.
Ribozymes can be chemically synthesized in the laboratory and structurally modified to increase their stability and catalytic activity using methods known in the art. Alternatively, ribozyme encoding nucleotide sequences can be introduced into host cells through gene-delivery mechanisms known in the art. U.S. Patent No. 8,592,368 and 5,093,246. Haselhoff et al., Nature 334: 585-591 (1988).

Other aspects of the invention include vectors (e.g., viral vectors, expression cassettes, plasmids) comprising or encoding polynucleotides of the subject invention (e.g., siRNA, antisense nucleic acids, and ribozymes), and host cells genetically modified with polynucleotides or vectors of the subject invention.

**Polypeptides**

The present inhibitors can also be a polypeptide exhibiting inhibitory activity toward any component of the MAPK signaling pathway. For example, a receptor decoy may be used. A peptide corresponding to the amino-terminal 13 amino acids of MEK1 (MPKKKPTPIQLNP; SEQ ID NO: 1), can be used to inhibit the activation of ERK1/2 (Kelemen B. R. et al., J. Biol. Chem., 2002, 277: 87841-8748).

Various means for delivering polypeptides to a cell can be utilized to carry out the methods of the subject invention. For example, protein transduction domains (PTDs) can be fused to the polypeptide, producing a fusion polypeptide, in which the PTDs are capable of transducing the polypeptide cargo across the plasma membrane (Wadia, J. S. and Dowdy, S. F., Curr. Opin. Biotechnol., 2002, 13(1)52-56).

According to the methods of the subject invention, recombinant cells can be administered to a patient, wherein the recombinant cells have been genetically modified to express a nucleotide sequence encoding an inhibitory polypeptide.

**Antibodies**

The present inhibitors can be an antibody or antigen-binding portion thereof that is specific to any component of the MAPK signaling pathway, thereby inhibiting the MAPK signaling.

The antibody or antigen-binding portion thereof may be the following: (a) a whole immunoglobulin molecule; (b) an scFv; (c) a Fab fragment; (d) an F(ab')2; and (e) a disulfide
linked Fv. The antibody or antigen-binding portion thereof may be monoclonal, polyclonal, chimeric and humanized. The antibodies may be murine, rabbit or human antibodies.

**Vectors**

The tumor-associated antigen(s) may be introduced into antigen-presenting cells using any vectors.

As used herein, the term "vector" refers to a polynucleotide capable of transporting another nucleic acid to which it has been linked. The present vectors can be, for example, a plasmid vector, a single- or double-stranded phage vector, or a single- or double-stranded RNA or DNA viral vector. Such vectors include, but are not limited to, chromosomal, episomal and virus-derived vectors, e.g., vectors derived from bacterial plasmids, bacteriophages, yeast episomes, yeast chromosomal elements, and viruses such as baculoviruses, papova viruses, SV40, vaccinia viruses, adenoviruses, fowl pox viruses, pseudorabies viruses and retroviruses, and vectors derived from combinations thereof, such as those derived from plasmid and bacteriophage genetic elements, cosmids and phagemids.

Expression vectors can be used to replicate and/or express the nucleotide sequence encoding a therapeutic agent in a target mammalian cell. A variety of expression vectors useful for introducing into cells the polynucleotides of the inventions are well known in the art. Viral vectors include, but are not limited to, adeno-associated virus, adenovirus, vaccinia virus, alphavirus, retrovirus and herpesvirus vectors.

In one embodiment, the vector is an adeno-associated virus (AAV, or adenovirus-associated virus) vector. See also, e.g., Walsh et al., 1993, Proc. Soc. Exp. Biol. Med. 204:289-300; U.S. Pat. No. 5,436,146).

Any of the AAV serotypes may be used, including, but not limited to, AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, etc.

The present vector may comprise wild-type or mutant AAV capsid (e.g., AAV2, AAV8, etc.) encoded by the AAV cap open reading frame (ORF).


Greater detail about retroviral vectors is available in Boesen et al., 1994, Biotherapy 6:291-302. In one embodiment, the present expression vector is a lentivirus (including human immunodeficiency virus (HIV)), which is a sub-type of retrovirus.

Plasmids that may be used as the present expression vector include, but are not limited to, pGL3, pCDM8 (Seed, 1987, "An LFA-3 cDNA encodes a phospholipid-linked membrane protein homologous to its receptor CD2", Nature. 840-842) and pMT2PC (Kaufman et al., 1987, "Translational efficiency of polycistronic mRNAs and their utilization to express heterologous genes in mammalian cells", EMBO J. 6:187-193). Any suitable plasmid may be used in the present invention.

Certain vectors are capable of autonomous replication in a host cell into which they are introduced (e.g., episomal mammalian vectors). Other vectors (e.g., non-episomal mammalian vectors) are integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome.

**Antigen-presenting Cells (APCs)**

At least one tumor-associated antigen (or at least one tumor-associated peptide antigen (TAPA) derived from at least one tumor-associated antigen) may be introduced into, or loaded onto, the APCs of the present invention. Alternatively or simultaneously, nucleic acids encoding at least one tumor-associated antigen (or a fragment thereof) may be introduced into the APCs.

Non-limiting examples of antigen-presenting cells including dendritic cells, macrophages, B cells, cells of myeloid lineage, Langerhans cells, epithelial cells, or any nucleated cells.

The APC may be autologous or allogeneic. The APC may be isolated from a subject. The APC may also be derived from cells isolated from a subject.

In some embodiments, the present invention provides a method for eliciting in a subject an immune response to a cell that expresses a tumor-associated antigen, the method comprising administering to the subject antigen-presenting cells (e.g., dendritic cells) comprising at least one tumor-associated antigen, or administering to the subject antigen-presenting cells comprising nucleic acids encoding at least one tumor-associated antigen, wherein the antigen-presenting cells (e.g., dendritic cells), when administered to the subject, elicits the immune response to the
cell.

The tumor-associated antigen-containing antigen-presenting cells may also be used to activate T lymphocytes. As described herein, the present antigen-presenting cells and/or T lymphocytes may be used for prophylactic or therapeutic applications.

Dendritic Cells (DCs)

DCs can be generated in vivo or ex vivo from immature precursors (e.g., monocytes). For example, for ex vivo DC generation, a cell population enriched for DC precursor cells (e.g., peripheral blood mononuclear cells (PBMCs)) is obtained from a patient, and then the DC precursor cells are differentiated ex vivo into mature DCs. Typically, to generate immature dendritic cells (DC), one must first purify or enrich the monocytic precursors from other cell types. For example, peripheral blood mononuclear cells (PBMCs) are extracted from whole blood (e.g., over Ficoll density gradient centrifugation). Then the PBMCs will be used to generate monocytic dendritic cell precursors.

DCs can be generated from monocytes, CD34+ cells (i.e., cells expressing CD34), etc.

In certain embodiments, monocytic dendritic cell precursors are isolated by adherence to a monocyte-binding substrate. For example, a population of leukocytes (e.g., isolated by leukapheresis) can be contacted with a monocytic dendritic cell precursor adhering substrate. When the population of leukocytes is contacted with the substrate, the monocytic dendritic cell precursors in the leukocyte population preferentially adhere to the substrate. In one embodiment, monocytes are isolated through adherence of the monocytic precursors to a plastic (polystyrene) surface, as the monocytes have a greater tendency to stick to plastic than other cells found in, for example, peripheral blood, such as lymphocytes and natural killer (NK) cells.

Methods for isolating cell populations enriched for dendritic cell precursors and immature dendritic cells from various sources, including blood and bone marrow, are known in the art. For example, dendritic cell precursors and immature dendritic cells can be isolated by phlebotomy, by apheresis or leukapheresis, by collecting heparinized blood, by preparation of buffy coats, rosetting, centrifugation, density gradient centrifugation (e.g., using Ficoll, Percoll (colloidal silica particles of 15-30 mm diameter coated with polyvinylpyrrolidone (PVP)), sucrose, and the like), differential lysis of cells, filtration, and the like. In one embodiment,
dendritic cell precursors can be selected using CD14 selection of G-CSF mobilized peripheral blood. U.S. Patent No. 8,728,806.

Before the subject's blood or bone marrow is obtained to isolate dendritic cell precursors, the subject may be administered granulocyte macrophage colony stimulating factor (GM-CSF) to increase bone marrow production of monocytes and dendritic cell precursors. In certain embodiments, GM-CSF is administered at a dose ranging from about 10 µg/day to about 500 µg/day, from about 20 µg/day to about 300 µg/day, from about 50 µg/day to about 250 µg/day, from about 100 µg/day to about 300 µg/day, from about 200 µg/day to about 300 µg/day, about 200 µg/day, or about 250 µg/day. The dose of GM-CSF can also be lower or higher. In certain embodiments, GM-CSF may be administered for about 1 day, about 2 days, about 3 days, about 4 day, about 5 days, about 6 days, about 1 week, about 1.5 weeks, about 2 weeks, or longer.

The effect of GM-CSF may be potentiated by another immunostimulant (such as plerixafor).

Variations on this method include different methods of purifying monocytes, including, for example, tangential flow filtration (TFF), or by binding antibodies attached to beads to surface molecules on the monocytes. The beads with the bound cells are then concentrated in a column, or on a magnetic surface, such that contaminating cells can be washed away, after which the monocytes are eluted off the beads. In yet another method to obtain dendritic cells precursors, cells expressing the stem cell marker CD34, either from blood (U.S. Patent No. 5,994,126, incorporated herein by reference) or from the bone marrow are purified. These cells can be cultured with the essential cytokine GM-CSF to differentiate into immature DC. These DC apparently have very similar characteristics and functional properties as immature DC generated from monocytes.

Isolated dendritic cell precursors can be cultured ex vivo for differentiation, maturation and/or expansion. In certain embodiments, the monocytic dendritic cells precursors are differentiated to form immature dendritic cells. The end result of this process is a cell which expresses T cell costimulatory molecules, as well as high levels of molecules of the major histocompatibility complex (MHC), but does not express the dendritic cell maturation marker CD83. These cells are similar to Langerhans cells in the skin, and their prime physiological function is to capture invading microorganisms.
The dendritic cell precursors and/or immature dendritic cells can be cultured and differentiated in suitable culture conditions. The tissue culture media can be supplemented with, e.g., plasma, serum, amino acids, vitamins, cytokines (e.g., granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukins such as Interleukin 4 (IL-4), Interleukin 13 (IL-13), Interleukin 15 (IL-15), or combinations thereof), purified proteins (such as serum albumin), divalent cations (e.g., calcium and/or magnesium ions), growth factors, and the like, to promote differentiation of the cells. Sallustio et al., J. Exp. Med., 179: 1109-1 118, 1994, incorporated herein by reference. For example, the use of type I interferons and Toll-like receptor agonists to induce DC maturation ex vivo have been shown to stimulate generation of immunogenic, rather than tolerogenic, DCs [58, 59]. In certain embodiments, the blood plasma or serum can be heat-inactivated. The plasma or serum can be autologous, allogeneic or heterologous to the cells. In certain embodiments, the dendritic cell precursors can be cultured in the serum-free media. Such culture conditions can optionally exclude any animal-derived products. In one embodiment, a dendritic cell culture medium contains about 200 units/ml to about 1500 units/ml (e.g., about 1000 units/ml, about 500 units/ml, etc.) of GM-CSF and about 200 units/ml to about 1500 units/ml (e.g., about 800 units/ml, about 500 units/ml, etc.) IL-4.

**Loading of APCs/Introducing tumor-associated antigen into APCs**

Immature DC have a high capacity for taking up and processing antigens, but have a limited ability to initiate immune responses. The ability to initiate an immune response is acquired by maturation of the immature DC. This maturation is also referred to as activating, or activation of, the DC. The maturation process may be initiated and/or induced through contact with, or intake of, maturation-inducing cytokines, tumor-associated antigens or tumor-associated peptide antigens and/or nucleic acids encoding tumor-associated antigens or tumor-associated peptide antigens, and the like, as described herein.

APCs (e.g., dendritic cells) can be loaded with one or more tumor-associated antigen, or peptide antigens (e.g., a tumor-associated peptide antigen). In certain aspects, 1, 2, 3, 4, 5, 6, 7, 8, 9, or more tumor-associated peptide antigens using any combinations of the tumor-associated peptide antigens of SEQ ID Nos. 7-15. As used herein, a cell or membrane bound composition (e.g., a liposome) "loaded" (or "pulsed") with a peptide shall mean that the cell or membrane bound composition has been incubated with the peptide under conditions permitting entry into
and/or attachment onto the cell or membrane bound composition of the peptide. For example, APCs (e.g., dendritic cells) can be incubated with one or more tumor-associated peptide antigens under conditions that are needed to load the MHC of the APC (e.g., the dendritic cell). Suitable conditions for antigen loading are provided that permit an APC to contact, process and/or present one or more antigens on its MHC, whether intracellularly or on the cell surface. The incubation time may range from about 10 minutes to about 3 days or longer, from about 30 minutes to about 36 hours, from about 1 hour to about 28 hours, from about 2 hours to about 24 hours, from about 4 hours to about 24 hours, from about 4 hours to about 16 hours, from about 16 hours to about 24 hours, from about 20 hours to about 28 hours, from about 2 hours to about 4 hours, from about 1 hour to about 12 hours, from about 2 hours to about 8 hours, from about 3 hours to about 5 hours, for less than about a week, illustratively, for about 1 minute to about 48 hours, about 2 minutes to about 36 hours, about 3 minutes to about 24 hours, about 4 minutes to about 12 hours, about 6 minutes to about 8 hours, about 8 minutes to about 6 hours, about 10 minutes to about 5 hours, about 15 minutes to about 4 hours, about 20 minutes to about 3 hours, about 30 minutes to about 2 hours, about 40 minutes to about 1 hour, about 16 hours, about 20 hours, about 24 hours, about 28 hours, about 1 hour, about 2 hours, or about 4 hours. The incubation temperature may range from about 4°C to about 37°C, from about 25°C to about 37°C, about 4°C, about 25°C, or about 37°C.

The concentration of the peptide for loading may range from about 1 µg/ml to about 1 mg/ml, from about 5 µg/ml to about 800 µg/ml, from about 10 µg/ml to about 600 µg/ml, from about 15 µg/ml to about 400 µg/ml, from about 10 µg/ml to about 200 µg/ml, from about 10 µg/ml to about 100 µg/ml, from about 20 µg/ml to about 100 µg/ml, from about 20 µg/ml to about 20 µg/ml, about 20 µg/ml, about 30 µg/ml, about 50 µg/ml, about 60 µg/ml, about 80 µg/ml, or about 100 µg/ml.

In one embodiment, one or more tumor-associated antigens can be coupled to a cytolysin to enhance the transfer of the antigens into the cytosol of an antigen-presenting cell for delivery to the MHC class I pathway. Exemplary cytolysins include saponin compounds such as saponin-containing Immune Stimulating Complexes (ISCOM5), pore-forming toxins (e.g., an alpha-toxin), and natural cytolysins of gram-positive bacteria such as listeriolysin O (LLO), streptolysin O (SLO), and perfringolysin O (PFO).
A number of methods for delivery of antigens to the endogenous processing pathway of antigen-presenting cells may be optionally used. Such methods include, but are not limited to, methods involving pH-sensitive liposomes, coupling of antigens to potent adjuvants, apoptotic cell delivery, pulsing cells onto dendritic cells, delivering recombinant chimeric virus-like particles (VLPs) comprising antigen to the MHC class I processing pathway of a dendritic cell line.

One or more tumor-associated antigens may be introduced into APCs. Methods of introducing a tumor-associated antigen into an APC include, but are not limited to, non-covalent complex formation (Chariot), osmotic lysis of pinocytic vesicles (Influx pinocytic cell-loading reagent), electric power (electroporation), lipid-based delivery system (Bioporter), microinjection, small protein transduction domains (PTDs) from viral proteins, bacterial secretion system type III (T3SS), cell-penetrating peptide (CPP) conjugation, and protein transfection.

APCs may be contacted with nucleic acids encoding one or more tumor-associated antigens. For example, antigen-presenting cells (e.g., dendritic cells) can be transfected with expression vectors or infected with viral vectors for introducing nucleic acids encoding tumor-associated antigens into the APCs.

Non-limiting viral vectors include adeno-associated viruses, lentiviruses, retroviruses, herpes viruses, adenoviruses, vaccinia viruses, baculoviruses, Fowl pox, AV-pox, modified vaccinia Ankara (MVA) and other recombinant viruses.

Other techniques for introducing nucleic acids into APCs include, but are not limited to, electroporation, microinjection, hypotonic shock, scrape loading, cationic liposomes, and calcium phosphate coprecipitation.

Expression can be optionally effected by targeting the expression construct to specific cells, such as with a viral vector or a receptor ligand, or by using a tissue-specific promoter, or combinations thereof.

The time and amount of antigens, or nucleic acids encoding the antigens, necessary for the antigen presenting cells to process and present the antigens can be determined, for example, by assaying T cell cytotoxic activity in vitro or using antigen-presenting cells as targets of CTLs. Other methods that can detect the presence of antigen on the surface of antigen-presenting cells.
are also contemplated by the presented invention. The antigen-presenting cells loaded with the antigen can be used to stimulate CTL proliferation in vivo or ex vivo. The ability of the loaded dendritic cells to stimulate a CTL response can be measured by assaying the ability of the effector cells to lyse target cells. For example, the non-radioactive LDH cytotoxicity assay or the europium release assay can be used. Volgmann et al., J. Immunol. Methods 119:45-51, 1989.

Ex vivo or in vitro maturation of DCs can be induced by various maturation factors, including, but not limited to, IL-1β, tumor necrosis factor alpha (TNF-a), interferon alpha (IFN-a), poly (I:C), interferon gamma (IFN-γ), Interleukin 1 beta (TL-β), Interleukin 6 (IL-6), prostaglandin E2 (PGE2), poly-dldC, vasointestinal peptide (VIP), bacterial lipopolysaccharide (LPS), mycobacteria or components of mycobacteria (such as cell wall constituents), or combinations thereof. Additional maturation factors include, for example, an imidazoquinoline compound, e.g., R848 (WO 00/47719, incorporated herein by reference in its entirety), a synthetic double stranded polyribonucleotide, agonists of a Toll-like receptor (TLR), such as TLR3, TLR4, TLR7 and/or TLR9, a sequence of nucleic acids containing unmethylated CpG motifs known to induce the maturation of DC, and the like. Further, a combination of any of the above agents can be used in inducing the maturation of immature dendritic cells or dendritic precursor cells. In certain embodiments, a dendritic maturation cocktail includes (comprises, consists essentially of, or consists of) IL-1β, TNF-a, IFN-a and poly (I:C).

The maturation factors can be added to the dendritic cells before, during or after peptide loading of the dendritic cells.

Immature dendritic cells are matured to form mature dendritic cells. Mature DCs lose the ability to take up antigen and display up-regulated expression of costimulatory cell surface molecules and various cytokines. Maturation of dendritic cells can be monitored by methods known in the art. Mature dendritic cells can be selected by expression of one or more markers. The markers include, but are not limited to, CD86, CD80, CD83, CD58, CD1a, HLA-DR, CD40, CD1 lc, IL-2-beta, TLR-4 and combinations thereof. The dendritic cells can also be identified as lacking or expressing low levels of markers such as CD14. In one embodiment, mature dendritic cells are identified as being CD80+, CD83+, CD86+, and CD14-. Greater MHC expression leads to an increase in antigen density on the DC surface, while up-regulation of costimulatory
molecules CD80 and CD86 strengthens the T cell activation signal through the counterparts of the costimulatory molecules, such as CD28 on the T cells.

Cell surface markers can be detected in suitable assays, such as flow cytometry, immunohistochemistry, and the like. The cells can also be monitored for cytokine production (e.g., by ELISA, FACS, or other immune assay). Dendritic cell precursors, immature dendritic cells, and mature dendritic cells, either primed or unprimed with antigens, can be cryopreserved for use at a later date.

The mature DCs of the invention either can be used immediately after their generation (and, optionally, purification) or stored frozen for future use. In certain embodiments, enough mature DCs or T cells are generated to provide an initial dose for the subject as well as cells that can be frozen and stored for future use if necessary.

In certain embodiments of the adoptive immunotherapy methods described above, the cells of interest (i.e., mature DCs) can be purified prior to administration to the subject. Purification of the cells can be done using a variety of methods known in the art, including methods in which antibodies to specific cell surface molecules are employed. These methods include both positive and negative selection methods. For example, cells generated in vitro can be isolated by staining the cells with fluorescently labeled antibodies to cell surface markers followed by sorting of the cells that express both of these markers on their cell surface using fluorescence activated cell sorting (FACS). These and other purification/isolation methods are well known to those of skill in the art.

In certain other embodiments, mature DCs or T cells can be expanded in vitro from freshly isolated or frozen cell stocks to generate sufficient numbers of cells for effective adoptive immunotherapy. The expansion of the cells can be achieved by any means that maintains their functional characteristics. The phenotypic and functional properties of the resultant expanded cells can be tested prior to their therapeutic use and/or storage to verify that the expansion process has altered their activity.

**Immunotherapy**

Methods are provided for administration of dendritic cells to a subject in need of immunostimulation. In certain embodiments, such methods are performed by obtaining dendritic
cell precursors or immature dendritic cells, differentiating and maturing those cells in the presence of a tumor-associated antigen or a tumor-associated peptide antigen (or a nucleic acid composition) to form a mature dendritic cell population. The immature dendritic cells can be contacted with antigen prior to or during maturation.

The DC administration (vaccination) may be given once, twice, three times, four times, five times, six times, seven times, eight times, nine times, ten times, eleven times, twelve times, thirteen times, fourteen times, fifteen times, or more, within a treatment regime to a subject/patient. The DC administration (vaccination) may be given every 2 days, every 3 days, every 4 days, every 5 days, every 6 days, every 7 days, every 8 days, every 9 days, every 10 days, every 11 days, every 12 days, every 13 days, every 14 days, every 16 days, every 18 days, every 20 days, every 1 month, every 2 months, every 3 months, every 6 months, or at different frequencies.

The DC may be administered at a dose ranging from about 1 X 10^3 DCs to about 1 X 10^{12} DCs, from about 1 X 10^4 DCs to about 1 X 10^{10} DCs, from about 1 X 10^5 DCs to about 1 X 10^9 DCs, from about 1 X 10^6 DCs to about 1 X 10^8 DCs, from about 1 X 10^6 DCs to about 1 X 10^7 DCs, from about 1 X 10^7 DCs to about 1 X 10^8 DCs, about 1 X 10^6 DCs, about 1 X 10^7 DCs, about 1 X 10^8 DCs, or about 1 X 10^9 DCs.

In a related embodiment, the mature dendritic cells can be contacted with, and thus activate, lymphocytes. The activated, polarized lymphocytes, optionally followed by clonal expansion in cell culture, can be administered to a subject in need of immunostimulation.

The present invention provides a method for eliciting in a subject an immune response to a cell expressing at least one tumor-associated antigen. The method comprises administering to the subject antigen-presenting cells (e.g., dendritic cells) comprising at least one tumor-associated antigen (or loaded with at least one tumor-associated peptide antigen), or antigen-presenting cells (e.g., dendritic cells) comprising nucleic acids encoding at least one tumor-associated antigen. The antigen-presenting cells may also be treated with at least one inhibitor of the MAPK signaling pathway. The antigen-presenting cells, when administered to the subject, may elicit an immune response to the cell that expresses at least one tumor-associated antigen.

In some aspects, the present invention provides a method of treating a tumor cell, the method comprising administering to a subject a therapeutically or prophylactically effective amount of a pharmaceutical composition to reduce or inhibit growth or spread of the cell in the
subject, wherein the composition comprises: an antigen-presenting cell comprising at least one tumor-associated antigen or a fragment thereof, a lymphocyte primed against the tumor-associated antigen, or a combination thereof, where the antigen-presenting cells may also be treated with at least one inhibitor of the MAPK signaling pathway.

The antigen-presenting cells comprising one or more tumor-associated antigens (or a fragment thereof) may be used to contact lymphocytes under conditions sufficient to produce tumor-associated antigen-specific lymphocyte capable of eliciting an immune response against a tumor cell. Thus, the antigen-presenting cells also can be used to provide lymphocytes, including T lymphocytes and B lymphocytes, for eliciting an immune response against a cell that expresses a tumor-associated antigen. In one embodiment, a preparation of T lymphocytes is contacted with the antigen-presenting cells described above for a period of time, preferably for at least about 24 hours, for priming the T lymphocytes to the at least one tumor-associated antigen presented by the antigen-presenting cells. In another embodiment, a population of antigen-presenting cells can be co-cultured with a heterogeneous population of peripheral blood T lymphocytes together with at least one tumor-associated antigen, or nucleic acids comprising the at least one tumor-associated antigen. The cells can be co-cultured for a period of time and under conditions sufficient for the tumor-associated antigens or their processed forms to be presented by the antigen-presenting cells and the antigen-presenting cells to prime a population of T lymphocytes to respond to cells that express a tumor-associated antigen. Accordingly, T lymphocytes and B lymphocytes that are primed to respond to cells that express a tumor-associated antigen can be prepared.

The ability to induce lymphocytes to exhibit an immune response can be determined by any method including, but not limited to, determining T lymphocyte cytolytic activity in vitro using for example tumor-associated antigen-specific antigen-presenting cells as targets of tumor-associated antigen-specific cytolytic T lymphocytes (CTL); assaying tumor-associated antigen-specific T lymphocyte proliferation; and determining B cell response to cells expressing a tumor-associated antigen using, for example, ELISA methods.

T lymphocytes can be obtained from any suitable source such as peripheral blood, spleen, and lymph nodes. The T lymphocytes can be used as crude preparations or as partially purified or substantially purified preparations, which can be obtained by standard techniques including, but not limited to, methods involving immunomagnetic or flow cytometry techniques using
antibodies.

In another embodiment, T cells can be removed from an individual and treated in vitro with the antigen(s) or peptide(s), wherein the resulting CTL are reinfused autologously or allogeneically to the subject. In various other embodiments, the tumor-associated antigen(s) or peptide(s) of the present invention also may be administered to the subject, or in vitro to T cells, in the form of a nucleic acid vaccine, wherein one or more suitable gene transfer vectors, such as a plasmid or an engineered viral vector that contains DNA encoding the peptide fragment(s), is administered to the subject or to T cells in vitro.

In one aspect, the present invention provides a method of treating tumor cells, the method comprising administering to a subject (or contacting the tumor cells) antigen-presenting cells, T lymphocytes, or both, where the antigen-presenting cells comprising at least one tumor-associated antigen (or a fragment thereof), or where the antigen-presenting cells comprise nucleic acids encoding at least one tumor-associated antigen. The antigen-presenting cells may also be treated with at least one inhibitor of the MAPK signaling pathway. The T lymphocytes have been contacted with antigen-presenting cells.

Accordingly, the antigen-primed antigen-presenting cells of the present invention and the antigen-specific T lymphocytes generated with these antigen-presenting cells can be used as immunomodulating compositions for prophylactic or therapeutic applications for cancer. In some embodiments, the tumor-associated antigen-primed antigen-presenting cells of the invention can be used for generating CD8+ CTL, CD4+ CTL, and/or B lymphocytes for adoptive transfer to the subject. Thus, for example, tumor-associated antigen-specific CTLs can be adoptively transferred for therapeutic purposes in subjects afflicted with cancer.

The present compositions or methods may function to provide or enhance an immune response. Generally, the immune response can include humoral immune response, cell-mediated immune response, or both. For example, antigen presentation through an immunological pathway involving MHC class II molecules or direct B-cell stimulation can produce a humoral response; and, antigens presented through a pathway involving MHC I molecules can elicit cell-mediated immune response. A humoral response can be determined by a standard immunoassay for antibody levels in a serum sample from the subject receiving the pharmaceutically acceptable composition. A cellular immune response is a response that involves T cells and can be
determined in vitro or in vivo. For example, a general cellular immune response can be
determined as the T cell proliferative activity in cells (e.g., peripheral blood leukocytes (PBLs))
sampled from the subject at a suitable time following the administering of a pharmaceutically
acceptable composition. Following incubation of e.g., PBMCs with a stimulator for an
appropriate period, [3H]thymidine incorporation can be determined. The subset of T cells that is
proliferating can be determined using flow cytometry. T cell cytotoxicity can also be
determined.

The immune response that is elicited or enhanced may be sufficient for prophylactic or
therapeutic treatment of a neoplastic disease, or a symptom associated therewith, particularly
cancer. Accordingly, a beneficial effect of the present compositions and/or methods will
generally at least in part be immune-mediated, although an immune response need not be
positively demonstrated in order for the compositions and methods described herein to fall
within the scope of the present invention.

The immunological efficacy of the present methods and compositions may be determined
based on the Distribution Free Resampling (DFR) method proposed and described by Moodie et
al. [66].

The release of cytokines (e.g., IFN-γ, TNF-a, and/or IL-17) may be assayed by, e.g.,
ELISpot assay, to determine immune responses.

The cytokine ELISPOT (Enzyme-Linked ImmunoSPOT) assay is designed to enumerate
cytokine-secreting cells. The assay has the advantage of detecting only activated/memory T cells
and has the ability to detect cytokine release in response to antigen by a single cell thereby
permitting direct calculation of responder T cell frequencies. The high sensitivity and easy
performance, allowing the determination of peptide-reactive T cells without prior in vitro
expansion, makes the ELISPOT assay well suited to monitor T cell responses. Tanguay et al.,

Briefly, cells are incubated in the wells of the ELISPOT plate pre-coated with a high-
affinity monoclonal antibody to which the cytokine, produced during incubation, will bind.
Subsequently, cells are washed away. Areas in which the cytokines have been bound are detected
with a combination of biotinylated anti-cytokine detection antibodies and φ-labeled goat anti-
biotin antibodies. The last step in the assay is the addition of a reagent allowing the precipitation
of silver on φ revealing the site of cytokine secretion (i.e., spot formation).

Treating a subject using the present compositions and methods may refer to reducing the symptoms of the disease, reducing the occurrence of the disease, reducing the severity of the disease, and/or preventing a disease from occurring. As such, to treat a subject means both preventing disease occurrence (prophylactic treatment) and treating a subject that has a disease (therapeutic treatment). In particular, treating a subject is accomplished by providing or enhancing an immune response in the subject.

One or more antigens or antigenic peptides may be introduced into (or loaded onto) the present antigen-presenting cells (e.g., dendritic cells), including 2, 3, 4, 5, 6, 7, 8, 9, 10 or more antigens or antigenic peptides. Additionally, multiple independently generated DCs can be administered to a subject. Furthermore, administration of DCs to a subject can be done as often as is required to ameliorate the symptoms associated with the disease state.

The antigen-presenting cells and/or lymphocytes described above can be administered to a subject for eliciting or enhancing an immune response (such as against tumor cells that express at least one tumor-associated antigen). Such cell-based compositions are useful for treating or preventing cancer.

The APCs (e.g., dendritic cells) and/or T lymphocytes may be autologous, allogenic (e.g., from a different donor subject that is MHC matched or mismatched with the recipient subject) or heterologous to the recipient subject. For example, immature dendritic cells can be harvested from an organ donor and treated in vitro with at least one tumor-associated peptide antigen. The resultant allogeneic mature DCs can then be administered to the subject to promote the cure or treatment of disease in that subject. The antigen-presenting cells and/or lymphocytes described above can be administered to a subject, either by themselves or in combination, for eliciting an immune response, particularly for eliciting an immune response to cells that express a tumor-associated antigen. Such cell-based compositions are useful, therefore, for treating or preventing cancer. The cells can be introduced into a subject by any mode that elicits the desired immune response to cells that express a tumor-associated antigen. Furthermore, the antigen-presenting cells and/or lymphocytes can be derived from the subject (i.e., autologous cells) or from a different subject that is MHC matched or mismatched with the subject (e.g., allogeneic).

In certain embodiments, the present methods induce an immune response to a tumor in a
patient. Such methods can comprise one or more steps of (a) obtaining monocytes (which may act as monocytic dendritic cell precursors) from a patient; (b) culturing the monocytes (e.g., with specific cytokines) to induce differentiation into immature dendritic cells; (c) differentiating the immature dendritic cells into mature dendritic cells by contacting the immature dendritic cells with at least one tumor-associated peptide antigen (or tumor-associated antigen); and (d) administering the mature dendritic cells to the patient. The antigen-presenting cells may also be treated with at least one inhibitor of the MAPK signaling pathway.

**Immunosuppressive agents**

Prior to the present immunotherapy, one or more immunosuppressive agents may be administered to the patient. In one embodiment, the immunosuppressive agent inhibits or decreases the activity of suppressive T-cell populations, such as suppressor regulatory T-cells (Treg).

Immunosuppressive agents are substances that inhibit or prevent activity of the immune system. This includes substances that suppress cytokine production, down-regulate or suppress self-antigen expression, or mask the MHC antigens. Immunosuppressive agents can be glucocorticoids, cytostatics, antibodies, drugs acting on immunophilins, etc. These may be used alone or in combination.

Cytostatics include, but are not limited to, alkylating agents, antimetabolites, etc. Non-limiting examples of alkylating agents include nitrogen mustards (e.g., cyclophosphamide), nitrosoureas, platinum compounds, and others. Non-limiting examples of antimetabolites include folic acid analogues (e.g., methotrexate), purine analogues (e.g., azathioprine and mercaptopurine), pyrimidine analogues (e.g., fluorouracil), protein synthesis inhibitors, cytotoxic antibiotics (e.g., dactinomycin, anthracyclines, mitomycin C, bleomycin, mithramycin, etc.).
Antibodies include polyclonal antibodies and monoclonal antibodies. For example, the antibodies may be T-cell receptor directed antibodies (e.g., CD3-directed antibodies), or JL-2 receptor directed antibodies (e.g., CD25-directed antibodies).

Drugs acting on immunophilins include, but are not limited to, ciclosporin, tacrolimus, sirolimus, etc.

Other immunosuppressive drugs include, interferons (e.g., IFN-β, IFN-γ, etc.), opioids, TNF binding proteins, mycophenolate, and small biological agents (e.g., fingolimod, myriocin etc.).

Non-limiting examples of immunosuppressive agents include 2-amino-6-aryl-5-substituted pyrimidines; mycophenolate mofetil; azathioprine; 6-mercaptopurine; bromocryptine; danazol; dapsone; glutaraldehyde; anti-idiotypic antibodies for MHC antigens and MHC fragments; cyclosporin A; steroids such as corticosteroids and glucocorticosteroids, e.g., prednisone, prednisolone (e.g., prednisolone sodium phosphate), methylprednisolone, and dexamethasone; methotrexate; hydroxyclooroquine; chloroquine; sulfasalazine; leflunomide; cytokine or cytokine receptor antagonists including anti-interferon-gamma, -beta, or -alpha antibodies, anti-tumor necrosis factor-alpha antibodies, anti-TNF-alpha immunoadhesin, anti-tumor necrosis factor-beta antibodies, anti-interleukin-2 antibodies and anti-IL-2 receptor antibodies; anti-LFA-1 antibodies, including anti-CD1 and anti-CD18 antibodies; anti-L3T4 antibodies; heterologous anti-lymphocyte globulin; polyclonal or pan-T antibodies, or monoclonal anti-CD3 or anti-CD4/CD4a antibodies; soluble peptide containing a LFA-3 binding domain; streptokinase; TGF-beta; streptodornase; FK506; RS-61443; deoxyspergualin; rapamycin; T-cell receptor (Cohen et al., U.S. Pat. No. 5,114,721); T-cell receptor fragments (Offner et al. Science 251: 430-432 (1991); WO 90/1294; laneway, Nature 341:482 (1989); and WO 91/01133); T cell receptor antibodies; cyclophosphamide; dapsone; penicillamine; plasma exchange; or intravenous immunoglobulin (IVIG).

In one embodiment, prior to the present immunotherapy, cyclophosphamide (CYP) may be administered to the patient. Cyclophosphamide (CYP) may exert cytotoxic and/or immunosuppressive effects, depending on the dose used. For example, studies have shown that low-dose cyclophosphamide (CYP) decreases Treg number and functionality [53]. Greten and colleagues evaluated single-agent CYP doses of 150, 250, and 350 mg/m² in patients with hepatocellular carcinoma and reported that the two (2) lower doses induced a decrease in the
The absolute and relative frequency of Tregs in the blood of patients, and the 250 mg/m² dose impaired suppressor function and showed decreased Treg frequency up to day 71. Alpha-fetoprotein-specific T-cell responses were also induced in the lower treatment arms [54]. In a different study, breast cancer patients received 50 mg CYP orally daily for 3 months. Tregs were reduced within 14 days of treatment and remained decreased until day 42, returning to pretreatment levels by day 84. Interestingly, endogenous breast tumor-reactive T cells were detected in 27% of patients before CYP treatment and increased to 73% on day 14, 80% on day 42, and 88% on day 84, indicating enhanced T-cell function after the use of metronomic doses of CYP [55]. More recently, the use of metronomic CYP combined with active immunotherapy has been reported [56]. In this study, patients with advanced solid tumors were treated with 3 different regimens of low-dose CYP in combination with an oncolytic adenovirus. CYP was given either as oral metronomic (50 mg/day), a single i.v. injection (1,000 mg), or both. Metronomic CYP was given starting 1 week before the adenovirus, and i.v. cyclophosphamide was given 1 hour prior to the adenovirus. All CYP regimens resulted in higher rates of disease control when compared with the rates for the adenovirus vaccine only, and the metronomic groups were most effective in decreasing Treg numbers. Studies are being conducted combining metronomic doses of CYP with active vaccination strategies for a variety of cancers [57].

In certain embodiments, prior to 1, 2, 3, 4, 5, 6, 7, 8, 9 or all of administration of the tumor-associated antigen loaded DCs, the subject will be administered cyclophosphamide at a dose ranging from about 10 mg/day to about 500 mg/day, from about 20 mg/day to about 400 mg/day, from about 30 mg/day to about 300 mg/day, from about 40 mg/day to about 200 mg/day, from about 50 mg/day to about 150 mg/day, from about 40 mg/day to about 120 mg/day, about 150 mg/day, about 50 mg/day, or about 100 mg/day. The dose of cyclophosphamide can also be lower or higher. In certain embodiments, cyclophosphamide may be administered for about 1 day, about 2 days, about 3 days, about 4 days, about 5 days, about 6 days, about 1 week, about 1.5 weeks, about 2 weeks, or longer. In certain embodiments, administration of cyclophosphamide may be started about 1 day, about 2 days, about 3 days, about 4 days, about 5 days, about 6 days, about 1 week, about 1.5 weeks, about 2 weeks, or earlier, prior to administration of the present loaded DCs to the subject. Administration of the present loaded DCs may be within about 1 day, within about 2 days, within about 3 days, within
about 4 days, within about 5 days, within about 6 days, after the cyclophosphamide administration.

**Adjuvants**

After each vaccination, adjuvants may be administered. The adjuvant may enhance DC migration and activation *in vivo*. The adjuvant may provide for increased immunogenicity.

Adjuvants include, but are not limited to, immunomodulatory molecules (e.g., cytokines), oil and water emulsions, aluminum hydroxide, glucan, dextran sulfate, iron oxide, sodium alginate, Bacto-Adjuvant, synthetic polymers such as poly amino acids and co-polymers of amino acids, saponin, paraffin oil, and muramyl dipeptide. U.S. Patent No. 9,01 1,835.

In one embodiment, the adjuvant is an immunomodulatory molecule. For example, the immunomodulatory molecule can be a cytokine, chemokine, or immunostimulatory agent, or nucleic acids encoding cytokines, chemokines, or immunostimulatory agents designed to enhance the immunologic response.

Cytokines include, but are not limited to, chemokines, interferons, interleukins, lymphokines, tumor necrosis factor, etc. Cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF) are known to induce DC development and serve as an immune adjuvant, both *in vitro* and *in vivo* [60, 61]. In one embodiment, low-dose GM-CSF is administered to the subject post vaccination to enhance vaccine-based immune stimulation in patients [62-65]. Examples of immunomodulatory cytokines include interferons (e.g., IFN-a, IFN-β and IFN-γ), interleukins (e.g., IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-15, IL-20, and IL-21), tumor necrosis factors (e.g., TNF-a and TNF-β), erythropoietin (EPO), FLT-3 ligand, glpLO, TCA-3, MCP-1, MIF, MIP-1α, MIP-1β, Rantes, macrophage colony stimulating factor (M-CSF), granulocyte colony stimulating factor (G-CSF), and granulocyte-macrophage colony stimulating factor (GM-CSF), as well as functional fragments of any of the foregoing.

In certain embodiments, GM-CSF is administered at a dose ranging from about 10 μg/day to about 500 μg/day, from about 20 μg/day to about 300 μg/day, from about 50 μg/day to about 250 μg/day, from about 25 μg/day to about 100 μg/day, from about 30 μg/day to about 80 μg/day, from about 100 μg/day to about 300 μg/day, from about 200 μg/day to about 300 μg/day, about 200 μg/day, about 250 μg/day, about 40 μg/day, about 50 μg/day, about 60
μg/day, or about 70 μg/day. The dose of GM-CSF can also be lower or higher. In certain embodiments, GM-CSF may be administered after the administration of the present loaded DCs for about 1 day, about 2 days, about 3 days, about 4 days, about 5 days, about 6 days, about 1 week, about 1.5 weeks, about 2 weeks, or longer. Any immunomodulatory chemokine that binds to a chemokine receptor, i.e., a CXC, CC, C, or CX3C chemokine receptor, also can be used in the context of the present invention. Examples of chemokines include, but are not limited to, Mipla, Mip-1β, Mip-3a (Larc), Mip-3β, Rantes, Hcc-1, Mpi-1, Mpi-2, Mcp-1, Mcp-2, Mcp-3, Mcp-4, Mcp-5, Eotaxin, Tare, Elc, 1309, IL-8, Gcp-2 Gro-a, Gro-β, Gro-γ, Nap-2, Ena-78, Gcp-2, Ip-10, Mig, I-Tac, Sdf-1, and Bca-1 (Blc), as well as functional fragments of any of the foregoing.

The adjuvant may be expressed from a vector, or may be administered simultaneously or sequentially, in any order.

**Pharmaceutical Compositions**

The present invention provides a pharmaceutical composition comprising antigen-presenting cells (or lymphocytes) comprising at least one tumor-associated antigen, or antigen-presenting cells (or lymphocytes) comprising nucleic acids encoding at least one tumor-associated antigen, described herein, where the dendritic cells are treated with at least one MAPK signaling pathway inhibitor. In some embodiments, the composition further comprises an adjuvant as described above. When administered to a subject, the pharmaceutical composition elicits or enhance an immune response to a cell expressing the tumor-associated antigen.

In one embodiment, the present pharmaceutical composition comprises antigen-presenting cells contacted in vitro or ex vivo with at least one tumor-associated antigen (or tumor-associated peptide antigen). In another embodiment, the present invention provides a composition comprising antigen-presenting cells contacted in vitro with nucleic acids encoding at least one tumor-associated antigen.

The pharmaceutically acceptable compositions of the present invention can be useful as vaccine compositions for prophylactic or therapeutic treatment of a neoplastic disease or symptoms thereof, particularly for preventing or treating cancer in the subject.

In certain embodiments, the present invention concerns formulation of one or more dendritic cell compositions disclosed herein in pharmaceutically acceptable carriers for
administration to a cell or a subject, either alone, or in combination with one or more other modalities of therapy.

The pharmaceutical composition may further comprise a pharmaceutically acceptable carrier, diluent, or excipient. Techniques for formulating and administering also can be found in Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, Pa., latest edition.

Pharmaceutically acceptable carriers known in the art include, but are not limited to, sterile water, saline, glucose, dextrose, or buffered solutions. Agents such as diluents, stabilizers (e.g., sugars and amino acids), preservatives, wetting agents, emulsifying agents, pH buffering agents, additives that enhance viscosity, and the like. Preferably, the medium or carrier will produce minimal or no adverse effects.

The pharmaceutical composition may further comprise an adjuvant. Preferably, the adjuvant employed provides for increased immunogenicity. The adjuvant can be one that provides for slow release of antigen (e.g., a liposome), or it can be an adjuvant that is immunogenic in its own right thereby functioning synergistically with antigens. For example, the adjuvant can be a known adjuvant or other substance that promotes nucleic acid uptake, recruits immune system cells to the site of administration, or facilitates the immune activation of responding lymphoid cells. Adjuvants include, but are not limited to, immunomodulatory molecules (e.g., cytokines), oil and water emulsions, aluminum hydroxide, glucan, dextran sulfate, iron oxide, sodium alginate, Bacto-Adjuvant, synthetic polymers such as poly amino acids and co-polymers of amino acids, saponin, paraffin oil, and muramyl dipeptide. U.S. Patent No. 9,011,835.

In one embodiment, the adjuvant is an immunomodulatory molecule. For example, the immunomodulatory molecule can be a recombinant protein cytokine, chemokine, or immunostimulatory agent or nucleic acid encoding cytokines, chemokines, or immunostimulatory agents designed to enhance the immunologic response.

Examples of immunomodulatory cytokines include interferons (e.g., IFN-a, IFN-β and IFN-γ), interleukins (e.g., IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-15, IL-20, and IL-21), tumor necrosis factors (e.g., TNF-a and TNF-β), erythropoietin (EPO), FLT-3 ligand, glpLO, TCA-3, MCP-1, MIF, MIP-1α, MIP-1β, Rantes, macrophage colony stimulating factor (M-CSF), granulocyte colony stimulating factor (G-CSF), and granulocyte-macrophage colony stimulating factor (GM-CSF), as well as functional fragments of any of the
foregoing.

Any immunomodulatory chemokine that binds to a chemokine receptor, i.e., a CXC, CC, C, or CX3C chemokine receptor, also can be used in the context of the present invention. Examples of chemokines include, but are not limited to, Mipla, Mip-1β, Mip-3α (Larc), Mip-3β, Rantes, Hcc-1, Mpi̇f-1, Mpi̇f-2, Mcp-1, Mcp-2, Mcp-3, Mcp-4, Mcp-5, Eotaxin, Tare, Etc, 1309, IL-8, Gcp-2 Gro-a, Gro-β, Gro-γ, Nap-2, Ena-78, Gcp-2, Ip-10, Mig, I-Tac, Sdf-1, and Bca-1 (Bc), as well as functional fragments of any of the foregoing.

In some embodiments, the adjuvant is comprised of incomplete Freund's adjuvant (Montanide ISA 51) or Corynebacterium granulosum P40.

The pharmaceutical composition can be administered in a therapeutically or a prophylactically effective amount. Administering the pharmaceutically acceptable composition of the present invention to the subject can be carried out using known procedures, and at dosages and for periods of time sufficient to achieve a desired effect. For example, a therapeutically or prophylactically effective amount of the pharmaceutical composition can vary according to factors such as the age, sex, and weight of the subject. Dosage regime can be adjusted by one of ordinary skill in the art to elicit the desired immune response including immune responses that provide therapeutic or prophylactic effects.

The pharmaceutically acceptable composition can be administered to the subject at any suitable site, for example, a site that is distal to or proximal to a primary tumor. The route of administering can be parenteral, intramuscular, subcutaneous, intradermal, intraperitoneal, intranasal, intravenous (including via an indwelling catheter), via an afferent lymph vessel, or by any other route suitable in view of the neoplastic disease being treated and the subject's condition. Preferably, the dose will be administered in an amount and for a period of time effective in bringing about a desired response, be it eliciting the immune response or the prophylactic or therapeutic treatment of the neoplastic disease and/or symptoms associated therewith.

Administering can be properly timed, and can depend on the clinical condition of the subject, the objectives of administering, and/or other therapies also being contemplated or administered. In some embodiments, an initial dose can be administered, and the subject monitored for an immunological and/or clinical response. Suitable means of immunological
monitoring include using patient's peripheral blood lymphocyte (PBL) as responders and neoplastic cells as stimulators. An immunological reaction also can be determined by a delayed inflammatory response at the site of administering.

One or more doses subsequent to the initial dose can be given as appropriate, typically on a monthly, semimonthly, or a weekly basis, until the desired effect is achieved. Thereafter, additional booster or maintenance doses can be given as required, particularly when the immunological or clinical benefit appears to subside.

Single or multiple administrations of the antigen-presenting cells and lymphocytes can be carried out with cell numbers and treatment being selected by a care provider (e.g., a physician). Preferably, the antigen-presenting cells and/or lymphocytes are administered in a pharmaceutically acceptable carrier. Suitable carriers can be the growth medium in which the cells were grown, or any suitable buffering medium such as phosphate buffered saline. The cells can be administered alone or as an adjunct therapy in conjunction with other therapeutics.

In one embodiment, the antigen-presenting cells or the lymphocytes are administered systemically, e.g., by injection. Alternately, one can administer locally rather than systemically, for example, via injection directly into tissue. The pharmaceutical composition may be in a depot or sustained release formulation. Furthermore, one can administer in a targeted drug delivery system, for example, in a liposome that is coated with tissue-specific antibody. The liposomes can be targeted to and taken up selectively by the tissue.

Pharmaceutical compositions may be administered directly, endoscopically, intratracheally, intratumorally, intravenously, intrallesionally, intramuscularly, intraperitoneally, regionally, percutaneously, topically, intrarterially, intravesically, or subcutaneously. Compositions may be administered 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more times, and they may be administered every 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24 hours, or 1, 2, 3, 4, 5, 6, 7 days, or 1, 2, 3, 4, 5 weeks, or 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 months.

**Combination Therapy**

The present APCs comprise at least one tumor-associated antigen (or a fragment thereof) and are also treated with at least one inhibitor of the MAPK signaling pathway.
The combination of introducing the tumor-associated antigen (or a fragment thereof) into the APCs and treating the APCs with the inhibitor of the MAPK pathway may produce an additive or synergistic effect (i.e., greater than additive effect) in treating the cancer compared to the effect of introducing the tumor-associated antigen (or a fragment thereof) into the APCs alone or treating the APCs with the inhibitor of the MAPK pathway alone. For example, the combination may result in a (synergistic or additive) increase in apoptosis of cancer cells, a (synergistic or additive) reduction in tumor volume, and/or a (synergistic or additive) increase in survival time. In different embodiments, the combination can inhibit tumor growth, achieve tumor stasis, or achieve substantial or complete tumor regression.

In certain embodiments, our data shows the induction of potent anti-tumor CD8 T-cell and CD4 T-cell mediated responses, providing increased survival rates and long-term prevention of tumor growth and dissemination, associated with diminished accumulation of CD4+Foxp3+ regulatory T cells, and enhanced accumulation of CD4+ IL17+ cells in the spleens of vaccinated subjects. P38 blockade in the presence of rAAV SP17 transduction is superior to rAAV Spl7 transduction alone, in terms of activation of human DC and their ability to generate effector lymphocytes from autologous PBMCs, in vitro. Human monocyte-differentiated DC that the optimum DC activation status is achieved by rAAV in the presence of p38 blockade. Vaccination with rAAV-SP17 transduced autologous DC treated with p38 MAPK inhibitor efficiently eliminates tumors and prevents dissemination.

In various embodiments, the present invention provides methods to reduce cancer cell growth, proliferation, and/or metastasis, as measured according to routine techniques in the diagnostic art. Specific examples of relevant responses include reduced size, mass, or volume of a tumor, or reduction in cancer cell number.

The present compositions and methods can have one or more of the following effects on cancer cells or the subject: cell death; decreased cell proliferation; decreased numbers of cells; inhibition of cell growth; apoptosis; necrosis; mitotic catastrophe; cell cycle arrest; decreased cell size; decreased cell division; decreased cell survival; decreased cell metabolism; markers of cell damage or cytotoxicity; indirect indicators of cell damage or cytotoxicity such as tumor shrinkage; improved survival of a subject (e.g., increased survival time of a subject); preventing, inhibiting or ameliorating the cancer in the subject, such as slowing progression of the cancer, reducing or ameliorating a sign or symptom of the cancer; reducing the rate of tumor growth in a
patient; preventing the continued growth of a tumor, reducing the size of a tumor; and/or disappearance of markers associated with undesirable, unwanted, or aberrant cell proliferation.

U.S. Patent Publication No. 20080275057 (incorporated herein by reference in its entirety). The effects may be compared with treating the cancer cells or the subject with APCs comprising at least one tumor-associated antigen (or a fragment thereof), compared with treating the cancer cells or the subject with APCs treated with at least one inhibitor of the MAPK pathway.

Methods and compositions of the present invention can be used for prophylaxis as well as amelioration of signs and/or symptoms of cancer.

In some embodiments, the combination therapy results in a synergistic or additive effect, for example, introducing at least one tumor-associated antigen (or a fragment thereof) into the APCs and treating the APCs with at least one inhibitor of the MAPK pathway may act synergistically or additively, for example, in the apoptosis of cancer cells, inhibition of proliferation/survival of cancer cells, in the production of tumor stasis.

As used herein, the term "synergy" (or "synergistic") means that the effect achieved with the methods and combinations of this invention is greater than the sum of the effects that result from using the individual agents alone, e.g., introducing at least one tumor-associated antigen (or a fragment thereof) into the APCs alone, and treating the APCs with at least one inhibitor of the MAPK pathway alone.

The effect (e.g., apoptosis of cells or an increase in apoptosis of cells, a decrease in cell viability, cytotoxicity or an increase in cytotoxicity, a decrease in cell proliferation, a decrease in cell survival, inhibition of tumor growth, a reduction in tumor volume, the subject's survival time or an increase in the subject's survival time, the amount of the antibodies specific to the tumor associated antigen or an increase in the subject's amount of the antibodies specific to the tumor associated antigen, levels of cytokines such as IFN-γ and/or TNF-a or an increase in levels of cytokines such as IFN-γ and/or TNF-a, levels of TNFa+ or IFNy+ cells or an increase in levels of TNFa+ or IFNy+ cells, a decrease in levels of cytokines such as IL-10 and/or IL-4, the migration index of monocytes, macrophages or splenocytes or an increase in the migration index of monocytes, macrophages or splenocytes, levels of T helper 17 cells (Th17) or an increase in levels of Th17 cells, levels of IL-17 or an increase in levels of IL-17, a decrease in levels of T-reg cells, a decrease in levels of Foxp3 or Foxp3+ T cells, and/or tumor stasis, etc. as described herein) achieved by the present composition or method (e.g., the combination of introducing the
tumor-associated antigen (or a fragment thereof) into the APCs and treating the APCs with the inhibitor of the MAPK pathway) may be about 1.1 fold, about 1.2 fold, about 1.3 fold, about 1.4 fold, about 1.5 fold, about 1.6 fold, about 1.7 fold, about 1.8 fold, about 1.9 fold, about 2 fold, about 2.5 fold, about 3 fold, about 3.5 fold, about 4 fold, about 4.5 fold, about 5 fold, about 5.5 fold, about 6 fold, about 6.5 fold, about 7 fold, about 8 fold, about 9 fold, about 10 fold, about 12 fold, about 15 fold, about 20 fold, about 25 fold, about 30 fold, about 50 fold, about 100 fold, at least about 1.2 fold, at least about 1.5 fold, at least about 2 fold, at least about 2.5 fold, at least about 3 fold, at least about 3.5 fold, at least about 4 fold, at least about 4.5 fold, at least about 5 fold, at least about 5.5 fold, at least about 6 fold, at least about 6.5 fold, at least about 7 fold, at least about 8 fold, at least about 9 fold, at least about 10 fold, of the corresponding effects that result from treating the cancer cells or the subject with APCs comprising at least one tumor-associated antigen (or a fragment thereof) alone, or of the corresponding effects that result from treating the cancer cells or the subject with APCs treated with at least one inhibitor of the MAPK pathway alone.

Synergistic effects of the combination may also be evidenced by additional, novel effects that do not occur when either agent is administered alone, or by reduction of adverse side effects when either agent is administered alone.

Cytotoxicity effects can be determined by any suitable assay, including, but not limited to, assessing cell membrane integrity (using, e.g., dyes such as trypan blue or propidium iodide, or using lactate dehydrogenase (LDH) assay), assaying cell lysis, measuring enzyme activity, measuring cell adherence, measuring ATP production, measuring co-enzyme production, measuring nucleotide uptake activity, crystal violet method, Tritium-labeled Thymidine uptake method, measuring lactate dehydrogenase (LDH) activity, 3-(4, 5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) or MTS assay, sulforhodamine B (SRB) assay, WST assay, clonogenic assay, cell number count, monitoring cell growth, etc.

Apoptosis of cells may be assayed by any suitable method, including, but not limited to, TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) assay, assaying levels of cytochrome C release, assaying levels of cleaved/activated caspases, assaying 5-bromo-2'-deoxyuridine labeled fragmented DNA, assaying levels of survivin etc.

Other methods that can be used to show the synergistic effects of the present methods, pharmaceutical compositions and combinations include, but are not limited to, clonogenic assay
(colony formation assay) to show decrease in cell survival and/or proliferation, studying tumor volume reduction in animal models (such as in mice, etc.)

In one embodiment, advantageously, such synergy provides greater efficacy at the same doses, lower side effects, and/or prevents or delays the build-up of multi-drug resistance.

Introducing at least one tumor-associated antigen (or a fragment thereof) into the APCs and treating the APCs with the inhibitor of the MAPK signaling pathway may be carried out simultaneously, separately or sequentially. They may exert an advantageously combined effect (e.g., additive or synergistic effects).

For sequential administration, either at least one tumor-associated antigen (or a fragment thereof) is introduced into the APCs first and then the APCs are treated with an MAPK pathway inhibitor, or the APCs are treated with an MAPK pathway inhibitor first and then at least one tumor-associated antigen (or a fragment thereof) is introduced into the APCs. In embodiments where the two treatments of the APCs are carried out separately, the first treatment can precede the second treatment by seconds, minutes, hours, days, or weeks. The time difference in non-simultaneous treatments may be greater than 1 minute, and can be, for example, precisely, at least, up to, or less than 5 minutes, 10 minutes, 15 minutes, 30 minutes, 45 minutes, 60 minutes, 2 hours, 3 hours, 6 hours, 9 hours, 12 hours, 24 hours, 36 hours, or 48 hours, or more than 48 hours. The two treatments can be carried out within minutes of each other or within about 0.5, about 1, about 2, about 3, about 4, about 6, about 9, about 12, about 15, about 18, about 24, or about 36 hours of each other or within about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14 days of each other or within about 2, 3, 4, 5, 6, 7, 8, 9, or 10 weeks of each other. In some cases, longer intervals are possible.

The pharmaceutical composition can be given subsequent to, preceding, or contemporaneously with other therapies including therapies that also elicit an immune response in the subject. For example, the subject may previously or concurrently be treated by chemotherapy, radiation therapy, surgery, other forms of immunotherapy, anti-angiogenic agents, anti-viral agents, and hormonal agents. Such other therapies preferably are provided in such a way so as not to interfere with the immunogenicity of the compositions of the present invention.

The pharmaceutically acceptable composition can be administered at any time that is
appropriate. For example, the administering can be conducted before or during traditional therapy of a subject having a tumor burden, and continued after the tumor becomes clinically undetectable. The administration also can be continued in a subject showing signs of recurrence.

The chemotherapeutic agent may be naturally occurring or synthetic, for example as described in "Cancer Chemotherapeutic Agents", American Chemical Society, 1995, W. O. Foye Ed. The chemotherapeutic agents may be compounds interacting with or binding tubulin, growth factor receptor antagonists, alkylating agents or platinum compounds, anthracyclines, as DNA intercalators or as DNA cross-linking agents, including DNA minor-groove binding compounds, anti-metabolites, bleomycin type antibiotics, inhibitors of DNA transcribing enzymes, and especially the topoisomerase I or topoisomerase II inhibitors, chromatin modifying agents, anti-mitotic agents, cell-cycle inhibitors, proteasome inhibitors, enzymes, hormones, hormone antagonists, hormone inhibitors, inhibitors of steroid biosynthesis, steroids, cytokines, hypoxia-selective cytotoxins, inhibitors of cytokines, lymphokines, antibodies directed against cytokines, oral and parenteral tolerance induction agents, supportive agents, chemical radiation sensitizers and protectors, photo-chemically activated drugs, synthetic poly- or oligonucleotides, optionally modified or conjugated, non-steroidal anti-inflammatory drugs, cytotoxic antibiotics, antibodies targeting the surface molecules of cancer cells, antibodies targeting growth factors or their receptors, inhibitors of metalloproteinases, metals, inhibitors of oncogenes, inhibitors of gene transcription or of RNA translation or protein expression, complexes of rare earth elements, and photo-chemotherapeutic agents.

Non-limiting examples of chemotherapeutic agents include paclitaxel (taxol), docetaxel, a vinca alkaloid such as navelbine, vinblastin, vincristin, vindesine or vinorelbine, an alkylating agent or a platinum compound such as melphalan, cyclophosphamide, an oxazaphosphorine, cisplatin, carboplatin, oxaliplatin, satraplatin, tetraplatin, iproplatin, mitomycin, streptozocin, carmustine (BCNU), lomustine (CCNU), busulfan, ifosfamide, streptozocin, thiotepa, chlorambucil, a nitrogen mustard such as mechlorethamine, an immunomodulatory drug such as thalidomide and its derivatives, or revimid (CC-5013), an ethyleneimine compound, an alkylsulphonate, daunorubicin, doxorubicin (adriamycin), liposomal doxorubicin (doxil), epirubicin, idarubicin, mitoxantrone, amsacrine, daclimycin, distamycin or a derivative thereof, netropsin, pibenzmol, mitomycin, CC-1065, a duocarmycin, mithramycin, chromomycin, olivomycin, a phtalanilide such as propamidine or stilbamidine, an anthramycin,
an aziridine, a nitrosourea or a derivative thereof, a pyrimidine or purine analogue or antagonist or an inhibitor of the nucleoside diphosphate reductase such as cytarabine, 5-fluorouracile (5-FU), uracil mustard, fludarabine, gemcitabine, capecitabine, mercaptopurine, cladribine, thioguanine, methotrexate, pentostatin, hydroxyurea, or folic acid, an acridine or a derivative thereof, a rifamycin, an actinomycin, adramycin, a camptothecin such as irinotecan (camptosar) or topotecan, an amsacrine or analogue thereof, a tricyclic carboxamide, an histonedeacetylase inhibitor such as SAHA, MD-275, trichostatin A, CBHA, LAQ824, or valproic acid, a proteasome inhibitor such as bortezomib, a small molecule VEGF receptor antagonist such as vatalanib (PTK-787/ZK222584), SU-5416, SU-6668, SU-1 1248, SU-14813, AZD-6474, AZD-2171, CP-547632, CEP-7055, AG-013736, IM-842 or GW-786034, an antagonist of the mitogen-activated protein kinase such as BAY-43-9006 or BAY-57-9006, a dual EGFR/HER2 antagonist such as gefitinib, erlotinib, CI-1033 or GW-2016, an EGFR antagonist such as irressa (ZD-1839), tarceva (OSI-774), PKI-166, EKB-569, HKI-272 or herceptin, a quinazoline derivative such as 4-{3-chloro-4-fluorophenyl]amino]-6-\{\Sigma-(N, N-dimethylamino)-L-oxo-2-but-\-en-1-yl]amino]-7-\{(S\)-tetrahydrofuran-3-yl)oxy\}-quinazoline or 4-{3-chloro-4-fluorophenyl]amino]-6-\{4-(homomorpholin-4-yl)-L-oxo-2-bu-\-ten-1-yl]amino]-7-\{(S\)-\(\Sigma\)-tetrahydrofuran-3-yl)oxy\} -quinazoline, or a pharmaceutically acceptable salt thereof, an inhibitor of the transcription factor complex ESX/DRIP130/Sur-2, an inhibitor of FIER-2 expression, such as the heat shock protein HSP90 modulator geldanamycin and its derivative 17-allylamino-geldanamycin or 17-AAG, a protein kinase receptor antagonist which is not classified under the synthetic small molecules such as atrasentan, rituximab, cetuximab, Avastin.TM. (bevacizumab), IMC-1C1 1, erbitux (C-225), DC-101, EMD-72000, vitaxin, imatinib, and an antibody targeting the surface molecules of cancer cells such as apolizumab or 1D09C3.

**Cancer**

The cancer that can be treated or prevented by the present methods and compositions includes, but is not limited to, solid malignancies and hematologic malignancies. The cancer may be primary or metastatic. The cancer may be Stage 0, Stage I, Stage II, Stage III, or Stage IV. The cancer also can be characterized as benign or malignant. The cancer may be metastatic, progressive and/or refractory. The cell that expresses a tumor-associated antigen can be any type
of cell. The cell can be a cancer cell, a precancerous cell, or a cell-type predisposed to developing cancer.

The subject can either have a neoplastic disease (e.g., a tumor), or be at risk of developing the neoplastic disease. Subjects can be characterized by clinical criteria, for example, those with advanced neoplastic disease or high tumor burden exhibiting a clinically measurable tumor. A clinically measurable tumor is one that can be detected on the basis of tumor mass (e.g., by palpation, MRI, CAT scan, X-ray). Thus, for example, the pharmaceutically acceptable composition in accordance with the present invention can be administered to subjects with advanced disease with the objective of mitigating their condition. Preferably, a reduction in tumor mass occurs as a result of administering the pharmaceutically acceptable composition of the present invention, but any clinical improvement constitutes a benefit. Clinical improvement includes decreased risk or rate of progression or reduction in pathological consequences of a tumor, for example.

By way of another example, the subject can be one that has a history of cancer and has been responsive or irresponsive to another mode of therapy. The other therapy may have included e.g., surgical resection, radiotherapy, chemotherapy, and other modes of immunotherapy whereby as a result of the other therapy, the subject presents no clinically measurable tumor. However, the subject can be one determined to be at risk for recurrence or progression of the cancer, either near the original tumor site, or by metastases. Such subjects can be further categorized as high-risk and low-risk subjects. The subdivision can be made on the basis of features observed before or after the initial treatment. These features are known in the clinical arts, and are suitably defined for each different cancer. Features typical of high risk subgroups are those in which the tumor has invaded neighboring tissues, or which show involvement of lymph nodes. Thus, for example, a pharmaceutical composition of the present invention can be administered to the subject to elicit an anti-cancer response primarily as a prophylactic measure against recurrence. Preferably, administering the composition delays recurrence of the cancer, or more preferably, reduces the risk of recurrence (i.e., improves the cure rate). Such parameters can be determined in comparison with other patient populations and other modes of therapy.

Cancers that may be evaluated by methods and compositions of the invention include cancer of the bladder, blood, bone, bone marrow, brain, breast, colon, esophagus, gastrointestinal,
gum, head, kidney, liver, lung, nasopharynx, neck, ovary, pancreas, prostate, skin, stomach, testis, tongue, or uterus. In addition, the cancer may specifically be of the following histological type, though it is not limited to these: neoplasm, malignant; carcinoma; undifferentiated; giant and spindle cell carcinoma; small cell carcinoma; papillary carcinoma; squamous cell carcinoma; lymphoepithelial carcinoma; basal cell carcinoma; pilomatrix carcinoma; transitional cell carcinoma; papillary transitional cell carcinoma; adenocarcinoma; gastrinoma, malignant; cholangiocarcinoma; hepatocellular carcinoma; combined hepatocellular carcinoma and cholangiocarcinoma; trabecular adenocarcinoma; adenoid cystic carcinoma; adenocarcinoma in adenomatous polyp; adenocarcinoma, familial polyposis coli; solid carcinoma; carcinoïd tumor, malignant; branchiolo-alveolar adenocarcinoma; papillary adenocarcinoma; chromophobe carcinoma; acidophil carcinoma; oxyphilic adenocarcinoma; basophil carcinoma; clear cell adenocarcinoma; granular cell carcinoma; follicular adenocarcinoma; papillary and follicular adenocarcinoma; nonencapsulating sclerosing carcinoma; adrenal cortical carcinoma; endometrioid carcinoma; skin appendage carcinoma; apocrine adenocarcinoma; sebaceous adenocarcinoma; ceruminous adenocarcinoma; mucoepidermoid carcinoma; cystadenocarcinoma; papillary cystadenocarcinoma; papillary serous cystadenocarcinoma; mucinous cystadenocarcinoma; mucinous adenocarcinoma; signet ring cell carcinoma; infiltrating duct carcinoma; medullary carcinoma; lobular carcinoma; inflammatory carcinoma; paget's disease, mammary; acinar cell carcinoma; adenosquamous carcinoma; adenocarcinoma w/squamous metaplasia; thymoma, malignant; ovarian stromal tumor, malignant; thecoma, malignant; granulosa cell tumor, malignant; androblastoma, malignant; Sertoli cell carcinoma; leydig cell tumor, malignant; lipid cell tumor, malignant; paraganglioma, malignant; extra-mammary paraganglioma, malignant; pheochromocytoma; glomangiosarcoma; malignant melanoma; amelanotic melanoma; superficial spreading melanoma; malig melanoma in giant pigmented nevus; epithelioid cell melanoma; blue nevus, malignant; sarcoma; fibrosarcoma; fibrous histiocytoma, malignant; myxosarcoma; liposarcoma; leiomyosarcoma; rhabdomyosarcoma; embryonal rhabdomyosarcoma; alveolar rhabdomyosarcoma; stromal sarcoma; mixed tumor, malignant; mullerian mixed tumor; nephroblastoma; hepatoblastoma; carcinosarcoma; mesenchymoma, malignant; brenner tumor, malignant; phyllodes tumor, malignant; synovial sarcoma; mesothelioma, malignant; dysgerminoma; embryonal carcinoma; teratoma, malignant; struma ovarii, malignant;
choriocarcinoma; mesonephroma, malignant; hemangiosarcoma; hemangioendothelioma, malignant; kaposi's sarcoma; hemangiopericytoma, malignant; lymphangiosarcoma; osteosarcoma; juxtacortical osteosarcoma; chondrosarcoma; chondroblastoma, malignant; mesenchymal chondrosarcoma; giant cell tumor of bone; ewing's sarcoma; odontogenic tumor, malignant; ameloblastic odontosarcoma; ameloblastoma, malignant; ameloblastic fibrosarcoma; pinealoma, malignant; chordoma; glioma, malignant; ependymoma; astrocytoma; protoplasmic astrocytoma; fibrillary astrocytoma; astroblastoma; glioblastoma; oligodendroglioma; oligodendroblastoma; primitive neuroectodermal; cerebellar sarcoma; ganglioneuroblastoma; neuroblastoma; retinoblastoma; olfactory neurogenic tumor; meningioma, malignant; neurofibrosarcoma; neurilemroma, malignant; granular cell tumor, malignant; malignant lymphoma; Hodgkin's disease; Hodgkin's lymphoma; paragranuloma; malignant lymphoma, small lymphocytic; malignant lymphoma, large cell, diffuse; malignant lymphoma, follicular; mycosis fungoides; other specified non-Hodgkin's lymphomas; malignant histiocytosis; mast cell sarcoma; immunoproliferative small intestinal disease; leukemia; lymphoid leukemia; plasma cell leukemia; erythroleukemia; lymphosarcoma cell leukemia; myeloid leukemia; basophilic leukemia; eosinophilic leukemia; monocytic leukemia; mast cell leukemia; megakaryoblastic leukemia; myeloid sarcoma; and hairy cell leukemia.

In one embodiment, the cancer treated or diagnosed by the present methods or compositions is multiple myeloma. In another embodiment, the cancer treated or diagnosed by the present methods or compositions is lymphoma. In a third embodiment, the cancer treated or diagnosed by the present methods or compositions is breast cancer. In a fourth embodiment, the cancer treated or diagnosed by the present methods or compositions is metastatic solid malignancy which may be from breast, colon, liver, stomach, etc. In a fifth embodiment, the cancer treated or diagnosed by the present methods or compositions is metastatic solid malignancy which may or may not demonstrate a measurable response to first-line, conventional systemic therapy. In a sixth embodiment, the cancer treated or diagnosed by the present methods or compositions is progressive and/or refractory solid malignancy.

**Kits**

The present invention further pertains to a kit containing the present pharmaceutical composition. The kit or container holds an effective amount of a pharmaceutical composition for
carrying out the methods or producing the compositions described herein and/or instructions for producing or using the compositions for therapy of a patient or subject having or suspected of having or at risk of developing cancer.

The compositions of the present invention can be supplied in unit dosage or kit form. Kits can comprise various components of the pharmaceutically acceptable composition or vaccines thereof provided in separate containers as well as various other active ingredients or agents including chemotherapeutic agents.

Administering to both human and non-human vertebrates is contemplated within the scope of the present invention. Veterinary applications also are contemplated. Generally, the subject is any living organism in which an immune response can be elicited. Examples of subjects include, without limitation, humans, livestock, dogs, cats, mice, rats, and transgenic species thereof.

The following examples of specific aspects for carrying out the present invention are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

**Example 1 Engineered dendritic cell vaccination for long-term protection against ovarian cancer**

Immunotherapy for ovarian cancer may prove effective for long-term control of the disease. We show that adoptive transfer of dendritic cells treated with a p38 MAPK inhibitor, and transduced with a recombinant adenovirus associated virus vector (rAAV) expressing sperm protein 17 (SP17), is highly effective in controlling ovarian cancer in the ID8 murine model of epithelial ovarian cancer, a system that reproduces the peritoneal dissemination seen in advanced human OC. Roby et al., Development of a syngeneic mouse model for events related to ovarian cancer. *Carcinogenesis* 21, 585-591 (2000). Our data shows the induction of potent anti-tumor CD8 T-cell and CD4 T-cell mediated responses, providing increased survival rates and long-term prevention of tumor growth and dissemination, associated with diminished accumulation of CD4+Foxp3+ regulatory T cells, and enhanced accumulation of CD4+ IL17+ cells in the spleens of vaccinated animals. We demonstrated that p38 blockade in the presence of rAAV SP17
transduction is superior to rAAV Spl7 transduction alone, in terms of activation of human DC and their ability to generate effector lymphocytes from autologous PBMCs, in vitro. Finally, we validated our results using human monocyte-differentiated DC that the optimum DC activation status is achieved by rAAV in the presence of p38 blockade.

Materials and Methods

Analysis for mSPl 7 expression

SP17 expression was analyzed in DC and peritoneal tissue by reverse-transcription quantitative PCR (RT-qPCR). RNA was isolated by TRIzol (Gibco BRL Life Sciences Technologies, Rockville, MD) and Oligotex mRNA kit (Quiagen, Valencia, CA). Following cDNA synthesis, qPCR was performed with mSP17 (murine Sp17) primer sets upstream 5'-AGATCTATGTGCATCTCTCCACACCC (SEQ ID NO. 2), downstream 5'-CTGCAGTCATCTGCCTCTTTTCTCAACCC (SEQ ID NO. 3) and β-actin (upstream 5'-ATGGATGACGATATCGCTGCGC-3' (SEQ ID NO. 4), downstream 5'-GGAACCGCTCGTTCCTTTCCATG-3') (SEQ ID NO. 5). SYBR Green (Bio-Rad)-based qPCR was performed using a Bio-Rad iCycler thermocycler, as we have recently described. Mirandola et al., Novel antigens in non-small cell lung cancer: SP17, AKAP4, and PTTG1 are potential immunotherapeutic targets. Oncotarget 6, 2812-2826 (2015). Results were calculated out of triplicate analyses and expressed as 2^\Delta Ct (\Delta Ct= Ct_{act-in}-Ct_{m^{sp17}}).

Construction of rAAV-mSPl 7 virus

The AAV-mSP17 genome was constructed as a plasmid as previously described. Chiriva-Internati et al., Testing recombinant adeno-associated virus-gene loading of dendritic cells for generating potent cytotoxic T lymphocytes against a prototype self-antigen, multiple myeloma HM1.24. Blood 102, 3100-3107 (2003). Yu et al, rAAV/Her-2/neu loading of dendritic cells for a potent cellular-mediated MHC class I restricted immune response against ovarian cancer. Viral Immunol 21, 435-442 (2008). The mSP17 (murine SP17) cDNA was inserted into the rAAV vector, dl6-95. Figure 1a shows a structural map of the rAAV/mSP17 vector. The mSP17 gene was expressed from the rAAV p5 promoter, which has been described to be active in DC. Santin et al, In vitro induction of tumor-specific human lymphocyte antigen class I-restricted CD8 cytotoxic T lymphocytes by ovarian tumor antigen-pulsed autologous dendritic cells from
patients with advanced ovarian cancer. *Am J Obstet Gynecol* 183, 601-609 (2000). We evaluated the ability of the rAAV-mSP17 construct to infect HEK293 cells. The rAAV vector infected cells expressed the target antigens, as confirmed by RT-PCR (Fig. 1b). The human rAAV-SP17 vector was produced following the same scheme described for mSP17. Human full length SP17 coding sequence was obtained by gene synthesis from GenScript. Amino acid sequences of human SP17 may be found under the following NCBI Reference Sequence (RefSeq) accession number: NP_059121 (SEQ ID NO:6). Nucleic acid sequences encoding human SP17 may be found under the following NCBI RefSeq accession number: NM_017425, or GenBank accession number BC032457.

**Titration of virus stocks**

DNA was extracted from virus crude lysates, and the titer of virus stocks was determined by real-time PCR. Briefly, we used serial dilutions of the corresponding rAAV vector for construction of a standard curve (Fig. 1c shows a representative result for rAAV-mSp17). The real-time PCR was performed on an ABI Prism 7000 instrument (Applied Biosystems, Darmstadt, Germany) in a 50-μl reaction volume.

**Generation of dendritic cells (DC)**

Murine DC were generated from splenocytes. We infected adherent monocytes with rAAV as previously described. Chiriva-Internati *et al.*, Testing recombinant adeno-associated virus-gene loading of dendritic cells for generating potent cytotoxic T lymphocytes against a prototype self-antigen, multiple myeloma HM1.24. *Blood* 102, 3100-3107 (2003). Chiriva-Internati *et al.*, Efficient generation of cytotoxic T lymphocytes against cervical cancer cells by adeno-associated virus/human papillomavirus type 16 E7 antigen gene transduction into dendritic cells. *Eur J Immunol* 32, 30-38 (2002). Yu *et al.*, Protective CD8+ T-cell responses to cytomegalovirus driven by rAAV/GFP/IE1 loading of dendritic cells. *J TranslMed* 6, 56 (2008). 10 μM p38 MAP Kinase inhibitor III (p38i, also called ML3403, (RS)-{4-[5-(4-Fluorophenyl)-2-methylsulfanyl-3H-imidazol-4-yl]pyridin-2-yl}-(1-phenylethyl)amine], EMD Chemicals, La Jolla, CA) was added at days 0, 3 and 5.

Human mature DCs were differentiated from PBMCs of five ovarian cancer patients. In one embodiment, isolation of PBMCs and generation of DCs were conducted as follows.
Peripheral blood mononuclear cells (PBMCs) were separated from heparinized venous blood by Ficoll-Hypaque (Sigma) density gradient centrifugation and either cryopreserved in RPMI 1640 plus 10% dimethyl sulfoxide and 30% autologous plasma or immediately used for DC generation. Briefly, PBMC obtained from 42 ml of peripheral blood were placed into six-well culture plates (Costar, Cambridge, Mass.) containing 3 ml of AIM-V (Gibco-BRL) at 0.5 \( \times \) \( 10^7 \) to \( 1 \times 10^7 \) per well. After 2 h at 37°C, nonadherent cells were removed, and adherent cells were cultured at 37°C in a humidified 5% CO\(_2\) 95% air incubator with medium supplemented with recombinant human GM-CSF (800 U/ml; Immunex, Seattle, Wash.) and IL-4 (1,000 U/ml; Genzyme, Cambridge, Mass.). In early experiments, only GM-CSF (800 U/ml) was used. Every 2 days, 1 ml of spent medium was replaced by 1.5 ml of fresh medium containing 1,600 U of GM-CSF per ml and 1,000 U of IL-4 per ml to yield final concentrations of 800 and 500 U/ml, respectively. After 6 or 7 days of culturing, DC were harvested for loading with SP17 as described below. Santin et al., Induction of human papillomavirus-specific CD4(+) and CD8(+) lymphocytes by E7-pulsed autologous dendritic cells in patients with human papillomavirus type 16- and 18-positive cervical cancer. Journal of virology 73, 5402-5410 (1999). In another embodiment, peripheral blood leukocytes (PBL) were recovered by gradient centrifugation over Lymphoprep (Greiner Bio-One). For the preparation of DC, PBL were placed in 12-well plates (Costar) at a concentration of up to \( 5 \times 10^6 \)/well in DC medium (CellGenix GmbH). After incubation for 2-3 h at 37 °C, non-adherent cells were removed, the adherent cells were rinsed with phosphate-buffered saline (PBS) and 1.5 ml of DC medium was added to each well, plus 800 U/ml GM-CSF (Sargramostim, Genzyme), and 500 U/ml IL-4 (CellGenix). On days 3 and 5, half the medium was removed and replaced with DC medium plus the same concentration of cytokines. Maturation cytokines (1 μM/ml PGE\(_2\) (Sigma), 1,000 U/ml TNF (CellGenix), and 500 U/ml IL-1 (National Cancer Institute Biological Response Modifiers Program) were added on day 5. Cannon et al., Modulation of p38 MAPK signaling enhances dendritic cell activation of human CD4+ Thl7 responses to ovarian tumor antigen. Cancer immunology, immunotherapy: CII 62, 839-849 (2013).

rAAV-transduced human DC were differentiated from rAAV-infected monocytes as we have previously described. Chiriva-Internati et al., Testing recombinant adeno-associated virus-gene loading of dendritic cells for generating potent cytotoxic T lymphocytes against a prototype self-antigen, multiple myeloma HM1.24. Blood 102, 3100-3107 (2003). Chiriva-Internati et al,
Efficient generation of cytotoxic T lymphocytes against cervical cancer cells by adeno-associated virus/human papillomavirus type 16 E7 antigen gene transduction into dendritic cells. *Eur J Immunol* **32**, 30-38 (2002). $10^7$ rAAV-SP17 encapsidated genomes (eg) were used to transduce adherent monocytes in 6-well plates. Liu *et al*, Rapid induction of cytotoxic T-cell response against cervical cancer cells by human papillomavirus type 16 E6 antigen gene delivery into human dendritic cells by an adeno-associated virus vector. *Cancer gene therapy* **8**, 948-957 (2001). To block p38, we used the same schedule and concentration detailed above for murine cells. Namely, 10 µM p38 MAP Kinase inhibitor III (p38i, also called ML3403, (RS)-{4-[5-(4-Fluorophenyl)-2-methylsulfanyl-3H-imidazol-4-yl]pyridin-2-yl}-(1-phenylethyl)amine], EMD Chemicals, La Jolla, CA) was added at days 0, 3 and 5.

**Mice and ID8 cell line**

Six-week-old female C57BL/6 mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). Cells (e.g., ID8 cells) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum in 5% C0₂ at 37 °C.

**Immunization and tumor challenge**

Female C57BL/6 mice (6 weeks old) were challenged i.p. (intraperitoneally) with $1\times10^6$ ID8 cells. Chiriva-Internati *et al*, Cancer testis antigen vaccination affords long-term protection in a murine model of ovarian cancer. *PLoS One* **5**, e10471 (2010). 30 days after tumor challenge, mice were i.m. injected (intramuscular injection) once a month for 10 months with rAAV or rAAV-mSP17 transduced DC treated or not with the p38 MAPK inhibitor. Each mouse was injected with $10^6$ DC.

**Flow cytometry**

Flow-cytometric analyses were performed with a Beckman-Coulter FC500 flow cytometer and tetraCXp Software. Mirandola *et al*, Anti-Notch treatment prevents multiple myeloma cells localization to the bone marrow via the chemokine system CXCR4/SDF-1. *Leukemia* **27**, 1558-1566 (2013). For lymphocyte phenotyping, murine splenocytes from tumor-challenged mice or human non-adherent PBMCs from ovarian cancer patients were washed twice in PBS, incubated with 10% autologous serum in PBS for 30 minutes on ice to block Fc
receptors, then incubated with phycoerythrin-conjugated anti-CD3 PE-CF-594 antibody (BD Biosciences) for 1 h on ice in staining buffer (0.5% BSA in PBS). After washing twice in 0.5 mL staining buffer, cells were fixed with 4% buffered paraformaldehyde at 4°C for 30 minutes in the dark. Then, cells were washed twice with staining buffer and permeabilized with 0.5% saponin in staining buffer (permeabilization buffer) for 10 minutes on ice, washed once with 0.5 mL permeabilization buffer and resuspended in 50 µL buffer/200,000 cells. Anti-Foxp3-FITC and IL-17-Alexafluor 488 antibodies (BD Biosciences), anti-IFNy Alexafluor 647 (Bio Legend), anti-TNFa Alexafluor 700 (BD Biosciences), were added and allowed to incubate for 1 h on ice. Cells were washed twice with permeabilization buffer (200 µL) and resuspended in 400 µL staining buffer supplemented with 1% W/V paraformaldehyde and stored at 4°C in the dark until data acquisition.

Preparation of lymphocytes

Briefly, the spleen was meshed with a sterile filter, and then centrifuged at 800 x g for 5 minutes at 4°C. Splenocytes were then isolated by Lympholyte-M (Cedarlane Ltd, Burlington, NC). Human lymphocytes were recovered from the non-adherent fraction of purified PBMCs after a 3-hour incubation in 6-well plates.

Generation of human DC-primed lymphocytes

For experiments with human cells, non-adherent PBMCs from the same subject were washed in PBS and resuspended in serum-free DC-medium (CellGenix) at 10⁶ cells/well in 6-well culture plates, with autologous DC (20:1 PBMCs to DC ratio). Chiriva-Internati et al., Efficient generation of cytotoxic T lymphocytes against cervical cancer cells by adeno-associated virus/human papillomavirus type 16 E7 antigen gene transduction into dendritic cells. *Eur J Immunol* 32, 30-38 (2002). Santin et al., Induction of human papillomavirus-specific CD4(+) and CD8(+) lymphocytes by E7-pulsed autologous dendritic cells in patients with human papillomavirus type 16- and 18-positive cervical cancer. *Journal of virology* 73, 5402-5410 (1999). Cultures were supplemented with 800 U/mL GM-CSF and 10 U/mL IL-2 for 7 days prior to analysis.
**Immunofluorescence**

A standardized technique for detecting mSP17 in ID8 cells was performed as previously described. Chiriva-Internati et al., Successful generation of sperm protein 17 (Spl7)-specific cytotoxic T lymphocytes from normal donors: implication for tumour-specific adoptive immunotherapy following allogeneic stem cell transplantation for Spl7-positive multiple myeloma. *Scand J Immunol* 56, 429-433 (2002).

**Immunohistochemistry**

After tissue de-paraffinization and re-hydration, 30-minute antigen retrieval was performed at 98°C for in 1 mM EDTA. Immunostaining was performed as described. Chiriva-Internati et al, Cancer testis antigen vaccination affords long-term protection in a murine model of ovarian cancer. *PLoS One* 5, el0471 (2010).

**ELISA for anti-mSP17 levels**

Serum anti-mSP17 IgGi levels were measured using commercial ELISA kit (U-CyTech, Utrecht, Netherlands), according to the manufacturer's directions. The absorbance was read at 450 nm using a Victor2 1420 Multilabel Counter (Perkin Elmer, Waltham, MA).

**ELISA for cytokine measurement**

Sera were collected post-mortem, and cytokine levels were measured by using commercial ELISA kits (R&D Systems, Minneapolis, MN), in accordance with the manufacturer's instructions.

**ELISPOT**

Cytokine expression by splenocytes was evaluated using an ELISPOT assay (U-CyTech, Utrecht, Netherlands), according to the manufacturer's directions. Positive control for cellular activation was Con-A (5 μg/mL), and background wells contained RPMI 1640 medium only. Spot counts were performed with the AID ELISPOT Reader System (Cell Technology, Inc., MD).
Cytotoxicity assay

We performed a EUROPIUM-based cytotoxicity assay using the DELFIA® EuTDA system according to the manufacturer's instructions with DC-co-cultured splenocytes. Paillard et al., NK cytotoxicity and alloreactivity against neuroblastoma cell lines in vitro: comparison of Europium fluorometry assay and quantification by RT-PCR. *J Immunol Methods* **380**, 56-64 (2012). To assay the memory response, we performed an identical assay using unprimed splenocytes taken from mice at the time of necropsy.

Migration assay

The bottom chambers of polycarbonate Corning® Transwell™ Permeable Supports (5 µm pore size, Cole-Parmer, Vernon Hills, Illinois) were coated with ID8 cells. 200,000 splenocytes were added in the upper chamber in complete medium. After 4 hours, the density of migrated cells in the bottom wells was determined. The assay was performed in triplicate and mean ±SEM were determined.

Statistical analyses

Tumor growth, cytotoxicity assays, ELISPOT, ELISA, migration assays and flow-cytometry were analyzed by a two-tailed, paired Student's test and survival rates were analyzed by the log-rank test.

Results

Characterization of DC phenotype

We analyzed the maturation of DC obtained from splenic monocytes after 7 days of culture (Fig. 2), by measuring the levels of CD80, CD86, MHC class I (MHC-I), MHC class II (MHC-II), and B7-H1. DC were treated as follows: infection with rAAV alone, infection with rAAV-mSP17 alone, or infection with rAAV-mSP17 plus a p38 MAP-kinase inhibitor treatment. Compared with rAAV and rAAV-mSp17, the addition of p38i (i.e., the rAAV-mSp17 + p38i sample) did not significantly affect the expression of CD80, CD86 and MHC-II. On the contrary, rAAV-mSp18 + p38i DC expressed significantly higher levels of MHC-I and lower levels of B7-
HI (One-way ANOVA and Bonferroni’s post-test p<0.001 for both markers). MHC-I increase was 52%, while B7-H1 decrease was 30%, compared with rAAV and rAAV-mSP17 DC.

Vaccination with rAAV-mSP17 transduced autologous DC treated with p38 MAPK inhibitor efficiently eliminates tumors and prevents dissemination, thereby providing long-term protection.

A total of 50 C57BL/6 female mice were included in the study per experiment. 40 mice were i.p. injected with \(10^6\) ID8 cells and randomly assigned to the following groups after 30 days: animals in group 1 received i.p. injection with \(10^6\) rAAV-mSP17 engineered DC pre-treated with p38 MAPK inhibitor (rAAV-mSP17 DC + p38i), group 2 received \(10^6\) rAAV-mSP17 DC without pre-treatment (rAAV-mSP17), group 3 received \(10^6\) DC transduced with rAAV vector alone, while group 4 was not treated. Injections were performed every 30 days. Treatment outcomes were assayed through in vivo and in vitro analyses. Two independent experiments were performed. To avoid excessive discomfort for the mice, animals were euthanized at different time points, at which point post-mortem analyses of tissues and spleens were performed.

Analysis of survival shows that rAAV-mSP17 DC + p38i vaccine prevents mortality for at least 10 months, representing a dramatic improvement in survival rates compared with tumor-bearing mice vaccinated with rAAV-mSP17 DC or rAAV DC (Fig. 3a). Specifically, 95% of rAAV-mSP17 DC + p38i vaccinated animals survived up to 300 days, while rAAV-mSP17 DC-vaccinated mice or rAAV DC-vaccinated mice died within 98 and 60 days, respectively.

Figure 3b shows the macroscopic effects of vaccination in five mice randomly chosen from each group. Reduction of body enlargement and ascites was evident in ID8 tumor-bearing mice given rAAV-mSP17 DC vaccination, relative to non-vaccinated mice, but a greater effect was seen in tumor-bearing mice vaccinated with rAAV-mSP17 DC + p38i, in which no macroscopic evidence of tumor growth could be found. Involvement of peritoneal lymph nodes was detected in ID8-injected mice but not in rAAV-mSP17 DC + p38i vaccinated mice.

Immuno-histochemical analyses revealed mSP17 expression in the peritoneum and lymph nodes of ID8-injected mice but not of rAAV-mSP17 DC + p38i vaccinated mice or tumor-free controls (Fig. 3b). RT-qPCR analysis for mSP17 in peritoneal tissue confirmed mSP17-positive ID8 cell dissemination (Fig. 3c). In contrast, mSP17 mRNA levels were reduced by over 40 folds in the peritoneum of mice that received rAAV-mSP17 DC + p38i.
rAAV-mSP17DC+p38i vaccination increases anti-SP17 IgG levels in serum

ELISA assays revealed a significant increase in anti-SP17 IgG levels following rAAV-mSP17 DC vaccination compared with empty rAAV vector-transduced DC, but even higher levels were detected with rAAV-mSP17 DC + p38i (Fig. 4a; two-tailed t-test rAAV-mSP17 + p38i versus rAAV or rAAV-mSP17 p<0.01).

Th1 cytokine expression is elevated in rAAV-mSP17 DC +p38 vaccinated mice

ELISA assays showed that, compared to untreated DC vaccinated mice, sera collected from rAAV-mSP17 DC + p38i vaccinated animals contained elevated levels of IFN-γ and TNF-α, and relatively lower levels of Th2-type cytokines, namely IL-10 and IL-4 (Fig. 4b). Expression of Th1 cytokines correlated with detection of TNF-a and IFN-γ secreting lymphocytes in the spleen taken from mice vaccinated with rAAV-mSP17 DC + p38i (Fig. 4c).

rAAV-mSP17DC+p38i vaccination induces potent cytotoxic responses against ID8 cells

Cytotoxicity assays were performed using ID8 cells as targets and splenocytes taken post-mortem from control or rAAV DC, rAAV-mSP17 DC or rAAV-mSP17 DC + p38i mice after priming with autologous DC as effectors. rAAV-mSP17 DC vaccine was effective in increasing lysis of ID8 cells in comparison with rAAV DC (two-tailed t-test p=0.03 for E:T ratio 20:1 and 10:1), but significantly higher lysis rates were obtained following vaccination with rAAV-mSP17 DC + p38i (Fig. 5a; two-tailed t-test rAAV-mSP17 + p38i versus rAAV-mSP17 or rAAV p<0.01 for E:T ratio 20:1 and 10:1).

rAAV-mSP17DC+p38i vaccination amplifies splenocytes migration towards ID8 cells

To evaluate the ability of splenocytes to traffic toward tumor cells we used a transwell migration assay to test migration of splenocytes from mice treated with different vaccine formulations (rAAV-mSP17 DC + p38i or control rAAV-mSP17 DC). In the presence of ID8 cells, we observed a 2.5-fold increase in migrating splenocytes from rAAV-mSP17 DC + p38i vaccinated mice compared with splenocytes from rAAV-mSP17 DC vaccinated mice.
rAAV-mSP17DC +p38i vaccination amplifies Thl7 frequencies and hampers T-reg expansion

Analysis of CD4+ Thl7 and T-reg cell populations was performed by flow-cytometry, using IL-17 as a Thl7 marker, and Foxp3 as a T-reg marker (Fig. 6). Tumor growth without vaccination or following rAAV DC vaccination was associated with elevated T-reg frequencies compared with healthy controls (no tumor). Both rAAV-mSP17 DC and rAAV-mSP17 DC + p38i vaccination increased Thl7 and diminished T-reg frequencies, with the biggest gain in Thl7 frequencies being observed following vaccination with p38 inhibitor-treated DC. Notably, T-reg frequencies in rAAV-mSP17 DC + p38i vaccinated mouse spleens were still higher than in healthy controls, but were significantly lower than in untreated animals (it should be noted that this analysis was conducted with splenocytes rather than with tumor-infiltrating lymphocyte populations, for the simple reason that tumors could not be detected in ID8-injected mice vaccinated with rAAV-mSP17 DC +p38i). Reduced Treg and increased Thl7 frequencies thus correlated with improved survival (Fig. 3a).

p38 inhibition improves the activation phenotype and the T cell activation potential of human DC derivedfrom rAAV-transducedmonocytes in vitro

To evaluate the validity of our results in human cells, human peripheral blood adherent monocytes were used to differentiate DC in vitro, and their activation status was assayed by flow-cytometry for CD80, CD83, CD86, and B7-H1. Figure 7 shows representative histogram plots, while figure 8 depicts the MFI values and results of statistical analysis. Two sets of DCs were generated, namely rAAV-SP17 transduced DCs, and rAAV-SP17 + p38i transduced DCs. In the presence of p38i, DCs showed statistically significant down-regulation of B7-H1 only, whereas CD80, CD83, and CD86 were unchanged, compared with rAAV-SP17 transduced DC (figure 8).

Non-adherent autologous PBMCs were then co-cultured with different DC preparations detailed above for 7 days, as described in Methods, and their activated versus regulatory phenotype was assayed by flow-cytometry for Foxp3 in the CD3 population (suppressor T cells) and for IFNy/TNFα (activated effector T cells). Figure 9A shows representative plots from one subject and figure 9B shows the statistical analyses. The blockade of p38 did not affect the frequency of TNFα+ cells, but resulted in an increase of IFNγ+ and consequently of TNFα+IFNγ+ cells (figures
9B). Consistently with these findings, p38i significantly reduced the frequency of Foxp3+ T cells (Foxp3+CD3+, figure 9B).

Discussion

In this report, we describe the efficacy of an innovative therapeutic vaccine based on DC treated with a p38 MAP-kinase inhibitor, and transduced with an mSpI7 expression rAAV vector.

Here we tested two different vaccine strategies based on autologous DC transduced with rAAV-mSP17, with or without exposure to a p38 MAPK inhibitor (rAAV-mSP17 DC ± p38i), and compared those with two controls, i.e., rAAV-transduced DC (without mSP17 coding sequence) or absence of vaccination.

Our results clearly demonstrate macroscopic and microscopic benefits of DC vaccination. rAAV-mSP17 DC and rAAV-mSP17 DC + p38i vaccines resulted in increased survival rates, but long-term protection was achievable only when p38 MAPK signaling within the DC was blocked. Macroscopic evidence of tumor burden was markedly diminished and survival was extended (300 days) with p38-blocked DC engineered with rAAV-mSP17 compared to all the other groups. We have previously shown that rAAV-based antigen transduction of DC could induce potent MHC class I-restricted in vitro immune responses against OC and multiple myeloma, overcoming immune tolerance. Chiriva-Internati et al., Testing recombinant adenovirus-gene loading of dendritic cells for generating potent cytotoxic T lymphocytes against a prototype self-antigen, multiple myeloma HM1.24. Blood 102, 3100-3107 (2003). Yu et al, rAAV/Her-2/neu loading of dendritic cells for a potent cellular-mediated MHC class I restricted immune response against ovarian cancer. Viral Immunol 21, 435-442 (2008). Here, we extended our study to show that rAAV-transduced DC vaccination provides excellent therapeutic results in vivo. The efficacy of DC vaccination was clearly enhanced through p38 MAPK inhibition, allowing exceptional levels of protection against tumor growth in syngeneic recipients. These observations are highly significant, since long-term protection against OC progression is critical to control fatal disease relapse. Bak et al, Scavenger receptor-A-targeted leukocyte depletion inhibits peritoneal ovarian tumor progression. Cancer Res 67, 4783-4789 (2007).
Based on our previous report indicating that SP17 is a suitable marker for monitoring OC progression in vivo, we unequivocally detected the spread of SP17-positive tumor cells in the peritoneum and lymph nodes of non-vaccinated mice. Chiriva-Internati et al., A NOD/SCID tumor model for human ovarian cancer that allows tracking of tumor progression through the biomarker Sp17. *J Immunol. Methods* 321, 86-93 (2007). Importantly, parallel quantitative PCR analysis revealed over 40-folds reduction of mSP17 expression levels in the peritoneum of rAAV-mSP17 DC + p38i vaccinated animals compared to untreated mice, demonstrating that this vaccine formulation prevented mSP17-positive ID8 tumor cell dissemination.

When evaluating the humoral immune response following vaccination, we also detected increased anti-mSP17 IgG in the sera of rAAV-mSP17 DC + p38i vaccinated mice relative to the other treatment groups. Of note, the occurrence of tumor-reactive immunoglobulins has been associated with improved prognosis in OC patients. Oei et al., Induction of IgG antibodies to MUC1 and survival in patients with epithelial ovarian cancer. *Int J Cancer* 123, 1848-1853 (2008). Taylor et al., Patient-derived tumor-reactive antibodies as diagnostic markers for ovarian cancer. *Gynecol Oncol* 115, 112-120 (2009).

We showed that rAAV-mSP17 DC + p38i injected mouse splenocytes expressed more Th1 (IFN-γ and TNF-a) and less Th2 (IL-10, IL-4) cytokines compared to rAAV-mSP17 DC or AAV DC groups. IFN-γ and TNF-α have been shown to provide protective effects in OC patients, while IL-10 and IL-4 are associated with reduced immune response and worse prognosis. Bamias et al., Correlation of NK T-like CD3+CD56+ cells and CD4+CD25+(hi) regulatory T cells with VEGF and TNFalpha in ascites from advanced ovarian cancer: Association with platinum resistance and prognosis in patients receiving first-line, platinum-based chemotherapy. *Gynecol Oncol* 108, 421-427 (2008). Barber et al., Chimeric NKG2D expressing T cells eliminate immunosuppression and activate immunity within the ovarian tumor microenvironment. *J Immunol* 183, 6939-6947 (2009). De Cesare et al., Ascites regression and survival increase in mice bearing advanced-stage human ovarian carcinomas and repeatedly treated intraperitoneally with CpG-ODN. *J Immunother* 33, 8-15. Santin et al., Induction of tumour-specific CD8(+) cytotoxic T lymphocytes by tumour lysate-pulsed autologous dendritic cells in patients with uterine serous papillary cancer. *Br J Cancer* 86, 151-157 (2002). Zhang et al., Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer. *N Engl J Med* 348, 203-213 (2003). Giuntoli et al., Ovarian cancer-associated ascites demonstrates altered immune
environment: implications for antitumor immunity. Anticancer Res 29, 2875-2884 (2009). These results suggest that p38 MAPK inhibition directs DC differentiation towards a Th1-polarizing profile. Neves et al, Differential roles of PI3-Kinase, MAPKs and NF-kappaB on the manipulation of dendritic cell T(h)1/T(h)2 cytokine/chemokine polarizing profile. Mol Immunol 46, 2481-2492 (2009). We also found that both CD4+ and CD8+ T-cells from rAAV-mSP17 DC + p38i vaccinated mice displayed significantly higher IFN-γ production compared to rAAV-DC and rAAV-DC vaccinated animals. Compared with unvaccinated and rAAV DC vaccinated mice, both CD4+ and CD8+ T-cells from rAAV-mSP17 DC treated animals displayed increased IFN-γ expression. Altogether, T cell IFN-γ expression correlates with splenocytes ELISPOT and serum ELISA assays, indicating that the rAAV-mSP17 transduction alone provides partial DC programming towards a Th1-polarizing profile, but inhibition of p38 MAPK is required to achieve maximum effects. Our observations are in accordance with the superior anti-tumor cytotoxic activity displayed by the splenocytes of rAAV-mSP17+p38 inhibitor treated mice, compared to all of the other groups. Of note, the cytotoxic response was achieved without re-stimulation of splenocytes with autologous dendritic cells in vitro, proving that our vaccine strategy induced a memory response, which was dramatically evident in the rAAV-mSP17+p38 inhibitor group, which persisted for 300 days.

Since lymphocytic tumor infiltration is considered a positive prognostic factor in OC patients, the observation that only rAAV-mSP17 DC + p38i splenocytes were able to migrate towards ID8 OC cells in vitro highlights the potential clinical relevance of our results.

The finding that IL-17 expression in ovarian tumors is associated with improved survival contrasts with the correlation of Treg infiltration with increased mortality, and suggests Th17 responses play a protective role in OC immunity. It suggests that DC vaccination strategies that diminish Treg activation and expansion and increase Th17 responses can result in strong anti-tumor immune reactivity and increased survival in OC patients. Leveque et al, Interleukin 2-mediated conversion of ovarian cancer-associated CD4+ regulatory T cells into proinflammatory interleukin 17-producing helper T cells. J Immunother 32, 101-108 (2009). Jarnicki et al., Attenuating regulatory T cell induction by TLR agonists through inhibition of p38 MAPK signaling in dendritic cells enhances their efficacy as vaccine adjuvants and cancer immunotherapeutics. J Immunol 180, 3797-3806 (2008). Wang et al., Optimizing immunotherapy in multiple myeloma: Restoring the function of patients’ monocyte-derived

We found that vaccination of ID8-bearing mice with rAAV-mSP17 DC + p38i resulted in diminished splenic Treg and increased Thl7 cells, when compared with rAAV-mSP17 DC-vaccinated or unvaccinated mice. It is thus possible that an SP17-targeted DC vaccine formulation, together with p38 MAPK inhibition, may redirect Treg differentiation towards more favorable Thl7 expansion. Indeed, we found that diminished Treg and increased Thl7 are associated with strong anti-tumor cytotoxicity following rAAV-mSP17 DC + p38i vaccination, and correlated with abrogation of tumor growth and long-term survival. The DC phenotype alterations induced by rAAV-mSpl7 infection in the presence of p38i partially explain these findings. While CD80 and CD86 co-stimulatory molecules and MHC-II levels were unaffected, we detected a significant increase in MHC-I and a decrease in B7-H1, compared with rAAV and rAAV-mSpl7 infected DC. Higher MHC-I expression suggests that rAAV-mSpl7 + p38i DC were more efficient in presenting Spl7 antigen to CD8+ T-cells, accounting for improved CTL differentiation. B7-H1 has been shown to be expressed in DC and myeloid cells in OC and this expression suppresses T effector functions by engaging PD-1 receptor expressed on T-cells. Cannon *et al.*, Dendritic cell vaccination against ovarian cancer tipping the Treg/T(H)17 balance to therapeutic advantage? *Expert Opin Biol Ther* **28**, 28 (2011). Liu *et al.*, B7-H1 on myeloid-derived suppressor cells in immune suppression by a mouse model of ovarian cancer. *Clin Immunol* **129**, 471-481 (2008). Accordingly, B7-H1 blockade on tumor-derived DC has been shown to improve T-cell stimulation *in vitro*. Curiel *et al.*, Blockade of B7-H1 improves myeloid dendritic cell-mediated antitumor immunity. *Nat Med* **9**, 562-567 (2003). Thus, down-regulation of B7-H1 is highly significant and we hypothesize that it accounts, in part, for the reduced Treg frequency we detected *in vivo*.

According to our previous report, p38 inhibition in differentiated DC affords for a dramatic down-regulation of the T-cell inhibitory signaling molecule, B7-H1. Consistent with our results in mouse DC used in this study (figure 2), we found that p38 inhibition did not significantly alter CD80/83/86 expression levels in autologous human rAAV-Spl7 DC (figures 7 and 8). When analyzing DC ability to induce activated T cells versus Treg cells *in vitro*, we found that, in accordance with the DC phenotype described above, non-adherent PBMCs co-cultured with DC differentiated in the presence of p38i displayed both a reduced expression of the Treg marker
Foxp3 and up-regulation of the T cell activation marker, IFNγ (figures 6, 9A and 9B). While it is known that rAAV transduction offers advantages over standard DC antigen pulsing methods, in terms of up-regulation of co-stimulatory molecules and consequently of T-cell activation due to the known pro-inflammatory mechanisms triggered by AAV entry (Liu et al, Molecular basis of the inflammatory response to adenovirus vectors. Gene therapy 10, 935-940 (2003)), the molecular mechanism explaining the different outcome of p38 blockade on B7-H1 and CD80/83/86 remains to be elucidated. Indeed, we have previously demonstrated that p38 inhibition alone, without rAAV, results in a down-regulation of CD80/83/86 expression along with B7-H1 in DC. Cannon et al., Modulation of p38 MAPK signaling enhances dendritic cell activation of human CD4+ Thl7 responses to ovarian tumor antigen. Cancer immunology, immunotherapy: CII 62, 839-849 (2013). Our hypothesis is that CD80/83/86 expression in p38i-treated DC in the presence of rAAV is rescued by the virus ability to activate the NF-κB pathway, which has been proven to increase the levels of co-stimulatory molecules in DC. Liu et al, Akt/protein kinase B activation by adenovirus vectors contributes to NFKbB-dependent CXCL10 expression. Journal of virology 79, 14507-14515 (2005). Ade et al, NF-kappaB plays a major role in the maturation of human dendritic cells induced by NiSO(4) but not by DNCB. Toxicological sciences: an official journal of the Society of Toxicology 99, 488-501 (2007).

In conclusion, our results show that rAAV-Spl7 engineered DC vaccination that incorporates p38 inhibition prevents tumor dissemination and induces long-term protection in a murine model of OC. Our results suggest that the use of this rAAV-Spl7 transduced DC vaccine, together with p38 inhibition, may prove a suitable strategy to generate potent anti-tumor responses in OC patients. Therefore, our findings support using rAAV-Spl7 transduced DC vaccination, together with p38 inhibition, to promote immunity and reduce regulatory T cells in human OC.

Example 2 Phase I/II study of low-dose cyclophosphamide, tumor associated peptide antigen-pulsed dendritic cell therapy and low dose granulocyte-macrophage colony stimulating factor, as consolidation therapy in patients with metastatic solid malignancies, or in patients with progressive and/or refractory solid malignancies

In this phase I/II study we will examine the feasibility, toxicity, immune response and antitumor activity of TAPA-pulsed DC therapy in patients with metastatic SM who may or may
not demonstrate a measurable response to first-line, conventional systemic therapy, or in patients with progressive and/or refractory SM.

Selected TAPAs will be used to pulse DCs *ex vivo*, in the presence of a highly immunogenic maturation cocktail [9,12,18,19]. Patients will also be treated with low-dose CYP prior to each DC vaccination, in an attempt to decrease the number and activity of Tregs. Low-doses of GM-CSF will be administered following each DC vaccination, in order to optimize immune responses in patients with relapsed/refractory SM. In a recent study conducted by others, dendritic cells loaded with tumor lysates have been combined with GM-CSF, pegylated IFN and cyclophosphamide to treat patients with refractory SM [9].

The regimen to treat patients with metastatic SM (who may or may not demonstrate a tumor response to conventional first-line systemic therapy) or patients with relapsed and/or refractory SM, and whose tumor cells express at least one TAPA, include, using low-dose CYP followed by an autologous, monocyte-derived, TAPA-pulsed DC vaccine and low-dose GM-CSF. This treatment regimen will result in TAPA-specific CD4+ T-cell and CD8+ CTL responses without significant toxicities. CD4+ T-cell and CD8+ CTL responses generated against specific TAPAs may translate into clinical antitumor activity.

The methods disclosed herein may be used to treat or prevent solid malignancy or hematologic malignancy. In one embodiment, the malignancy is lymphoma. In another embodiment, the malignancy is multiple myeloma. In a third embodiment, the malignancy is breast cancer.

**Study Design**

Patients diagnosed with metastatic solid malignancies (SM), who have responded to first-line conventional therapy, will be candidates for this Phase I/II study. Following confirmation of disease response to first-line systemic therapy, eligible patients will have their tumor cells analyzed for the expression of a specific panel of Tumor Associated Peptide Antigens (TAPAs), including Spl7, Ropporin, AKAP-4, PTTG1, Span-xb, Her-2/neu, HM1.24, NY-ESO-1 and MAGE-1. Patients whose tumors express one or more of these TAPAs will receive three (3) days of subcutaneous Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) to increase bone marrow production of monocytes and dendritic cell (DC) precursors, and whole blood will be obtained by phlebotomy and/or leukapheresis performed for generation of
autologous DCs. Patient's DCs will be generated, and activated by pulsing/loading them with the TAPA(s) relevant for each particular patient. Patients will receive 5 days of low-dose cyclophosphamide prior to each vaccination with TAPA-pulsed DCs to decrease Treg activity. TAPA-pulsed DCs will be administered at a dose of 1 x 10^7 DCs at least two (2) days following cyclophosphamide administration. DC vaccination schedule will be once every 14 days via subcutaneous (SC) and intradermal (ID) injections for a total of 6 vaccinations. Low dose GM-CSF will also be administered SC for 5 consecutive days, starting six (6) hours after each TAPA-pulsed DC treatment, to optimize immune responses. Patients will be followed on a weekly basis (or more frequently if required) to evaluate treatment-related toxicity. Immune responses and anti-tumor responses will also be evaluated. Continuation and stopping rules for the study will be defined based on toxicity/tolerability (Phase I) and/or immune responses (Phase II).

The more specific protocol of the study is as follows. After patients are enrolled in the study program due to cancer cell expression of one or more of the relevant TAPAs by RT-PCR and/or Western blot, IHC, ELISA, they consent for either leukapheresis or phlebotomy. The leukapheresis or phlebotomy product is processed. The PBMCs are separated over Ficoll density gradient centrifugation. PBMC are pelleted and resuspended in CellGro DC serum free media (CellGenix, NH, USA) with L-glutamine. PBMCs are counted using a hemacytometer and viability determined using trypan blue (1:1) exclusion. For generation of a total DC vaccine bank all PBMCs are transferred to T150 flasks for monocyte sorting and iDC (immature DC) generation. For generation of only one fresh DC vaccine dose five/sixths (5/6th) of the total number of PBMCs are cryopreserved in five (5) or more 2 ml NUNC vials. The remaining one-sixth (1/6th) of the Ficoll-purified PBMC are utilized for generation of iDCs. PBMCs are washed and resuspended in T-150 tissue culture flasks at 1.0 x 10^8 per flask in CellGro DC serum free media with L-glutamine and incubated at 37°C for 2 hours in a 5% CO_2 incubator.

Following incubation, non-adherent cells are removed by three gentle washes with CellGro DC serum free media and adherent cells cultured in CellGro DC serum free media plus 10% autologous (patient) plasma, 800 U/ml of IL-4 and 1000 U/ml of GM-CSF, and incubated at 37°C and 5% CO_2 for six (6) to eight (8) days (average seven (7) days). Fresh IL-4/GM-CSF are added on days two (2), four (4) and six (6). DC cultures are observed every other day. Morphology and level of confluence are noted. On days six (6) or seven (7) immature DCs will be counted to determine Total Volume Count (TVC). 1 x 10^6 DCs with supernatant are
removed for the gram stain, fungal, aerobic and anaerobic culture on days six (6) or seven (7) (prior to peptide loading/maturation cytokine cocktail pulsing). QC samples are removed after the cells have been washed and the TVC determined.

DCs are kept on T-150 flasks at a density of approximately 5 X 10^5 DC cells/ml for peptide pulsing or alternatively, washed with phosphate buffered saline (PBS) and transferred (adherent and non-adherent DCs) to 50 ml conical tubes. DC culture is then pulsed with 20 μg/mL of one or more of the relevant tumor associated peptide antigens (TAPAs) (i.e., one or more peptides derived from Spl7, AKAP-4, Roppolin, PTTG-1, HML24, Her-2/neu, NY-ESO-1, MAGE-1, SPAN-Xb; see SEQ ID Nos. 7-15) for 4 hours followed by the addition of DC maturation cocktail (IL-1β and TNFa at 50 ng/ml, INFα at 1000 IU/ml and poly (I:C) at 20 μg/ml). DC culture is then incubated at 37°C and 5% CO2 for 16-24 hrs (average 20 hrs). After 16-24 hrs of incubation, pulsing/maturation treatment is stopped by centrifugation and peptide/cytokine containing medium removed. DCs are washed twice and resuspend DCs in DPBS IX (Gibco). Number of pulsed/mature DCs is determined (based on DC phenotype release assay results) and the appropriate dose loaded into a syringe. For patients for whom a total DC vaccine bank is generated and cryopreserved, DC vials containing the appropriate number of DCs will be thawed, DC viability determined and contents transferred into one or more syringe(s) with a 23 gauge needle and sterile saline solution for a total volume of 1 cc. The DC vaccine release criteria include: a passing result for the phenotype release assay is defined as cells expressing > 70% CD86, CD80, CD83, CD58, CD1a, HLA-DR and < 10% CD14, within the DC gate, DC viability of more than 80%, and negative test results for endotoxin, mycoplasma, fungal, aerobic and anaerobic cultures.

DC vaccination strategies have been studied clinically in many different diseases. Both monocyte-derived DCs and CD34+-derived DCs have been used in the presence of serum-free mediums, autologous serum-containing mediums, or fetal calf serum-containing mediums. Because these cells have been generated from autologous cells, their administration either intravenously (IV), subcutaneously (SC) or intradermally (ID) has not been associated with any significant adverse effects. Minor adverse effects may include low grade fever and local reactions, such as erythema, at the sites of injection.

In a recently conducted phase I clinical trial of DC vaccination of patients with early-
stage cervical cancer (BB-IND 11307), no adverse side effects were observed or reported by
subjects following immunization beyond the immediate discomfort associated with injection [10]. The patients were monitored during treatment with complete blood counts and serum
chemistries that included liver and renal function tests and electrolytes. No alterations in liver
and renal function were detected. The safety of DC vaccination was also recently demonstrated
in a study combining autologous DC vaccine against the melanoma antigen MART-1 and the
cytotoxic T lymphocyte associated antigen 4 (CTL-4) antagonist tremelimumab [67]. The only
toxicity reported in this study was related to grade I-II pruritic skin eruptions previously
associated with CTLA4 antagonists. No toxicity attributed to DC vaccination alone was
reported. One of the major concerns regarding DC vaccination with self-tumor antigens is the
possible induction of autoimmunity. Vitiligo has been seen in some melanoma patients, but no
cases of severe autoimmune reactions have been reported.

Study Materials and Procedures

TAPA-Pulsed Dendritic Cells (DCs)

DCs will be derived from monocyte precursors present in peripheral blood mononuclear
cells (PBMC) cultures following phlebotomy and/or leukapheresis. Monocyte precursors will be
cultured in CellGro serum free media (CellGenix, USA), 10% plasma from patients or human
AB serum (Biowhittaker) tested for endotoxin, 800 U/ml of IL-4 and 1000 U/ml of GM-CSF
(CellGenix, USA) for seven (7) days. DC vaccine will be prepared by "pulsing" immature DCs
with relevant, recombinant TAPAs (20 μg/ml) for four (4) hours followed by the addition of a
DC maturation cytokine cocktail containing IL-1β and TNFa at 50 ng/ml (CellGenix, USA),
poly (I:C) at 20 μg/ml (Hemisphere or InvivoGene, USA) and INFα at 1000 IU/ml
(Humanzyme, USA) for an additional 16 to 24 hours. Successful generation of mature DCs will
be confirmed in each case by immunophenotyping for classical DC markers, including CD1a,
CD1 lα, CD40, CD80, CD83, CD86 and HLA-DR. Recombinant, clinical grade TAPAs will be
obtained from CS Bio Company, Inc., Menlo Park, CA. Following the maturation period, and
after extensive washing to remove any unbound protein, DCs will be aliquoted into six or more
equal volumes for patient administration or cryopreservation until further. Alternatively, DCs
for the first vaccine dose will be generated from one sixth (l/6th) of the original pool of PBMCs,
with subsequent DC vaccine doses generated from cryopreserved PBMCs prior to vaccination

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day.

Mature DCs (or PBMCs for later generation of fresh mature DCs) will be cryopreserved and stored in liquid nitrogen until use.

A minimum of 6 and a maximum of 12 vials of TAPA-pulsed DCs (containing no less than 1 X 10^7 DCs each) will be cryopreserved as a master vaccine bank for each patient. TAPA-pulsed DC vaccine will be frozen in DC medium plus 90% heat-inactivated autologous plasma (or AB human serum) and 10% dimethyl sulfoxide. Alternatively, 5/6th of the original pool of PBMCs will be cryopreserved, as described above, for subsequent thawing and generation of fresh DCs prior to each vaccination schedule. This process may improve the viability of DCs.

**Generation of Tumor Associated Peptide Antigen-Pulsed Dendritic Cells (DCs)**

**Isolation of Monocytes from Peripheral Blood Mononuclear Cells (PBMC)**

For the specimens which arrive as a phlebotomy or leukopack product from a leukapheresis, the cells are manually separated over Ficoll-HyPaque density gradient centrifugation.

PBMC are pelleted and resuspended in CellGro DC serum free media (CellGenix, NH, USA) with L-glutamine.

The PBMCs are counted with a hemacytometer and viability determined using trypan blue 1:1. DCs are generated either as a total vaccine bank prior to patient administration or individually prior to each administration. For generation of a total DC vaccine bank, all PBMCs isolated from phlebotomy and/or leukapheresis are processed and the final DC vaccine product cryopreserved until use. For generation of individual DC vaccine doses, excess PBMCs that will not be used for the generation of the first vaccine dose, are cryopreserved in 5 or more 2 ml NUNC vials. The remaining Ficoll-purified PBMC will be utilized for generation of fresh DCs, for the first DC vaccine injection. The subsequent five injections/doses of DCs will be prepared from frozen PBMC.

**Generation of iDCs (Immature DCs) from Monocytes**

PBMCs are harvested by Ficoll Hypaque density gradient centrifugation. The PBMCs are washed and resuspended in DC-medium and transferred in T-150 tissue culture flasks in CellGro DC serum free media (CellGenix, NH, USA) with L-glutamine. The cells are incubated
at 37 °C for 2 to 4 hours in a 5% CO₂ incubator. Following incubation, non-adherent cells are removed by one to four gentle washes with DPBS and adherent cells are cultured in CellGro DC medium plus 1% to 10% autologous (patient) plasma or 1% to 10% heat inactivated normal human AB serum, 800 U/ml of GM-CSF and 1000 U/ml of IL-4, and incubated at 37 °C and 5% CO₂ for 4 to 7 days. Fresh IL-4/GM-CSF are added on days two (2), four (4), and six (6). Human AB serum (Sigma-Aldrich, USA) will be used only when patients' autologous plasma is unavailable.

In one embodiment, 35 mL of peripheral blood obtained from a healthy individual is purified using Ficoll. 70 x 10⁶ cells were left to adhere in a T-150 flask as indicated above (DC medium +1% autologous plasma) for 3 hours in a cell culture incubator. Viability after Ficoll purification was 98%. The non-adherent cells were collected and frozen in 90% normal human AB serum + 10% DMSO to be used in the cytotoxicity assay. The adherent fraction represented about 40% of the total amount of PBMCs isolated. The blood may be from a subject having solid malignancy or hematologic malignancy. In one embodiment, the malignancy is lymphoma. In another embodiment, the malignancy is multiple myeloma. In a third embodiment, the malignancy is breast cancer.

On days four (4), five (5), or six (6), immature DCs are counted to determine Total Volume Count (TVC).

0.5 X 10⁶ DCs with supernatant are removed for gram stain, aerobic, anaerobic and fungal culture, mycoplasma testing and mycoplasma testing on days four (4), five (5), or six (6) (prior to peptide loading/maturation cytokine cocktail pulsing). QC samples will be removed after the cells have been washed and the TVC determined. Remove a 100 µl aliquot for cell counting on the hemacytometer with Trypan blue staining.

**Harvest of iDCs**

Wash non-adherent iDCs from each flask by gently flushing the flask with the cell suspension 5 to 10 times, then transfer the cell suspension to a 50 mL tube. Take an aliquot for trypan-blue counting.
Keep iDC at a density of approximately 2 to 10 X 10^6 cells/ml. If necessary, concentrate the cells by centrifuging at 100 to 300 X g for 5 to 10 minutes at 10-20 °C and discard the excess medium before resuspending the pellet.

**Maturation of DC and peptide pulsing**

Keep iDC at a density of approximately 2 to 10 X 10^6 cells/ml. If necessary, concentrate the cells by centrifuging at 100 to 300 X g for 5 to 10 minutes at 10-20 °C and discard the excess medium before resuspending the pellet.

Transfer the cell suspension in a conical 50 mL or 15 mL tube and add the maturation cytokine cocktail, then transfer the cell suspension to a new flask (up to 50 x 10^6 cells in a 75 cm^2 growth area).

The maturation cytokine cocktail contains IL-1β and TNFα at 50 ng/ml, INF-α at 1,000 U/ml and poly (I:C) at 20 μg/ml. DC culture is then incubated at 37 °C and 5% CO₂ for 16 to 72 hours.

DC culture is then harvested (the adherent cells, if present, will be harvested by washing with PBS and by the use of a cell scraper), and re-adjusted at a density of 2 to 10 X 10^6 cells/mL.

DCs are pulsed with 20 μg/mL of one or more of the relevant tumor associated peptide antigens (TAPAs) (i.e., peptides derived from Spl7, AKAP-4, Ropporin, PTTG-1, HMI.24, Her-2/neu, NY-ESO-1, MAGE-1, and/or SPAN-Xb. See SEQ ID Nos. 7-15 for the peptide sequences) for two (2) to four (4) hours. Working stock for the above TAPAs is 1 to 10 mg/ml, depending on the solubility of each TAPA.

After two (2) to four (4) hours of incubation, the pulsing is stopped by centrifuging the DC in the tubes at 100 to 300 X g for 5 to 10 minutes at 10-20 °C and eliminating the supernatant. If using multi-wells, transfer the DC suspension in 15 or 14-mL conical tubes before centrifuging. Pulsed DC are then resuspended in 1 to 10 mL with fresh DC-medium 1% to 10% autologous (patient) plasma or 1% to 10% heat-inactivated normal human AB serum. Following pulsing and after viability count (trypan-blue), 0.9 X 10^6 DCs are collected to perform flow-cytometry quality control analysis, and 1.5 x 10^6 DCs with medium to perform sterility QC tests.

As an alternative, the pulsing with TAPAs can be performed prior to exposure to the cytokine maturation cocktail. iDCs are pulsed and subsequently matured, as follows. Keep iDCs (derived from the GM-CSF/IL-4 culture) at a density of approximately 2 to 10 X 10^6 cells/ml. If
necessary, concentrate the cells by centrifuging at 100 to 300 X g for 5 to 10 minutes at 10-20 °C and discard the excess medium before resuspending the pellet.

Then, DC is pulsed as described above. After two (2) to four (4) hours of incubation, the pulsing is stopped as described above. Pulsed DC are then resuspended at 2 to 10 X 10^6 cells/mL in CellGro DC medium plus 1% to 10% autologous (patient) plasma or 1% to 10% heat inactivated normal human AB serum, 800 U/ml of GM-CSF and 1000 U/ml of IL-4. Then, the maturation cocktail is added as described above and incubated at 37 °C and 5% CO_2 for 16 to 72 hours. Matured DC are then centrifuged at 100 to 300 X g for 5 to 10 minutes at 10-20 °C, the supernatant is discarded and the DC are resuspended in 1 to 10 mL with fresh DC-medium 1% to 10% autologous (patient) plasma or 1% to 10% heat inactivated normal human AB serum.

Following pulsing and after viability count (trypan-blue), 0.9 X 10^6 DCs are collected to perform flow-cytometry quality control analysis, and 1.5 x 10^6 DC with medium to perform sterility QC test.

**Harvest of pulsed/mature DCs**

Calculate the proportion of DCs based on flow cytometry (see DC Phenotype Release Assay) and determine if the culture contains the appropriate number/dose of DCs for the entire treatment (approximately up to 1-6 X 10^7 DCs depending on the generation of one dose vs total vaccine bank).

After washing the DC with 2 volumes of PBS, resuspend the DC pellet in PBS at 10^6 to 10^7/mL in PBS and load the appropriate number of DCs into a syringe with a 23-gauge needle for a total volume of 1 cc.

**DC Phenotype release assay and cell viability**

An aliquot from the harvested, pulsed mDC culture is stained and the flow-cytometry analysis performed. These are performed from mature/pulsed DC on days 5, 6, 7, 8, 9, or 10 from the beginning of the iDC differentiation, depending on the day when the maturation is performed.

A passing result for the phenotype release assay is defined as cells expressing > 70% CD86, CD80, CD83, CD58, HLA-DR and < 10% CD14, within the DC gate.
Viability for fresh cells are determined by Trypan blue exclusion. A viability of more than 80% is required for release.

The DC population is understood to be larger and more internally granular than the lymphocyte population. Therefore, the DC population lies above and over from the lymphocyte population in a FS/SS scattergram.

The release assay has two sections; the first determines the percentage of live cells that are DCs, and the second determines the percentage of DCs that are positive for certain cell surface markers.

Following 5 days of culture with GM-CSF and IL-4, non-adherent, immature DC (20 x 10^6 were harvested as described above and concentrated to 2 x 10^6/mL in the same medium. Then, the maturation cocktail was added, and the DC suspension was transferred to a T-75 flask and incubated for 24 hours in 5% CO_2 at 37 °C. Then, suspension and adherent cells were collected and pulsed with TAPAs as described above, for 2 hours in 14-mL polypropylene tubes (2 mL/tube with 2 x 10^6 cells/tube). Then, an aliquot of 0.4 x 10^6 cells was removed for flow-cytometry quality control.

The following staining is performed: Isotype control FITC, Isotype control PE, CD86 (FITC), CD58 (PE), HLA-DR (FITC), CD83 (PE), CD14 (FITC), and CD80 (PE). Cells will be stained according to standard protocol. Approximately 0.9 X 10^6 cells will be required for the assay.

Draw one bitmap around the entire DC population. Draw a second bitmap around the entire lymphocyte population. For the DC only bitmap perform a separate single color analysis for CD86, CD83, CD80, CD58, CD1a, HLA-DR and CD14. For the lymphocyte only bitmap perform a single color analysis for CD86, CD83, CD80, CD58, CD1a, HLA-DR and CD14.

**Determining percent DCs**

From the isotype control tube, the percent DC is the percentage of cells within the DC only bitmap, as opposed to all of the cells in the FS/SS scattergram. The % DC is used in various sections of the DC process to determine the total number of DC in culture.

**Analysis of cell surface markers for release assay**
From the DC bitmap only, acceptance criteria are: CD86 greater than or equal to 70% positive; CD80 greater than or equal to 70% positive; CD83 greater than or equal to 70% positive; CD58 greater than or equal to 70% positive; HLA-DR greater than or equal to 70% positive; and CD14 less than or equal to 10% positive.

After 2 or 5 days of GM-CSF stimulation, and after 20 μg/mL SP17(103-1 11) peptide pulse, the DC population increased in dimensions, as depicted by the FSC/FSC dot-plot, and that the maturation successfully induced the expected up-regulation of the maturation markers, CD80, CD83, CD86, CD58, and HLA-DR, while reduced the expression of the monocyte marker, CD14.

**QC sampling after harvest of pulsed/mature DCs**

These are performed from mature/pulsed DC on days 5, 6, 7, 8, 9, or 10 from the beginning of the iDC differentiation, depending on the day when the maturation is performed.

**Endotoxin test:** 0.5 X 10^6 cells with supernatant are removed for the endotoxin Limulus Amoebolysate (LAL) testing. The LAL test is performed using the QCL-1000 kit by the chromogenic method. A passing result of less than or equal to 1.0 IU/mL of treatment aliquot tested is required for release of the fresh DCs and administration to patients.

**Mycoplasma test:** 0.5 X 10^6 cells with supernatant are removed for the mycoplasma assay. A mycoplasma culture is done. A 96-hour DNA fluorochrome results (Hoechst) is optional and is not required for administration of the fresh DC infusion. If performed, a passing result for the 96 hour Hoechst assay is "negative."

**Sterility Test:** 0.5 X 10^6 cells with supernatant are removed for the fungal, aerobic and anaerobic bacterial culture, sensitivity and stat gram stain. Samples are observed on a continuous basis for 14 days. A negative gram stain on the day of harvest and negative culture at 24 hours (removed prior to peptide pulsing) is required for release of the initial fresh DC culture. A passing result for sterility testing is "negative" for the presence of microbial contamination in fungal and aerobic and anaerobic bacterial canisters.
**Final record**

To evaluate the ability of matured/pulsed DC to induce specific autologous cytotoxic lymphocytes (CTL), an *in vitro* cytotoxicity was performed using the autologous pulsed dendritic cells as targets and *in vitro* generated CTL as effectors. To obtain CTL, 0.5 x 10^6 mature/pulsed DC were mixed with 5 x 10^6 autologous non-adherent PBMCs (PBL) obtained after the adhesion of monocytes (described above) on day 0. Such co-cultures were maintained in DC medium + 2% autologous plasma, and they were supplemented with 10 μg/mL corresponding peptide and 10 U/mL IL-2. Every 2 days, 50% of medium was replaced with fresh medium, and fresh peptides and IL-2 were added. After 10 days, cells were counted and incubated with target cells at different ratios (from 40 to 20: 1). As a negative control, CTL generated with DC pulsed with different TAPAs were incubated with autologous DC pulsed with the irrelevant antigen, E6 (HPV E6 protein). In the co-cultures, but not when PBL were cultured alone (without DC), we detected the formation of rosetta-like structure, which indicate interaction between T cells and DC.

**Cryopreservation of Mature DCs**

For cryopreservation of DCs, when a total DC vaccine bank is generated, centrifuge cells at 100 to 300 X g for 5 to 10 minutes at 10-20 °C, then resuspend in 5 to 10 mL of cold freezing medium (90% normal human AB serum + 10% DMSO).

**Thawing of Frozen PBMC for DC Generation**

Frozen PBMCs are thawed in the 37 °C water bath for 2 to 5 minutes. The product is then diluted in 9 volumes of DC medium (pre-warmed at 37 °C) supplemented with 2% autologous plasma, centrifuged at 100 to 300 X g for 5 to 10 minutes at 10-20 °C, and then transferred to the appropriate number of T-150 flasks.

**Thawing of Frozen Mature DCs for Patient Administration**

Frozen mature DCs is thawed in the 37 °C water for 2 to 5 minutes. The product is then diluted in 9 volumes of DC medium (pre-warmed at 37 °C) supplemented with 2% autologous plasma Diluted in DC-medium and counted by Trypan blue exclusion method. A DC viability of more than 80% is required for release of vaccine dose. The product is then centrifuged at 100 to 300 X g for 5 to 10 minutes at 10-20 °C, resuspended in sterile PBS at the concentration of up
tolO^7 cells/mL, then transferred to one or more syringe(s) with a 23-gauge needle, each one to a volume of 1 ml for patient administration.

**Treatment**

*Cyclophosphamide (CYP)*

Patients will be treated with CYP orally at a dose of 100 mg/day for 5 days, beginning seven (7) days prior to each TAPA-pulsed DC vaccine dose (day -7 though day -3, days 7-11, days 21-25, days 35-39, days 49-53, days 63-67 corresponding to 6 treatments).

*Selection of TAPA-Pulsed DC Dose Levels*

A phase I dose escalation clinical trial of DC vaccination in patients with cervical cancer indicated optimal stimulation of tumor antigen-specific cytotoxic T cell responses with a dose of 1.0 x 10^7 DCs, in injection-grade saline containing 30% heat-inactivated autologous serum, and delivered SC and ID at 21 day intervals [10]. Thus, in this study we will explore one (1) dose of TAPA-pulsed DC vaccination (1 X 10^7 DCs) and determine the toxicity, immune efficacy (IE) and clinical response in patients with progressive and/or refractory SM.

Preliminary studies indicate that leukapheresis (10 liter volume) followed by CliniMACS isolation of CD14+ cells and high density DC culture in G-Rex flasks provided an optimal yield of 2.5 x10^8 mature DCs from 10^9 CD14+ cells. This would be sufficient for cryopreservation of 12 vials at 1 x10^7 DC/vial. Phlebotomy of 250 ml of whole blood yields approximately 1.2 X 10^8 monocytes and 3 X 10^7 mature DCs.

*TAPA-Pulsed DC Vaccination*

If a total vaccine bank is generated, TAPA-pulsed DCs will be thawed out, washed once with sterile saline and resuspended in up to 1 ml injection-grade saline containing 10% autologous human serum. The vaccine volume will be up to 1.0 ml and half the volume (0.5 ml) will be administered SC and ID in the patient’s inguinal or axillary fold, in order to increase proximity to local lymph node draining basins and optimize access of the TAPA-pulsed DCs to secondary lymphoid organs and propagation of the immune response. A maximum of 1.0 ml will be injected in a single site. Approximately half the volume per injection (0.5 ml) will be delivered SC and half ID, on a single site. The same site(s) will be used for repeated vaccinations.
unless a grade 2 or greater injection site reaction occurs, in which case a new site in the inguinal fold will be selected. Six DC vaccines will be administered at 14 day intervals, plus or minus 3 days, to maximize patient convenience and protocol adherence. Alternatively, TAPA-pulsed DCs will be generated prior to each vaccination and administered to patients every two (2) weeks, as planned. Patients will be observed for up to six (6) hours following each vaccine dose administration.

This is a phase I/II safety and efficacy study. The first six (6) patients will receive 1 x $10^7$ DCs divided in a subcutaneous (SC) and intradermal (ID) administration, every fourteen (14) days, for up to a maximum of six (6) treatments. The SC and ID DC vaccinations will be administered in normal saline with a total volume of 0.5 ml per injection (total vaccination dose-volume 1.0 ml). Prior to receiving the DC vaccination, all patients will receive premedication with diphenhydramine (50 mg) intravenously and acetaminophen (1000 mg) orally.

If one (1) or less than six (6) patients develops DLT, the Phase II/efficacy level will proceed. If two or more (> 2) of the first six (6) patients develop DLTs, the study will be terminated. A minimum of six (6) patients will be treated for evaluation of safety/toxicity (Phase I level). A maximum of seventeen (17) patients will be treated in the study for evaluation of immune efficacy and clinical response (Phase II level).

**GM-CSF Treatment**

Following each DC vaccination, each patient will receive SC injections of low dose GM-CSF (50 meg) daily for five (5) consecutive days, beginning six hours after the DC administration (days 0-4, days 14-18, days 28-32, days 42-46, days 56-60, days 70-74 corresponding to 6 treatments).

**Study Drug Quality and Safety Measures: TAPA-Pulsed Dendritic Cells (DCs)**

TAPA-pulsed DCs will be administered SC and ID at 14 day intervals plus or minus 3 days. Safety tests before cryopreservation or release of TAPA-pulsed DCs including, Agar sterility test; mycoplasma test by approved kit; and/or endotoxin test by an approved independent testing laboratory. Endotoxin must be less than 1 IU/ml by the LAL method. Safety tests before administration of TAPA-pulsed DCs including, Gram stain prior to administration; cell viability by trypan blue exclusion; and/or broth culture sterility test.
Immunosuppressive or anti-inflammatory drugs (including hydrocortisone) that inhibit cellular immune responses should not be taken, unless otherwise indicated for the management of study-related toxicities or adverse events.

The scope of the present invention is not limited by what has been specifically shown and described hereinabove. Those skilled in the art will recognize that there are suitable alternatives to the depicted examples of materials, configurations, constructions and dimensions. Numerous references, including patents and various publications, are cited and discussed in the description of this invention. The citation and discussion of such references is provided merely to clarify the description of the present invention and is not an admission that any reference is prior art to the invention described herein. All references cited and discussed in this specification are incorporated herein by reference in their entirety. Variations, modifications and other implementations of what is described herein will occur to those of ordinary skill in the art without departing from the spirit and scope of the invention. While certain embodiments of the present invention have been shown and described, it will be obvious to those skilled in the art that changes and modifications may be made without departing from the spirit and scope of the invention. The matter set forth in the foregoing description is offered by way of illustration only and not as a limitation.
References


61. Dang Y, Wagner W, Gad E, et al. Dendritic cell-activating vaccine adjuvants differ in the ability to elicit antitumor immunity due to adjuvant-specific induction of


What is claimed is:

1. A method of treating and/or preventing cancer in a subject, the method comprising the steps of:

   (a) introducing at least one tumor-associated antigen or a fragment thereof into antigen-presenting cells, wherein the tumor-associated antigen comprises SP17, Ropporin, AKAP-4, PTTG1, Span-xb, Her-2/neu, HM1.24, NY-ESO-1, MAGE-1 or combinations thereof;

   (b) treating the antigen-presenting cells with at least one inhibitor of the mitogen-activated protein kinase (MAPK) signaling pathway; and

   (c) administering the antigen-presenting cells to the subject.

2. The method of claim 1, wherein the antigen-presenting cells are dendritic cells.

3. The method of claim 2, wherein the dendritic cells are derived from autologous monocytes.

4. The method of claim 3, wherein the monocytes are cultured in vitro to induce differentiation into dendritic cells.

5. The method of claim 4, wherein differentiation into dendritic cells is facilitated by a composition comprising granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4.

6. The method of claim 4, wherein differentiation into dendritic cells is facilitated by a composition comprising IL-1β, TNFα, IFN-a and poly (I:C).

7. The method of claim 3, wherein the monocytes are isolated from the subject's blood.
8. The method of claim 7, wherein granulocyte-macrophage colony-stimulating factor (GM-CSF) is administered to the subject before the subject's blood is obtained.

9. The method of claim 1, wherein the inhibitor is an inhibitor of RAF, an inhibitor of MEK, an inhibitor of ERK, an inhibitor of RAS, an inhibitor of receptor tyrosine kinases (RTKs), or combinations thereof.

10. The method of claim 1, wherein the inhibitor is an inhibitor of p38.

11. The method of claim 1, wherein the inhibitor is a small molecule, a polynucleotide, a polypeptide, or an antibody or antigen-binding portion thereof.

12. The method of claim 1, wherein the inhibitor is ML3403, PLX4720, PD325901, GW5074, BAY 43-9006, ISIS 5132, PD98059, PD184352, U0126, Ro 09-2210, L-783,277, GSK-1120212, trametinib, vemurafenib, purvalanol, or imidazolium trans-imidazolium sulfoxide-tetrachlororuthenate (NAMI-A).

13. The method of claim 11, wherein the polynucleotide is a small interfering RNA (siRNA) or an antisense molecule.

14. The method of claim 1, wherein the cancer is ovarian cancer, melanoma, breast cancer, colon cancer, pancreatic cancer, cervical cancer, thyroid cancer or bladder cancer.

15. The method of claim 1, wherein the cancer is ovarian cancer.

16. The method of claim 1, wherein in step (a) the antigen-presenting cells are infected with viral vectors encoding the tumor-associated antigen.

17. The method of claim 16, wherein the viral vectors are adeno-associated viral vectors.
18. The method of claim 1, wherein step (a) is conducted before, after, simultaneously with or overlapping with step (b).

19. The method of claim 1, wherein an immunosuppressive agent is administered to the subject before step (c).

20. The method of claim 19, wherein the immunosuppressive agent is an alkylating agent.

21. The method of claim 20, wherein the alkylating agent is cyclophosphamide.

22. The method of claim 1, wherein granulocyte-macrophage colony-stimulating factor (GM-CSF) is administered to the subject after step (c).

23. A pharmaceutical composition comprising dendritic cells comprising at least one tumor-associated antigen or a fragment thereof, wherein the dendritic cells are treated with at least one MAPK signaling pathway inhibitor.

24. The pharmaceutical composition of claim 23, wherein the tumor-associated antigen comprises Spl7, Ropporin, AKAP-4, PTTG1, Span-xb, Her-2/neu, HM1.24, NY-ESO-1, MAGE-1 or combinations thereof.

25. The pharmaceutical composition of claim 23, wherein the dendritic cells are derived from autologous monocytes.

26. The pharmaceutical composition of claim 23, wherein the inhibitor is an inhibitor of RAF, an inhibitor of MEK, an inhibitor of ERK, an inhibitor of RAS, an inhibitor of receptor tyrosine kinases (RTKs), or combinations thereof.

27. The pharmaceutical composition of claim 23, wherein the inhibitor is an inhibitor of p38.
28. The pharmaceutical composition of claim 23, wherein the inhibitor is a small molecule, a polynucleotide, a polypeptide, or an antibody or antigen-binding portion thereof.

29. The pharmaceutical composition of claim 1, wherein the inhibitor is ML3403, PLX4720, PD325901, GW5074, BAY 43-9006, ISIS 5132, PD98059, PD184352, U0126, Ro 09-2210, L-783,277, GSK-1 120212, trametinib, vemurafenib, purvalanol, or imidazolium trans-imidazoledimethyl sulfoxide-tetrachlororuthenate (NAMI-A).

30. A pharmaceutical composition comprising dendritic cells comprising nucleic acids encoding at least one tumor-associated antigen, wherein the tumor-associated antigen is Spl7, Ropporin, AKAP-4, PTTG1, Span-xb, Her-2/neu, HM1.24, NY-ESO-1, MAGE-1 or combinations thereof, wherein the dendritic cells are treated with at least one MAPK signaling pathway inhibitor.

31. The pharmaceutical composition of claim 30, wherein the inhibitor is an inhibitor of RAF, an inhibitor of MEK, an inhibitor of ERK, an inhibitor of RAS, an inhibitor of receptor tyrosine kinases (RTKs), or combinations thereof.

32. The pharmaceutical composition of claim 30, wherein the inhibitor is an inhibitor of p38.

33. The pharmaceutical composition of claim 30, wherein the inhibitor is ML3403, PLX4720, PD325901, GW5074, BAY 43-9006, ISIS 5132, PD98059, PD184352, U0126, Ro 09-2210, L-783,277, GSK-1 120212, trametinib, vemurafenib, purvalanol, or imidazolium trans-imidazoledimethyl sulfoxide-tetrachlororuthenate (NAMI-A).
Survival Proportions

- No Treatment
- rAAV
- rAAV-mSP17
- rAAV-mSP17 + p38i

Time (days)

FIG. 3A

Tumor Mass

Peritoneum

Nodes

SP17 mRNA (Peritoneum)

2^ΔCt(x100)

No Tumor  No Vaccine  DC AAV- mSP17+p38i

FIG. 3C
5/11
CTL Activity Assay

![Graph showing CTL activity at different effector:target ratios and treatments](image)

**FIG. 5A**

Splenocyte Migration Assay

![Graph showing splenocyte migration index](image)

**FIG. 5B**
FIG. 9A
FIG. 9B
<table>
<thead>
<tr>
<th>Box No. 1</th>
<th>Nucleotide and/or amino acid sequence(s) (Continuation of item Ic of the first sheet)</th>
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<tbody>
<tr>
<td>1.</td>
<td>With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:</td>
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<tr>
<td></td>
<td>a. [x] forming part of the international application as filed:</td>
</tr>
<tr>
<td></td>
<td>[x] in the form of an Annex C/ST.25 text file.</td>
</tr>
<tr>
<td></td>
<td>[x] on paper or in the form of an image file.</td>
</tr>
<tr>
<td></td>
<td>b. [ ] furnished together with the international application under PCT Rule 1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.</td>
</tr>
<tr>
<td></td>
<td>c. [x] furnished subsequent to the international filing date for the purposes of international search only:</td>
</tr>
<tr>
<td></td>
<td>[x] in the form of an Annex C/ST.25 text file (Rule 13(a)).</td>
</tr>
<tr>
<td></td>
<td>[x] on paper or in the form of an image file (Rule 1(b) and Administrative Instructions, Section 713).</td>
</tr>
<tr>
<td>2.</td>
<td>In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.</td>
</tr>
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<td>3.</td>
<td>Additional comments:</td>
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INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

<table>
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<tr>
<th>IPC(8)</th>
<th>CPC</th>
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</thead>
<tbody>
<tr>
<td>A61K 39/00; C12N 5/0784; C07K 14/705; A61P 35/00 (2016.01)</td>
<td>A61K 39/00, 39/001; C12N 5/0639, 5/064; C07K 14/705</td>
</tr>
</tbody>
</table>

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

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<th>IPC(8) Classifications:</th>
<th>CPC</th>
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</thead>
<tbody>
<tr>
<td>C07K 14/47, 14/705; A61K 39/00, 35/12; C12N 9/12, 5/0784, 5/0783; A61P 35/00 (2016.01)</td>
<td>C07K -273 0300 C T 03P. - T Z - T</td>
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</tbody>
</table>

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

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

Patent (US, EP, WO, JP, DE, GB, CN, FR, KR, ES, AU, IN, CA, INPADOC Data); Google; Google Scholar; PubMed; EBSCO; Cancer "; Tumor "; malignan"; associat"; antigen"; SP17; "Sperm protein 17"; Roppin, Ropni; "Rhophilin associated protein 1"; ODF6, RHPNAP1, Akap-4; "a-kinase anchoring protein 4"; Pitg, EAP1, Htpg, Pitg, Tutif, "Pituitary tumor transforming 1"; Span-xb, Her-2/neu, Erb-b2

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y</td>
<td>US 2015/0065255 A1 (TEXAS TECH UNIVERSITY SYSTEM) 26 February 2015; paragraphs [0013], [0016], [0019], [0033], [0037], [0043], [0066], [0073].</td>
<td>1-33</td>
</tr>
<tr>
<td>Y</td>
<td>WO 2004/067941 A1 (THE GENERAL HOSPITAL CORPORATION) 14 October 2004; page 3, lines 31-32; page 4, lines 3-5, 8; page 5, lines 16-17; page 8, lines 11-15; page 11, lines 18-20; page 12, lines 12-16; page 15, lines 6-9, 27-28; page 16, lines 16-17; page 24, lines 29-32.</td>
<td>1, 9, 11-21, 23, 24, 26, 28-31, 33</td>
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<td>Y</td>
<td>US 2005/0197314 A1 (KRIEG, et al.) 8 September 2005; paragraphs [0008], [0017], [0027], [0038], [0139].</td>
<td>8, 22</td>
</tr>
</tbody>
</table>

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

* Special categories of cited documents:
  "A" document defining the general state of the art which is not considered to be of particular relevance
  "E" earlier application or patent but published on or after the international filing date
  "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
  "O" document referring to an oral disclosure, use, exhibition or other means
  "P" document published prior to the international filing date but later than the priority date claimed
  "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
  "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
  "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
  "Z" document member of the same patent family

Date of the actual completion of the international search: 26 July 2016 (26.07.2016)

Date of mailing of the international search report: 02 Sep 2016

Name and mailing address of the ISA:
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450
Facsimile 571-272-3000

Authorized officer: Shane Thorias

Form PCT/ISA/210 (second sheet) (January 2015)
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<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
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