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(54) Title: TLR-9 AGONIST WITH TLR-7 AND/OR TLR-8 AGONIST FOR TREATING TUMORS

(57) Abstract: Methods for treating a tumor, such as a benign or malignant tumor, are disclosed herein. The methods include administering to the subject a therapeutically effective amount of a TLR-9 agonist, such as a CpG ODN, and a therapeutically effective amount of a TLR-7 and/or TLR-8 agonist, such as an imidazoquinoline compound. These compounds act synergistically to treat the tumor in the subject. The methods are also of use to decrease the size and/or number of metastases.



**TLR-9 AGONIST WITH TLR-7 AND/OR TLR-8 AGONIST FOR TREATING TUMORS****CROSS REFERENCE TO RELATED APPLICATION**

This claims the benefit of U.S. Application No. 61/834,375, filed June 12, 2013, which is  
5 incorporated by reference herein.

**FIELD OF THE DISCLOSURE**

This relates to the field of tumor biology, specifically to methods for treating tumors that  
include the use of a Toll-like receptor (TLR)-9 agonist and a TLR 7/TLR-8 agonist, such as with an  
10 imidazoquinoline compound and a CpG oligodeoxynucleotide.

**PARTIES TO JOINT RESEARCH AGREEMENT**

The United States of America, as represented by the Secretary, Department of Health and  
Human Services ("the Government") and the 3M Company are parties to a Joint Research  
15 Agreement.

**BACKGROUND**

Toll-like receptor (TLR)-7 and TLR-8 are structurally related members of the TLR family.  
At least 13 different TLRs have been identified in mammals, with TLRs 7, 8, and 9 being similar in  
20 their recognition of nucleic acid motifs and expression within endosomal compartments (Janeway  
and Medzhitov, *Annu Rev Immunol* 20:197-216.:197-216, 2002; Hemmi et al., *Nature* 408:740-745,  
2000; Akira et al., *Cell* 124:783-801, 2006).

Small molecule agonists of TLR-7 and TLR-8 have anti-tumor activity. The lead compound  
of this family is imiquimod, that is marketed as a topical agent. The imidazoquinoline compounds  
25 are used alone or in combination with other therapies (such as anti-virals, anti-bacterials, other  
immune modulators or in therapeutic vaccine antigens) for treatment of chronic infections such as  
those caused by the human immunodeficiency virus (HIV), the hepatitis C virus (HCV), the  
hepatitis B virus (HBV), the herpes simplex virus (HSV), and *H. pylori*. Imiquimod (R837, 1-(2-  
methylpropyl)-1 H-imidazo[4,5-c]quinolin-4-amine), and Resiquimod (R848, 4-amino- $\alpha,\alpha$ -  
30 dimethyl-2-ethoxymethyl-1H-imidazo[4,5-c]quinoline-1-ethanol) are selective ligands for TLR-7  
and TLR-8, and are also used to treat many types of skin cancer and cutaneous metastases.  
Cutaneous tumors that have responded well to topical treatment with imiquimod include basal cell  
carcinomas, keratoacanthomas, actinic keratosis, Bowen's disease, melanoma and cutaneous T-cell

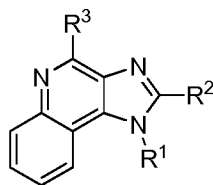
lymphomas (reviewed in Schon and Schon, *Oncogene* 27: 190-9, 2008).

Cancer is the second leading cause of human death next to coronary disease in the United States. Worldwide, millions of people die from cancer every year. In the United States alone, as reported by the American Cancer Society, cancer causes the death of well over a half-million people annually, with over 1.2 million new cases diagnosed per year. Death from cancer is increasing; cancer is soon predicted to become the leading cause of death in the United States. A need remains for therapeutic agents, such as combination therapies, that are effective for the treatment of cancer.

### SUMMARY OF THE DISCLOSURE

Methods for treating a tumor, such as a benign or malignant tumor, are disclosed herein. These methods include administering to the subject a therapeutically effective amount of a TLR-9 agonist, such as a CpG ODN, and a therapeutically effective amount of a TLR-7 and/or TLR-8 agonist, such as an imidazoquinoline compound, for example a lipophilic imidazoquinoline compound (for example, wherein the imidazoquinoline compound comprises an aliphatic tail having from 1 to 23 carbon atoms, such as 11-23 carbon atoms, 15-23 carbon atoms, or 15-19 carbon atoms). The CpG ODN and the imidazoquinoline compound, for example a lipophilic imidazoquinoline compound, can be administered intratumorally. In some embodiments, these compounds act synergistically to treat the tumor in the subject.

In some embodiments, methods are provided for treating a subject with a tumor that include administering to the subject a therapeutically effective amount of a imidazoquinoline compound and an immunostimulatory CpG oligodeoxynucleotide. The methods can include administering to the subject a therapeutically effective amount of a imidazoquinoline compound having a formula:



wherein  $R^1$  is selected from hydrogen,  $C_{12-24}$  alkyl,  $C_{11-24}$  heteroalkyl, substituted  $C_{1-10}$  heteroalkyl, carboxyl, and hydroxyl;  $R^2$  is selected from hydrogen,  $C_{1-10}$  alkyl, and substituted  $C_{1-10}$  alkyl; and  $R^3$  is selected from hydrogen, amine, substituted amine, hydroxyl, and  $C_{1-10}$  alkoxy. The methods also include administering to the subject a therapeutically effective amount of an immunostimulatory CpG oligodeoxynucleotide, thereby treating the tumor in the subject.

In some embodiments, the method includes administering a K-type CpG ODN, a D-type CpG ODN, and/or a C-type CpG ODN. In one specific non-limiting example, one or more K-type

CpG ODNs is administered to the subject. In additional embodiments, the imidazoquinoline compound is N-(4-{[4-amino-2-butyl-1H-imidazo[4,5-c]quinolin-1-yl]oxy}butyl) octadecanamide, also known as 3M-052. In other non-limiting examples, the CpG ODN is a K-type CpG ODN.

The foregoing and other features and advantages of the invention will become more apparent from the following detailed description of a several embodiments which proceeds with reference to the accompanying figures.

### BRIEF DESCRIPTION OF THE FIGURES

**Fig. 1.** A set of line graphs showing the effect of TLR agonists on the growth of small tumors.  $10^5$  CT26 colon carcinoma cells were implanted into the flank of syngeneic BALB/c mice. When the tumors reached  $\approx 200 \text{ mm}^3$  in volume they were injected with 100  $\mu\text{g}$  of CpG or control ODN and/or 50  $\mu\text{g}$  of 3M-052 or 3M control. Each treatment was repeated 2 days later. Data show the change in tumor volume (mean + SE) of 6-8 mice from 2 independent experiments. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$  compared with the control group

**Fig. 2.** A set of bar graphs showing the effect of TLR agonists on the frequency of tumor infiltrating MDSC, NK and CD8 T cells. Mice were treated as described in Fig. 1. The frequency of tumor-infiltrating MDSC, NK and CD8<sup>+</sup> T cells was determined one day after the second treatment. Results show the mean + SD of each cell type as a percentage of total CD45<sup>+</sup> tumor infiltrating cells analyzed independently in 6 mice from 2 independent experiments. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$

**Fig. 3.** A set of bar graphs showing the effect of TLR agonists on tumor-specific CTL. CT26 tumors were implanted into BALB/c mice as described in Fig 1. Spleen cells and tumor infiltrating lymphocytes were isolated one day after the second treatment, stimulated *ex vivo* with AH-1 peptide, and monitored for IFN $\gamma$  secretion by ELISpot assay. Results represent the mean + SD of 6 mice from 2 independent experiments. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$

**Fig. 4.** A line graph showing the effect of depleting CD8 T cells on immune mediated protection. CT26 tumors were implanted into BALB/c mice and treated with 100  $\mu\text{g}$  of CpG and 50  $\mu\text{g}$  of 3M as described in Fig 1. The effect of depleting CD4 or CD8 T cells on tumor growth was determined as described in the methods section. Data show the change in tumor volume (mean + SE) of 5 mice/group. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$  compared with the control group

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**Fig. 5.** A set of line graphs showing the effect of TLR agonists on large established CT26 tumors.  $10^5$  CT26 colon cancer cells were implanted into the flank of syngeneic BALB/c mice. When the tumors reached  $\approx 800 \text{ mm}^3$  in volume they were injected with 200  $\mu\text{g}$  of CpG or control ODN and/or 100  $\mu\text{g}$  of 3M-052 or 3M control twice weekly for one month. The change in tumor volume of 15 mice/group is shown (mean + SE).

**Fig. 6.** A set of line graphs showing the effect of TLR agonists on large established B16-F10 tumors.  $10^5$  B16-F10 melanoma cancer cells were implanted into the flank of syngeneic C57Bl/6 mice. When the tumors reached  $\approx 500 \text{ mm}^3$  in volume they were injected with 200  $\mu\text{g}$  of CpG or control ODN and/or 100  $\mu\text{g}$  of 3M-052 or 3M control twice weekly for one month. The change in tumor volume of 8 mice/group is shown (mean + SE).

**Figs. 7A-7B.** TLR agonist therapy induces persistent immunity.  $10^5$  CT26 cells were implanted into the flank of syngeneic BALB/c mice. Large established tumors ( $\approx 800 \text{ mm}^3$  in volume) were treated as described in Fig 5. Fig. 7A) Cells from the tumor draining LN were isolated one day after the third treatment, stimulated *ex vivo* with AH-1 peptide, and monitored for  $\text{IFN}\gamma$  secretion by ELISpot assay. Results represent the mean + SD of four independently studied mice/group. Fig. 7B) Mice cured of their CT26 tumors by treatment with CpG ODN plus 3M-052 (a cure being defined as being free of detectable tumor for  $\geq 2$  months after the cessation of therapy) were re-challenged with  $10^6$  CT26 cells. Their survival compared to naive mice challenged with the same tumor dose is shown (N = 6 mice/group). \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ , \*\*\*,  $p < 0.001$

**Fig. 8.** A line graph showing the *in vivo* persistence of 3M-052. Serum levels of 3M-05 and Resiquimod were measured at multiple time points after subcutaneous administration of 1 mg/kg of each agent. Blood was collected before and at various time post-delivery. % maximal serum concentration was calculated by the formula: serum level / maximum serum level X 100%. Results represent the mean of 5 independently studied animals/group.

**Fig. 9.** A set of plots showing the gating strategy used to identify the immune cells. Single cell suspensions were prepared as described in the methods section. Live cells isolated by density gradient centrifugation were stained and analyzed using an LSR-II flow cytometer. The gates used to identify specific cell subpopulations are shown.

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**Fig. 10.** A bar graph showing that TLR agonist therapy does not affect T<sub>reg</sub> frequency.

Mice were treated as described in Fig. 1. The frequency of tumor-infiltrating T<sub>reg</sub> was determined one day after the second treatment by staining for Foxp3<sup>+</sup> cells. Results show the mean + SD of as a percentage of total CD45<sup>+</sup> tumor infiltrating cells analyzed independently in 6 mice from 2 independent experiments.

**Figs. 11A-11B.** A set of survival plots showing the effect of TLR agonists on large established tumors. Survival curves are provided for mice challenged with CT26 colon cancer cells (A) or B16-F10 melanoma cancer cells (B) and treated with 200 µg of CpG or control ODN and/or 100 µg of 3M-052 or 3M control twice weekly for one month as described in Figs 4, 5. \*\*; p <.01 vs all 3 control groups.

### SEQUENCE LISTING

The nucleic and amino acid sequences listed in the accompanying sequence listing are shown using standard letter abbreviations for nucleotide bases, and three letter code for amino acids, as defined in 37 C.F.R. 1.822. Only one strand of each nucleic acid sequence is shown, but the complementary strand is understood as included by any reference to the displayed strand. The Sequence Listing is submitted as an ASCII text file [4239-90644-02\_Sequence\_Listing.txt, June 12, 2014, 15.2 KB], which is incorporated by reference herein.

In the accompanying sequence listing:

SEQ ID NO: 1 is a D-type CpG oligodeoxynucleotide (ODN).

SEQ ID NOS: 2-34 are K-type CpG ODNs.

SEQ ID NOS: 35-36 are control ODNs.

SEQ ID NOS: 37-63 are D-type CpG ODN.

### DETAILED DESCRIPTION OF SEVERAL EMBODIMENTS

Methods for treating a tumor, such as a benign or malignant tumor, are disclosed herein.

The methods are also of use to decrease the size and/or number of metastases.

The methods include administering to the subject a therapeutically effective amount of a TLR-9 agonist, such as a CpG ODN, and a therapeutically effective amount of a TLR-7 and/or TLR-8 agonist, such as an imidazoquinoline compound, such as a lipophilic imidazoquinoline

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compound. These compounds act synergistically to treat the tumor in the subject.

Thus, methods are provided for treating a subject with a tumor that include administering to the subject a therapeutically effective amount of an imidazoquinoline compound and an immunostimulatory CpG oligodeoxynucleotide. In some embodiments, the methods include administering one or more K-type and/or D-type CpG oligodeoxynucleotides (ODNs). In specific non-limiting examples, the imidazoquinoline compound can be N-(4- {[4-amino-2-butyl-1H-imidazo[4,5-c]quinolin-1-yl]oxy}butyl) octadecanamide, also known as 3M-052. In other non-limiting examples, the CpG ODN is a K-type CpG ODN. In further non-limiting examples, the imidazoquinoline compound and an immunostimulatory CpG oligodeoxynucleotide are administered intratumorally.

### *Terms*

**Alkyl:** A saturated or unsaturated monovalent hydrocarbon radical having a number of carbon atoms ranging from one to 30 (e.g., C<sub>1-30</sub> alkyl), which is derived from removing one hydrogen atom from one carbon atom of a parent compound (e.g., alkane, alkene, alkyne). An alkyl group may be branched, straight-chain, or cyclic.

**Alkenyl:** An unsaturated monovalent hydrocarbon radical having a number of carbon atoms ranging from two to 30 (e.g., C<sub>2-30</sub> alkenyl), which has at least one carbon-carbon double bond and is derived from removing one hydrogen atom from one carbon atom of a parent alkene. An alkenyl group may be branched, straight-chain, cyclic, cis, or trans.

**Alkynyl:** A unsaturated monovalent hydrocarbon radical having a number of carbon atoms ranging from two to 30 (e.g., C<sub>2-30</sub> alkynyl), which has at least one carbon-carbon triple bond and is derived from removing one hydrogen atom from one carbon atom of a parent alkyne. An alkynyl group may be branched, straight-chain, or cyclic.

**Animal:** Living multi-cellular vertebrate organisms, a category that includes, for example, mammals and birds. The term mammal includes both human and non-human mammals. Similarly, the term "subject" includes both human and veterinary subjects.

**"C" class oligodeoxynucleotides (ODNs):** ODNs that resemble K ODNs and are composed of only phosphorothioate nucleotides. Typically, C class ODNs have a TCGTCG motif at the 5' end and have a CpG motif imbedded in a palindromic sequence. Backbone modifications like 2'-O-methyl modifications especially in the 5' part of the ODN influence IFN- $\alpha$ -producing capacity of these ODN. C class ODNs have combined properties of D- and K-type CpG ODNs. This class of ODNs stimulates B cells to secrete IL-6 and stimulates plasmacytoid dendritic cells to

produce interferon- $\alpha$ . C class ODNs also induce IP-10 production and strong NK activation.

**CpG or CpG motif:** A nucleic acid having a cytosine followed by a guanine linked by a phosphate bond in which the pyrimidine ring of the cytosine is unmethylated. The term

"methylated CpG" refers to the methylation of the cytosine on the pyrimidine ring, usually

5 occurring at the 5-position of the pyrimidine ring. A CpG motif is a pattern of bases that include an unmethylated central CpG surrounded by at least one base flanking (on the 3' and the 5' side of) the central CpG. Without being bound by theory, the bases flanking the CpG confer a significant part of the activity to the CpG oligodeoxynucleotide. A CpG oligodeoxynucleotide is an

oligodeoxynucleotide that is at least about ten nucleotides in length and includes an unmethylated

10 CpG. CpG oligodeoxynucleotides include both D and K-type oligodeoxynucleotides (see below).

CpG oligodeoxynucleotides are single-stranded. The entire CpG oligodeoxynucleotide can be unmethylated or portions may be unmethylated. In one embodiment, at least the C of the 5' CG 3' is unmethylated.

**Cancer:** A malignant tumor that has undergone characteristic anaplasia with loss of

15 differentiation, increase rate of growth, invasion of surrounding tissue, and is capable of metastasis.

For example, thyroid cancer is a malignant tumor that arises in or from thyroid tissue, and breast cancer is a malignant tumor that arises in or from breast tissue (such as a ductal carcinoma).

Residual cancer is cancer that remains in a subject after any form of treatment given to the subject to reduce or eradicate the cancer. Metastatic cancer is a tumor at one or more sites in the body other  
20 than the site of origin of the original (primary) cancer from which the metastatic cancer is derived.

Cancer includes, but is not limited to, solid tumors.

**Chemotherapy; chemotherapeutic agents:** As used herein, any chemical agent with therapeutic usefulness in the treatment of diseases characterized by abnormal cell growth. Such

diseases include tumors, neoplasms, and cancer as well as diseases characterized by hyperplastic

25 growth such as psoriasis. In one embodiment, a chemotherapeutic agent is an agent of use in

treating neoplasms such as solid tumors. In one embodiment, a chemotherapeutic agent is

radioactive molecule. One of skill in the art can readily identify a chemotherapeutic agent of use

(e.g. see Slapak and Kufe, *Principles of Cancer Therapy*, Chapter 86 in Harrison's Principles of

Internal Medicine, 14th edition; Perry et al., *Chemotherapy*, Ch. 17 in Abeloff, Clinical Oncology

30 2<sup>nd</sup> ed., © 2000 Churchill Livingstone, Inc; Baltzer L., Berkery R. (eds): Oncology Pocket Guide to

Chemotherapy, 2nd ed. St. Louis, Mosby-Year Book, 1995; Fischer DS, Knopf MF, Durivage HJ

(eds): The Cancer Chemotherapy Handbook, 4th ed. St. Louis, Mosby-Year Book, 1993).

Chemotherapeutic agents include those known by those skilled in the art, including but not limited

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to: 5-fluorouracil (5-FU), azathioprine, cyclophosphamide, antimetabolites (such as Fludarabine), antineoplastics (such as Etoposide, Doxorubicin, methotrexate, and Vincristine), carboplatin, cis-platinum and the taxanes, such as taxol. Rapamycin has also been used as a chemotherapeutic.

**Colon cancer:** Cancers, also called large bowel cancers, that include cancerous growths in the colon, rectum and appendix. With 655,000 deaths worldwide per year, it is the third most common form of cancer and the second leading cause of cancer-related death in the Western world. Many colorectal cancers are thought to arise from adenomatous polyps in the colon. These mushroom-like growths are usually benign, but some may develop into cancer over time. The majority of the time, the diagnosis of localized colon cancer is through colonoscopy. Therapy is usually through surgery, which in many cases is followed by chemotherapy. The first symptoms of colon cancer are usually vague, such as bleeding, weight loss, and fatigue (tiredness). Local (bowel) symptoms are rare until the tumor has grown to a large size. Generally, the nearer the tumor is to the anus, the more bowel symptoms are present.

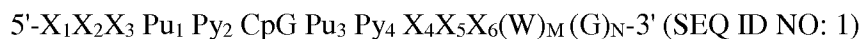
**Cytokine:** Proteins made by cells that affect the behavior of other cells, such as lymphocytes. In one embodiment, a cytokine is a chemokine, a molecule that affects cellular trafficking. Specific non-limiting examples of cytokines are interferon (IFN) $\gamma$ , IL-6, and IL-10.

**D-type Oligodeoxynucleotide (D ODN):** An oligodeoxynucleotide including an unmethylated CpG motif that has a sequence represented by the formula:



wherein the central CpG motif is unmethylated, R is A or G (a purine), and Y is C or T (a pyrimidine). D-type oligodeoxynucleotides include an unmethylated CpG dinucleotide. Inversion, replacement or methylation of the CpG reduces or abrogates the activity of the D oligodeoxynucleotide.

In one embodiment, a D-type CpG ODN is at least about 16 nucleotides in length and includes a sequence represented by Formula III:



wherein the central CpG motif is unmethylated, Pu is a purine nucleotide, Py is a pyrimidine nucleotide, X and W are any nucleotide, M is any integer from 0 to 10, and N is any integer from 4 to 10. Additional detailed description of D ODN sequences and their activities can be found in

Verthelyi et al., *J. Immunol.* 166:2372-2377, 2001, which is herein incorporated by reference.

Generally D ODNs can stimulate a cellular response. In some examples D ODNs can be up to 30, 35, 40, 45 or 50 nucleotides in length. For example, D ODNs stimulate natural killer cells and the maturation of dendritic cells.

**Heteroalkyl/Heteroalkenyl/Heteroalkynyl:** An alkyl, alkenyl, or alkynyl group, respectively, wherein one or more of the carbon atoms are each independently replaced with the one or more heteroatoms selected from oxygen, sulfur, and nitrogen.

**Immune response:** A response of a cell of the immune system, such as a B cell or T cell to a stimulus. In one embodiment, the response is specific for a particular antigen (an “antigen-specific response”). A “parameter of an immune response” is any particular measurable aspect of an immune response, including, but not limited to, cytokine secretion (IL-6, IL-10, IFN $\gamma$ , etc.), immunoglobulin production, dendritic cell maturation, and proliferation of a cell of the immune system. One of skill in the art can readily determine an increase in any one of these parameters, using known laboratory assays. In one specific non-limiting example, to assess cell proliferation, incorporation of  $^3\text{H}$ -thymidine can be assessed. A “substantial” increase in a parameter of the immune response is a significant increase in this parameter as compared to a control. Specific, non-limiting examples of a substantial increase are at least about a 50% increase, at least about a 75% increase, at least about a 90% increase, at least about a 100% increase, at least about a 200% increase, at least about a 300% increase, and at least about a 500% increase. One of skill in the art can readily identify a significant increase using known statistical methods. One, specific, non-limiting example of a statistical test used to assess a substantial increase is the use of a Z test to compare the percent of samples that respond to an imidazoquinoline compound and a K-type CpG ODN as compared to the percent of samples that respond using the another type of ODN, such as a D-type CpG ODN, or as compared to the K-type CpG ODN alone (without the imidazoquinoline compound). A non-parametric ANOVA can be used to compare differences in the magnitude of the response induced by a imidazoquinoline compound a K-type CpG ODN as compared to the percent of samples that respond using the K-type CpG ODN alone or the imidazoquinoline compound alone. In this example,  $p \leq 0.05$  is significant, and indicates a substantial increase in the parameter. One of skill in the art can readily identify other statistical assays of use.

**Isolated:** An “isolated” biological component (such as a nucleic acid, peptide or protein) has been substantially separated, produced apart from, or purified away from other biological components in the cell of the organism in which the component naturally occurs, i.e., other chromosomal and extrachromosomal DNA and RNA, and proteins. Nucleic acids, peptides and proteins which have been “isolated” thus include nucleic acids and proteins purified by standard purification methods. The term also embraces nucleic acids, peptides and proteins prepared by recombinant expression in a host cell as well as chemically synthesized nucleic acids.

**K-Type Oligodeoxynucleotide (K ODN):** An oligodeoxynucleotide including an unmethylated CpG motif that has a sequence represented by the formula:



wherein the central CpG motif is unmethylated, D is T, G or A, W is A or T, and N<sub>1</sub>, N<sub>2</sub>, N<sub>3</sub>, N<sub>4</sub>, N<sub>5</sub>, and N<sub>6</sub> are any nucleotides. In one embodiment, D is a T. Additional description of K-type CpG ODN sequences and their activities can be found in the description below. Generally K-type CpG ODNs can stimulate a humoral response. For example, K-type CpG ODNs stimulate the production of immunoglobulins, such as IgM and IgG. K-type CpG ODNs can also stimulate proliferation of peripheral blood mononuclear cells and increase expression of IL-6 and/or IL-12, amongst other activities. In some embodiments, ad K-type CpG ODN is up to 30, 35, 40, 45, or 50 nucleotides in length.

**Mammal:** This term includes both human and non-human mammals. Similarly, the term “subject” includes both human and veterinary subjects.

**Melanoma:** A form of cancer that originates in melanocytes (cells that make the pigment melanin). Melanocytes are found primarily in the skin, but are also present in the bowel and eye. Melanoma in the skin includes superficial spreading melanoma, nodular melanoma, acral lentiginous melanoma, and lentigo maligna (melanoma). Any of the above types may produce melanin or can be amelanotic. Similarly, any subtype may show desmoplasia (dense fibrous reaction with neurotropism) which is a marker of aggressive behavior and a tendency to local recurrence. Other melanomas include clear cell sarcoma, mucosal melanoma, and uveal melanoma.

Features that affect prognosis are tumor thickness in millimeters (Breslow's depth), depth related to skin structures (Clark level), type of melanoma, presence of ulceration, presence of lymphatic/perineural invasion, presence of tumor infiltrating lymphocytes (if present, prognosis is better), location of lesion, presence of satellite lesions, and presence of regional or distant metastasis.

**Nucleic acid:** A deoxyribonucleotide or ribonucleotide polymer in either single or double stranded form, and unless otherwise limited, encompasses known analogues of natural nucleotides that hybridize to nucleic acids in a manner similar to naturally occurring nucleotides.

**Oligonucleotide or “oligo”:** Multiple nucleotides (i.e. molecules comprising a sugar (e.g. ribose or deoxyribose) linked to a phosphate group and to an exchangeable organic base, which is either a substituted pyrimidine (Py) (e.g. cytosine (C), thymine (T) or uracil (U)) or a substituted purine (Pu) (e.g. adenine (A) or guanine (G)). The term “oligonucleotide” as used herein refers to both oligoribonucleotides (ORNs) and oligodeoxynucleotides (ODNs). The term “oligonucleotide”

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also includes oligonucleosides (i.e. an oligonucleotide minus the phosphate) and any other organic base polymer. Oligonucleotides can be obtained from existing nucleic acid sources (e.g. genomic or cDNA), but are preferably synthetic (e.g. produced by oligonucleotide synthesis).

5 A “stabilized oligonucleotide” is an oligonucleotide that is relatively resistant to *in vivo* degradation (for example via an exo- or endo-nuclease). In one embodiment, a stabilized oligonucleotide has a modified phosphate backbone. One specific, non-limiting example of a stabilized oligonucleotide has a phosphorothioate modified phosphate backbone (wherein at least one of the phosphate oxygens is replaced by sulfur). Other stabilized oligonucleotides include: nonionic DNA analogs, such as alkyl- and aryl- phosphonates (in which the charged phosphonate  
10 oxygen is replaced by an alkyl or aryl group), phosphodiester and alkylphosphotriesters, in which the charged oxygen moiety is alkylated. Oligonucleotides which contain a diol, such as tetraethyleneglycol or hexaethyleneglycol, at either or both termini have also been shown to be substantially resistant to nuclease degradation.

An “immunostimulatory oligodeoxynucleotide,” “immunostimulatory CpG containing  
15 oligodeoxynucleotide,” “CpG ODN,” refers to an oligodeoxynucleotide, which contains a cytosine, guanine dinucleotide sequence and (e.g. has a mitogenic effect or induces cytokine production) vertebrate immune cells. In one embodiment, an immunostimulatory CpG ODN stimulates a parameter of an immune response in a subject. The cytosine, guanine is unmethylated.

An “oligonucleotide delivery complex” is an oligonucleotide associated with (e.g. ionically  
20 or covalently bound to or encapsulated within) a targeting agent (e.g. a molecule that results in a higher affinity binding to a target cell (e.g. B-cell or natural killer (NK) cell) surface and/or increased cellular uptake by target cells). Examples of oligonucleotide delivery complexes include oligonucleotides associated with: a sterol (e.g. cholesterol), a lipid (e.g. cationic lipid, virosome or liposome), or a target cell specific binding agent (e.g. a ligand recognized by a target cell specific  
25 receptor). Preferred complexes must be sufficiently stable *in vivo* to prevent significant uncoupling prior to internalization by the target cell. However, the complex should be cleavable or otherwise accessible under appropriate conditions within the cell so that the oligonucleotide is functional. (Gursel, *J. Immunol.* 167:3324, 2001).

**Pharmaceutical agent or drug:** A chemical compound or composition capable of inducing  
30 a desired therapeutic or prophylactic effect when properly administered to a subject. Pharmaceutical agents include, but are not limited to, chemotherapeutic agents and anti-infective agents.

**Pharmaceutically acceptable carriers:** The pharmaceutically acceptable carriers useful in the methods and compositions disclosed herein are conventional. *Remington's Pharmaceutical Sciences*, by E. W. Martin, Mack Publishing Co., Easton, PA, 15th Edition (1975), describes compositions and formulations suitable for pharmaceutical delivery of the fusion proteins herein disclosed.

In general, the nature of the carrier will depend on the particular mode of administration being employed. For instance, parenteral formulations usually comprise injectable fluids that include pharmaceutically and physiologically acceptable fluids such as water, physiological saline, balanced salt solutions, aqueous dextrose, glycerol or the like as a vehicle. For solid compositions (e.g., powder, pill, tablet, or capsule forms), conventional non-toxic solid carriers can include, for example, pharmaceutical grades of mannitol, lactose, starch, or magnesium stearate. In addition to biologically-neutral carriers, pharmaceutical compositions to be administered can contain minor amounts of non-toxic auxiliary substances, such as wetting or emulsifying agents, preservatives, and pH buffering agents and the like, for example sodium acetate or sorbitan monolaurate.

**Preventing or treating a disease:** "Preventing" a disease refers to inhibiting the full development of a disease, for example in a person who is known to have a predisposition to a disease such as a cancer. An example of a person with a known predisposition is someone with a history of breast cancer in the family, or who has been exposed to factors that predispose the subject to a condition, such as melanoma. "Treatment" refers to a therapeutic intervention that ameliorates a sign or symptom of a disease or pathological condition after it has begun to develop. In several embodiments, treatment refers to a reduction in size of a tumor, a decrease in the number and/or size of metastases, or a decrease in a symptom of the tumor.

**Purified:** The term purified does not require absolute purity; rather, it is intended as a relative term. Thus, for example, a purified peptide preparation is one in which the peptide or protein is more enriched than the peptide or protein is in its natural environment within a cell. Preferably, in a purified preparation, the protein or peptide represents at least 50% of the total peptide or protein content of the preparation. Similarly, in a purified preparation of oligodeoxynucleotides, the oligodeoxynucleotide represents at least 50% of the total nucleic acid content of the preparation.

**Self-complementary nucleic acid sequence:** A nucleic acid sequence that can form Watson-Crick base pairs. The four bases characteristic of deoxyribonucleic acid unit of DNA are the purines (adenine and guanine) and the pyrimidines (cytosine and thymine). Adenine pairs with thymine via two hydrogen bonds, while guanine pairs with cytosine via three hydrogen bonds. If a

nucleic acid sequence includes two or more bases in sequence that can form hydrogen bonds with two or more other bases in the same nucleic acid sequence, then the nucleic acid includes a self-complementary sequence. In several embodiments, a self-complementary nucleic acid sequence includes 3, 4, 5, 6 or more bases that could form hydrogen bonds with 3, 4, 5, 6 or more bases, respectively, of the same nucleic acid sequence.

**Specific binding:** Binding which occurs between such paired species as enzyme/substrate, receptor/agonist, receptor/ligand, antibody/antigen, and lectin/carbohydrate which may be mediated by covalent or non-covalent interactions or a combination of covalent and non-covalent interactions. When the interaction of the two species produces a non-covalently bound complex, the binding that occurs is typically electrostatic, hydrogen-bonding, or the result of lipophilic interactions. Accordingly, "specific binding" occurs between a paired species where there is interaction between the two that produces a bound complex having the characteristics of an antibody/antigen or enzyme/substrate interaction. In particular, the specific binding is characterized by the binding of one member of a pair to a particular species and to no other species within the family of compounds to which the corresponding member of the binding member belongs. Thus, for example, an antibody preferably binds to a single epitope and to no other epitope within the family of proteins. A TLR-9 agonist binds to TLR-9 and not to other TLRs, such as TLR-7 or TLR-8. Similarly, a TLR-7 agonist binds to TLR-7 and not to other TLRs, such as TLR-9.

**Substituted:** A fundamental compound, such as an aryl or aliphatic compound, or a radical thereof, having coupled thereto, typically in place of a hydrogen atom, a second substituent. For example, substituted aryl compounds or substituents may have an aliphatic group coupled to the closed ring of the aryl base, such as with toluene. Again solely by way of example and without limitation, a long-chain hydrocarbon may have a substituent bonded thereto, such as an aryl group, a cyclic group, a heteroaryl group or a heterocyclic group.

**Therapeutically effective dose:** A dose sufficient to prevent advancement, or to cause regression of a disease, or which is capable of relieving symptoms caused by a disease, such as pain.

**Tumor:** An abnormal growth of cells, which can be benign or malignant. Cancer is a malignant tumor, which is characterized by abnormal or uncontrolled cell growth. Other features often associated with malignancy include metastasis, interference with the normal functioning of neighboring cells, release of cytokines or other secretory products at abnormal levels and suppression or aggravation of inflammatory or immunological response, invasion of surrounding or distant tissues or organs, such as lymph nodes, etc. "Metastatic disease" refers to cancer cells that

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have left the original tumor site and migrate to other parts of the body for example *via* the bloodstream or lymph system.

The amount of a tumor in an individual is the “tumor burden” which can be measured as the number, volume, or weight of the tumor. A tumor that does not metastasize is referred to as “benign.” A tumor that invades the surrounding tissue and/or can metastasize is referred to as “malignant.” Examples of hematological tumors include leukemias, including acute leukemias (such as 11q23-positive acute leukemia, acute lymphocytic leukemia, acute myelocytic leukemia, acute myelogenous leukemia and myeloblastic, promyelocytic, myelomonocytic, monocytic and erythroleukemia), chronic leukemias (such as chronic myelocytic (granulocytic) leukemia, chronic myelogenous leukemia, and chronic lymphocytic leukemia), polycythemia vera, lymphoma, Hodgkin's disease, non-Hodgkin's lymphoma (indolent and high grade forms), multiple myeloma, Waldenstrom's macroglobulinemia, heavy chain disease, myelodysplastic syndrome, hairy cell leukemia and myelodysplasia.

Examples of solid tumors, such as sarcomas and carcinomas, include fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, and other sarcomas, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, lymphoid malignancy, pancreatic cancer, breast cancer (including basal breast carcinoma, ductal carcinoma and lobular breast carcinoma), lung cancers, ovarian cancer, prostate cancer, hepatocellular carcinoma, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, medullary thyroid carcinoma, papillary thyroid carcinoma, pheochromocytomas, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, Wilms' tumor, cervical cancer, testicular tumor, seminoma, bladder carcinoma, and CNS tumors (such as a glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, melanoma, neuroblastoma and retinoblastoma). In several examples, a tumor is melanoma, lung cancer, lymphoma breast cancer or colon cancer.

An “established” or “existing” tumor is an existing tumor that can be discerned by diagnostic tests. In some embodiments, and established tumor can be palpated. In some embodiments, and “established tumor” is at least 500 mm<sup>3</sup>, such as at least 600 mm<sup>3</sup>, at least 700 mm<sup>3</sup>, or at least 800 mm<sup>3</sup> in size. In other embodiments, the tumor is at least 1 cm long. With regard to a solid tumor, and established tumor generally has an robust blood supply, and has induced Tregs and myeloid derived suppressor cells (MDSC).

Unless otherwise explained, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure belongs. The singular terms “a,” “an,” and “the” include plural referents unless context clearly indicates otherwise. The term “comprises” means “includes.” Therefore, comprising “A” or “B” refers to including A, including B, or including both A and B. It is further to be understood that all base sizes or amino acid sizes, and all molecular weight or molecular mass values, given for nucleic acids or polypeptides are approximate, and are provided for description. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present disclosure, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including explanations of terms, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

#### *CpG Oligodeoxynucleotides (ODNs)*

Several types of CpG ODN are known in the art and can be used in the methods disclosed herein. These include K-type CpG ODN, D-type CpG ODN, and C ODN.

Combinations of ODNs can also be used, including multiple K-type CpG ODN, multiple D-type CpG ODN, and multiple C-type ODN. In some examples, more than one K-type CpG ODN are administered to the subject. In other examples, more than one D-type CpG ODN are administered to administered to the subject. In yet other examples, more than one C-type ODN are administered to the subject. In further examples, at least one K-type CpG ODN and at least one C-type ODNs are administered to the subject. In some embodiments, a K-type CpG ODN is not administered in combination with a D-type CpG ODN.

#### *A. K-type CpG ODN*

In several embodiments, a K-type CpG ODN or a mixture of K-type CpG ODNs is utilized in the methods disclosed herein. Briefly, the K-type CpG ODN nucleic acid sequences useful in the methods disclosed herein are represented by the formula:



wherein at least one nucleotide separates consecutive CpGs; D is adenine, guanine, or thymidine; Y is cytosine or thymine, N is any nucleotide and  $N_1 + N_2$  is from about 0-26 bases. In one

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embodiment,  $N_1$  and  $N_2$  do not contain a CCGG quadmer or more than one CGG trimer; and the nucleic acid sequence is from about 8-30 bases in length, such as about 10 to 30 nucleotides in length. However, nucleic acids of any size (even many kb long) can be used in the methods disclosed herein if CpGs are present. In one embodiment, synthetic oligonucleotides of use do not include a CCGG quadmer or more than one CCG or CGG trimer at or near the 5' or 3' terminals and/or the consensus mitogenic CpG motif is not a palindrome. A "palindromic sequence" or "palindrome" means an inverted repeat (i.e., a sequence such as ABCDEE'D'C'B'A', in which A and A' are bases capable of forming the usual Watson-Crick base pairs).

In another embodiment, the methods include the use of an ODN which contains a CpG motif represented by the formula:



wherein at least one nucleotide separates consecutive CpGs; RD is selected from the group consisting of GpT, GpG, GpA, ApT and ApA; YT is selected from the group consisting of TpT or CpT; N is any nucleotide and  $N_1 + N_2$  is from about 0-26 bases, such that the ODN is about 8 to 30 nucleotides in length.

In several embodiments, the methods disclosed herein include the use of an effective amount of at least one K-type CpG ODN, wherein the K-type CpG ODNs include an unmethylated CpG motif that has a sequence represented by the formula:



wherein the central CpG motif is unmethylated, D is T, G or A, W is A or T, and  $N_1$ ,  $N_2$ ,  $N_3$ ,  $N_4$ ,  $N_5$ , and  $N_6$  are any nucleotides. In one embodiment, D is a T. The K ODN(s) can be 10 to 30 nucleotides in length. A K ODN can include multiple CpG motifs. In some embodiments, at least one nucleotide separates consecutive CpGs;  $N_3D$  is selected from the group consisting of GpT, GpG, GpA, ApT and ApA;  $WN_4$  is selected from the group consisting of TpT or CpT; N is any nucleotide and  $N_1 + N_2$  is from about 0-26 bases

In one embodiment,  $N_1$ , and  $N_2$  do not contain a CCGG quadmer or more than one CCG or CGG trimer. CpG ODN are also in the range of 8 to 50 bases in length, such as 8 to 30 bases in length, but may be of any size (even many kb long) if sufficient motifs are present. In several examples, the K-type CpG ODN is 10 to 20 nucleotides in length, such as 12 to 18 nucleotides in length. In one embodiment, synthetic ODNs of this formula do not include a CCGG quadmer or more than one CCG or CGG trimer at or near the 5' and/or 3' terminals and/or the consensus CpG motif is not a palindrome. Other CpG ODNs can be assayed for efficacy using methods described

herein. It should be noted that exemplary K-type CpG ODNs are known in the art, and have been fully described, for example in PCT Publication No. WO 98/18810A1, and WO 01/22972, which are incorporated herein by reference. The K type OD can be stabilized.

5 Exemplary K ODN are listed below:

|    |     |                      |                 |
|----|-----|----------------------|-----------------|
|    | K X | ATAATCGACGTTCAAGCAAG | (SEQ ID NO: 3)  |
|    | K22 | CTCGAGCGTTCTC        | (SEQ ID NO:4)   |
|    | K21 | TCTCGAGCGTTCTC       | (SEQ ID NO:5)   |
| 10 | K82 | ACTCTGGAGCGTTCTC     | (SEQ ID NO:6)   |
|    | K30 | TGCAGCGTTCTC         | (SEQ ID NO: 7)  |
|    | k31 | TCGAGGCTTCTC         | (SEQ ID NO:8)   |
|    | K39 | GTCGGCGTTGAC         | (SEQ ID NO:9)   |
|    | K16 | TCGACTCTCGAGCGTTCTC  | (SEQ ID NO:10)  |
| 15 | K3  | ATCGACTCTCGAGCGTTCTC | (SEQ ID NO:11)  |
|    | k23 | TCGAGCGTTCTC         | (SEQ ID NO:12)  |
|    | K40 | GTCGGCGTCGAC         | (SEQ ID NO:13)  |
|    | K34 | GTCGACGTTGAC         | (SEQ ID NO:14)  |
|    | K83 | ACTCTCGAGGGTTCTC     | (SEQ ID NO:15)  |
| 20 | K19 | ACTCTCGAGCGTTCTC     | (SEQ ID NO:16)  |
|    | K73 | GTCGTTCGATGAC        | (SEQ ID NO:17)  |
|    | K46 | GTCGACGCTGAC         | (SEQ ID NO:18)  |
|    | K47 | GTCGACGTCGAC         | (SEQ ID NO:19)  |
|    | K72 | GTCATCGATGCA         | (SEQ ID NO:20)  |
| 25 | K37 | GTCAGCGTCGAC         | (SEQ ID NO:21)  |
|    | k25 | TCGAGCGTTCT          | (SEQ ID NO:22)  |
|    | K82 | ACTCTGGAGCGTTCTC     | (SEQ ID NO: 23) |
|    | K83 | ACTCTCGAGGGTTCTC     | (SEQ ID NO:24)  |
|    | K84 | ACTCTCGAGCGTTCTA     | (SEQ ID NO: 25) |
| 30 | K85 | CATCTCGAGCGTTCTC     | (SEQ ID NO: 26) |
|    | K89 | ACTCTTTCGTTCTC       | (SEQ ID NO: 27) |

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K109 TCGAGCGTTCT (SEQ ID NO: 28)

K123 TCGTTCGTTCTC (SEQ ID NO: 29)

K1555 GCTAGACGTTAGCGT (SEQ ID NO: 30)

K110 TCGAGGCTTCTC (SEQ ID NO: 31)

5 CpG10103 TCGTCGTTTTACGGCGCCGTGCCG (SEQ ID NO: 32)

CpG7909 TCGTCGTTTTGTCGTTTTGTCGTT (SEQ ID NO: 33)

K1826 TCCATGACGTTCTGACGTT (SEQ ID NO: 34)

CONTROL (not immunostimulatory, used for comparisons)

10 K1612 TAGAGCTTAGCTTGC (SEQ ID NO: 35)

K1745 TCCATGAGCTTCCTGAGTCT (SEQ ID NO: 36)

A single K-type CpG ODN can be used in the methods disclosed herein. In some embodiments, the K-type CpG ODN comprises or consists of the nucleic acid sequence set forth as one of SEQ ID NO: 3-34. The K-type CpG ODN can be any ODN listed above, including but not limited to K1555 or K3. However, it is also possible to use mixtures of K-type CpG ODNs having more than one K-type CpG ODN and an imidazoquinoline compound. Exemplary combinations that can be used include 1) K3, K19, K110; 2) K19, K23, K123; K3, 3) K110, K123; 4) K3, K23, K123; 5) K3, K19, K123; and 6) K19, K110, K123. Additional exemplary combinations include at least two different K-type CpG ODNs, wherein one of the K-type CpG ODNs is K1555, and/or wherein one of the K-type CpG ODNs is K3.

#### *B. D-type CpG ODN*

D-type CpG ODNs also can be used in the method disclosed herein. D-type CpG ODNs (also known as "A" class ODNs) differ both in structure and activity from K-type CpG ODNs (also known as "B" class ODNs) and a third type of ODNs, known as "C" class ODNs. For example, as disclosed herein, D-type CpG ODNs stimulate the release of cytokines from cells of the immune system, and induce the maturation of dendritic cells. In specific, non-limiting examples D-type CpG ODNs stimulate the release or production of interferon inducible protein (IP)-10 and IFN- $\alpha$  by monocytes and/or plasmacytoid dendritic cells.

With regard to structure, in one embodiment, a CpG motif in a D-type CpG ODN has been described by the formula:

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5' RY-CpG-RY 3'

wherein the central CpG motif is unmethylated, R is A or G (a purine), and Y is C or T (a pyrimidine). D-type oligonucleotides include an unmethylated CpG dinucleotide. Inversion, replacement or methylation of the CpG reduces or abrogates the activity of the D oligonucleotide.

5 In one embodiment, a D-type CpG ODN is at least about 16 nucleotides in length and includes a sequence represented by the formula:

$$5' X_1X_2X_3 Pu_1 Py_2 CpG Pu_3 Py_4 X_4X_5X_6(W)_M(G)_N-3' \text{ (SEQ ID NO : 1)}$$

wherein the central CpG motif is unmethylated, Pu is a purine nucleotide, Py is a pyrimidine nucleotide, X and W are any nucleotide, M is any integer from 0 to 10, and N is any integer from 4  
10 to 10.

The region  $Pu_1 Py_2 CpG Pu_3 Py_4$  is termed the CpG motif. The region  $X_1X_2X_3$  is termed the 5' flanking region, and the region  $X_4X_5X_6$  is termed the 3' flanking region. If nucleotides are included 5' of  $X_1X_2X_3$  in the D ODN, these nucleotides are termed the 5' far-flanking region. Nucleotides 3' of  $X_4X_5X_6$  in the D ODN are termed the 3' far-flanking region.

15 In one specific, non-limiting example,  $Py_2$  is a cytosine. In another specific, non-limiting example,  $Pu_3$  is a guanidine. In yet another specific, non-limiting example,  $Py_2$  is a thymidine and  $Pu_3$  is an adenine. In a further specific, non-limiting example,  $Pu_1$  is an adenine and  $Py_2$  is a tyrosine. In another specific, non-limiting example,  $Pu_3$  is an adenine and  $Py_4$  is a tyrosine.

In one specific, non-limiting example, N is from about 4 to about 8. In another specific,  
20 non-limiting example, N is about 6.

In several embodiments, the D-type CpG ODN is at least about 16 nucleotides in length. For example, the D-type CpG ODNs can be from about 16 to about 50 nucleotides in length, or from about 18 to about 50 nucleotides in length, or from about 18 to about 40 nucleotides in length, or from about 18 to about 30 nucleotides in length. Exemplary D-type CpG ODNs are disclosed  
25 below. D-type CpG ODNs can include modified nucleotides and/or can be stabilized. For example, modified nucleotides can be included to increase the stability of a D-type CpG ODN.

Without being bound by theory, because phosphothioate-modified nucleotides confer resistance to exonuclease digestion, CpG ODNs are "stabilized" by incorporating phosphothioate-modified nucleotides. In one embodiment, the CpG dinucleotide motif and its immediate flanking  
30 regions include phosphodiester rather than phosphothioate nucleotides. In one specific, non-limiting example, the sequence  $Pu_1 Py_2 CpG Pu_3 Py_4$  includes phosphodiester bases. In another

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specific, non-limiting example, all of the bases in the sequence Pu<sub>1</sub> Py<sub>2</sub> CpG Pu<sub>3</sub> Py<sub>4</sub> are phosphodiester bases. In yet another specific, non-limiting example, X<sub>1</sub>X<sub>2</sub>X<sub>3</sub> and X<sub>4</sub>X<sub>5</sub>X<sub>6</sub>(W)<sub>M</sub>(G)<sub>N</sub> include phosphodiester bases. In yet another specific, non-limiting example, X<sub>1</sub>X<sub>2</sub>X<sub>3</sub> Pu<sub>1</sub> Py<sub>2</sub> CpG Pu<sub>3</sub> Py<sub>4</sub> X<sub>4</sub>X<sub>5</sub>X<sub>6</sub>(W)<sub>M</sub>(G)<sub>N</sub> (SEQ ID NO: 1) include phosphodiester bases. In further non-limiting examples the sequence X<sub>1</sub>X<sub>2</sub>X<sub>3</sub> includes at most one or at most two phosphothioate bases and/or the sequence X<sub>4</sub>X<sub>5</sub>X<sub>6</sub> includes at most one or at most two phosphothioate bases. In additional non-limiting examples, X<sub>4</sub>X<sub>5</sub>X<sub>6</sub>(W)<sub>M</sub>(G)<sub>N</sub> includes at least 1, at least 2, at least 3, at least 4, or at least 5 phosphothioate bases. Thus, a D ODN can be a phosphothioate/phosphodiester chimera.

As disclosed herein, any suitable modification can be used to render a CpG ODN resistant to degradation *in vivo* (for example, via an exo- or endo-nuclease). In one specific, non-limiting example, a modification that renders the oligodeoxynucleotide less susceptible to degradation is the inclusion of nontraditional bases such as inosine and quesine, as well as acetyl-, thio- and similarly modified forms of adenine, cytidine, guanine, thymine, and uridine. Other modified nucleotides include nonionic DNA analogs, such as alkyl or aryl phosphonates (i.e., the charged phosphonate oxygen is replaced with an alkyl or aryl group, as set forth in U.S. Patent No. 4,469,863), phosphodiester and alkylphosphotriesters (i.e., the charged oxygen moiety is alkylated, as set forth in U.S. Patent No. 5,023,243 and European Patent No. 0 092 574). Oligonucleotides containing a diol, such as tetraethyleneglycol or hexaethyleneglycol, at either or both termini, have also been shown to be more resistant to degradation. The CpG ODNs can also be modified to contain a secondary structure (e.g., stem-loop structure). Without being bound by theory, it is believed that incorporation of a stem-loop structure renders an oligodeoxynucleotide more effective.

In a further embodiment, Pu<sub>1</sub> Py<sub>2</sub> and Pu<sub>3</sub> Py<sub>4</sub> are self-complementary. In another embodiment, X<sub>1</sub>X<sub>2</sub>X<sub>3</sub> and X<sub>4</sub>X<sub>5</sub>X<sub>6</sub> are self-complementary. In yet another embodiment X<sub>1</sub>X<sub>2</sub>X<sub>3</sub> Pu<sub>1</sub> Py<sub>2</sub> and Pu<sub>3</sub> Py<sub>4</sub> X<sub>4</sub>X<sub>5</sub>X<sub>6</sub> are self-complementary. Specific non-limiting examples of a D-type CpG ODN wherein Pu<sub>1</sub> Py<sub>2</sub> and Pu<sub>3</sub> Py<sub>4</sub> are self-complementary include, but are not limited to, ATCGAT, ACCCGGT, ATCGAC, ACCCGAT, GTCGAC, or GCCCGGC (wherein the CpG is underlined). Thus, in one non-limiting example, the D-type CpG ODN includes the motif X<sub>1</sub>X<sub>2</sub>X<sub>3</sub> ACCCGGT X<sub>4</sub>X<sub>5</sub>X<sub>6</sub> (SEQ ID NO: 37), wherein X<sub>1</sub>X<sub>2</sub>X<sub>3</sub> AT and AT X<sub>4</sub>X<sub>5</sub>X<sub>6</sub> are self-complementary. In another non-limiting example, the D-type CpG ODN includes the motif X<sub>1</sub>X<sub>2</sub>X<sub>3</sub> ATCGAT X<sub>4</sub>X<sub>5</sub>X<sub>6</sub> (SEQ ID NO: 38), wherein X<sub>1</sub>X<sub>2</sub>X<sub>3</sub> AT and AT X<sub>4</sub>X<sub>5</sub>X<sub>6</sub> are self-complementary.

Without being bound by theory, the self-complementary base sequences can help to form a stem-loop structure with the CpG dinucleotide at the apex to facilitate immunostimulatory

functions. Thus, in one specific, non-limiting example, D-type CpG ODNs wherein Pu<sub>1</sub> Py<sub>2</sub> and Pu<sub>3</sub> Py<sub>4</sub> are self-complementary induce higher levels of IFN- $\gamma$  production from a cell of the immune system. The self-complementarity need not be limited to Pu<sub>1</sub> Py<sub>2</sub> and Pu<sub>3</sub> Py<sub>4</sub>. Thus, in another embodiment, additional bases on each side of the three bases on each side of the CpG-containing hexamer form a self-complementary sequence (see above).

One specific, non-limiting example of a sequence wherein Pu<sub>1</sub> Py<sub>2</sub> and Pu<sub>3</sub> Py<sub>4</sub> are self-complementary but wherein the far-flanking sequences are not self-complementary is

GGTGCATCGATACAGGGGGG (DV113, SEQ ID NO: 39, see the Table below)

This oligodeoxynucleotide has a far-flanking region that is not self-complementary and induces high levels of IFN- $\gamma$  and IFN- $\alpha$ .

Another specific, non-limiting example of a D-type CpG ODN is:

GGTGCGTCGATGCAGGGGGG (DV28, SEQ ID NO: 40, see the Table below)

This D-type CpG ODN is of use for inducing production and/or release of cytokines from immune cells, although it lacks a self-complementary motif.

In one embodiment, the D-type CpG ODNs are at least about 16 nucleotides in length. In a second embodiment, a D-type CpG ODN is at least about 18 nucleotides in length. In another embodiment, a D-type CpG ODN is from about 16 nucleotides in length to about 100 nucleotides in length. In yet another embodiment, a D-type CpG ODN is from about 16 nucleotides in length to about 50 nucleotides in length. In a further embodiment, a D-type CpG ODN is from about 18 nucleotides in length to about 30 nucleotides in length.

In another embodiment, the D-type CpG ODN is at least 18 nucleotides in length, and at least two G's are included at the 5' end of the molecule, such that the oligodeoxynucleotide includes a sequence represented by the formula:

5' GGX<sub>1</sub>X<sub>2</sub>X<sub>3</sub> Pu<sub>1</sub> Py<sub>2</sub> CpG Pu<sub>3</sub> Py<sub>4</sub> X<sub>4</sub>X<sub>5</sub>X<sub>6</sub>(W)<sub>M</sub>(G)<sub>N</sub>-3' (SEQ ID NO : 41).

The D-type CpG ODN can include additional G's at the 5' end of the oligodeoxynucleotide. In one specific example, about 1 or about 2 G's are included at the 5' end of an oligodeoxynucleotide including a sequence as set forth as the above formula.

Examples of a D-type CpG ODN include, but are not limited to the sequence shown in the following table:

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|    | ODN   | SEQUENCE                              | SEQUENCE IDENTIFIER |
|----|-------|---------------------------------------|---------------------|
|    | DV113 | GGT <u>GCATCGATACAGGGGGG</u>          | (SEQ ID NO: 39)     |
|    | DV28  | GGT <u>GC</u> <b>GT</b> CGATGCAGGGGGG | (SEQ ID NO: 40)     |
|    | DV104 | GGT <u>GCATCGATGCAGGGGGG</u>          | (SEQ ID NO: 41)     |
| 5  | DV19  | GGT <u>GCATCGATGCAGGGGGG</u>          | (SEQ ID NO: 41)     |
|    | DV35  | GGT <u>GCATCGATGCAGGGGGG</u>          | (SEQ ID NO: 41)     |
|    | DV29  | GGTGCACCG <b>GT</b> GCAGGGGGG         | (SEQ ID NO: 42)     |
|    | DV106 | GGTGT <b>G</b> TCGATGCAGGGGGG         | (SEQ ID NO: 43)     |
|    | DV116 | TGCATCGATGCAGGGGGG                    | (SEQ ID NO: 44)     |
| 10 | DV34  | GGT <u>GCATCGATGCAGGGGGG</u>          | (SEQ ID NO: 45)     |
|    | DV102 | GGT <u>GCATCGTTGCAGGGGGG</u>          | (SEQ ID NO: 46)     |
|    | DV32  | GGT <u>GC</u> <b>GT</b> CGACGCAGGGGGG | (SEQ ID NO: 47)     |
|    | DV117 | GGT <u>TCGATCGATGCACGGGGG</u>         | (SEQ ID NO: 48)     |
|    | DV37  | GGTGC <b>A</b> TCGATGCAGGGGGG         | (SEQ ID NO: 49)     |
| 15 | DV25  | GGTGCATCGATGCAGGGGGG                  | (SEQ ID NO: 49)     |
|    | DV30  | GGTGCATCGACGCAGGGGGG                  | (SEQ ID NO: 50)     |
|    | dv120 | GGT <u>GCATCGATAGGCGGGGGG</u>         | (SEQ ID NO: 51)     |
|    | DV27  | GGTGCACCG <b>AT</b> GCAGGGGGG         | (SEQ ID NO: 52)     |
|    | dv119 | CCTGCATCGATGCAGGGGGG                  | (SEQ ID NO: 53)     |
| 20 | D142  | GGTATATCGATATAGGGGGG                  | (SEQ ID NO: 54)     |
|    | d143  | GGTGGAT <b>C</b> GATCCAGGGGGG         | (SEQ ID NO: 55)     |

Underlined bases are phosphodiester. Bold indicates self-complementary sequences. The corresponding sequence identifier is noted. Note that "DV" can also be abbreviated as "D."

25 Examples of a D-type CpG ODN also include, but are not limited to:

5'NNTGCATCGATGCAGGGGGG 3' (SEQ ID NO: 56)

5'NNTGCACCGGTGCAGGGGGG3' (SEQ ID NO: 57),

5'NNTGCGTCGACGCAGGGGGG3' (SEQ ID NO: 58),

5'NNTGCGTCGATGCAGGGGGG3' (SEQ ID NO: 59),

30 5'NNTGCGCCGGCGCAGGGGGG3' (SEQ ID NO: 60),

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5'NNTGCGCCGATGCAGGGGGG3' (SEQ ID NO: 61),

5'NNTGCATCGACGCAGGGGGG3' (SEQ ID NO: 62),

5'NNTGCGTCGGTGCAGGGGGG3' (SEQ ID NO: 63),

wherein N is any base, or is no base at all. In one specific, non-limiting example, N is a G.

5 Additional exemplary D ODN sequences can be found in U.S. Patent No. 6,977,245 and in Verthelyi et al., *J. Immunol.* 166:2372-2377, 2001, which are both herein incorporated by reference in their entireties. Thus, in some embodiments, the D-type CpG ODN includes, or consists of, the nucleic acid sequence set forth as one of SEQ ID NO: 39-63. The D-type CpG ODN can be any ODN listed above, including but not limited to DV35, DV19, DV28 or DV29.

10 D-type CpG ODN can be used in combination. Thus, multiple D-type CpG ODNs can be utilized in the methods disclosed herein. For example, two, three, four, five or more D-type CpG ODNs can be utilized to induce an immune response. In addition, a single ODN can be generated that includes the two or more D-type CpG motifs disclosed herein. Thus, DV35, DV19, DV28, DV29 or DV113, or two, three, four or five of these ODNs can be used in combination. In another  
15 example, DV35, DV29 and DV19 can be used in combination. Additional exemplary combinations include at least two different D-type CpG ODNs, wherein one of the D-type CpG ODNs is DV35, and/or wherein one of the D-type CpG ODNs is DV28. D-type and K-type CpG ODNs can also be used in combination.

### 20 C. C-type CpG ODN and Modifications

C-type ODNs also can be utilized in the methods disclosed herein. Typically, C class ODNs have a TCGTCG motif at the 5' end and have a CpG motif imbedded in a palindromic sequence. M362 is an exemplary C-type CpG ODN that contains a 5'-end 'TCGTCG-motif' and a 'GTCGTT-motif'. C-type ODNs resemble K-type as they are composed entirely of phosphorothioate  
25 nucleotides, but resemble D-type in containing palindromic CpG motifs. This class of ODNs stimulates B cells to secrete IL-6 and pDCs to produce IFN- $\alpha$  (see Hartmann et al., *Eur. J. Immunol.* 33: 1633-41, 2003, incorporated herein by reference). A palindromic sequence of at least 8 nucleotides increases activity, for example a palindrome of at least 12, such as 14, 16, 18 or 20 nucleotides, increases activity. In some embodiments, the CpG-C ODNs include one to two TCG  
30 trinucleotides at or close to the 5' end of the ODN and a palindromic region of at least 10-12 bases, which contains at least two additional CG dinucleotides preferably spaced zero to three bases apart. The CG dinucleotides in the palindrome are preferably spaced 1, 2, or 3 nucleotides apart, although

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sequences with four nucleotide spacings retained low levels of IFN- $\alpha$ -inducing activity (see Marshall et al., J. Leukocyte Biol. 73: 781-792, 2003, incorporated herein by reference). C-type ODNs are present in both early and late endosomes, and thus express properties in common with both K- and D-type CpG ODNs. C-type CpG ODNs include ODN2216, ODN M362, ODN 1668, and ODN2395, which are available from Invivogen and C274, see also Marshall et al., *supra*.

As noted above, any of the classes of CpG ODN (K, D and C-type ODNs) can be stabilized. In one embodiment, the stabilized oligodeoxynucleotide has a modified phosphate backbone. One specific, non-limiting example of a stabilized oligonucleotide has a phosphorothioate modified phosphate backbone (wherein at least one of the phosphate oxygens is replaced by sulfur). Other stabilized oligonucleotides include: nonionic DNA analogs, such as alkyl- and aryl- phosphonates (in which the charged phosphonate oxygen is replaced by an alkyl or aryl group), phosphodiester and alkylphosphotriesters, in which the charged oxygen moiety is alkylated. Oligonucleotides which contain a diol, such as tetraethyleneglycol or hexaethyleneglycol, at either or both termini have also been shown to be substantially resistant to nuclease degradation.

CpG ODN can be synthesized *de novo* using any of a number of procedures well known in the art. For example, the oligodeoxynucleotides can be synthesized as set forth in U.S. Patent No. 6,194,388, which is herein incorporated by reference in its entirety.

ODNs can be synthesized using any methods known to those of skill in the art. Automated synthesis of ODNs is routine. An ODN can be synthesized using, for example, the B-cyanoethyl phosphoramidite method or nucleoside H-phosphonate method. These chemistries can be performed by a variety of automated oligonucleotide synthesizers available in the market. Alternatively, oligodeoxynucleotides can be prepared from existing nucleic acid sequences (e.g. genomic or cDNA) using known techniques, such as employing restriction enzymes, exonucleases or endonucleases, although this method is less efficient than direct synthesis.

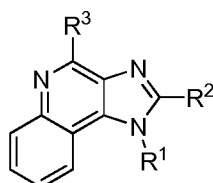
### *Imidazoquinoline Compounds*

Imidazoquinolines are of use in the methods disclosed herein. Imidazoquinolines are synthetic immunomodulatory drugs that act by binding toll-like receptors 7 and 8 (TLR7/TLR8) on dendritic cells, structurally mimicking these receptors' natural ligand, viral single-stranded RNA. Imidazoquinolines are heterocyclic compounds comprising a fused quinoline-imidazole skeleton. Derivatives, salts (including hydrates, solvates, and N-oxides), and prodrugs thereof also are contemplated by the present disclosure. Particular imidazoquinoline compounds are known in the

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art, see for example, U.S. Patent No. 6,518,265; and U.S. Patent No. 4,689,338. In some embodiments, the imidazoquinoline compound is not imiquimod and/or is not resiquimod. In additional embodiments, the imidazoquinoline compound is a lipophilic imidazoquinoline compound.

5 Certain embodiments of the compound may have a formula as illustrated below:



(Formula I)

With reference to Formula I, R<sup>1</sup> may be selected from hydrogen, C<sub>12-24</sub> alkyl, C<sub>11-24</sub> heteroalkyl, substituted C<sub>1-10</sub> heteroalkyl, carboxyl, and hydroxyl; R<sup>2</sup> may be selected from hydrogen, C<sub>1-10</sub> alkyl, C<sub>1-10</sub> heteroalkyl, and substituted C<sub>1-10</sub> alkyl; and R<sup>3</sup> may be selected from hydrogen, amino, aminoacyl, hydroxyl, and C<sub>1-10</sub> alkoxy.

In particular disclosed embodiments, R<sup>1</sup> may be C<sub>11-24</sub> heteroalkyl, such as C<sub>11-24</sub> alkoxy (e.g., -OC<sub>11-24</sub> alkyl), C<sub>11-24</sub> thioalkyl (e.g., -SC<sub>11-24</sub> alkyl), and C<sub>11-24</sub> aminoalkyl (e.g., -NR<sup>5</sup>C<sub>11-24</sub> alkyl, wherein R<sup>5</sup> is selected from hydrogen, alkyl, cycloalkyl, aryl, and the like). In other disclosed embodiments, R<sup>1</sup> may be C<sub>1-10</sub> heteroalkyl substituted with one or more substituents selected from amino (e.g., N(R<sup>5</sup>)<sub>2</sub>, wherein each R<sup>5</sup> independently is selected from hydrogen, alkyl, cycloalkyl, aryl, and the like), aminoacyl (e.g., -NC(O)C<sub>1-30</sub> alkyl, -NC(O)C<sub>1-23</sub> alkyl; such as -NC(O)C<sub>11-23</sub> alkyl; -NC(O)C<sub>15-23</sub> alkyl; -NC(O)C<sub>15-19</sub> alkyl, and -NC(O)C<sub>17</sub> alkyl), aminocarbonylamino (e.g., -NC(O)NR<sup>5</sup>C<sub>1-30</sub> alkyl, -NC(O)NR<sup>5</sup>C<sub>1-23</sub> alkyl, such as -NC(O)NR<sup>5</sup>C<sub>11-23</sub> alkyl, -NC(O)NR<sup>5</sup>C<sub>15-23</sub> alkyl, -NC(O)NR<sup>5</sup>C<sub>15-19</sub> alkyl, and -NC(O)NR<sup>5</sup>C<sub>17</sub> alkyl), aminocarbonyloxy (e.g., -NC(O)OC<sub>1-30</sub> alkyl, -NC(O)OC<sub>1-23</sub> alkyl, such as -NC(O)OC<sub>11-23</sub> alkyl, -NC(O)OC<sub>15-23</sub> alkyl, -NC(O)OC<sub>15-19</sub> alkyl, and -NC(O)OC<sub>17</sub> alkyl), ether (e.g., -OC<sub>1-30</sub> alkyl, -OC<sub>1-23</sub> alkyl, -OC<sub>11-23</sub> alkyl, -OC<sub>15-23</sub> alkyl, -OC<sub>15-19</sub> alkyl, and -OC<sub>17</sub> alkyl), ester (e.g., -OC(O)C<sub>1-30</sub> alkyl, -OC(O)C<sub>1-23</sub> alkyl, such as -OC(O)C<sub>11-23</sub> alkyl, -OC(O)C<sub>15-23</sub> alkyl, -OC(O)C<sub>15-19</sub> alkyl, and -OC(O)C<sub>17</sub> alkyl), aldehyde (e.g., -OC(O)H), carboxyl (e.g., -OC(O)H), thioether (e.g., -SC<sub>1-30</sub> alkyl, -SC<sub>1-23</sub> alkyl, such as -SC<sub>11-23</sub> alkyl, -SC<sub>15-23</sub> alkyl, -SC<sub>15-19</sub> alkyl, and -SC<sub>17</sub> alkyl), and thioester (e.g., -SC(O)C<sub>1-30</sub> alkyl, -SC(O)C<sub>1-23</sub> alkyl, -SC(O)C<sub>11-23</sub> alkyl, -SC(O)C<sub>15-23</sub> alkyl, -SC(O)C<sub>15-19</sub> alkyl, and -SC(O)C<sub>17</sub> alkyl).

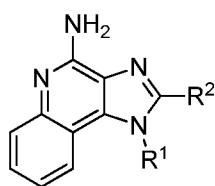
In particular disclosed embodiments, R<sup>1</sup> is -X-Y-Z-R<sup>6</sup> wherein X is selected from the group consisting of a bond, -O-, and -NH-; Y is selected from the group consisting of C<sub>1-10</sub>alkyl and C<sub>1-</sub>

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5alkylOC<sub>1-5</sub>alkyl; Z is selected from the group consisting of -NHC(O)-, -NHS(O)<sub>2</sub>-, -NHC(O)NH-; and R<sup>6</sup> is -C<sub>11-23</sub>alkyl. In particular disclosed embodiments, Y is C<sub>2-10</sub>alkyl or Y is C<sub>2-4</sub>alkylOC<sub>2-4</sub>alkyl, with certain embodiments being C<sub>2-5</sub>alkyl. X is selected from a group consisting of a bond and -O- in certain embodiments, with particular disclosed embodiments having X as -O-. In certain  
 5 embodiments, Z is -NHC(O)-. Particular disclosed compounds may have an R<sup>6</sup> substituent that is C<sub>15-23</sub>alkyl, more typically R<sup>6</sup> is C<sub>15</sub>-C<sub>19</sub>alkyl.

In particular disclosed embodiments, R<sup>1</sup> is selected from the group consisting of –  
 CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>NHC(O)C<sub>11-23</sub>alkyl, –OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>NHC(O)C<sub>11-23</sub>alkyl, –  
 NHCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>NHC(O)C<sub>11-23</sub>alkyl, –CH<sub>2</sub>CH<sub>2</sub>OCH<sub>2</sub>CH<sub>2</sub>NHC(O)C<sub>11-23</sub>alkyl. More typically,  
 10 R<sup>1</sup> is selected from the group consisting of  
 –CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>NHC(O)C<sub>15-23</sub>alkyl,  
 –OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>NHC(O)C<sub>15-23</sub>alkyl, –NHCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>NHC(O)C<sub>15-23</sub>alkyl,  
 –CH<sub>2</sub>CH<sub>2</sub>OCH<sub>2</sub>CH<sub>2</sub>NHC(O)C<sub>15-23</sub>alkyl.

In another disclosed embodiment, the imidazoquinoline compound has the formula:



wherein, R<sup>1</sup> is selected from –C<sub>12-24</sub>alkyl, –OC<sub>11-24</sub>alkyl, –NHC<sub>11-24</sub>alkyl, and –X-Y-Z-R<sup>6</sup>, wherein

X is selected from the group consisting of a bond, -O-, and -NH-;

Y is selected from the group consisting of C<sub>1-10</sub>alkyl and C<sub>1-5</sub>alkylOC<sub>1-5</sub>alkyl

20 Z is selected from the group consisting of:

-NHC(O) -,

-NHS(O)<sub>2</sub> -,

-NHC(O)NH-; and

R<sup>6</sup> is -C<sub>11-23</sub>alkyl;

25 R<sup>2</sup> is selected from the group consisting of hydrogen, alkyl, alkylaminoalkyl, alkoxyalkyl, and hydroxyalkyl.

In particular disclosed embodiments, R<sup>2</sup> may be C<sub>1-10</sub>alkyl, such as methyl, ethyl, propyl, butyl, pentyl, hexyl, heptyl, octyl, and the like. In other disclosed embodiments, R<sup>2</sup> may be C<sub>1-10</sub>alkyl substituted with one or more substituents selected from halogen (e.g., chloro, iodo, bromo, fluoro), trihaloalkyl (e.g., trifluoromethyl), aryl (e.g., C<sub>6-14</sub> aryl), heteroaryl (e.g., C<sub>6-14</sub> aryl wherein  
 30 one or more carbon atoms is replaced with a heteroatom selected from oxygen, sulfur, and

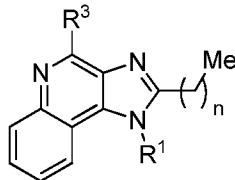
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nitrogen), amino (e.g.,  $-\text{N}(\text{R}^5)_2$ , wherein each  $\text{R}^5$  independently is selected from hydrogen, alkyl, cycloalkyl, aryl, and the like), aminoacyl (e.g.,  $-\text{NC}(\text{O})\text{C}_{1-10}$  alkyl), aminocarbonylamino (e.g.,  $-\text{NC}(\text{O})\text{NR}^5\text{C}_{1-10}$  alkyl), aminocarbonyloxy (e.g.,  $-\text{NC}(\text{O})\text{OC}_{1-10}$  alkyl), ether (e.g.,  $-\text{OC}_{1-10}$  alkyl), ester (e.g.,  $-\text{OC}(\text{O})\text{C}_{1-10}$  alkyl), hydroxyl ( $-\text{OH}$ ), aldehyde (e.g.,  $-\text{OC}(\text{O})\text{H}$ ), carboxyl (e.g.,  $-\text{OC}(\text{O})\text{H}$ ), thioether (e.g.,  $-\text{SC}_{1-10}$  alkyl), and thioester (e.g.,  $-\text{SC}(\text{O})\text{C}_{1-10}$  alkyl).

In particular embodiments,  $\text{R}^2$  is selected from the group consisting of hydrogen, alkyl, alkylaminoalkyl, alkoxyalkyl, and hydroxyalkyl. For example,  $\text{R}^2$  may be selected from hydrogen, methyl, ethyl, propyl, butyl, ethoxymethyl, methoxymethyl, 2-methoxyethyl, hydroxymethyl, and 2-hydroxyethyl. In certain embodiments,  $\text{R}^2$  is selected from the group consisting of ethyl, propyl, butyl, methoxyethyl, and ethoxymethyl. In exemplary embodiments,  $\text{R}^2$  is selected from the group consisting of butyl and ethoxymethyl.

In particular disclosed embodiments,  $\text{R}^3$  may be amine ( $-\text{NH}_2$ ), amino (e.g.,  $-\text{N}(\text{R}^5)_2$ , wherein each  $\text{R}^5$  independently is selected from hydrogen, alkyl, cycloalkyl, aryl, heterocyclyl, heteroaryl, and the like), or aminoacyl (e.g.,  $-\text{NC}(\text{O})\text{R}^6$  wherein  $\text{R}^6$  is selected from  $\text{C}_{1-10}$  alkyl,  $\text{C}_{1-10}$  haloalkyl, hydrogen,  $\text{C}_{6-14}$  aryl, and the like).

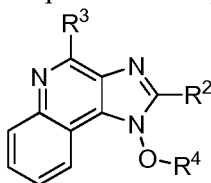
In other embodiments, the imidazoquinoline compound has a formula:



(Formula II)

wherein  $n$  ranges from zero to 10; and  $\text{R}^1$  and  $\text{R}^3$  may be selected from any of the particular groups recited above for Formula I.

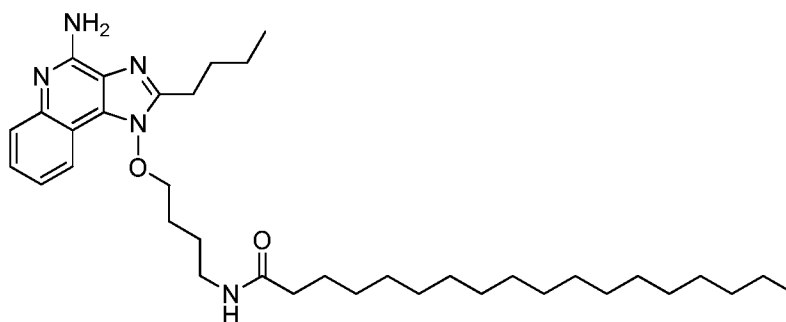
In further embodiments, the imidazoquinoline compound has a formula:



(Formula III)

wherein  $\text{R}^2$  and  $\text{R}^3$  may be selected from any of the particular groups recited above for Formula I, and  $\text{R}^4$  may be selected from  $\text{C}_{1-10}$  alkyl substituted with one or more of the substituents provided for substituted heteroalkyl, such as those provided for  $\text{R}^1$  in Formula I, above.

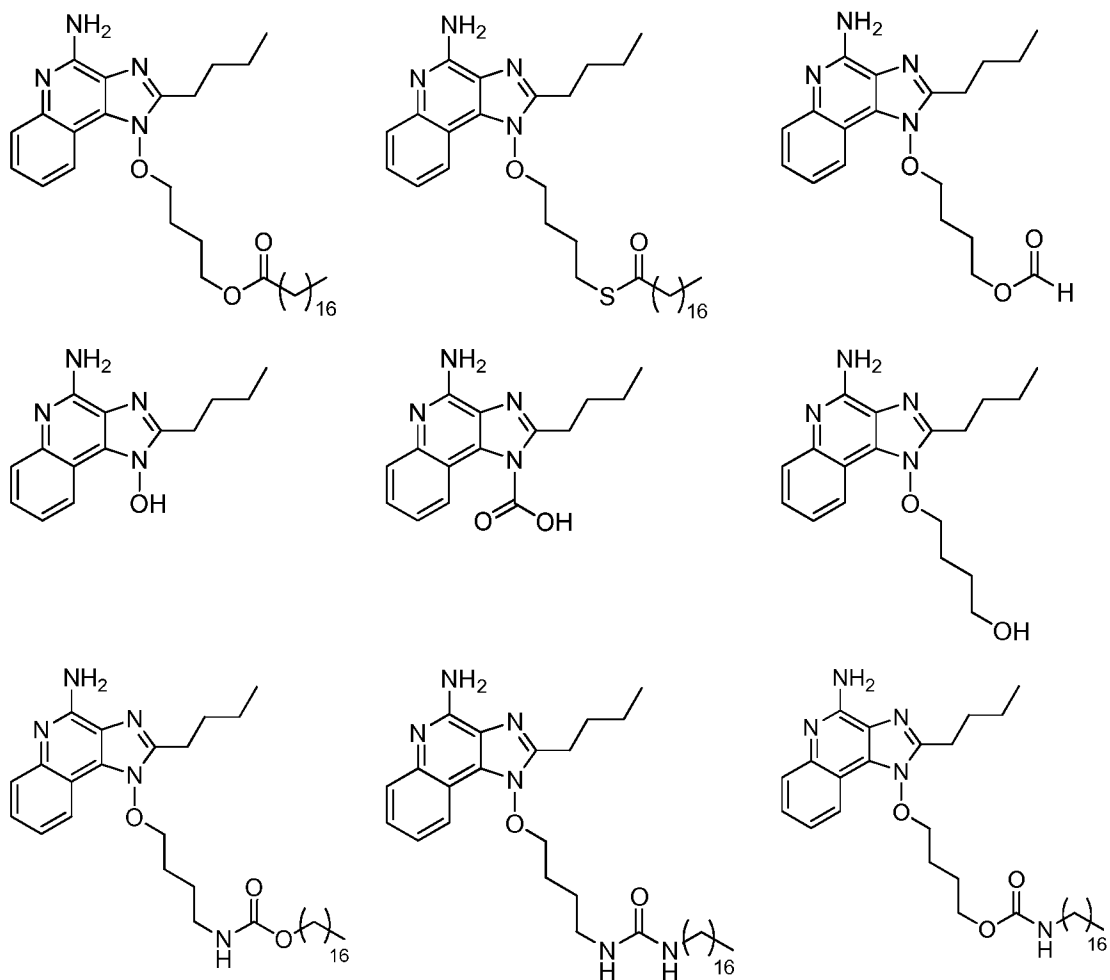
In further embodiments the imidazoquinoline compound is:



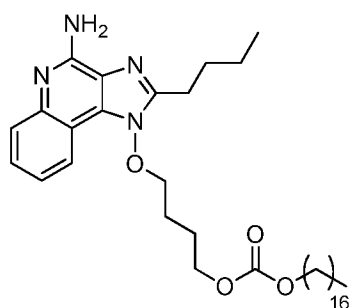
(Formula IV)

(i.e., N-(4- {[4-amino-2-butyl-1H-imidazo[4,5-c]quinolin-1-yl]oxy}butyl) octadecanamide, also known as 3M-052).

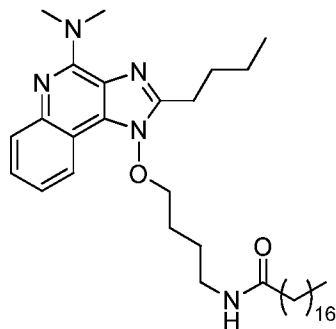
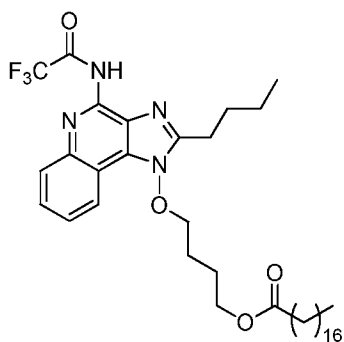
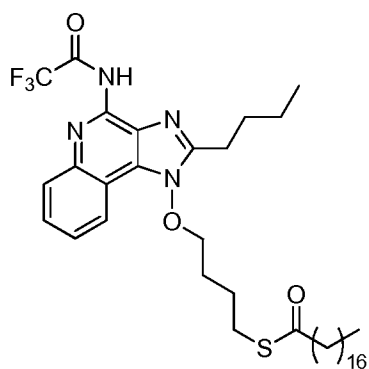
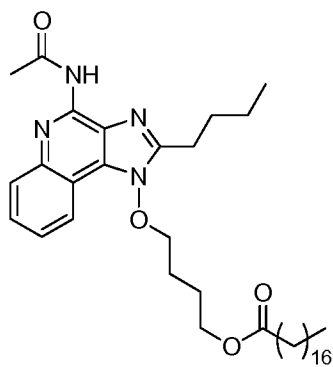
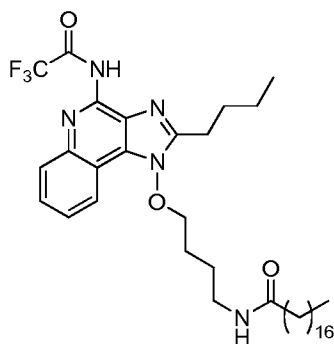
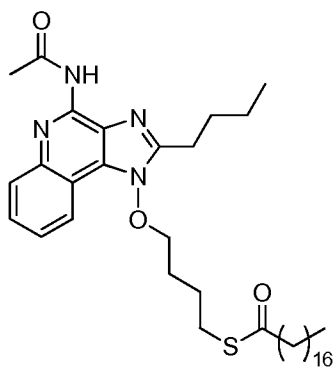
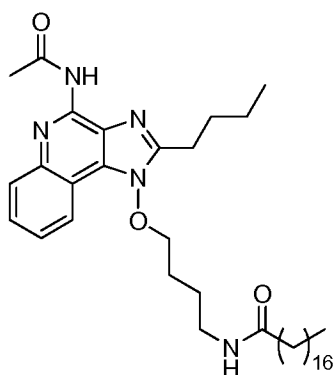
- 5 Other exemplary imidazoquinoline compounds include the following:



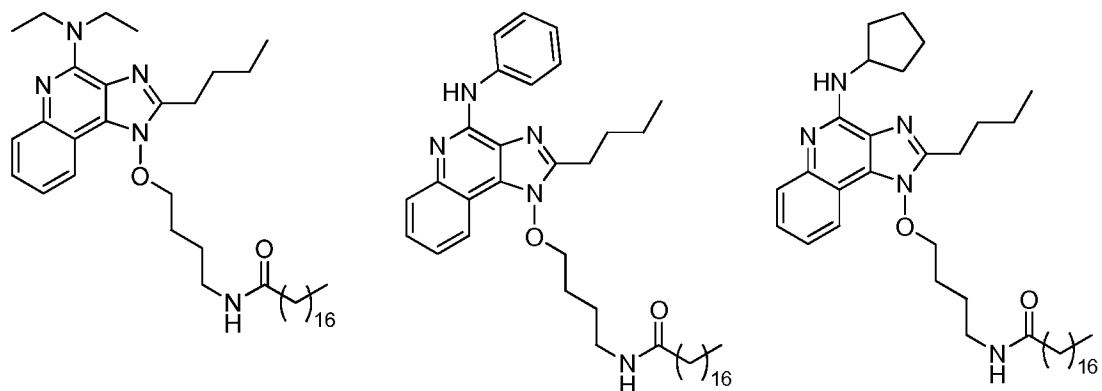
- 29 -



In other disclosed embodiments, the imidazoquinoline compounds can be selected from the following compounds:



- 30 -



This imidazoquinoline compound can be used in any of its pharmaceutically acceptable forms including solid, semi-solid, solvate (e.g., hydrate), wholly or partially dissolved (such as in a pharmaceutical composition), a prodrug, or dispersed in a pharmaceutically acceptable carrier. Any pharmaceutically acceptable salt of the imidazoquinoline compound can also be used, see PCT

5 Publication No. WO 2012/024284, which is incorporated herein by reference. Additional compounds are disclosed for example, in U.S. Patent No. 7,799,800, which is incorporated herein by reference.

In particular disclosed embodiments, the pharmaceutically acceptable salt may be selected from any suitable salt known in the art, such as (but not limited to) salts of organic and inorganic

10 counter ions and salts of organic or inorganic acids. In particular disclosed embodiments, the pharmaceutically acceptable salt may be a hydrochloride, hydrobromide, hydroiodide, nitrate, sulfate, bisulfate, phosphate, acetate, lactate, salicylate, citrate, tartrate, bitartrate, ascorbate, succinate, ammonium, potassium, sodium, calcium, magnesium, maleate, fumarate, gluconate, glucaronate, saccharate, formate, benzoate, glutamate, mesylate, tosylate, or besylate salt. One or

15 more of the functional groups provided in any one of Formulas I-III may be manipulated to provide the desired pharmaceutically acceptable salt. Methods of forming pharmaceutically acceptable salts are readily recognized by a person of ordinary skill in the art.

Also disclosed herein are prodrugs of the disclosed compounds. Exemplary prodrug moieties that may be used to functionalize one or more of the functional groups provided in any one

20 of Formulas I-III include, but are not limited to, carbonates, esters, amides, carbamates, oximes, imines, phosphates, and ethers. Methods of forming prodrugs are readily recognized by a person of ordinary skill in the art.

*Pharmaceutical Compositions and Methods of Use*

Methods are disclosed herein for producing an immune response to a tumor in a subject. Methods are also provided for treating a tumor in a subject. In some embodiments, the methods include treating an existing tumor in a subject. In additional embodiments, methods are disclosed  
5 herein for preventing conversion of a benign to a malignant lesion, or preventing metastasis in a subject. In some examples, the methods reduce a symptom of the tumor in the subject. In additional examples, the tumor is a solid tumor.

Generally, the methods include selecting a subject having a tumor, such as a benign or malignant tumor, and administering to the subject a therapeutically effective amount of (1) an  
10 imidazoquinoline compound and (2) a CpG ODN, such as K-type CpG ODN, D-type CpG ODN, C-type CpG ODN, or a combination thereof. The methods are of use for treating the tumor, preventing metastasis and/or preventing the conversion of a benign to a malignant tumor. The administration can be local. In some embodiments, the imidazoquinoline compound and the CpG ODN are administered intratumorally. In any of the embodiments disclosed herein, the  
15 imidazoquinoline compound can be a lipophilic imidazoquinoline compound, such as 3M-052. In any of the embodiments disclosed below, the CpG ODN can be one of SEQ ID NOs: 3-34 or 39-65; combinations thereof can also be used. Exemplary methods are disclosed below.

The methods disclosed herein include selecting a subject in need of treatment, such as a subject with a tumor, and administering to the subject a therapeutically effective amount of both (1)  
20 the imidazoquinoline compound and (2) the one or more CpG ODN, such as K-type CpG ODN, D-type CpG ODN, C-type CpG ODN, or a combination thereof. In some examples, more than one CpG ODN is utilized, such as two, three, four or five CpG ODN. These ODNs can be of the same type or can be different types. Additional agents can also be administered to the subject of interest, such as, but not limited to, chemotherapeutic agents. Additional treatments can also be  
25 administered to the subject, such as, but not limited to, surgical resection of the tumor. Additional chemotherapeutic agents can also be administered to the subject.

The tumor can be benign or malignant. The tumor can be any tumor of interest, including, but not limited to, melanoma and colorectal cancer. In other embodiments, the tumor is a lymphoma, breast cancer, lung cancer and colon cancer. Additional examples are skin tumors,  
30 breast tumors, brain tumors, cervical carcinomas, testicular carcinomas, head and neck tumors, gastrointestinal tract tumors, genitourinary system tumors, gynecological system tumors, breast, endocrine system tumors, skin tumors, a sarcoma of the soft tissue and bone, a mesothelioma, a melanoma, a neoplasm of the central nervous system, or a leukemia. In some embodiments, the

tumor is a head and neck tumor, such as tumors of the nasal cavity, paranasal sinuses, nasopharynx, oral cavity, oropharynx, larynx, hypopharynx, salivary glands and paragangliomas. In other embodiments, the tumor is a lung tumor, such as a non-small cell lung cancer or a small cell lung cancer. In further embodiments, the tumor can be a tumor of the gastrointestinal tract, such as cancer of the esophagus, stomach, pancreas, liver, biliary tree, small intestine, colon, rectum and anal region. In yet other embodiments, the tumor can be a tumor of the genitourinary system, such as cancer of the kidney, urethra, bladder, prostate, urethra, penis and testis. In some embodiments, the tumor is a gynecologic tumor, such as cancer of the cervix, vagina, vulva, uterine body, gestational trophoblastic diseases, ovarian, fallopian tube, peritoneal, or breast. In other embodiments, the tumor is an endocrine system tumor, such as a thyroid tumor, parathyroid tumor, adrenal cortex tumor, pancreatic endocrine tumor, carcinoid tumor and carcinoid syndrome. The tumor can be a sarcoma of the soft tissue and bone, a mesothelioma, a cancer of the skin, a melanoma, comprising cutaneous melanomas and intraocular melanomas, a neoplasm of the central nervous system, a cancer of the childhood, comprising retinoblastoma, Wilm's tumor, neurofibromatoses, neuroblastoma, Ewing's sarcoma family of tumors, rhabdomyosarcoma. The tumor can be a lymphoma, comprising non-Hodgkin's lymphomas, cutaneous T-cell lymphomas, primary central nervous system lymphoma, and Hodgkin's disease. The tumor can be a leukemia, such as acute leukemia, chronic myelogenous leukemia and lymphocytic leukemia. The tumor can be plasma cell neoplasms, a cancer of unknown primary site, a peritoneal carcinomatosis, a Kaposi's sarcoma, AIDS-associated lymphomas, AIDS-associated primary central nervous system lymphoma, AIDS-associated Hodgkin's disease and AIDS-associated anogenital cancers, a metastatic cancer to the liver, metastatic cancer to the bone, malignant pleural and pericardial effusions and malignant ascites. In specific non-limiting examples, the tumor is melanoma or colon cancer.

Treatment of the tumor is generally initiated after the diagnosis of the tumor, or after the initiation of a precursor condition (such as dysplasia or development of a benign tumor). Treatment can be initiated at the early stages of cancer, for instance, can be initiated before a subject manifests symptoms of a condition, such as during a stage I diagnosis or at the time dysplasia is diagnosed. However, treatment can be initiated during any stage of the disease, such as but not limited to stage I, stage II, stage III and stage IV cancers. In some examples, treatment is administered to these subjects with a benign tumor that can convert into a malignant or even metastatic tumor.

The presence of a tumor can be determined by methods known in the art, and typically include cytological and morphological evaluation. The tumor can be an established tumor. The

cells can be *in vivo* or *ex vivo*, including cells obtained from a biopsy.

Treatment initiated after the development of a condition, such as malignant cancer, may result in decreasing the severity of the symptoms of one of the conditions, or completely removing the symptoms, or reducing metastasis, tumor volume or number of tumors. In some example, the tumor becomes undetectable following treatment. Treatment can also include increasing the immune response to the tumor, such as by increasing the humoral response, or cytokines, NK cells, activated CTLs, such as CD9+ T cells, or MDSCs, such as mMDSCs.

In one aspect of the disclosure, the formation of tumors, such as metastasis, is delayed, prevented or decreased. In another aspect, the size of the primary tumor is decreased. In a further aspect, a symptom of the tumor is decreased. In yet another aspect, tumor volume is decreased.

In some examples, the methods are for the treatment of a subject with a tumor. A therapeutically effective amount of (1) an imidazoquinoline compound, such as a lipophilic imidazoquinoline compound, for example, 3M052, and (2) one or more CpG ODN, such as K-type CpG ODN, D-type CpG ODN, C-type CpG ODN, or a combination thereof, is administered to the subject. Exemplary K-type CpG ODN, D-type CpG ODN and C-type ODN are listed above, see for example, SEQ ID NOs: 3-34 and 39-63. The administration can be directly to the tumor. An immune response can be measured, tumor volume can be measured, the number of metastatic lesions can be measured, or a symptom of a tumor can be measured. A therapeutically effective dose can increase the immune response, decrease tumor volume, decrease the number and/or size of metastases, and/or decrease one or more symptoms of the tumor.

Treatment prior to the development of the condition, such as treatment upon detecting dysplasia or an early (benign) precursor condition, is referred to herein as treatment of a subject that is "at risk" of developing the condition. In some embodiments, administration of a composition can be performed during or after the occurrence of the conditions described herein.

Pharmaceutical compositions can include (1) an imidazoquinoline compound and (2) one or more CpG ODN, such as K-type CpG ODN, D-type CpG ODN, C-type CpG ODN, or a combination thereof as active ingredients. These compositions can also include an additional agent, such as an additional chemotherapeutic agent. The compositions described herein include both (1) the imidazoquinoline compound, such as a lipophilic imidazoquinoline compound (for example, 3M-052) and (2) the one or more CpG ODN, such as K-type CpG ODN, D-type CpG ODN, C-type CpG ODN, or a combination thereof. These compositions are of use for treating a tumor. In some embodiments, the composition includes 3M-052. These compositions can be formulated in a variety of ways for administration to a subject to induce an immune response to a tumor, or to

delay, prevent, reduce the risk of developing, or treat, any tumor of interest. The compositions described herein can also be formulated for application such that they prevent metastasis of an initial lesion. In some embodiments, the compositions are formulated for local administration, such as intratumoral administration. Pharmaceutical compositions are thus provided for both local use  
5 and for systemic use, formulated for use in human or veterinary medicine.

While the disclosed methods and compositions will typically be used to treat human subjects they may also be used to treat similar or identical diseases in other vertebrates, such as other primates, dogs, cats, horses, and cows. A suitable administration format may best be determined by a medical practitioner for each subject individually. Various pharmaceutically  
10 acceptable carriers and their formulation are described in standard formulation treatises, *e.g.*, *Remington's Pharmaceutical Sciences* by E. W. Martin. See also Wang, Y. J. and Hanson, M. A., *Journal of Parenteral Science and Technology*, Technical Report No. 10, Supp. 42: 2S, 1988. The dosage form of the pharmaceutical composition will be determined by the mode of administration chosen.

When locally administered into cells in an affected area or a tissue of interest, such as a tumor, the (1) the imidazoquinoline compound, such as a lipophilic imidazoquinoline compound (for example, 3M-052) and (2) the one or more CpG ODN, such as K-type CpG ODN, D-type CpG ODN, C-type CpG ODN, or a combination thereof can be administered in a composition that contains a synthetic or natural hydrophilic polymer as the carrier. Examples of such polymers  
20 include hydroxypropyl cellulose and polyethylene glycol. The active ingredients can be mixed with a hydrophilic polymer in an appropriate solvent. The solvent is then removed by methods such as air-drying, and the remainder is then shaped into a desired form (for example, a sheet) and applied to the target site. Formulations containing such hydrophilic polymers keep well as they have a low water-content. At the time of use, they absorb water, becoming gels that also store well. In the case  
25 of sheets, the firmness can be adjusted by mixing a polyhydric alcohol with a hydrophilic polymer similar to those above, such as cellulose, starch and its derivatives, or synthetic polymeric compounds. Hydrophilic sheets thus formed can be used.

The compositions or pharmaceutical compositions including (1) the imidazoquinoline compound, such as a lipophilic imidazoquinoline compound (for example, 3M-052) and (2) the one  
30 or more CpG ODN, such as K-type CpG ODN, D-type CpG ODN, C-type CpG ODN, or a combination thereof, can be administered by any route, including parenteral administration, for example, intravenous, intraperitoneal, intramuscular, intraperitoneal, intrasternal, or intraarticular injection or infusion, or by sublingual, oral, topical, intranasal, or transmucosal administration, or

by pulmonary inhalation. In some embodiments, the imidazoquinoline compound and the one or more CpG ODN are administered to a tissue wherein the tumor is located, or directly into the tumor. When ODNs are provided as parenteral compositions, *e.g.* for injection or infusion, they are generally suspended in an aqueous carrier, for example, in an isotonic buffer solution at a pH of about 3.0 to about 8.0, preferably at a pH of about 3.5 to about 7.4, 3.5 to 6.0, or 3.5 to about 5.0. Useful buffers include sodium citrate-citric acid and sodium phosphate-phosphoric acid, and sodium acetate-acetic acid buffers. A form of repository or “depot” slow release preparation may be used so that therapeutically effective amounts of the preparation are delivered into the bloodstream over many hours or days following transdermal injection or delivery.

Compositions including (1) the imidazoquinoline compound, such as a lipophilic imidazoquinoline compound (for example, 3M-052) and (2) the one or more CpG ODN, such as K-type CpG ODN, D-type CpG ODN, C-type CpG ODN, or a combination thereof are also suitably administered by sustained-release systems. Suitable examples of sustained-release compositions include suitable polymeric materials (such as, for example, semi-permeable polymer matrices in the form of shaped articles, *e.g.*, films, or microcapsules), suitable hydrophobic materials (such as, for example, an emulsion in an acceptable oil) or ion exchange resins, and sparingly soluble derivatives (such as, for example, a sparingly soluble salt). Sustained-release formulations may be administered orally, rectally, parenterally, intracisternally, intravaginally, intraperitoneally, topically (as by powders, ointments, gels, drops or transdermal patch), buccally, or as an oral or nasal spray, depending on the location of the tumor.

Preparations for administration can be suitably formulated to give controlled release of (1) the imidazoquinoline compound, such as a lipophilic imidazoquinoline compound (for example, 3M-052) and (2) the one or more CpG ODN, such as K-type CpG ODN, D-type CpG ODN, C-type CpG ODN, or a combination thereof over an extended period of time. For example, the pharmaceutical compositions may be in the form of particles comprising a biodegradable polymer and/or a polysaccharide jellifying and/or bioadhesive polymer, an amphiphilic polymer, an agent modifying the interface properties of the particles and a pharmacologically active substance. These compositions exhibit certain biocompatibility features which allow a controlled release of the active substance. See U.S. Patent No. 5,700,486.

The pharmaceutically acceptable carriers and excipients useful in the disclosed methods are conventional. For instance, parenteral formulations usually comprise injectable fluids that are pharmaceutically and physiologically acceptable fluid vehicles such as water, physiological saline, other balanced salt solutions, aqueous dextrose, glycerol or the like. Excipients that can be included

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are, for instance, proteins, such as human serum albumin or plasma preparations. If desired, the pharmaceutical composition to be administered may also contain minor amounts of non-toxic auxiliary substances, such as wetting or emulsifying agents, preservatives, and pH buffering agents and the like, for example sodium acetate or sorbitan monolaurate. Actual methods of preparing such dosage forms are known, or will be apparent, to those skilled in the art.

Generally, the formulations are prepared by contacting (1) the imidazoquinoline compound, such as a lipophilic imidazoquinoline compound (for example, 3M-052) and (2) the one or more CpG ODN, such as K-type CpG ODN, D-type CpG ODN, C-type CpG ODN, or a combination thereof each uniformly and intimately with liquid carriers or finely divided solid carriers or both. Then, if necessary, the product is shaped into the desired formulation. Optionally, the carrier is a parenteral carrier, and in some embodiments it is a solution that is isotonic with the blood of the recipient. Examples of such carrier vehicles include water, saline, Ringer's solution, and dextrose solution. Non-aqueous vehicles such as fixed oils and ethyl oleate are also useful herein, as well as liposomes.

The pharmaceutical compositions that comprise (1) the imidazoquinoline compound, such as a lipophilic imidazoquinoline compound (for example, 3M-052) and (2) the one or more CpG ODN, such as K-type CpG ODN, D-type CpG ODN, C-type CpG ODN, or a combination thereof can be formulated in unit dosage form, suitable for individual administration of precise dosages. The amount of active compound(s) administered will be dependent on the subject being treated, the severity of the affliction, and the manner of administration, and is best left to the judgment of the prescribing clinician. Within these bounds, the formulation to be administered will contain a quantity of the active component(s) in amounts effective to achieve the desired effect in the subject being treated. Multiple treatments are envisioned, such as over defined intervals of time, such as daily, bi-weekly, weekly, bi-monthly or monthly, such that chronic administration is achieved. As disclosed herein, therapeutically effective amounts of (1) the imidazoquinoline compound, such as a lipophilic imidazoquinoline compound (for example, 3M-052) and (2) the one or more CpG ODN, such as K-type CpG ODN, D-type CpG ODN, C-type CpG ODN, or a combination thereof, are of use for inducing an immune response to the tumor cells, treating a tumor, and/or preventing conversion of a benign to a malignant lesion, or preventing metastasis. Administration may begin whenever the suppression or prevention of disease is desired, for example, at a certain age of a subject, or prior to an environmental exposure.

The therapeutically effective amount of (1) the imidazoquinoline compound, such as a lipophilic imidazoquinoline compound (for example, 3M-052) and (2) the one or more CpG ODN,

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such as K-type CpG ODN, D-type CpG ODN, C-type CpG ODN, or a combination thereof, will be dependent on the CpG ODN(s) utilized, the subject being treated, the severity and type of the affliction, and the manner of administration. The exact dose is readily determined by one of skill in the art based on the potency of the specific compound (such as the ODN utilized and the

imidazoquinoline compound), the age, weight, sex and physiological condition of the subject.

Suitable concentrations include, but are not limited to, about 1 to about 100 µg/gm K-type CpG ODN, such as about 5 to about 50 µg/gm, such as about 50 µg/gm CpG ODN. Additional suitable concentrations include 1 to 100 mg/kg, such as about 5 to about 50 mg/kg, such as about 10 mg/kg.

Suitable does also include about 0.1 to about 2 mg/kg in humans. In other embodiments about 0.1

to about 10 mg/kg of the imidazoquinoline compound is administered, such as about 0.1 to about 1 mg/kg. In specific non-limiting examples, about 0.5 to about 5 mg/kg, about 1 to about 5 mg/kg, about 0.5 to 2 mg/kg, or about 1 ng/kg, about 2 mg/kg, about 3 mg/kg, about 4 mg/kg, or 5 mg/kg of the imidazoquinoline compound is administered. Thus, pharmaceutical compositions are

provided that include a therapeutically effective amount of one or more CpG ODNs and an

imidazoquinoline compound, such as a lipophilic imidazoquinoline compound (for example, 3M-052).

Additional agents can be administered, such as a cytokine, a chemokine, or a chemotherapeutic agent. These can be included in the disclosed pharmaceutical compositions. A cytokine can be administered, such as interleukin-2 (IL-2), granulocyte macrophage colony stimulating factor (GM-CSF), or interferon, such as interferon (IFN) β. In one example, for the prevention and treatment of cancer, surgical treatment can be administered to the subject. In one example, this administration is sequential. In other examples, this administration is simultaneous.

Examples of chemotherapeutic agents are alkylating agents, antimetabolites, natural products, or hormones and their antagonists. Examples of alkylating agents include nitrogen mustards (such as mechlorethamine, cyclophosphamide, melphalan, uracil mustard or chlorambucil), alkyl sulfonates (such as busulfan), nitrosoureas (such as carmustine, lomustine, semustine, streptozocin, or dacarbazine). Examples of antimetabolites include folic acid analogs (such as methotrexate), pyrimidine analogs (such as 5-FU or cytarabine), and purine analogs, such as mercaptopurine or thioguanine. Examples of natural products include vinca alkaloids (such as vinblastine, vincristine, or vindesine), epipodophyllotoxins (such as etoposide or teniposide), antibiotics (such as dactinomycin, daunorubicin, doxorubicin, bleomycin, plicamycin, or mitocycin C), and enzymes (such as L-asparaginase). Examples of miscellaneous agents include platinum coordination complexes (such as cis-diamine-dichloroplatinum II also known as cisplatin),

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substituted ureas (such as hydroxyurea), methyl hydrazine derivatives (such as procarbazine), and adrenocortical suppressants (such as mitotane and aminoglutethimide). Examples of hormones and antagonists include adrenocorticosteroids (such as prednisone), progestins (such as hydroxyprogesterone caproate, medroxyprogesterone acetate, and magesrol acetate), estrogens (such as diethylstilbestrol and ethinyl estradiol), antiestrogens (such as tamoxifen), and androgens (such as testosterone propionate and fluoxymesterone). Examples of the most commonly used chemotherapy drugs include Adriamycin, Alkeran, Ara-C, BiCNU, Busulfan, CCNU, Carboplatinum, Cisplatinum, Cytosin, Daunorubicin, DTIC, 5-FU, Fludarabine, Hydrea, Idarubicin, Ifosfamide, Methotrexate, Mithramycin, Mitomycin, Mitoxantrone, Nitrogen Mustard, Taxol (or other taxanes, such as docetaxel), Velban, Vincristine, VP-16, while some more newer drugs include Gemcitabine (Gemzar), Herceptin, Irinotecan (Camptosar, CPT-11), Leustatin, Navelbine, Rituxan STI-571, Taxotere, Topotecan (Hycamtin), Xeloda (Capecitabine), Zavelin and calcitriol. Non-limiting examples of immunomodulators that can be used include AS-101 (Wyeth-Ayerst Labs.), bropirimine (Upjohn), gamma interferon (Genentech), GM-CSF (granulocyte macrophage colony stimulating factor; Genetics Institute), IL-2 (Cetus or Hoffman-LaRoche), human immune globulin (Cutter Biological), IMREG (from Imreg of New Orleans, La.), SK&F 106528, and TNF (tumor necrosis factor; Genentech).

This disclosure is illustrated by the following non-limiting examples:

### EXAMPLES

Large solid tumors become infiltrated by a subpopulation of myeloid derived suppressor cells (mMDSC) that suppress anti-tumor immunity. These mMDSC express TLRs 7, 8 and 9.

Previous studies showed that intra-tumoral injection of the TLR9 agonist CpG ODN alone reduced the suppressive activity of these tumor infiltrating cells. This ability of the TLR7/8 agonist 3M-052, alone and in combination with CpG ODN, to alter mMDSC function and thereby influence tumor growth was investigated.

The results presented herein show that the combination of 3M-052 plus CpG ODN significantly increases CTL activity and Th1 cytokine production while down-regulating the activity of immunosuppressive MDSC. Although neither CpG ODN nor 3M-052 alone were effective against large tumors, the combination was highly active and mediated tumor eradication and the establishment of long-term immunity.

### Example 1

#### Materials and Methods

**Reagents:** 3M-052 was supplied by 3M Drug Delivery System with 4mg/ml stock solution in ethanol. Endotoxin free phosphorothioate ODN were synthesized at the Core Facility of the Center for Biologics Evaluation and Research, Food and Drug Administration (Bethesda, MD) with the following sequence, CpG ODN 1555 (5'-GCTAGACGTTAGCGT-3', SEQ ID NO: 30) and control ODN 1612 (5'-GCTAGAGCTTAGCGT-3', SEQ ID NO: 35). All ODN were dissolved in phosphate buffered saline (PBS) at a concentration of 4mg/ml.

**Mice and tumor cell lines:** Six to eight weeks wild type BALB/c and C57BL/6 mice were utilized. The CT26 colon cancer cell line, and the B16-F10 cell lines (purchased from American Type Culture collection (Manassas, VA), were utilized in the experiments disclosed below. Tumor cell lines were maintained in RPMI 1640 medium supplemented with 10% FCS, 100 U/ml penicillin, 100ug/ml streptomycin, 25 mM HEPES, 1.0 mM sodium pyruvate, nonessential amino acids, and 0.0035% 2-ME.

**Tumor experiments:** For tumor induction, Balb/c mice were injected subcutaneously (s.c.) with  $10^5$  CT26 tumor cells and C57BL/6 mice with  $10^5$  B16-F10 tumor cells on the right flank. Treatment was initiated on week 2 or 3 by intratumoral inject 100/200 ug ODN, 50/100 ug 3M-052 or same volume of control 3M. Tumor size was calculated by the formula: (length x width x width)/2 and the mice with tumor exceeded a diameter of 2.0 cm were immediately euthanized. Tumor growth curves were generated from five mice per group, and all results were derived by combining data from two to three independent experiments.

Two treatment regimens were used. For small tumors ( $<300 \text{ mm}^3$ ), two doses of 100  $\mu\text{g}$  of CpG ODN (4 mg/ml) and/or 50  $\mu\text{g}$  of 3M-052 (4 mg/ml) were injected intra-tumorally using a 30 g needle. To deplete  $\text{CD4}^+$  or  $\text{CD8}^+$  T cell subsets, mice were injected intraperitoneally (i.p.) with 25  $\mu\text{l}$  ascites of rat anti-mouse CD4 (L3/T4) or mouse anti-mouse CD8 (Ly2.2) Abs from Cedarlane labs (Burlington, NC) on day -2, 0, 3 and 6 post-tumor implantation. For large tumors (500 - 800  $\text{mm}^3$ ), 200  $\mu\text{g}$  of CpG ODN and/or 100  $\mu\text{g}$  of 3M-052 were injected intra-tumorally twice weekly for one month. Inactive controls for each TLR agonist were included in all experiments. Tumor growth curves were generated from five mice per group and all results were derived by combining data from 2-3 independent experiments.

**Flow cytometric analysis:** Leukocytic infiltrates of the tumor site were prepared by surgical removal of tumor tissue followed by homogenization using a GentleMACS Dissociator (Miltenyi Biotec) and then digestion in RPMI containing 5% fetal calf serum, 250 U/mL type IV collagenase (Invitrogen) and 100 mg/mL DNase I (Roche Molecular Biochemicals) at 37° C for 30 minutes. The resulting single cell suspension was passed through a 70 um cell strainer (BD Biosciences, Bedford, MA), and washed twice with RPMI. Live cells were isolated by density gradient centrifugation (Histopaque-1077, Sigma-Aldrich), washed, and stained using the following Abs from BD Pharmingen (clone names provided in parentheses). CD11b (M1/70) and Gr-1 (RB6-8C5) for MDSC, CD3 (145-2C11) and CD8 (53-6.7) for T cells and CD49b (DX5) for NK cells. CD45 (30-F11) was used as a leukocyte marker. Stained cells were analyzed using an LSR-II flow cytometer (Becton Dickinson).

**Enzyme linked immunosorbent spot (ELISpot) assay:** Single cell suspensions were prepared from whole spleen or tumor drain lymph node and  $1.5 - 3.0 \times 10^5$  cells/well were stimulated for 12 hours with AH-1 peptide (1 µg/ml) in 96 well IMMULON™ II plates (Millipore, Billerica, MA) which coated with the monoclonal antibody (mAb) anti-interferon (IFN)γ Ab (R4-6A2) (BD Biosciences). The plates were washed and treated with biotinylated polyclonal goat anti-IFN-γ Ab (R & D systems, MN) followed by streptavidin alkaline phosphatase. Spots were visualized by the addition of a 5-bromo-4-chloro-3-indolyl phosphatase solution (Sigma Aldrich) in low melt agarose (Sigma Aldrich) and counted manually under x40 magnification. The number of cytokine secreting cells was determined by a single blind reader, and all data was generated by analyzing 12 separate wells per sample.

**Quantitative real-time PCR analysis:** Total RNA was isolated from tumor homogenates by using TRIZOL® (Invitrogen), precipitated, and then reverse transcribed with a reverse transcription kit (Qiagen). Interleukin (IL)-12p40, IFNγ, Granzyme B, arginase (Arg)1, nitric oxide synthetase (Nos)2, cytotoxic T-lymphocyte antigen (CTLA)4 and transforming growth factor (TGF)β mRNA levels were examined by using TAQMAN® Gene Expression Master Mix and Applied Biosystems STEPONE™ reverse transcription polymerase chain reaction (RT-PCR) system. All primer sets were from the gene expression assay set (Applied Biosystems, Foster City, CA). Gene expression was normalized to the level of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) housekeeping gene. Data were analyzed by STEPONE™ software (Applied Biosystems) and

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expressed as a fold change in mRNA expression relative to control values. Ct values for all genes studied fell in the range of 22 - 35.

**Statistical analysis:** P values for each experimental group were determined by comparison to the PBS control group using an unpaired student's t test. A value of  $P < 0.05$  was considered statistically significant.

## Example 2

### Effect of TLR Agonists on the Growth of Small Tumors

CT26 colon cancer cells were implanted subcutaneously into the flank of syngeneic BALB/c mice. When these tumors reached  $\approx 200 \text{ mm}^3$  in volume, 100  $\mu\text{g}$  of ODN and/or 50  $\mu\text{g}$  of 3M-052 was delivered intra-tumorally. This procedure was repeated two days later. Tumors in untreated mice proliferated rapidly (Fig. 1). The rate of proliferation was significantly reduced by treatment with either CpG ODN (CpG ODN K1555, see above) or 3M-052 although the tumors persisted. In contrast, mice treated with the combination of CpG ODN plus 3M-052 completely rejected their tumors ( $p < 0.01$ ; Fig. 1).

To explore the mechanism underlying this tumor rejection, spleen cells were isolated from mice three days after treatment was initiated. These were stimulated in vitro with the CT26-derived AH-1 tumor peptide and interferon (IFN) $\gamma$  secretion (a surrogate for cytotoxic T lymphocyte (CTL) activity) monitored. The number of cells from mice treated with CpG ODN plus 3M-052 was significantly expanded when compared to controls ( $p < 0.001$ , Fig. 1).

## Example 3

### Effect of TLR Agonists on the Frequency of Tumor Infiltrating MDSC, Natural Killer (NK) Cells and CD8 T Cells

Immune cells in the tumor microenvironment profoundly influence the success of immunotherapy. A single cell suspension was prepared from tumor samples, and the frequency of various immune subsets evaluated by FACS (Fig. 9). The number of mMDSC is considered an important marker of immune suppression, as these cells suppress the tumoricidal activity of CTL and NK cells. Consistent with previous reports, the frequency of Gr1<sup>+</sup>CD11b<sup>+</sup> mMDSC was significantly elevated in mice bearing CT26 tumors (Fig 2). Treatment with either CpG ODN or

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3M-052 alone reduced the number of mMDSC infiltrating the tumor site by  $\approx 50\%$  ( $p < 0.05$ ). The combination of these two agonists resulted in a nearly 90% reduction in mMDSC frequency ( $p < 0.01$ , Fig 2). This effect was detectable by 1 day after the second treatment.

Previous studies showed that increased infiltration of NK and CD8 T cells into the tumor microenvironment correlated with improved survival (Shirota, *J Immunol* 2012, **188**:1592-1599, 2012). The effect of TLR agonist treatment on the frequency of tumoricidal cells was therefore analyzed. The number of NK cells was  $\approx 25\%$  higher in mice treated with 3M-052 or CpG ODN when compared to untreated controls ( $p < 0.05$ , Fig. 2). This increase was magnified in mice treated with the combination of both TLR agonists. By comparison, while CpG ODN or 3M-052 alone increased CD8 T cell frequency by approximately 2-fold, the combination of both agonists synergistically increased CD8 T cell numbers by  $>5$ -fold ( $p < 0.05$ , Fig. 2). No effect on the frequency of Foxp3+ regulatory T cells was observed (Fig. 10).

Two experiments were performed to explore the functional activity of these CD8 T cells. Splenocytes from mice in each group were isolated and stimulated *ex vivo* with the CT26-derived AH-1 tumor peptide. To monitor CTL activity, the frequency of IFN $\gamma$  secreting cells was determined by ELISpot assay. Consistent with changes in the frequency of CD8 T cells noted above, the number of cells stimulated by AH-1 peptide to produce IFN $\gamma$  was  $>8$ -fold higher in mice treated with CpG ODN plus 3M-052 than in controls by 3 days post treatment ( $p < 0.001$ , Fig. 3). To evaluate the relevance of these T cells *in vivo*, mice that had been challenged with tumor and treated with the combination CpG ODN plus 3M-052 were injected with anti-CD8 Abs. As shown in Fig. 4, protection was abrogated by depletion of CD8 $^+$  but not CD4 $^+$  T cells, indicating that tumor-specific CD8 T cells were critical mediators of tumor immunity.

#### Example 4

##### Effect of TLR agonists on gene up-regulation in the tumor microenvironment

Since significant change of infiltration immune cells was observed in TLR agonists treated tumor-bearing mice, it was hypothesized that this process would be associated with different levels of cytokines and chemokines induced by TLR agonists' treatment. Therefore, tumor infiltrating cells from treated mice were analyzed for gene expression by quantitative PCR (qPCR). CpG ODN and 3M-052 combination treatment significantly increased Th1 cytokines and CTL associated protein expression in the TIL, including IL-12 (Fig. 3), IFN- $\gamma$  (Fig. 3), Granzyme B (Fig. 3C) as compared with CpG ODN and 3M-052 alone treatment groups. CpG ODN or 3M-052 alone only

slightly induced production of these cytokines, as compared with the group treated with phosphate buffered saline (PBS).

### Example 5

#### 5 Effect of TLR agonists on gene down-regulation in the tumor microenvironment

Treatment with CpG ODN and/or 3M-052 led to a significant changes in the frequency of CD8 T cells, NK cells and MDSC (Fig. 2). To evaluate the activity of these cells, the expression of genes associated with their immunological function was examined by qPCR. The genes selected to evaluate CD8 and NK cell responses were IL-12 and IFN $\gamma$  (which contribute to the induction and maintenance of) and granzyme B (which mediates their cytotoxicity) (Trinchieri et al., *Blood* 1994, 84:4008-4027; Komita et al., *J Hepatol* 45:662-672, 2006; Packard et al., *J Immunol* 179:3812-3820, 2007). As seen in Table 1, cells isolated from the tumor of mice treated with either CpG ODN or 3M-052 had higher levels of expression of IL-12, IFN $\gamma$  and granzyme B than tumor infiltrating cells from untreated mice (p. <.05). In animals treated with a combination of both agonists, mRNA levels were significantly higher when compared to either agonist alone (see Table legend). This effect was additive for IL-12 and IFN $\gamma$  and supra-additive for Granzyme B.

Table I Effect of TLR agonists on gene regulation in the tumor microenvironment.

|    |              | Mean fold change in mRNA level (vs PBS treated controls) |                                |                            |
|----|--------------|----------------------------------------------------------|--------------------------------|----------------------------|
|    | <i>Gene</i>  | <b>CpG ODN<br/>+3M control</b>                           | <b>3M-052+<br/>CpG Control</b> | <b>CpG ODN+<br/>3M-052</b> |
|    | IL-12        | 2.23*+ 0.18                                              | 1.40 + 0.19                    | 3.39 + 0.11                |
| 25 | IFN $\gamma$ | 1.94* + 0.15                                             | 1.87*+ 0.25                    | 2.81**+0.24                |
|    | Granzyme B   | 1.69*+ 0.07                                              | 1.87*+ 0.08                    | 4.44*** + 0.23             |
|    | Arg1         | 1.01 + 0.09                                              | 0.63*+ 0.09                    | 0.43** + 0.08              |
|    | Nos2         | 1.83 + 0.05                                              | 0.39**+ 0.04                   | 0.09** + 0.04              |
|    | CTLA-4       | 0.85*+ 0.03                                              | 0.52**+ 0.01                   | 0.33***+ 0.02              |
| 30 | TGF $\beta$  | 0.43*+ 0.03                                              | 0.45*+ 0.01                    | 0.26** + 0.02              |

Mice were treated as described in Fig. 1. mRNA was isolated from tumor infiltrating cells one day after the second treatment and analyzed by RT-PCR. Each point represents the mean + SD fold difference in cells from treated vs untreated tumor bearing mice derived from independently studying 6 mice/group in 2 independent experiments. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ , \*\*\*,  $p < 0.001$  when compared to PBS treated controls. Note: the level of expression of all genes from mice treated with CpG ODN plus 3M-052 was also significantly different ( $p < 0.01 - 0.05$ ) from that of mice treated with CpG ODN alone or 3M-052 alone.

### Example 6

#### **TGF $\beta$ , Arg1 and Nos2 (which mediate the inhibitory activity of MDSC) and CTLA-4 (which acts as a negative regulator of T cell activation).**

The mechanism by which MDSC suppress T cell cytotoxicity in the tumor microenvironment is mediated by the production of L-arginine via arginase-1 and the release of iNOS (Rodriguez et al., *Immunol Rev* 222:180-191, 2008; Bronte and Zanoello, *Nat Rev Immunol* 5:641-654, 2005). The expression of Arg1 and Nos2 by tumor infiltrating immune cells was therefore evaluated by quantitative PCR (qPCR). Results show that 3M-052 but not CpG ODN reduced the level of expression of genes encoding these immunosuppressive agents (Table 1). The combination of CpG ODN plus 3M-052 further reduced expression levels of both genes, an effect culminating in a nearly 90% reduction in Nos2 mRNA ( $p < 0.05$ ).

Immune suppression in the tumor microenvironment can take many forms. One metric of the down-regulation of CTL activity is the expression of CTLA-4 by T cells and another is the production of the immunoinhibitory molecule TGF $\beta$ . CTLA-4, a homologue and antagonist of CD28 (Li et al., *Int J Cancer* 131:2584-2595, 2012; Walunas et al., *Immunity* 1:405-413, 1994); and acts as negative regulator of T cell activation by depriving them of CD28-mediated co-stimulation (Walunas et al., *supra*; Maasteller et al., *J Immunol*, 164:5319-5327, 2000). On the other hand, TGF $\beta$  suppresses both innate and adaptive immune responses in the tumor microenvironment. CTL-mediated tumor elimination is thus reduced by the presence of TGF $\beta$  (Gorelik and Flavell, *Nat Med* 7:1118-1122, 2001; Nam et al., *Cancer Res* 68:3915-3923, 2008). As both 3M-052 and CpG ODN tend to reduce the level of immune suppression in the tumor microenvironment, their effect on CTLA-4 and TGF $\beta$  expression was examined. When compared to cells isolated from tumors treated with PBS, both TLR agonists mediated a significant reduction in the level of expression of these genes (Table 1). The combination of both 3M-052 and CpG ODN was even more effective ( $p < 0.05$ ).

### Example 6

#### Effect of TLR Agonists on the Growth of Larger Tumors

To evaluate the effect of TLR agonists on tumors of clinically relevant size, CT26 cancer cells were implanted as described above and treatment initiated only after the resultant tumors reached  
5  $\approx 800 \text{ mm}^3$  in volume. Mice were then injected intra-tumorally twice weekly for one month with 200  $\mu\text{g}$  of CpG ODN and/or 100  $\mu\text{g}$  of 3M-052. Tumors in untreated mice proliferated rapidly over this period, reaching a volume of  $>2,000 \text{ mm}^3$  within 10 days (mandating their sacrifice as per ACUC guidelines, Fig 5). While both CpG ODN and 3M-052 therapy slowed tumor growth and prolonged survival, tumors in all animals reached the  $2,000 \text{ mm}^3$  by 3 weeks after the initiation of  
10 treatment (Fig 5). In contrast, 87% (13/15) of the mice treated with the combination of CpG ODN plus 3M-052 in 3 independent experiments completely rejected their tumors ( $p < 0.01$ ; Fig. 5).

To verify the utility of this combination against even more aggressive tumors, the studies were repeated in C57/BL6 mice challenged with B16-F10 tumor cells. Therapy was initiated when these tumors reached  $\approx 500 \text{ mm}^3$  in volume. These cancers grow so rapidly that they all reached the  $2,000$   
15  $\text{mm}^3$  endpoint in control mice and had to be sacrificed in less than one week (Fig 6). The same endpoint was reached by all animals treated with a single TLR agonist within 2 weeks. In contrast, nearly 90% recipients (8/9) of the combination therapy survived indefinitely, totally clearing their tumors (Fig. 6).

Two approaches were taken to verify that TLR-induced tumor-specific immunity was  
20 responsible for these cures. First, lymphocytes were isolated from the draining lymph node (LN) of mice challenged with CT26 tumors one week after the initiation of therapy. These cells were then stimulated *in vitro* with AH-1 peptide and their production of  $\text{IFN}\gamma$  monitored. As in Fig. 3, T cells from mice treated with the combination of CpG ODN plus 3M-052 generated significantly stronger tumor specific responses that did any of the controls ( $p < .001$ , Fig. 7A).

The twice weekly combination therapy with CpG ODN plus 3M-052 was discontinued when  
25 tumors could no longer be detected (generally after 1 month). There was no recurrence of these cancers through three months of follow up. To verify that these mice had developed long lasting tumor-specific immunity, they were re-challenged with a 10-fold higher dose of CT26 cells. As shown in Fig 7B, all of these animals survived whereas naive controls perished.

30 Whereas many forms of immunotherapy are effective against small tumors ( $<300 \text{ mm}^3$ ), activity wanes when larger tumors are targeted. A number of factors contribute to the resistance of established tumors. In addition to challenging the immune system with a larger number of target

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cells, organized tumors are better able to cloak themselves in immunosuppressive Tregs and MDSC (Zhou et al., *Blood* 107:628-636.; Lyman et al., *J Immunol* 172:6558-6567, 2004). In this context, MDSC from patients with advanced tumors are particularly effective at inhibiting tumor-specific CD8 T cells (Almand et al., *J Immunol* 166:678-689, 2001). Having found that intra-tumoral  
5 delivery of CpG ODN was considerably more effective than systemic administration for the treatment of tumors, our plan was to examine whether adding a TLR 7/8 agonist could further improve this therapeutic approach. Unfortunately, first generation TLR 7/8 agonists were water soluble and proved ineffective when co-administered with CpG ODN. A relatively new TLR 7/8 agonist was identified that contains a modified tail allowing it to persist *in vivo* after being injected  
10 into the tumor (3M-052) (Smirnov et al., *Vaccine* 29:5434-5442, 2011). Further studies therefore evaluated the activity of locally administered 3M-052 in combination with CpG ODN.

The value of combination therapy was initially examined under conditions where a single TLR agonist only delayed tumor growth (Fig. 1). Large tumors were then studied in which the  
15 combination of CpG ODN plus 3M-052 proved highly successful against both CT26 colon cancer and B10-F16 melanomas. Whereas each agonist alone barely delayed the progression of these large tumors, cure rates on the order of 80 - 90% were achieved by combination therapy. Indeed, as weeping of the injected material from the tumor site was sometimes observed, it is possible that even higher success rates might be achieved by technical improvements in TLR agonist delivery.  
20 Successful therapy of large tumors required twice-weekly treatment with CpG ODN plus 3M-052 over the course of  $\approx 1$  month. A single dose had no detectable effect on the growth of large tumors while 1 - 2 weeks of combination therapy resulted in only short-lived tumor regression. Systemic treatment was uniformly unsuccessful.

There are several mechanisms by which TLR agonists can support the elimination of  
25 established tumors. CD28 is a co-stimulatory molecule that enhances the proliferation, cytokine production and survival of TCR-activated T cells. This process is antagonized by CTLA-4, a surface receptor that is up-regulated when T cells become activated (Walunas, *Immunity* 1:405-413, 1994; Masteller et al., *J Immunol* 164:5319-5327, 2000). It was observed that the level of mRNA encoding CTLA-4 was significantly reduced in mice receiving combination therapy (Table 1). This  
30 down-modulation of CTLA-4 may help explain the improved activity of tumor-specific T cells found in the current work (Fig. 3). A decrease in mRNA encoding the immunosuppressive cytokine TGF $\beta$  was observed in mice treated with CpG ODN plus 3M-052. TGF $\beta$  is produced by tumor cells and Gr-1+ CD11b+ MDSC in the tumor microenvironment and serves to suppress both

innate and adaptive arms of the immune system (Li et al., *Int J Cancer* 2012, 131:2584-2595; Brierie et al., *Nat Rev Cancer* 6:506-520, 2006; Flavell et al., *Nat Rev Immunol* 10:554-567, 2012).

Consistent with current findings, reduced TGF $\beta$  signaling can enhance tumor elimination by improving CTL activity (Gorelik and Flavell, *Nat Med* 7:1118-1122, 2001; Nam et al. *Cancer Res* 5 68:3915-3923, 2008).

To establish the role of increased CTL function in recipients of combination therapy, cells from the tumor draining lymph node were isolated and stimulated *ex vivo* with the CT26-specific AH-1 peptide. While CpG ODN and 3M-052 alone boosted the number of cells secreting IFN $\gamma$ , significantly more cells from recipients of combination therapy were stimulated to produce that cytokine (Figs. 3, 7A). Consistent with the conclusion that these cells contribute to tumor eradication, the level of mRNA encoding cytokines that promote Th1 and cellular immunity (IL-12 and IFN $\gamma$ ) and the lytic activity of NK and CD8 T cells (granzyme B) were all significantly up-regulated in the tumor microenvironment (Table 1) as were the number of tumor infiltrating CD8 T cells and NK cells (Fig. 2).

A number of clinical trials have explored the activity of CpG ODN in cancer patients. CpG treatment induces a dose-related increase in serum levels of IP-10, IFN $\alpha$ , MIP-1 $\alpha$ , and IL-12p40 (Kreig, *Curr Oncol Rep* 2004, 6:88-95, 2004; Offersen et al., *Hum Vaccin Immunother* 2012, 8:1042-1047, 2001). While anti-tumor activity was observed in several phase II trials (Kreig et al., *J Immunother* 27:460-471, 2004) this finding was not reproduced in a definitive phase III study (Hirsh et al., *J Clin Oncol* 29:2667-2674, 2011; Manegold et al, *Ann Oncol* 23:72-77, 2012). However, none of these studies combined CpG ODN with a TLR7/8 agonist and generally administered the ODN systemically rather intra-tumorally.

The local delivery of combination TLR 7/8/9 agonists can be critical for improving the host's anti-tumor response by acting on multiple cell types in the tumor microenvironment, including mMDSC, CD8 T lymphocytes and NK cells. Of interest, MDSC express receptors for both agonists and play a vital role protecting tumors from immune aggression by inhibiting T and NK cell activity (see, for example, Li et al., *J Immunol* 182:240-249, 2009). The findings disclosed herein demonstrate that the combination of CpG ODN plus 3M-052 reduced mMDSC frequency by 10-fold when compared to untreated mice and 3-5 fold when compared to either agonist alone (Fig. 2). This reduction was associated with a significant decline iNOS and arginase-1 expression (Table 1), a constellation of findings that may explain the increase in tumor-specific CTL activity in mice treated with combination therapy (Figs. 3, 7A).

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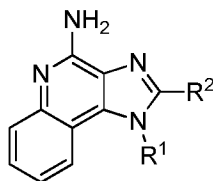
Thus, the experiments disclosed herein document that co-administering CpG ODN with 3M-052 is remarkably effective at eliminating large established tumors. This anti-tumor activity is associated with a significant diminution in the frequency of tumor resident MDSC and accumulation of tumor-lytic NK and CD8 T cells, resulting in persistent anti-tumor immunity.

5 These findings indicate that the disclosed methods can be of considerable benefit in cases of advanced cancer.

In view of the many possible embodiments to which the principles of our invention may be applied, it should be recognized that illustrated embodiments are only examples of the invention and should not be considered a limitation on the scope of the invention. Rather, the scope of the  
10 invention is defined by the following claims. We therefore claim as our invention all that comes within the scope and spirit of these claims.

We claim:

1. A method for treating a subject with a tumor, comprising administering directly into the tumor of the subject a therapeutically effective amount of an imidazoquinoline compound having a formula:



wherein,  $R^1$  is selected from  $-C_{12-24}alkyl$ ,  $-OC_{11-24}alkyl$ ,  $-NHC_{11-24}alkyl$ , and  $-X-Y-Z-R^6$ , wherein

X is selected from the group consisting of a bond,  $-O-$ , and  $-NH-$ ;

Y is selected from the group consisting of  $C_{1-10}alkyl$  and  $C_{1-5}alkylOC_{1-5}alkyl$

Z is selected from the group consisting of:

$-NHC(O)-$ ,

$-NHS(O)_2-$ ,

$-NHC(O)NH-$ ; and

$R^6$  is  $-C_{11-23}alkyl$ ;

$R^2$  is selected from the group consisting of hydrogen, alkyl, alkylaminoalkyl, alkoxyalkyl, and

hydroxyalkyl; and

a therapeutically effective amount of an immunostimulatory CpG oligodeoxynucleotide, thereby treating the tumor in the subject.

2. The method of claim 1 wherein  $R^2$  is selected from the group consisting of hydrogen, methyl, ethyl, propyl, butyl, ethoxymethyl, methoxymethyl, 2-methoxyethyl, hydroxymethyl, and 2-hydroxyethyl.

3. The method of claim 1 wherein  $R^2$  is selected from the group consisting of ethyl, propyl, butyl, methoxyethyl, and ethoxymethyl.

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4. The method of claim 1 wherein R<sup>2</sup> is selected from the group consisting of butyl and ethoxymethyl.

5. The method of claim 1 wherein Y is C<sub>2-10</sub>alkyl.

6. The method of claim 1 wherein Y is C<sub>2-4</sub>alkylOC<sub>2-4</sub>alkyl.

7. The method of claim 1 wherein Y is C<sub>2-5</sub>alkyl.

8. The method of claim 1 wherein X is selected from a group consisting of a bond and -O-.

9. The method of claim 1 wherein X is -O-.

10. The method of claim 1 wherein R<sup>6</sup> is C<sub>15-23</sub>alkyl.

11. The method of claim 1 wherein R<sup>6</sup> is C<sub>15</sub>-C<sub>19</sub>alkyl.

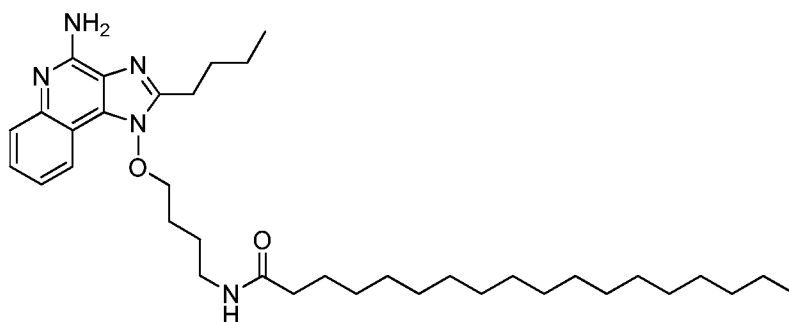
12. The method of claim 1 wherein Z is -NHC(O) -.

13. The method of claim 1 wherein R<sup>1</sup> is selected from the group consisting of -CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>NHC(O)C<sub>11-23</sub>alkyl, -OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>NHC(O)C<sub>11-23</sub>alkyl, -NHCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>NHC(O)C<sub>11-23</sub>alkyl, -CH<sub>2</sub>CH<sub>2</sub>OCH<sub>2</sub>CH<sub>2</sub>NHC(O)C<sub>11-23</sub>alkyl.

14. The method of claim 1 wherein R<sup>1</sup> is selected from the group consisting of -CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>NHC(O)C<sub>15-23</sub>alkyl, -OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>NHC(O)C<sub>15-23</sub>alkyl, -NHCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>NHC(O)C<sub>15-23</sub>alkyl, -CH<sub>2</sub>CH<sub>2</sub>OCH<sub>2</sub>CH<sub>2</sub>NHC(O)C<sub>15-23</sub>alkyl.

15. The method according to claim 1 wherein the imidazoquinoline compound is:

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16. The method of any one of claims 1-15, wherein the CpG oligodeoxynucleotide is a K-type CpG oligodeoxynucleotide or a D-type CpG oligodeoxynucleotide, and

wherein the K-type CpG oligodeoxynucleotide has a nucleic acid sequence set forth as:

5' N<sub>1</sub>N<sub>2</sub>N<sub>3</sub>D-CpG-WN<sub>4</sub>N<sub>5</sub>N<sub>6</sub> 3' (SEQ ID NO: 2)

wherein the central CpG motif is unmethylated, D is T, G or A, W is A or T, and N<sub>1</sub>, N<sub>2</sub>, N<sub>3</sub>, N<sub>4</sub>, N<sub>5</sub>, and N<sub>6</sub> are any nucleotide, wherein the CpG oligodeoxynucleotide is 10 to 30 nucleotides in length; and

wherein the D-type CpG oligodeoxynucleotide has a sequence

5' X<sub>1</sub>X<sub>2</sub>X<sub>3</sub> Pu<sub>1</sub> Py<sub>2</sub> CpG Pu<sub>3</sub> Py<sub>4</sub> X<sub>4</sub>X<sub>5</sub>X<sub>6</sub>(W)<sub>M</sub>(G)<sub>N</sub>-3' (SEQ ID NO : 1)

wherein the central CpG motif is unmethylated, Pu is a purine nucleotide, Py is a pyrimidine nucleotide, X and W are any nucleotide, M is any integer from 0 to 10, and N is any integer from 4 to 10, wherein the CpG ODN is 18 to 50 nucleotides in length.

17. The method of claim 16, wherein the CpG oligodeoxynucleotide is the D-type CpG oligodeoxynucleotide.

18. The method of claim 16, wherein the CpG oligodeoxynucleotide is the K-type CpG oligodeoxynucleotide.

19. The method of claim 16, wherein N<sub>3</sub>D is selected from the group consisting of GpT, GpG, GpA, ApT and ApA; and WN<sub>4</sub> is selected from the group consisting of TpT or CpT.

20. The method of claim 16, wherein the K-type CpG oligodeoxynucleotide comprises SEQ ID NO: 30 (1555) or SEQ ID NO: 11 (K3).

21. The method of claim 16, wherein the K-type CpG ODN comprises one of the

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nucleotide sequences set forth as SEQ ID NO: 3-34.

22. The method of claim 16, wherein Pu<sub>1</sub> Py<sub>2</sub> and Pu<sub>3</sub> Py<sub>4</sub> are self-complementary.

5 23. The method of claim 16, wherein the CpG oligodeoxynucleotide comprises at least one phosphate backbone modification.

24. The method of any one of claim 16-23, wherein the CpG oligodeoxynucleotide comprises at least one phosphorothioate base.

10

25. The method of claim 24, wherein the CpG oligodeoxynucleotide comprises plurality of phosphorothioate bases.

15 26. The method of any one of claims 16-23, wherein the CpG oligodeoxynucleotide comprises at least one phosphodiester base.

27. The method of claim 26, wherein the CpG oligodeoxynucleotide comprises a plurality of phosphodiester bases.

20 28. The method of any one of claims 16 or 22-27, wherein the D-type CpG oligodeoxynucleotide comprises one of the nucleotide sequences set forth as SEQ ID NO: 39-63.

29. The method of any one of claims 1-28, wherein the tumor is a solid tumor.

25 30. The method of claim 29, wherein the tumor is a melanoma or a colorectal cancer.

31. The method of any one of claims 1-30, wherein treating the tumor comprises decreasing tumor volume; decreasing the number or size of metastases; or lessening a symptom of the tumor.

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32. The method of any one of claims 1-31, further comprising surgically resecting the tumor.

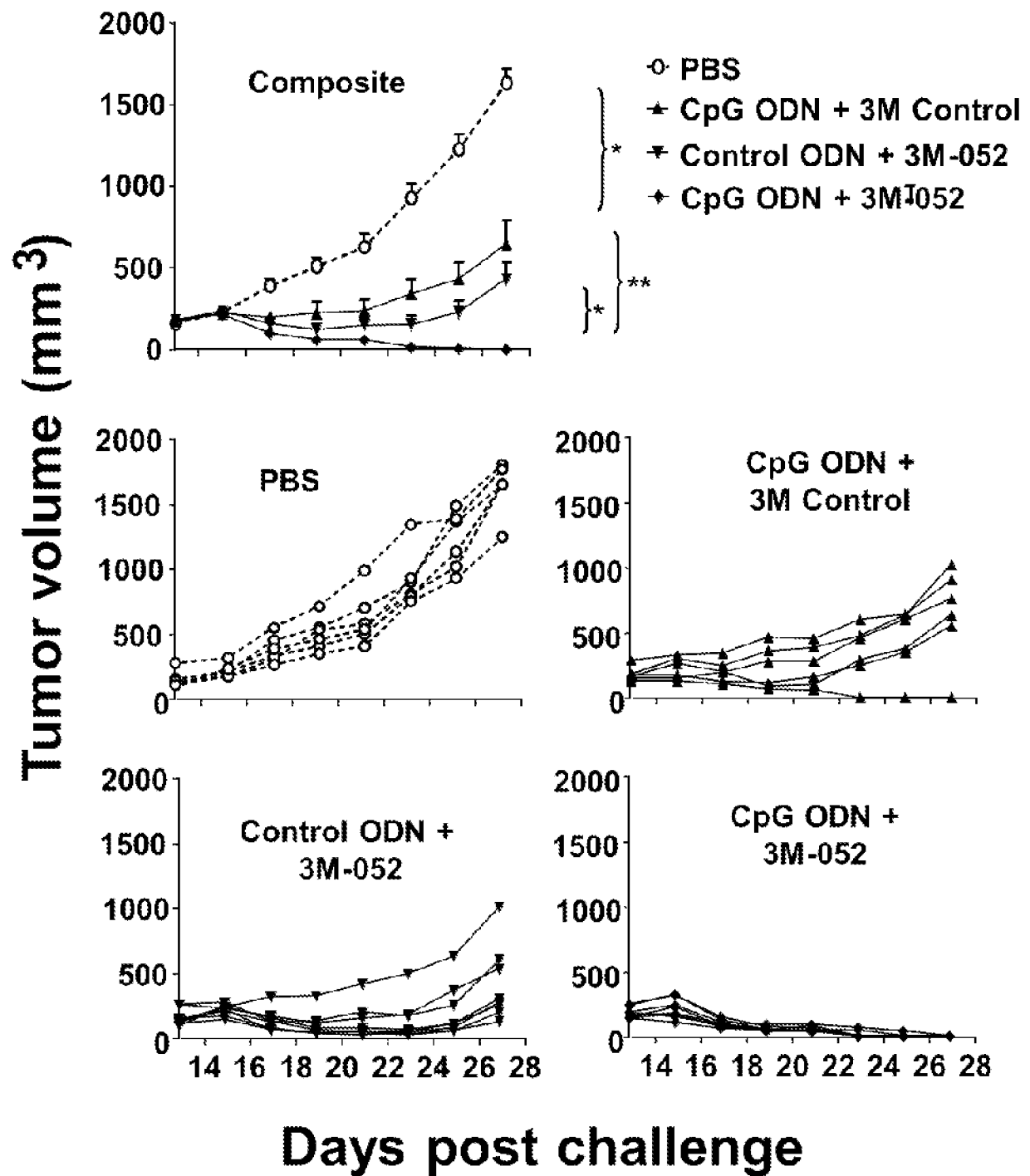
33. The method of any one of claims 1-32, further comprising administering to the subject  
5 an therapeutically effective amount of an additional chemotherapeutic agent.

34. The method of any one of claims 1-33, wherein the tumor is an established tumor.

35. The method of any one of claim 1-32, wherein administering the CpG  
10 oligodeoxynucleotide and the imidazoquinoline compound comprises intra-tumoral administration.

36. The method of any one of claims 1-35, wherein comprising administering to the subject  
a pharmaceutical composition comprising both the CpG oligodeoxynucleotide and the  
15 imidazoquinoline compound.

FIG. 1



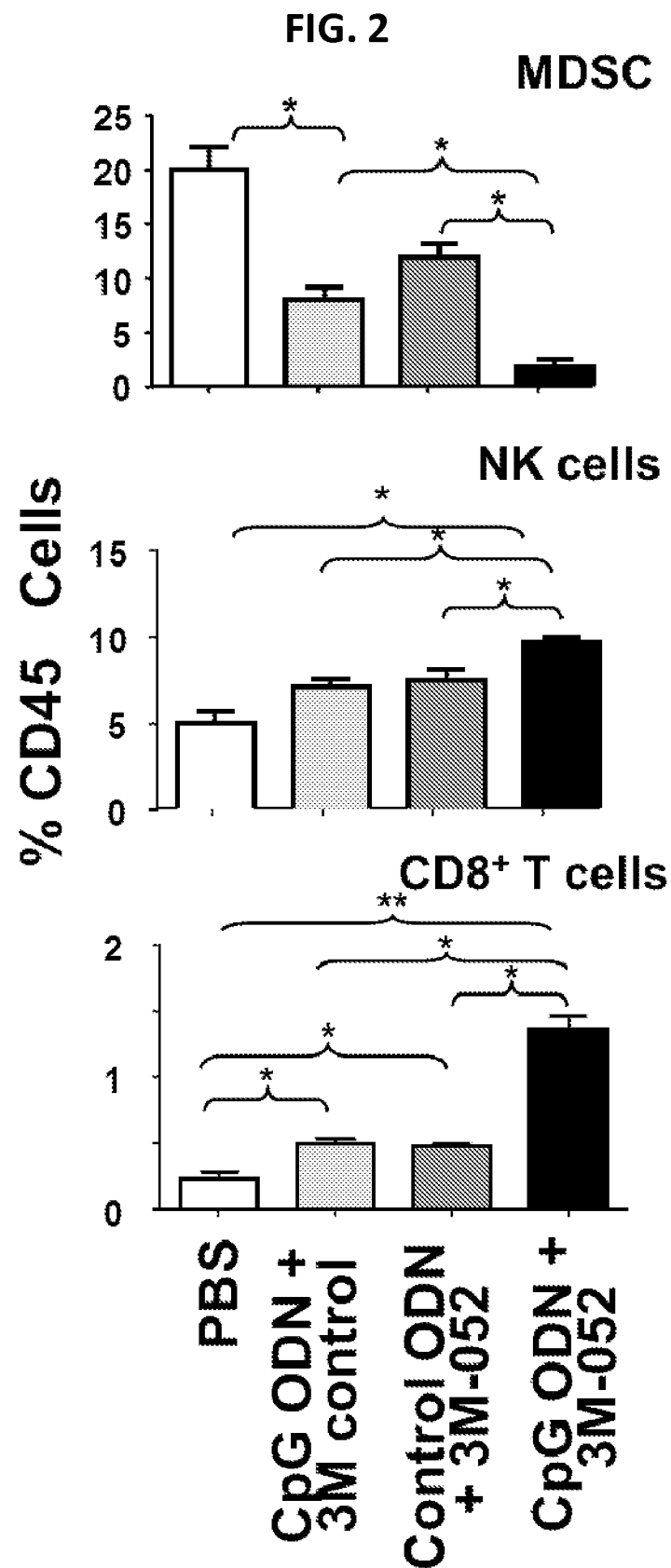


FIG. 3

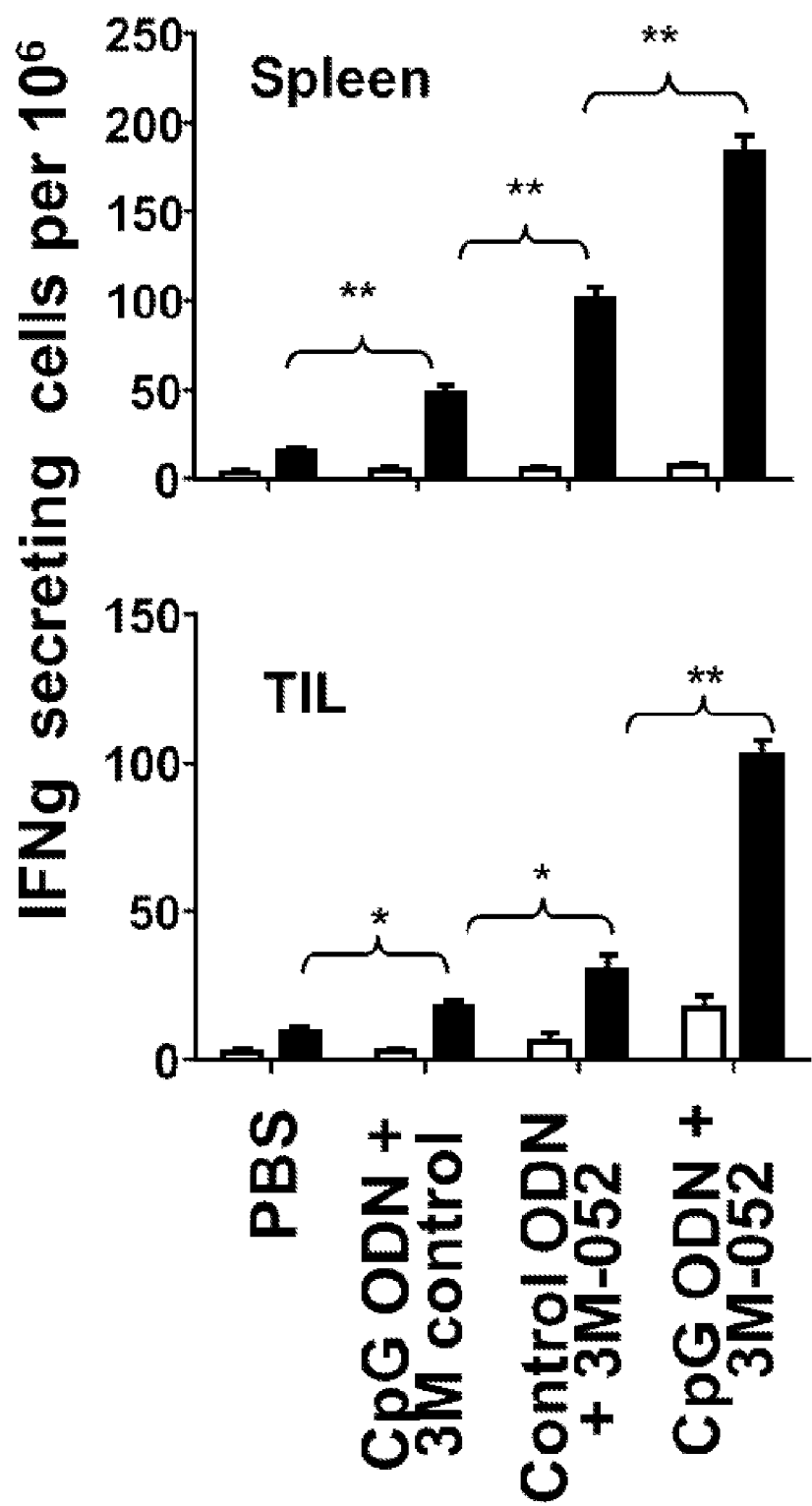
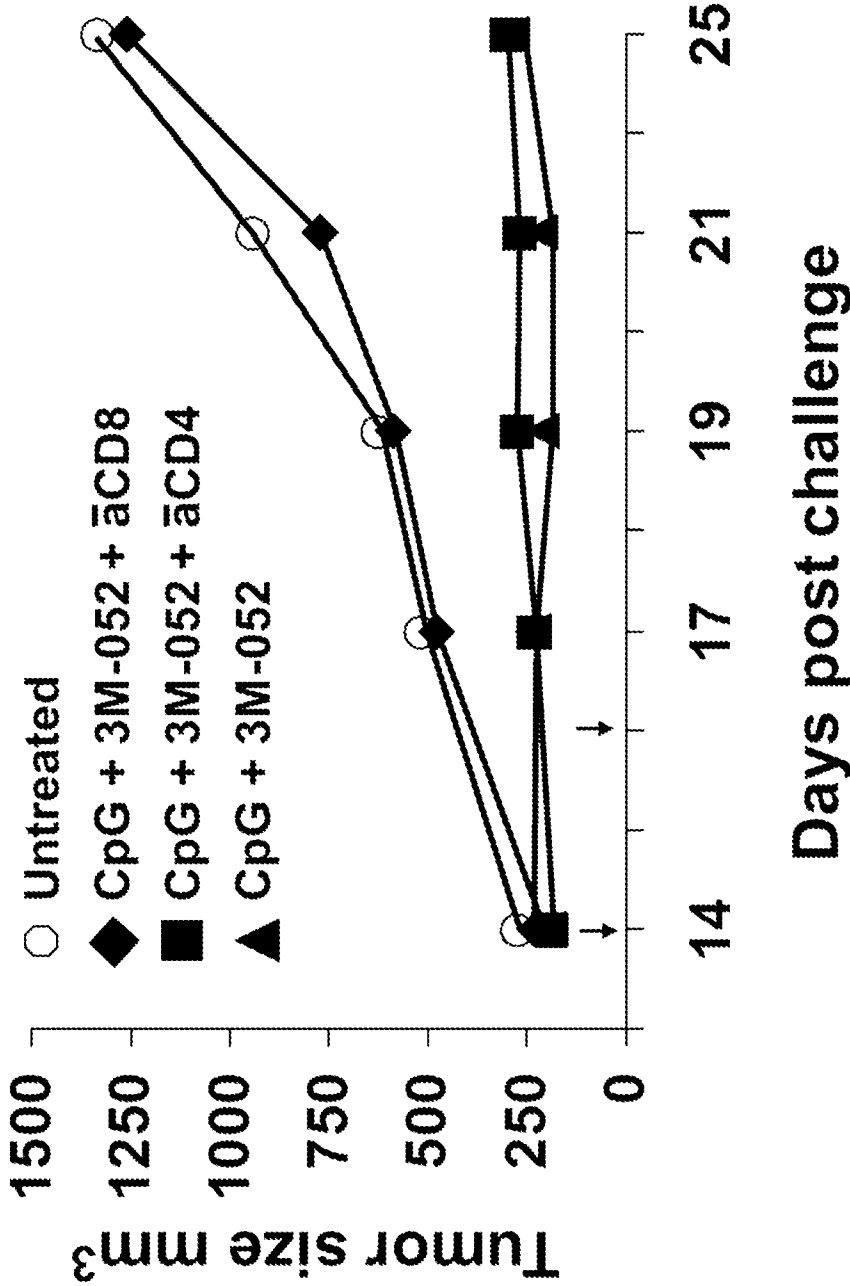


FIG. 4



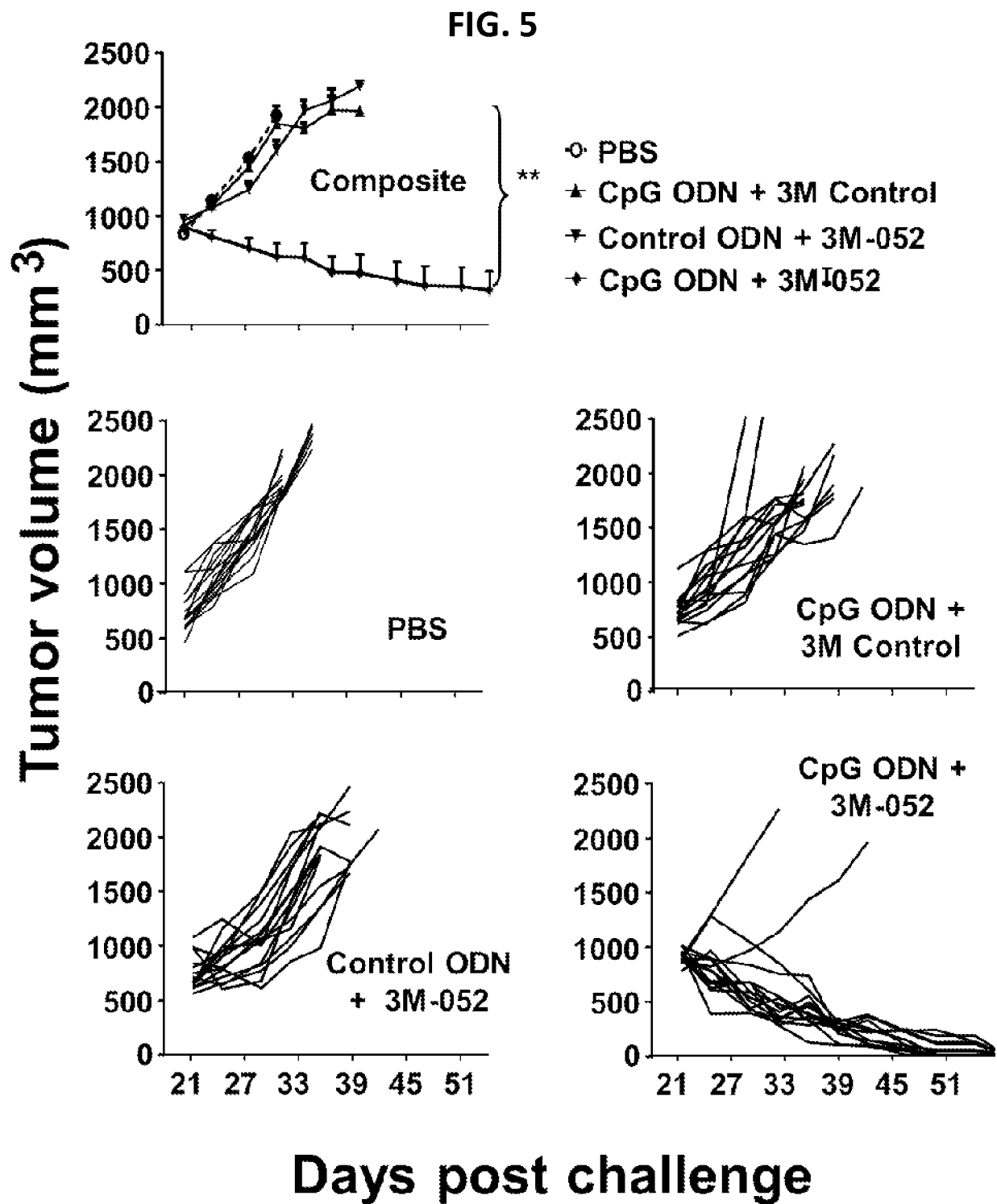


FIG. 6

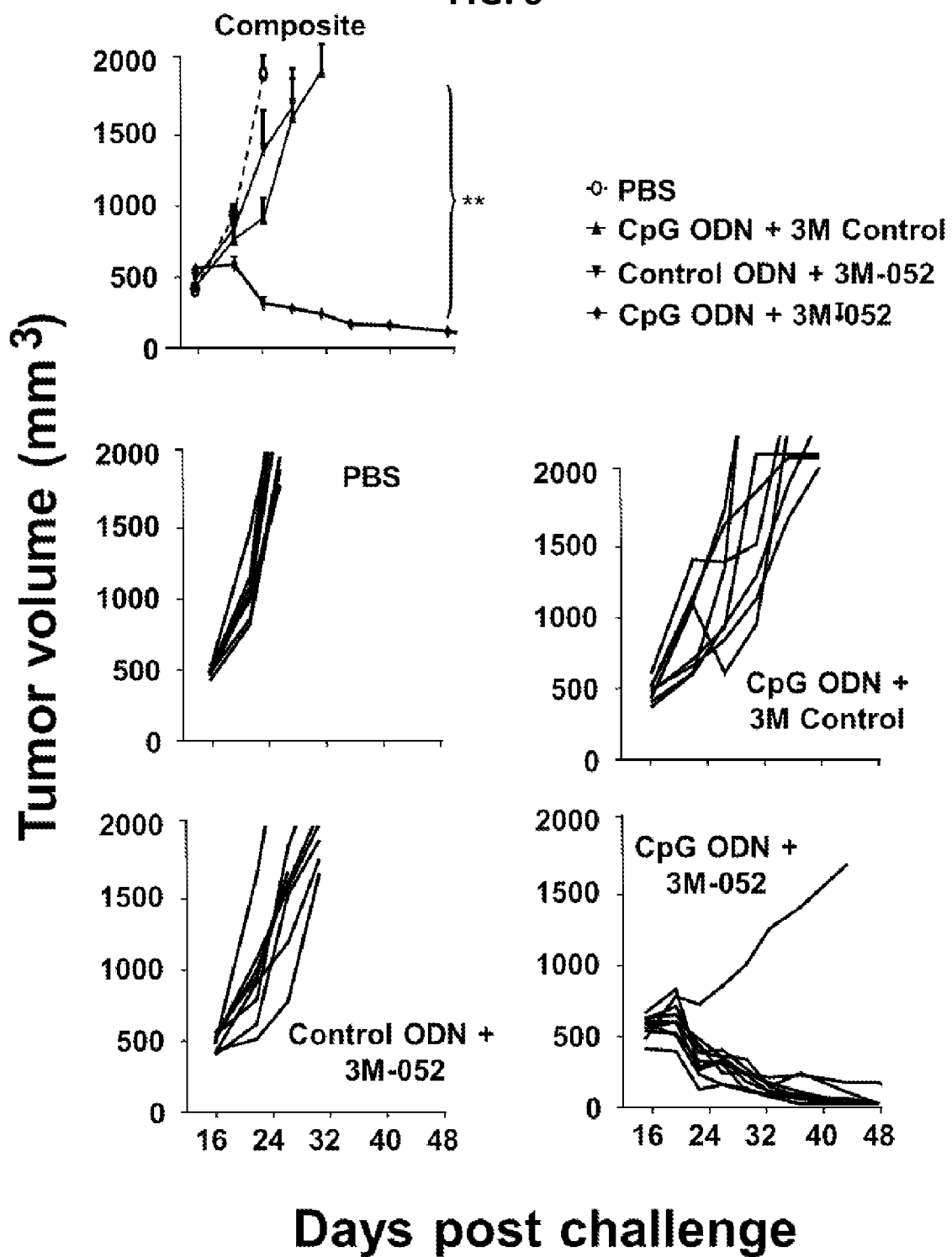


FIG. 7A

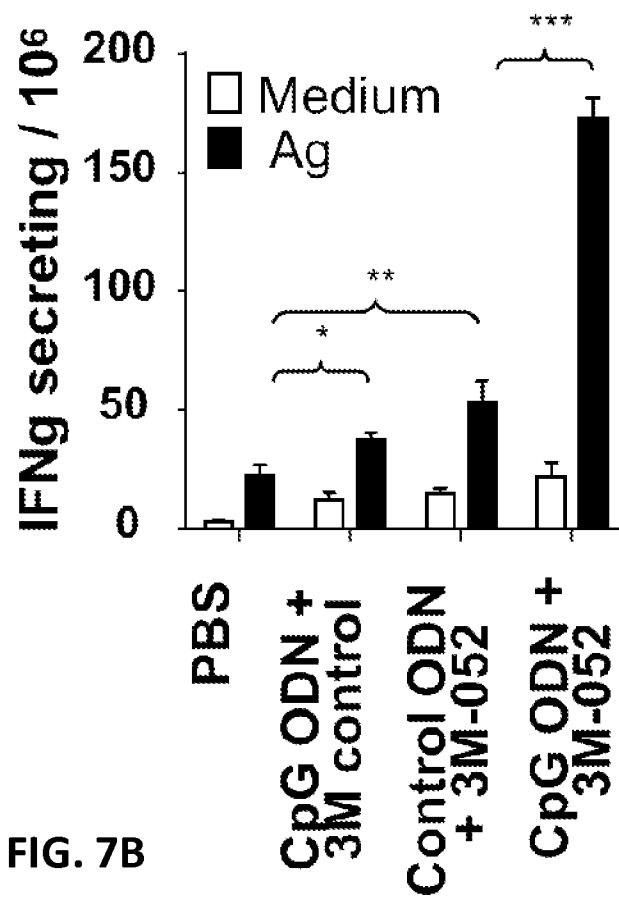


FIG. 7B

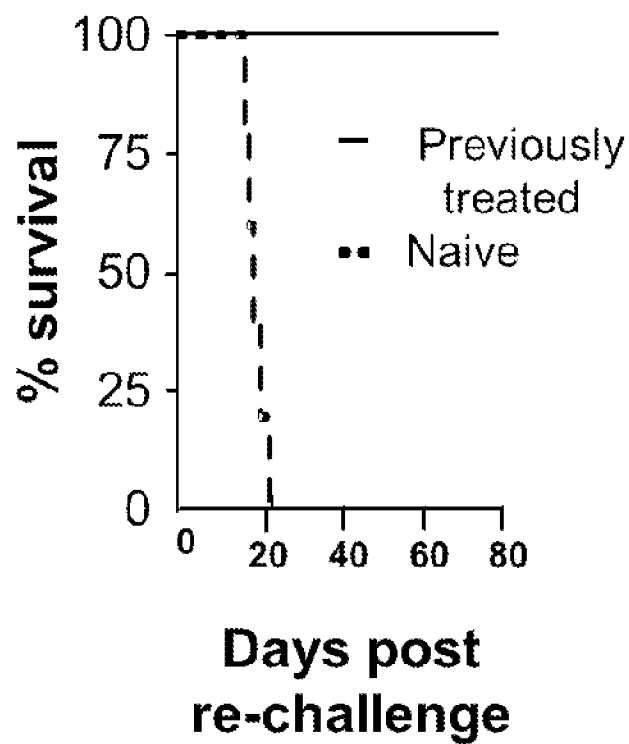
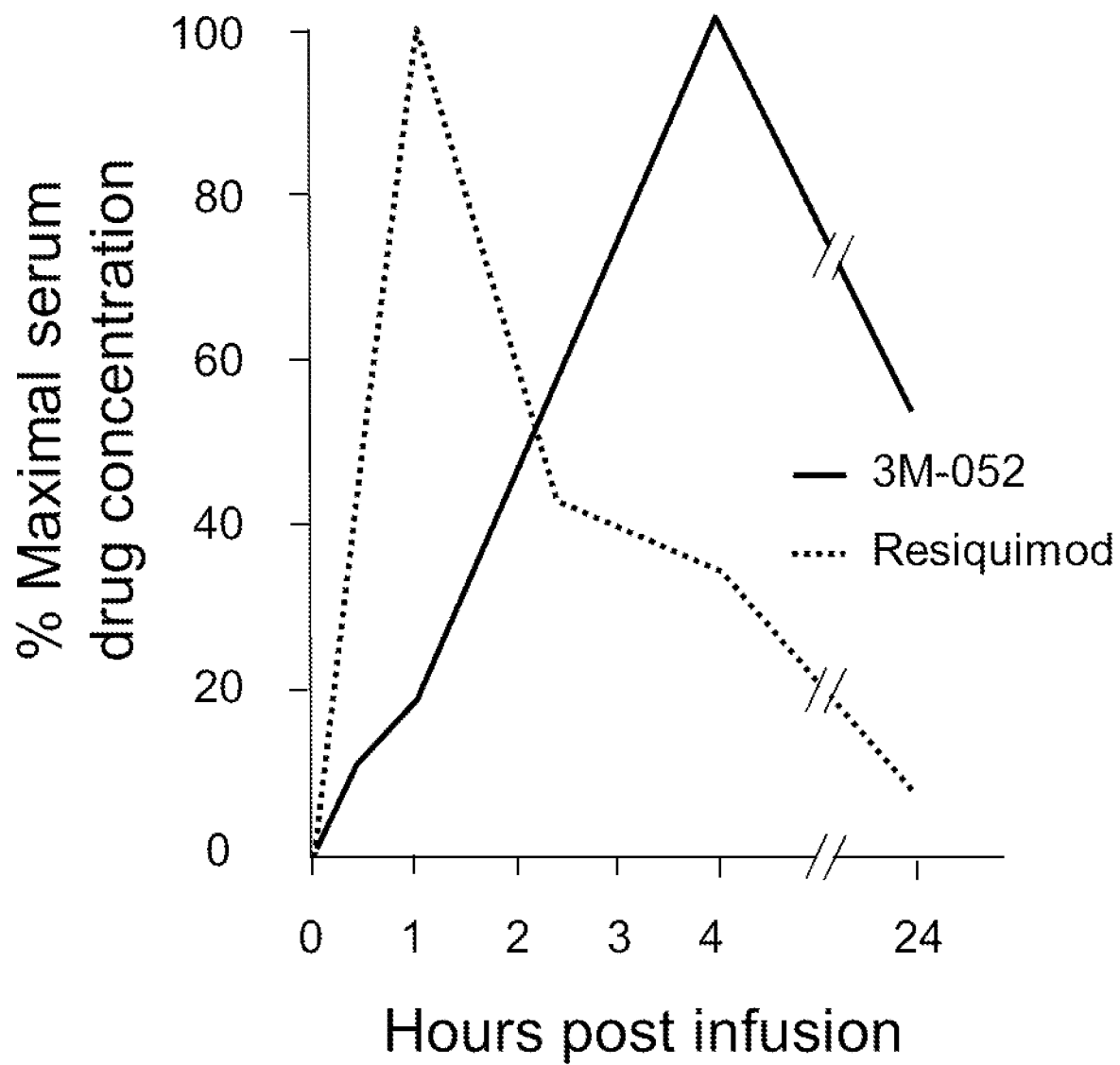


FIG. 8



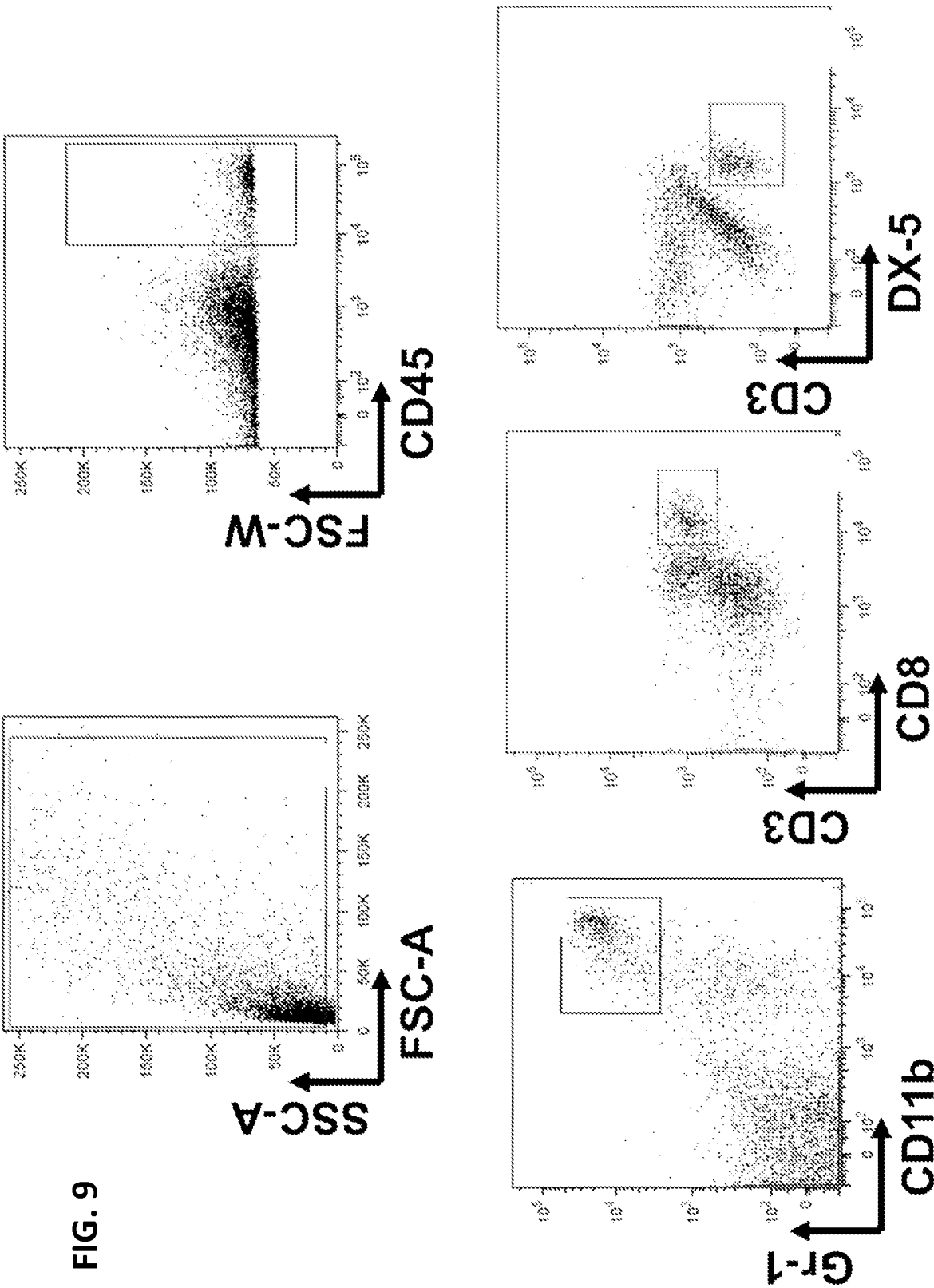


FIG. 10

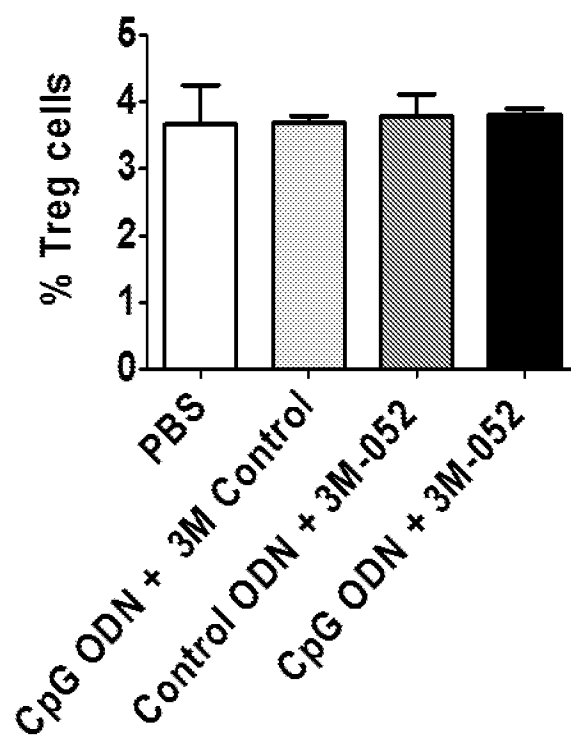


FIG. 11A

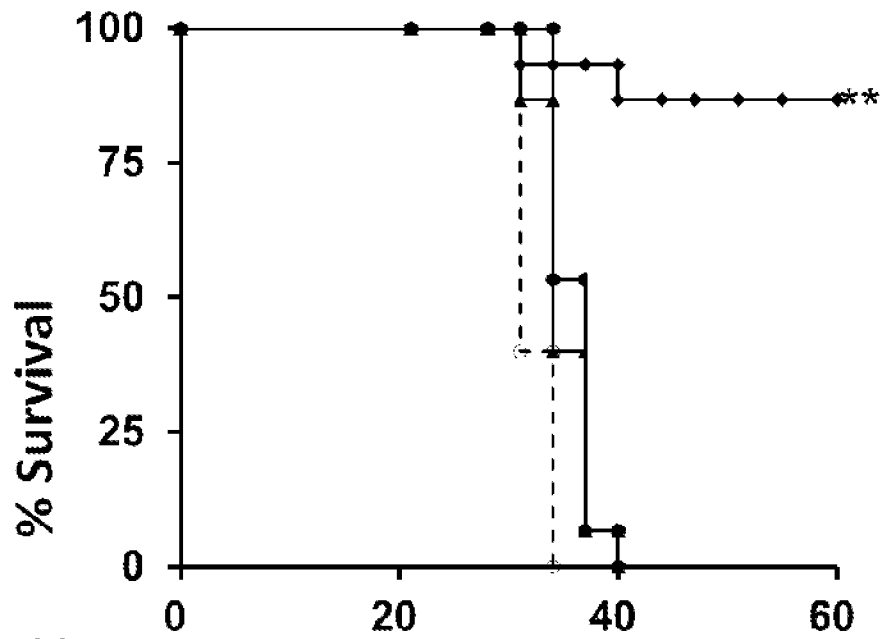
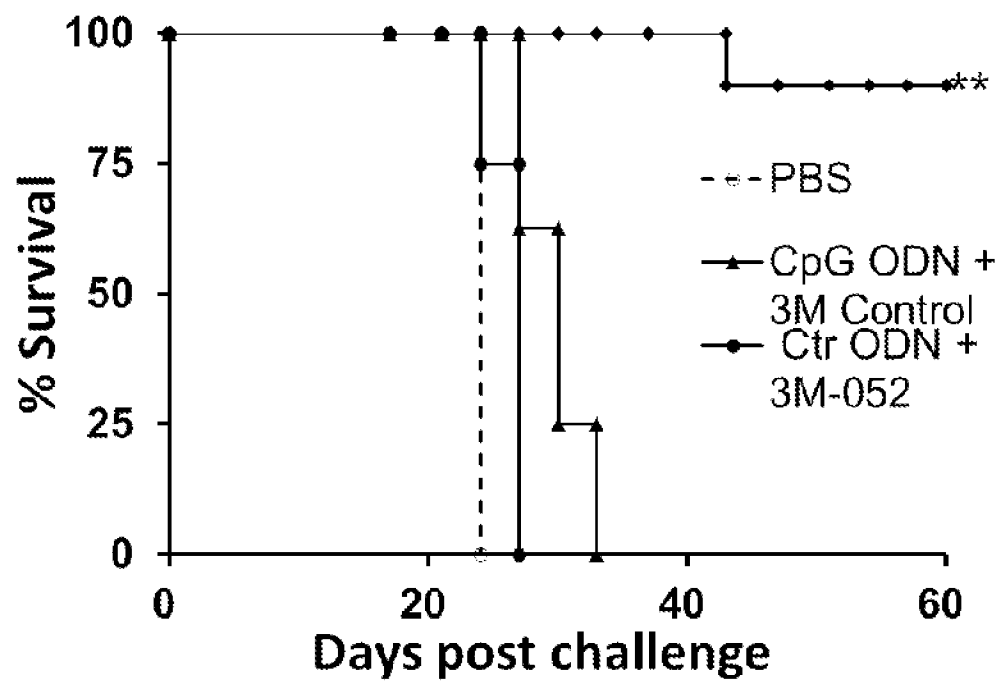


FIG. 11B



# INTERNATIONAL SEARCH REPORT

International application No  
PCT/US2014/042117

|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      |                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |                                                                                                                               |
|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------|
| <b>A. CLASSIFICATION OF SUBJECT MATTER</b><br>INV. A61K45/06      A61K31/4745      A61K31/7125      A61P35/00<br>ADD.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                |                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |                                                                                                                               |
| According to International Patent Classification (IPC) or to both national classification and IPC                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    |                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |                                                                                                                               |
| <b>B. FIELDS SEARCHED</b><br>Minimum documentation searched (classification system followed by classification symbols)<br>A61K                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       |                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |                                                                                                                               |
| 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)<br>EPO-Internal, BIOSIS, CHEM ABS Data, EMBASE, WPI Data                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                |                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |                                                                                                                               |
| <b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |                                                                                                                               |
| Category*                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                                                                                                                                                                                                                                                | Relevant to claim No.                                                                                                         |
| Y                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    | WO 2012/024284 A1 (3M INNOVATIVE PROPERTIES CO [US]; WIGHTMAN PAUL D [US])<br>23 February 2012 (2012-02-23)<br>cited in the application<br>claims; example 9<br><div style="text-align: center;">-----</div>                                                                                                                                                                                                                                                      | 1-36                                                                                                                          |
| Y                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    | DMITRI SMIRNOV ET AL: "Vaccine adjuvant activity of 3M-052: An imidazoquinoline designed for local activity without systemic cytokine induction", VACCINE, ELSEVIER LTD, GB, vol. 29, no. 33, 19 May 2011 (2011-05-19), pages 5434-5442, XP028240658, ISSN: 0264-410X, DOI: 10.1016/J.VACCINE.2011.05.061 [retrieved on 2011-05-25] cited in the application abstract<br><div style="text-align: center;">-----</div> <div style="text-align: center;">-/--</div> | 1-36                                                                                                                          |
| <div style="display: flex; justify-content: space-between;"> <span><input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C.</span> <span><input checked="" type="checkbox"/> See patent family annex.</span> </div>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |                                                                                                                               |
| <div style="display: flex;"> <div style="flex: 1;"> <p>* Special categories of cited documents :</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier application or patent but published on or after the international filing date</p> <p>"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)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="flex: 1;"> <p>*" 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</p> <p>*"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</p> <p>*"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</p> <p>*"&amp;" document member of the same patent family</p> </div> </div> |                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |                                                                                                                               |
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