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(54) **Title:** METHOD FOR RAPID GENERATION OF MATURE DENDRITIC CELLS

(57) **Abstract:** Novel methods for rapidly generating dendritic cells are disclosed herein. The methods include contacting a dendritic cell precursor with a D ODN to generate a mature dendritic cell. In one specific, non/limiting example, the method includes contacting the dendritic cell precursor or the mature dendritic cell with an antigen. The methods are of use both *in vitro* and *in vivo*.

METHOD FOR RAPID GENERATION OF
MATURE DENDRITIC CELLS

PRIORITY CLAIM

5   This application claims the benefit of U.S. Provisional Application No.
60/312,190, filed August 14, 2002, which is incorporated by reference herein in its
entirety.

FIELD

This disclosure relates to dendritic cells, specifically to the methods of

10   generating of mature dendritic cells using D type olidgodeoxynucleotides including
a CpG motif.

BACKGROUND

Dendritic cells (DC) have been identified as a pivotal antigen presenting cell

15   for initiation of an immune responses. It has been postulated that dendritic cells
provide the basis for more effective immune responses, particularly for antigens
wherein conventional vaccination is inadequate, or for use in producing a response
to tumor antigens.

"Antigen presentation" is the set of events whereby cells fragment antigens

20   into peptides, and then present these peptides in association with products of the
major histocompatibility complex, (MHC). The MHC is a region of highly
polymorphic genes whose products are expressed on the surfaces of a variety of
cells. T cells recognize foreign antigens bound to only one specific class I or class II
MHC molecule. The patterns of antigen association with either a class I or class II

25   MHC molecule determines which T cells are stimulated.

T cells do not effectively respond to antigen unless the antigen is processed
and presented to them by the appropriate antigen presenting cells (APC). The two
major classes of antigen presenting cells are dendritic cells (DC) and macrophages.
DC precursors migrate from bone marrow and circulate in the blood to specific sites

30   in the body where they mature. This trafficking is directed by expression of
chemokine receptors and adhesion molecules. Immature dendritic cells (DC) reside
in the periphery and act as sentinels, detecting invasion by pathogenic

microorganisms (Caetano, *Immunity* 14, 495-498, 2001). Exposure to certain agents trigger DC to differentiate and migrate to primary lymphoid organs where they present antigen to T cells and initiate a protective immune response (Banchereau, et al.,*Ann. Rev. Immunol.* 18, 767-811, 2000; Banchereau & Steinman, *Nature* **392**,

5 245-252, 1998). Tissue resident DC include Langerhans cells in skin, hepatic DC in the portal triads, mucosal DC and lung DC. Upon exposure to antigen and activation signals, the tissue resident DC are activated, and leave tissues to migrate via the afferent lymphatics to the T cell rich paracortex of the draining lymph nodes. The activated DC then secrete chemokines and cytokines involved in T cell homing

10 and activation, and present processed antigen to T cells. In summary, dendritic cell precursors migrate to the primary lymphoid organs where they differentiate into mature dendritic cells.

Mature DC have a distinct morphology characterized by the presence of numerous membrane processes. These processes can take the form of dendrites,

15 pseudopods or veils. DC are also characterized by the cell surface expression of large amounts of class II MHC antigens and the absence of lineage markers, including CD14 (monocyte), CD3 (T cell), CD19, 20, 24 (B cell), CD56 (natural killer), and CD66b (granulocyte). DC express a variety of adhesion and co-stimulatory molecules, e.g. CD80 and CD86, and molecules that regulate co-

20 stimulation, such as CD40. The phenotype of DC varies with the stage of maturation and activation, where expression of adhesion molecules, MHC antigens and co-stimulatory molecules increases with maturation. Antibodies that preferentially stain mature DC include anti-CD83 and CMRF-44.

Activated DC are uniquely capable of processing and presenting antigens to

25 naive T cells. The efficacy of DC in antigen presentation is widely acknowledged, but the clinical use of these cells is hampered by the fact that there are very few in any given organ. Animal studies demonstrate that mature DC can be generated *ex vivo*, loaded with antigen, and infused *in vivo* to trigger protective responses against tumors and pathogenic microorganisms (Fields et al., *Proc. Natl. Acad. Sci* 95:

30 9482-9487, 1998; Okada, H. et al. *Int. J. Cancer* 78: 196-201, 1998; Su et al., *J. Exp. Med.* 188: 809-818, 1998; DeMatos et al., *J. Surg. Oncol.* , 68: 79-91, 1998; Zhu et al., *J. Med. Primatol* 29: 182-192, 2000). Large numbers of mature DC are

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required for this type of immunotherapy. These are typically generated by incubating human peripheral blood monocytes with GM-CSF plus IL-4 for one week, followed by monocyte-conditioned medium for 2 - 7 days (Gluckman et al., *Cytokines Cell Mol Ther* 3: 187-196, 1997; Chapuis et al., *Eur. J. Immunol.* 27:

5   431-441, 1997; Palucka et al., *J. Immunol* 160: 4587-4595, 1999). In human blood, for example, about 1% of the white cells are DC. While DC can process foreign antigens into peptides that immunologically active T cells can recognize, the low numbers of DC makes their therapeutic use very difficult. Thus, this process is not only lengthy and complex, but does not uniformly generate DC with full functional

10   activity due to difficulties in standardizing the monocyte-conditioned medium (Tarte et al., *Leukemia* 14, 2182-2192, 2000; Verdijk et al., *J. Immunol.* 163, 57-61, 1999; Syme & Gluck, *J. Hematother. Stem Cell Res.* 10: 43-51, 2001). Thus, a need remains to generate mature dendritic cells in vitro.

15   **SUMMARY**

        Novel methods for rapidly generating dendritic cells is disclosed herein. The methods include contacting a dendritic cell precursor with a D ODN to generate a mature dendritic cell. In one specific, non-limiting example, the method includes contacting the dendritic cell with an antigen. In another specific, non-limiting

20   example, the method is a single step method wherein the D ODN is administered without other cytokines, such as GM-CSF and/or IL-4. These methods are of use both *in vitro* and *in vivo*.

        In one embodiment, a method for generating a mature dendritic cell is disclosed herein. The method includes contacting a dendritic cell precursor with an

25   effective amount of an oligodeoxynucleotide of at least about 16 nucleotides in length comprising a sequence represented by the following formula:

            5' $X_1X_2X_3$ Pu$_1$ Py$_2$ CpG Pu$_3$ Py$_4$ $X_4X_5X_6$(W)$_M$ (G)$_N$-3'
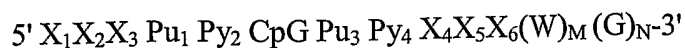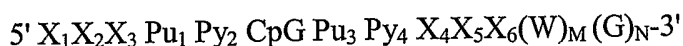
wherein the central CpG motif is unmethylated, Pu is a purine nucleotide, Py is a pyrimidine nucleotide, X and W are any nucleotide, M is any integer from 0 to 10,

30   and N is any integer from 4 to 10, to generate a mature dendritic cell.

        In another embodiment, a method is disclosed herein for producing a mature, antigen-presenting dendritic cell. The method includes contacting a

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dendritic cell precursor with an effective amount of an oligodeoxynucleotide of at least about 16 nucleotides in length comprising a sequence represented by the following formula:

$$5' \; X_1X_2X_3 \; Pu_1 \; Py_2 \; CpG \; Pu_3 \; Py_4 \; X_4X_5X_6(W)_M \; (G)_N\text{-}3'$$

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 to generate a mature dendritic cell. The method also includes contacting the mature dendritic cell with an antigen for a time sufficient to allow the antigen to be presented, thereby producing a mature antigen-presenting dendritic cell.

In a further embodiment, a single step method is disclosed for differentiating a dendritic precursor cell into a mature antigen presenting cell. The method includes contacting a dendritic cell precursor with an effective amount of an antigen and an oligodeoxynucleotide of at least about 16 nucleotides in length comprising a sequence represented by the following formula:

$$5' \; X_1X_2X_3 \; Pu_1 \; Py_2 \; CpG \; Pu_3 \; Py_4 \; X_4X_5X_6(W)_M \; (G)_N\text{-}3'$$
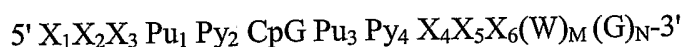
wherein the central CpG motif is unmethylated, Pu is a purine nucleotide, Py is a pyrimidine nucleotide, X and W are any nucleotide, M is any integer from 0 to 10, and N is any integer from 4 to 10, thereby differentiating a mature antigen presenting cell.

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

BRIEF DESCRIPTION OF THE FIGURES

Fig1 is a set of plots demonstrating that D ODN induce elutriated monocytes to differentiate into mature DC. Elutriated monocytes (95% pure) were incubated with 3 μM ODN for 24 - 96 h. Cells were fixed and stained for expression of CD83, CD86, CD80, CD40 and CD14 using fluorescence activated cell sorting. Results are

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representative of 5 independent experiments. The bottom panel provides a 600X magnification of Giemsa-stained cytospin preparations at each time point.

Fig2 is a set of plots and a bar graph showing that the contribution of pDC to D ODN induced monocyte differentiation. For the results shown in Fig. 2A, elutriated monocytes were depleted of, or enriched in, $CD123^{hi}$ $CD45RA^+$ pDC. For the results shown in Fig. 2B, the pDC depleted monocytes were incubated for 48 h with 3 μM ODN and their maturation monitored by the appearance of $CD83/CD86^+$ cells in the presence or absence of 5% pDC. The results shown in Fig. 2C demonstrate neutralizing anti-human IFNα Ab (10 μg /ml) significantly reduced DC maturation. Fig. 1D is a bar graph showing production of IFNα by pDC-enriched cultures stimulated with D ODN. Results are representative of 3 independent experiments.

Fig3 is a set of graphs showing that DC generated by D ODN treatment are active in mixed lymphocyte reaction. Fig. 3A is a set of line graphs of results obtained when elutriated monocytes were cultured in vitro for 2 days with 3 μM of control ODN (—○—), D ODN (—▲—) or medium (—□—). These DC were mixed with monocyte-depleted peripheral blood lymphocytes from an allogeneic donor for 5 days and proliferation monitored. Fig. 3B is a bar graph of results obtained when supernatants from the 1:20 stimulator/responder cell MLR cultures were tested for IFNγ levels by ELISA (▦ medium; □ control ODN; ▪ D ODN). Data represent the mean ± SD of triplicate cultures from two donors, studied independently.

Fig.4 is a bar graph demonstrating that DC generated by D ODN treatment can present tetanus toxoid to autologous T cells. PBMC from tetanus immune individuals were incubated with 3 μM of ODN for 2 days and then pulsed with tetanus toxoid for 4 h. After 12 h incubation with brefeldin A, the cells were fixed, permeabilized and stained with PE-conjugated anti-IFNγ plus FITC-conjugated anti-CD3. The mean ± SD of IFNγ producing $CD3^+$ T cells from four independently studied individuals is shown. Note that there was no increase in the number of IL-4 producing cells in these cultures □ no antigen; ▪, tetanus toxoid).

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Fig 5 is a line graph of the comparative activity of DC generated by treating monocytes with D ODN vs GM-CSF plus IL-4.

Elutriated human PBL were transferred into SCID mice to generate Hu-PBL-SCID recipients. Elutriated monocytes from the same donor were induced to mature into DC by 2 days of treatment with D ODN or 5 days of treatment with GM-CSF plus IL-4. Treated cells or naive monocytes were pulsed with OVA and injected into SCID recipients. Two weeks later, serum IgG anti-OVA titers were determined by ELISA. Hu-PBL-SCID mice immunized with free OVA (—▽—), or OVA-pulsed syngeneic monocytes (—◆—) generated no anti-OVA response, whereas OVA-pulsed DC produced by treatment with D ODN (—■—) or GM-CSF/IL-4 (—○—) stimulated equivalent anti-OVA responses. Data represent serum dilutions of individual mice (n=3, per group).

Fig. 6 is a bar graph showing the *in vivo* activity of D ODN. Rhesus monkeys were immunized subcutaneously and boosted 3 weeks later with OVA adsorbed onto alum (4 µg) or OVA plus a mixture of D ODN (250 µg) on alum. Serum IgG anti-OVA Ab levels were measured 10 days after immunization and 2 weeks post boost. Results represent the geometric mean ± SD of 3 animals/group □ OVA; ■ OVA+ D ODN). The dotted line shows the background anti-OVA titer of pre-immune sera. * P < 0.05 when compared to animals immunized with OVA in the absence of D ODN.

Fig. 7 is a set of plots showing a comparison of DC generation induced by GM-CSF + IL-4 to that of D ODN alone or D ODN in the presence of GM-CSF + IL-4.

Elutriated monocytes (95% pure) were incubated with for 48 h in medium alone or in the presence of 3 µM D ODN and/or 25 ng/ml IL-4 + 100 ng/ml GM-CSF. Cells were fixed and stained for expression of CD83, CD80 and CD40 Results are representative of 3 independent experiments.

Fig. 8 is a table of D ODN sequences.

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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.

SEQ ID NOs: 1-50 are oligodeoxynucleotide seqeunces.

DETAILED DESCRIPTION

I.   Abbreviations

Ab: antibody

APC: Antigen presenting cell

DC: dendritic cell

D ODN: D type oligodeoxynucleotide including an unmethylated CpG motif

Flt-3L: flt-3 ligand

GM-CSF: granulocyte macrophasge colony stimulating factor

H: hours

IFN: Interferon

IL: interleukin

K ODN: K type oliogdeoxynucleotide including an unmethylated CpG motif

ODN: Oligodeoxynucleotide

pDC: plasmacytoid dendritic cell

μg: microgram

II.   Terms

Unless otherwise noted, technical terms are used according to conventional usage. Definitions of common terms in molecular biology may be found in Benjamin Lewin, *Genes V*, published by Oxford University Press, 1994 (ISBN 0-19-854287-9); Kendrew *et al.* (eds.), *The Encyclopedia of Molecular Biology*, published by Blackwell Science Ltd., 1994 (ISBN 0-632-02182-9); and Robert A. Meyers

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(ed.), *Molecular Biology and Biotechnology: a Comprehensive Desk Reference*, published by VCH Publishers, Inc., 1995 (ISBN 1-56081-569-8).

In order to facilitate review of the various embodiments of this disclosure, the following explanations of specific terms are provided:

5

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.

10   **Antigen:** A compound, composition, or substance that can stimulate the production of antibodies or a T-cell response in an animal, including compositions that are injected or absorbed into an animal. An antigen reacts with the products of specific humoral or cellular immunity, including those induced by heterologous immunogens. The term "antigen" includes all related antigenic epitopes.

15   **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 occurring the 5-position of the pyrimidine ring. A CpG motif is a pattern of bases that include an unmethylated central CpG

20   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 part of the activity to the CpG oligodeoxynucleotide. A CpG oligonucleotide is an oligonucleotide that is at least about ten nucleotides in length and includes an unmethylated CpG. CpG oligonucleotides include both D and K type

25   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 neoplasm that has undergone characteristic anaplasia with loss of differentiation, increase rate of growth, invasion of surrounding tissue,

30   and is capable of metastasis. For example, thyroid cancer is a malignant neoplasm that arises in or from thyroid tissue, and breast cancer is a malignant neoplasm that arises in or from breast tissue (such as a ductal carcinoma). Residual cancer is

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

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 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 2nd 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, Knobf MF, Durivage HJ (eds): The Cancer Chemotherapy Handbook, 4th ed. St. Louis, Mosby-Year Book, 1993).

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.

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

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 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 ODN is at least about 16 nucleotides in length and includes a sequence represented by Formula III:

5' $X_1X_2X_3$ Pu_1 Py_2 CpG Pu_3 Py_4 $X_4X_5X_6(W)_M$ $(G)_N$-3'
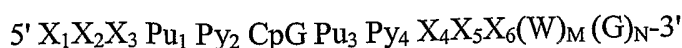
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wherein the central CpG motif is unmethylated, Pu is a purine nucleotide, Py
is a pyrimidine nucleotide, X and W are any nucleotide, M is any integer from 0 to
10, and N is any integer from 4 to 10. Additional detailed description of D ODN
sequences and their activities can be found in Verthelyi et al., *J. Immunol.* 166:2372-
5   2377, 2001, which is herein incorporated by reference. Generally D ODNs can
stimulate a cellular response. For example, an "effective amount" of a D ODN is an
amount of the D ODN sufficient to stimulate a response, such as the maturation of
dendritic cells.

Dendritic cell (DC): Dendritic cells are the principle antigen presenting
10   cells (APCs) involved in primary immune responses. Dendritic cells include
plasmacytoid dendritic cells and myeloid dendritic cells. Their major function is to
obtain antigen in tissues, migrate to lymphoid organs and present the antigen in
order to activate T cells. Immature dendritic cells originate in the bone marrow and
reside in the periphery as immature cells. In one embodiment, a dendritic cell is a
15   plasmacytoid dendritic cell. Plasmacytoid dendritic cells differentiate from
precursors called "DC2" while myeloid dendritic cells differentiate from precursors
termed "DC1."

DCs are capable of evolving from immature, antigen-capturing cells to
mature, antigen-presenting, T cell-priming cells; converting antigens into
20   immunogens and expressing molecules such as cytokines, chemokines,
costimulatory molecules and proteases to initiate an immune response.

DCs are derived from hematopoietic stem cells in the bone marrow and are
widely distributed as immature cells within all tissues, particularly those that
interface with the environment (*e.g.* skin, mucosal surfaces) and in lymphoid organs.
25   Immature DCs are recruited to sites of inflammation in peripheral tissues following
pathogen invasion. Chemokine responsiveness and chemokine receptor expression
are essential components of the DC recruitment process to sites of inflammation and
migration to lymphoid organs. "Immature" DCs may express the chemokine
receptors CCR1, CCR2, CCR5, CCR6 and CXCR1. Immature DCs capture
30   antigens by phagocytosis, macropinocytosis or via interaction with a variety of cell
surface receptors and endocytosis. Internalization of foreign antigens can

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subsequently trigger their maturation and migration from peripheral tissues to lymphoid organs (see below).

The ability of DCs to regulate immunity is dependent on DC differentiation, as it depends on their maturation state. A variety of factors can induce differentiation following antigen uptake and processing within DCs, including: whole bacteria or bacterial-derived antigens (*e.g.* lipopolysaccharide, LPS), inflammatory cytokines, ligation of select cell surface receptors (*e.g.* CD40) and viral products (*e.g.* double-stranded RNA). During their conversion from immature to mature cells, DCs undergo a number of phenotypical and functional changes. The process of DC maturation, in general, involves a redistribution of major histocompatibility complex (MHC) molecules from intracellular endocytic compartments to the DC surface, down-regulation of antigen internalization, an increase in the surface expression of costimulatory molecules, morphological changes (*e.g.* formation of dendrites), cytoskeleton re-organization, secretion of chemokines, cytokines and proteases, and surface expression of adhesion molecules and chemokine receptors.

Dendritic Cell Precursor: Immature cells that can differentiate into dendritic cells. In one embodiment a dendritic cell precursor is a DC1 cell that differentiates into myeloid cells (e.g. monocytes). In another embodiment, a dendritic cell precursor is a DC2 cell that differentiates into a plasmacytoid dendritic cell. Plasmacytoid dendritic cells and monocytes are also dendritic cell precursors as they differentiate into mature dendritic cells.

Differentiation: The process by which cells become more specialized to perform biological functions, and differentiation is a property that is totally or partially lost by cells that have undergone malignant transformation. For example, dendritic cell precursors such as monocytes or plasmacytoide dendritic cells can differentiate into dendritic cells under the inluence of certain cytokines and growth factors.

Epitope: An antigenic determinant. These are particular chemical groups or peptide sequences on a molecule that are antigenic, i.e. that elicit a specific immune response. An antibody binds a particular antigenic epitope.

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Functionally Equivalent: Sequence alterations, for example in a D type ODN, that yield the same results as described herein. Such sequence alterations can include, but are not limited to, deletions, base modifications, mutations, labeling, and insertions.

5   **Flt-3 ligand (flt-3L)**: A factor that binds to the flt-3 receptor. The flt-3 ligand promotes long-term expansion and differentiation of human pro-B-cells in the presence of IL-7, or IL-7 and IL-3. The flt-3 ligand is known to support the survival of precursor cell types in the lineage of blood-forming cells, such as highly proliferative potential colony forming cells (e.g. see Lyman et al., *Cell* 75: 1157-67,

10   1993).

       Granulocyte/macrophage colony-stimulating factor (GM-CSF): A factor which modulates the maturation and function of denritic cells, Witmer-Pack et al., J. Exp. Med. 166:1484-98, 1987).

       GM-CSF is a monomeric protein of 127 amino acids with two glycosylation

15   sites. The protein is synthesized as a precursor of 144 amino acids, which included a hydrophobic secretory signal sequence at the aminoterminal end. The human gene has a length of approximately 2. 5 kb and contains four exons. The distance between the GM-CSF gene and the IL-3 gene is approximately 9 kb. The human GM-CSF gene maps to chromosome 5q22-31.

20   GM-CSF was isolated initially as a factor stimulating the growth of macrophage/granulocyte-containing colonies in soft agar cultures. GM-CSF is also involved in the growth and development of granulocyte and macrophage progenitor cells. It stimulates myeloblasts and monoblasts and triggers irreversible differentiation of these cells. GM-CSF synergises with erythropoietin in the

25   proliferation of erythroid and megakaryocytic progenitor cells.

       GM-CSF has been used clinically for the physiological reconstitution of hematopoiesis in diseases characterized either by an aberrant maturation of blood cells or by a reduced production of leukocytes. The usual dose, route and schedules for GM-CSF are 5-10 micrograms/kg/day either by 4-6 hours intravenous infusion

30   or by subcutaneous injection.

       Mobilization Agent: A compound such as a naturally occurring protein or a derivative thereof, that acts on hematopoietic progenitor or stem cells to mobilize

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precursor cells. A mobilizing agent causes DC precursors to migrate from their tissue of origin such as the bone marrow, and move into other tissues and the peripheral blood. Mobilization agents include, but are not limited to, FLT-3 ligand or GM-CSF.

Immune response: A response of a cell of the immune system, such as a B cell, or a 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-γ, 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}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 a D ODN as compared to the percent of samples that respond using a K ODN. A non-paramentric ANOVA can be used to compare differences in the magnitude of the response induced by D ODN as compared to the percent of samples that respond using a K ODN. In this example, p ≤ 0.05 is significant, and indicates a substantial increase in the parameter of the immune response. One of skill in the art can readily identify other statistical assays of use.

Infectious agent: An agent that can infect a subject, including, but not limited to, viruses, bacteria, and fungi.

Examples of infectious virus include: *Retroviridae*; *Picornaviridae* (for example, polio viruses, hepatitis A virus; enteroviruses, human coxsackie viruses,

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rhinoviruses, echoviruses); *Calciviridae* (such as strains that cause gastroenteritis); *Togaviridae* (for example, equine encephalitis viruses, rubella viruses); *Flaviridae* (for example, dengue viruses, encephalitis viruses, yellow fever viruses); *Coronaviridae* (for example, coronaviruses); *Rhabdoviridae* (for example, vesicular

5   stomatitis viruses, rabies viruses); *Filoviridae* (for example, ebola viruses); *Paramyxoviridae* (for example, parainfluenza viruses, mumps virus, measles virus, respiratory syncytial virus); *Orthomyxoviridae* (for example, influenza viruses); *Bungaviridae* (for example, Hantaan viruses, bunga viruses, phleboviruses and Nairo viruses); *Arena viridae* (hemorrhagic fever viruses); *Reoviridae* (e.g.,

10 reoviruses, orbiviurses and rotaviruses); *Birnaviridae*; *Hepadnaviridae* (Hepatitis B virus); *Parvoviridae* (parvoviruses); *Papovaviridae* (papilloma viruses, polyoma viruses); *Adenoviridae* (most adenoviruses); *Herpesviridae* (herpes simplex virus (HSV) 1 and HSV-2, varicella zoster virus, cytomegalovirus (CMV), herpes viruses); *Poxviridae* (variola viruses, vaccinia viruses, pox viruses); and *Iridoviridae*

15 (such as African swine fever virus); and unclassified viruses (for example, the etiological agents of Spongiform encephalopathies, the agent of delta hepatitis (thought to be a defective satellite of hepatitis B virus), the agents of non-A, non-B hepatitis (class 1=internally transmitted; class 2=parenterally transmitted (i.e., Hepatitis C); Norwalk and related viruses, and astroviruses).

20   Examples of infectious bacteria include: *Helicobacter pyloris, Borelia burgdorferi, Legionella pneumophilia, Mycobacteria sps* (such as. *M. tuberculosis, M. avium, M. intracellulare, M. kansaii, M. gordonae), Staphylococcus aureus, Neisseria gonorrhoeae, Neisseria meningitidis, Listeria monocytogenes, Streptococcus pyogenes* (Group A *Streptococcus), Streptococcus agalactiae* (Group

25 B *Streptococcus), Streptococcus* (*viridans* group), *Streptococcus faecalis, Streptococcus bovis, Streptococcus (anaerobic sps.), Streptococcus pneumoniae,* pathogenic *Campylobacter sp., Enterococcus sp., Haemophilus influenzae, Bacillus antracis, corynebacterium diphtheriae, corynebacterium sp., Erysipelothrix rhusiopathiae, Clostridium perfringers, Clostridium tetani, Enterobacter aerogenes,*

30 *Klebsiella pneumoniae, Pasturella multocida, Bacteroides sp., Fusobacterium nucleatum, Streptobacillus moniliformis, Treponema pallidium, Treponema pertenue, Leptospira,* and *Actinomyces israelli.*

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Examples of infectious fungi include, but are not limited to, *Cryptococcus neoformans, Histoplasma capsulatum, Coccidioides immitis, Blastomyces dermatitidis, Chlamydia trachomatis, Candida albicans.*

Other infectious organisms (such as protists) include: *Plasmodium*

5   *falciparum* and *Toxoplasma gondii.*

Interferon alpha: At least 23 different variants of IFN-α are known. The individual proteins have molecular masses between 19-26 kDa and consist of proteins with lengths of 156-166 and 172 amino acids. All IFN-α subtypes possess a common conserved sequence region between amino acid positions 115-151 while

10   the amino-terminal ends are variable. Many IFN-α subtypes differ in their sequences at only one or two positions. Naturally occurring variants also include proteins truncated by 10 amino acids at the carboxy-terminal end.

There are at least 23 different IFN-α genes. They have a length of 1-2 kb and are clustered on human chromosome 9p22. Based upon the structures two types

15   of IFN-alpha genes, designated class I and II, are distinguished. They encode proteins of 156-166 and172 amino acids, respectively.

IFN-α is assayed by a cytopathic effect reduction test employing human and bovine cell lines. Minute amounts of IFN-α can be assayed also by detection of the Mx protein specifically induced by this interferon. A sandwich ELISA employing

20   bi-specific monoclonal antibodies for rapid detection is also available.

Interferon gamma: IFN-γ is a dimeric protein with subunits of 146 amino acids. The protein is glycosylated at two sites, and the pI is 8.3-8.5. IFN-γ is synthesized as a precursor protein of 166 amino acids including a secretory signal sequence of 23 amino acids. Two molecular forms of the biologically active protein

25   of 20 and 25 kDa have been described. Both of them are glycosylated at position 25. The 25 kDa form is also glycosylated at position 97. The observed differences of natural IFN-γ with respect to molecular mass and charge are due to variable glycosylation patterns. 40-60 kDa forms observed under non-denaturing conditions are dimers and tetramers of IFN-γ. The human gene has a length of approximately 6

30   kb. It contains four exons and maps to chromosome 12q24.1.

IFN-γ can be detected by sensitive immunoassays, such as an ELSA test that allows detection of individual cells producing IFN-γ. Minute amounts of IFN-γ can

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be detected indirectly by measuring IFN-induced proteins such as Mx protein. The
induction of the synthesis of IP-10 has been used also to measure IFN-gamma
concentrations. In addition, bioassays can be used to detect IFN-γ, such as an assay
that employs induction of indoleamine 2,3-dioxygenase activity in 2D9 cells.

5   **Interferon Inducible Protein 10**: A cytokine that is 98 amino acids in
length that has homology to platelet factor-4, and is a chemokine. The human IP-10
genes contains four exons and maps to chromosome 4q12-21.

         Interleukin-10: IL-10 is a homodimeric protein with subunits having a
length of 160 amino acids that is a cytokine. Human IL-10 shows 73 percent amino
10   acid homology with murine IL-10. The human IL-10 gene contains four exons.

         IL10 inhibits the synthesis of a number of cytokines such as IL-2 and IFN-γ
in Th1 subpopulations of T-cells but not of Th2. IL10 can be detected with an
ELISA assay. In addition, the murine mast cell line D36 can be used to bioassay
human IL10. The intracellular factor can be detected also by flow cytometry.

15   **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
20   "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
25   including an unmethylated CpG motif that has a sequence represented by the
formula:

$$5' \ N_1N_2N_3Q\text{-}CpG\text{-}WN_4N_5N_6 \ 3'$$

         wherein the central CpG motif is unmethylated, Q 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, Q is a T.
30   Additional detailed description of K ODN sequences and their activities can be
found in the description below. Generally K ODNs can stimulate a humoral
response. For example, K ODNs stimulate the production of immunoglobulins, such

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as IgM and IgG. K ODNs can also stimulate proliferation of peripheral blood mononuclear cells and increase expression of IL-6 and/or IL-12, amongst other activities.

     Leukocyte: Cells in the blood, also termed "white cells," that are involved in defending the body against infective organisms and foreign substances. Leukocytes are produced in the bone marrow. There are 5 main types of white blood cell, subdivided between 2 main groups: polymorphomnuclear leukocytes (neutrophils, eosinophils, basophils) and mononuclear leukocytes (monocytes and lymphocytes). When an infection is present, the production of leukocytes increases.

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

     Maturation: The process in which an immature cell, such as dendritic cell precursor, changes in form or function to become a functionally mature dendritic cell, such as an antigen presenting cell (APC).

     Neoplasm: An abnormal cellular proliferation, which includes benign and malignant tumors, as well as other proliferative disorders.

     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 oligodeoxyribonucleotides (ODNs). The term "oligonucleotide" 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).

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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 phophorothioate 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- phophonates (in which the charged phosphonate oxygen is replaced by an alkyl or aryl group), phophodiester 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 oligonucleotide," "immunostimulatory CpG containing oligodeoxynucleotide," "CpG ODN," refers to an oligodeoxynucleotide, which contains a cytosine, guanine dinucleotide sequence and stimulates (e.g. has a mitogenic effect or induces cytokine production) vertebrate immune cells. The cytosine, guanine is unmethylated.

An "oligonucleotide delivery complex" is an oligonucleotide associated with (e.g. ionically or covalently bound to; or encapsulated within) a targeting means (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 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 a desired therapeutic or prophylactic effect when properly

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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 this invention 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 an autoimmune disorder. An example of a person with a known predisposition is someone with a history of diabetes in the family, or who has been exposed to factors that predispose the subject to a condition, such as lupus or rheumatoid arthritis. "Treatment" refers to a therapeutic intervention that ameliorates a sign or symptom of a disease or pathological condition after it has begun to develop.

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, a preparation is purified such that the protein or peptide represents at least 50% of the total peptide or protein content of the preparation.

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Self-complementary nucleic acid sequence: A nucleic acid sequence that can form Watson-Crick base pairs. The four bases characteristic of deoxyribonucleic 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.

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

Vaccine: A preparation of attenuated microorganisms (including but not limited to bacteria and viruses), living microorganisms, antigen, or killed microorganisms, administered for the prevention, amelioration or treatment of infectious disease.

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. Similarly, the word "or" is intended to include "and" unless the context clearly indicates otherwise. 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 compositions, suitable methods and materials are described below. The term "comprises" means "includes." All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their

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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.

5   *III. Specific embodiments*

A method is provided herein for generating mature dendritic cells. The method includes contacting a dendritic cell precursor with a D ODN (see below), thereby generating mature dendritic cells. This method provides for enhancement of T cell responses by increasing dendritic cell maturation, and thus enhancing antigen

10   presentation. In one embodiment, a method is provided for inducing production of interferon alpha (IFN-α) by plasmacytoid dendritic cells. The method includes contacting a dendritic cell precursor or a plasmacytoid dendritic cell with a D ODN, thereby inducing production of IFN-α.

Mature dendritic cells are of use for immunotherapy, such as in situations in

15   which a host response to an antigen is sub-optimal. Thus, in one embodiment, the methods disclosed herein are of use in augmenting a response to an antigen. In one specific, non-limiting example, the antigen is a tumor antigen (e.g. an antigen used in tumor immunotherapy). In another embodiment, the methods disclosed herein are of use in augmenting an immune response to an infectious agent. In a further

20   embodiment, the methods disclosed herein are of use in augmenting an immune response to a vaccine. In yet another embodiment, the method disclosed herein are of use in decreasing an allergic response.

A.   CpG Oligodeoxynucleotides

25   K type CpG ODNs (K ODNs) have been previously described. K ODNs which exhibit the greatest immunostimulatory activity share specific characteristics. These characteristics differ from those of the Formula II or D ODN (see below). In addition, K ODN have specific effects on the cells of the immune system, which differ from the effects of D ODN. For example, K ODN stimulate proliferation of B

30   cells and stimulate the production of IL-6.

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The K ODNs at least about 10 nucleotides and include a sequence represented by either Formula I:

$$5' \ N_1N_2N_3T\text{-}CpG\text{-}WN_4N_5N_6 \ 3'$$

wherein the central CpG motif is unmethylated, W is A or T, and N_1, N_2, N_3, N_4, N_5, and N_6 are any nucleotides.

These Formula I or K ODN, stimulate B cell proliferation and the secretion of IgM and IL-6, processes involved in the body's humoral immunity, such as the production of antibodies against foreign antigens. In one embodiment, the K ODNs induce a humoral immune response.

In one embodiment, K type oligonucleotides of the formula

$$5' \ N_1N_2N_3T\text{-}CpG\text{-}WN_4N_5N_6 \ 3'$$

contain a phosphate backbone modification. In one specific, non-limiting example, the phosphate backbone modification is a phosphorothioate backbone modification (i.e., one of the non-bridging oxygens is replaced with sulfur, as set forth in International Patent Application WO 95/26204, herein incorporated by reference). In one embodiment, K ODNs have a phophorothioate backbone, and at least one unmethylated CpG dinucleotide. Eliminating the CpG dinucleotide motif from the K ODN significantly reduces immune activation. Incorporating multiple CpGs in a single K ODN increases immune stimulation. Preferably, the K ODN are at least 12 bases long. In addition, K ODN containing CpG motifs at the 5' end are the most stimulatory, although at least one base upstream of the CpG is required. More particularly, the most active K ODNs contain a thymidine immediately 5' from the CpG dinucleotide, and a TpT or a TpA in a position 3' from the CpG motif. Modifications which are greater than 2 base pairs from the CpG dinucleotide motif appear to have little effect on K ODN activity.

D ODNs differ both in structure and activity from K ODNs. The unique activities of D ODNs are disclosed below (see section C). For example, as disclosed herein, D 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 ODNs stimulate the release or production of IP-10 and IFN-α by monocytes and/or plasmacitoid dendritic cells.

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With regard to structure, in one embodiment, a CpG motif in a D ODN has been described by Formula II:

5' RY-CpG-RY 3'

5

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.

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

5' $X_1X_2X_3$ Pu_1 Py_2 CpG Pu_3 Py_4 $X_4X_5X_6(W)_M (G)_N$-3'
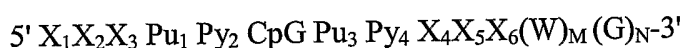
wherein the central CpG motif is unmethylated, Pu is a purine nucleotide, Py is a pyrimidine nucleotide, X and W are any nucleotide, M is any integer from 0 to 15 10, and N is any integer from 4 to 10.

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 20 ODN are termed the 3' far flanking region.

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 25 example, Pu_3 is an adenine and Py_4 is a tyrosine.

In one specific not limiting example, N is from about 4 to about 8. In another specific, non-limiting example, N is about 6.

D ODNs can include modified nucleotides. Without being bound by theory, modified nucleotides can be included to increase the stability of a D ODN. Without 30 being bound by theory, because phosphorothioate-modified nucleotides confer resistance to exonuclease digestion, the D ODN are "stabilized" by incorporating

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phosphorothioate-modified nucleotides. In one embodiment, the CpG dinucleotide motif and its immediate flanking regions include phosphodiester rather than phosphorothioate nucleotides. In one specific non-limiting example, the sequence $Pu_1 Py_2$ CpG $Pu_3 Py_4$ includes phosphodiester bases. In another specific, non-

5   limiting example, all of the bases in the sequence $Pu_1 Py_2$ CpG $Pu_3 Py_4$ are phosphodiester bases. In yet another specific, non-limiting example, $X_1X_2X_3$ and $X_4X_5X_6(W)_M (G)_N$ include phosphodiester bases. In yet another specific, non-limiting example, $X_1X_2X_3 Pu_1 Py_2$ CpG $Pu_3 Py_4 X_4X_5X_6(W)_M (G)_N$ include phosphodiester bases. In further non-limiting examples the sequence $X_1X_2X_3$

10   includes at most one or at most two phosphothioate bases and/or the sequence $X_4X_5X_6$ includes at most one or at most two phosphotioate bases. In additional non-limiting examples, $X_4X_5X_6(W)_M (G)_N$ 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 phosphorothioate/phosphodiester chimera.

15   As disclosed herein, any suitable modification can be used in the present invention to render the D ODN resistant to degradation in vivo (e.g., 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

20   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), phosphodiesters and alkylphosphotriesters (i.e., the charged oxygen moiety is alkylated, as set forth in

25   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 D type oligodeoxynucleotides can also be modified to contain a secondary structure (e.g., stem loop structure). Without being bound by theory, it is believed that

30   incorporation of a stem loop structure renders and oligodeoxynucleotide more effective.

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In a further embodiment, $Pu_1 Py_2$ and $Pu_3 Py_4$ are self-complementary. In another embodiment, $X_1X_2X_3$ and $X_4X_5X_6$ are self complementary. In yet another embodiment $X_1X_2X_3 Pu_1 Py_2$ and $Pu_3 Py_4 X_4X_5X_6$ are self complementary.

Specific non-limiting examples of a D ODN wherein $Pu_1 Py_2$ and $Pu_3 Py_4$
5   are self-complementary include, but are not limited to, AT<u>CG</u>AT, AC<u>CG</u>GT, AT<u>CG</u>AC, AC<u>CG</u>AT, GT<u>CG</u>AC, or GC<u>CG</u>GC (wherein the CpG is underlined). 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
10   ODNs wherein $Pu_1 Py_2$ and $Pu_3 Py_4$ are self-complementary induce higher levels of IFN-γ production from a cell of the immune system. The self-complementary need not be limited to $Pu_1 Py_2$ and $Pu_3 Py_4$. 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).

15   One specific, non-limiting example of a sequence wherein $Pu_1 Py_2$ and $Pu_3 Py_4$ are self-complementary but wherein the far-flanking sequences are not self-complementary is

GGTGCATCGATACAGGGGGG (ODN D 113, SEQ ID NO:3).

This oligodeoxynucleotide has a far flanking region that is not self
20   complementary and induces high levels of IFN-γ and IFN-α.

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

GGTGCGTCGATGCAGGGGGG (D28, SEQ ID NO:4).

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

25   In one embodiment, the D ODN disclosed herein are at least about 16 nucleotides in length. In a second embodiment, a D ODN is at least about 18 nucleotides in length. In another embodiment, a D ODN is from about 16 nucleotides in length to about 100 nucleotides in length. In yet another embodiment, a D ODN is from about 16 nucleotides in length to about 50 nucleotides in length.

In a further embodiment, a D ODN is from about 18 nucleotides in length to about 30 nucleotides in length.

In another embodiment, the D 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 Formula IV:

$$5' \ GGX_1X_2X_3 \ Pu_1 \ Py_2 \ CpG \ Pu_3 \ Py_4 \ X_4X_5X_6(W)_M \ (G)_N\text{-}3'.$$

The D 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 olgiodeoxynucleotide including a sequence as set forth as Formula IV.

Examples of a D ODN include, but are not limited to:

5'XXTGCATCGATGCAGGGGGG 3' (SEQ ID NO: 5)

5'XXTGCACCGGTGCAGGGGGG3' (SEQ ID NO: 6),

5'XXTGCGTCGACGCAGGGGGG3' (SEQ ID NO: 7),

5'XXTGCGTCGATGCAGGGGGG3' (SEQ ID NO: 13),

5'XXTGCGCCGGCGCAGGGGGG3' (SEQ ID NO: 8),

5'XXTGCGCCGATGCAGGGGGG3' (SEQ ID NO: 9),

5'XXTGCATCGACGCAGGGGGG3' (SEQ ID NO: 10),

5'XXTGCGTCGGTGCAGGGGGG3' (SEQ ID NO: 11),

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

Other specific, non-limiting examples of a D ODN include:

GGTGCATCGATGCAGGGGGG (SEQ ID NO: 1);or

GGTGCACCGGTGCAGGGGGG (SEQ ID NO:2).

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Additional exemplary D ODN sequences can be found Fig. 6 (Table I) and in U.S. Patent Application No. U.S. Patent Application No. 10/068,160, and Verthelyi et al., *J. Immunol.* 166:2372-2377, 2001, which are both herein incorporated by reference in their entirety).

5   The oligodeoxynucleotides disclosed herein 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. A D ODN can be synthesized using, for example, the B-cyanoethyl phophoramidite method or

10   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

15   efficient than direct synthesis.

          As disclosed herein, K and D ODN have disparate effects on purified monocytes. K ODN stimulate $CD14^+$ monocytes to proliferate and secrete IL-6, while D ODN has no effect in these assays. Instead, D (but not K) ODN stimulates monocytes to mature into $CD83^+/CD86^+$ dendritic cells. The divergent effects of K

20   versus D ODN on monocytes persists throughout the physiologic concentration range of both types of ODN, and is observed using a variety of D and K ODN, indicating that these differences are not due to variation in ODN binding or uptake. Although both types of ODN increased CD69 and CD25 expression on monocytes, D ODN up-regulates these activation markers in monocytes significantly more

25   effectively.

          B.   Methods for Inducing Dendritic Cell Maturation
          Methods for generating mature dendritic cells are disclosed herein. The method includes contacting a dendritic cell precursor with an effective amount of an

30   D ODN, thereby inducing differentiation of the dendritic cell precursor. In one embodiment, an agent that enhances dendritic cell maturation is administered in

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conjunction with the D ODN. Specific, non-limiting examples of agents of use are GM-CSF, or a combination of IL-4 and GM-CSF. In another embodiment, the dendritic cell precursor is contacted with an effective amount of a D ODN in the absence of an additional agent. Thus, the dendritic cell precursor is not contacted

5   with GM-CSF and/or IL-4.

In one embodiment, a dendritc cell precursor is contacted with a D ODN *in vitro* to produce dendritic cells. One of skill in the art can readily identify tissue sources of dendritic precursor cells, such as fetal or umbilical cord blood, or bone marrow. To increase the number of dendritic cell precursor cells in animals,

10   including humans, the subject can be treated with substances which stimulate hematopoiesis, such as GM-CSF. For example, U.S. Patent No. 5,994,126 discloses methods for isolating dendritic cell precursors and methods for increasing the number of dendritic cell precursors in a sample.

Thus, a dendritic cell precursor, such as a monocyte or a plasmacytoid

15   dendritic cell precursor are contacted with an effective amount of a D ODN for a sufficient period of time to differentiate a mature dendritic cell *in vitro*. In one specific, non-limiting example, peripheral blood mononuclear cell cultures (PBMCs) are contacted with an effective amount of one or more D ODNs for a sufficient period of time to differentiate into mature dendritic cells *in vitro*. In one specific,

20   non-limiting example, a culture of isolated peripheral blood monocytes containing about 1 to about 4×10^6 cells/ml are treated with an effective amount D ODN *in vitro*. In this example an amount of D type ODN effective to induce the maturation of the monocytes is about 1 to about 10 μM, or about 1 to about 6 μM. In one specific, non-limiting example, the D ODN is present at a concentration of about

25   3μM. In one embodiment, the culture is maintained for at least one day. In another embodiment, the culture is maintained for about 1 to about 7 days. In another embodiment, the culture is maintained for about 1 to about 4 days.

In yet a further embodiment, a method is disclosed herein for inducing differentiation of an antigen-presenting cell in vitro. The method includes

30   contacting a dendritic cell precursor, or a dendritic cell with an effective amount of a D ODN and an effective amount of an antigen, thereby differentiating an antigen presenting cell in vitro. The dendritic cell precursor can be contacted with the D

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ODN and the antigen sequentially or simultaneously. The antigen can be any antigen, including, but not limited to, a tumor antigen, an antigen from an infectious agent, or an antigen of use in a vaccine. Thus, in one embodiment, a dendritic cell precursor is contacted with an effective amount of a D ODN to produce a mature

5   dendritic cell. The mature dendritic cells is contacted with an effective amount of an antigen to induce presentation of the antigen by the mature dendrtic cell. Thus, a mature antigen-presenting dendritic cell is produced by this method.

Exemplary antigens include, but are not limited to, epitopes or antigens from tumors, viruses, parasites, fungi or allergens. These antigens may be composed of

10   protein, DNA, RNA, lipid, sugar, whole cell lysates, apoptotic cells, or any combination thereof. Some preferred antigens include, tetanus toxoid, soluble tumor protein antigens, tumor-derived RNA, unfractionated acid-eluted peptides from the MHC class I molecules of tumor cells, and recombinant, purified, or inactivated HIV-1 proteins. Antigens of interest include polypeptides and other immunogenic

15   biomolecules, which can be produced by recombinant methods or isolated from natural sources. Complex antigens such as cell lysates inactivated (e.g. heat killed) viruses, bacterial cells or fractions thereof are also of use.

The antigen can be delivered to the dendritic cells or to dendritic cell precursors via any method known in the art, including, but not limited to, pulsing

20   dendritic cells directly with antigen, or utilizing a broad variety of antigen delivery vehicles, such as, for example, liposomes, or other vectors known to deliver antigen to cells. In one specific, non-limiting example an antigenic formulation includes about 0.1 µg to about 1,000 µg, or about 1 to about 100 µg of a selected antigen. An antigen preparation can also contain buffers, excipients, and preservatives, amongst

25   other ingredients.

In one specific, non-limiting example, a culture comprising a monocyte and a plasmacytoid dendritic cell with a D ODN, in the presence or the absence of an antigen thereby inducing the plamacytoid dendritic cell to producing interferon-alpha. Without being bound by theory, secretion of IFN-α by the plamacytoid

30   dendritic cell is important in the induction the differentiation of the monocyte into an antigen presenting dendritic cell.

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In another embodiment, a method is disclosed herein for of inducing differentiation of a monocyte *in vitro*. The method includes contacting a culture comprising a monocyte and a plasmacytoid dendritic cell with an effective amount of a D ODN, thereby differentiating the monocyte. Without being bound by theory, The D ODN induces the plamacytoid dendritic cell to produce interferon-alpha. Secretion of IFN-α, alone or in combination with another factor, by the plamacytoid dendritic cell then induces the differentiation of the monocyte into a dendritic cell.

In one embodiment, a single step method is also disclosed herein for inducing differentiation of a dendritic cell precursor. The dendritic cell precursor can be contacted with an effective amount of a D type ODN *in vivo* or *in vitro*.

In one embodiment, to induce dendritic cell differentiation, a dendritic cell precursor is contacted with an effective amount of a D type ODN in the absence other agents that affect dendritic cell differentiation. In another specific, non-limiting example, to induce differentiation a dendritic cell precursor is contacted with an effective amount of a D ODN in the absence of a dendritic cell mobilization agent.

In a further embodiment, a dendritic cell precursor, such as a monocyte, is cultured in the presence of D type ODN and cytokines or factors which promote dendritic cell maturation, to further enhance dendritic cell differentiation. Examples of such agents include, but are not limited to, granulocyte macrophae colony stimulating factor (GM-CSF), flt-3, and interleukin-4 (IL-4). Other examples include, but are not limited to, M-CSF, TNF-α, IL-6, IL-13, IL-7, IFN-α, heparan sulfate, calcium ionophore, and polyriboinosinic polyribocytidylic acid (poly (I:C)). One of skill in the art can readily identify the concentration of cytokine of use. In one specific, non-limiting example, cytokines are present in concentrations ranging from about 25 to about 100 ng/ml, depending on the specific cytokine used. Without being bound by theory, it is believed that agents such as GM-CSF, IL-4, and flt-3 act synergistically with D ODN to induce and enhance dendritic cell maturation.

In another embodiment, a dendritic cell precursor is contacted with D type ODN and an antigen to produce an antigen-presenting differentiated dendritic cell. The cells are contacted with antigen for a time sufficient to allow the antigen to be

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internalized, processed, and presented by the mature dendritic cell. Accordingly, the present invention also relates to methods for generating enriched populations of mature, antigen-presenting dendritic cells that can function to present antigen to T cells. In one specific non-limiting example, the dendritic cell precursor is contacted

5   with the D ODN and the antigen simultaneously. In another embodiment, the dendritic cell precursor is contacted with a D ODN to produce a mature dendritic cell, which is subsequently or simultaneously contacted with an antigen to generate an antigen presenting mature dendritic cell. -In yet another embodiment the dendritic cell precursor is contacted with an agent such as a cytokine to expand the number of

10   dendritic cell precursors, and then with the D ODN and the antigen.

In one specific, non-limiting example, mature dendritic cells are obtained *in vitro* by culturing dendritic cell precursors (e.g. monocytes) with D ODN for about 24 to about 48 hrs. In another specific, non-limiting example, antigen-presenting mature dendritic cells are obtained *in vitro* by culturing dendritic cell precursors

15   (e.g. monocytes) with D ODN for about 12 to about 72 hrs, and then contacting the mature dendritic cells with antigen for a time sufficient to allow the antigen to be internalized, processed, and presented by the mature dendritic cell, thereby producing antigen presenting dendritic cells.

One of skill in the art can readily identify mature dendritic cells and antigen

20   presenting dendritic cells. These techniques include, but are not limited to, analysis of cell morphology, detection of specific antigens present on mature dendritic cells with monoclonal antibodies, or and assays for mixed lymphocyte reactions.

In a one embodiment, the presence of mature dendritic cells can be confirmed by antibodies specific for various mature dendritic cell surface markers,

25   such as CD83 and CD86. Among the specific monoclonal antibodies suitable for identifying mature dendritic cells include, but are not limited to, CD83, in combination with CD86, CD40, CD80 or HLA-DR. Typically, labeled antibodies specifically directed to the marker are used to identify the cell population. The antibodies can be conjugated to other compounds including, but not limited to,

30   enzymes, magnetic beads, colloidal magnetic beads, haptens, fluorochromes, metal compounds, radioactive compounds or drugs. The enzymes that can be conjugated to the antibodies include, but are not limited to, alkaline phosphatase, peroxidase,

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urease and ß-galactosidase. The fluorochromes that can be conjugated to the
antibodies include, but are not limited to, fluorescein isothiocyanate,
tetramethylrhodamine isothiocyanate, phycoerythrin, allophycocyanins and Texas
Red. For additional fluorochromes that can be conjugated to antibodies see

5   Haugland, R. P., *Molecular Probes: Handbook of Fluorescent Probes and Research
Chemicals* (1992-1994). The metal compounds that can be conjugated to the
antibodies include, but are not limited to, ferritin, colloidal gold, and particularly,
colloidal superparamagnetic beads.

Mature dendritic cells may also be identified histologically, by assessing

10   nuclear reorganization, vacuole formation, cytoplasmic enlargement, and membrane
ruffling. In addition, one skilled in the art can assess typical mature dendritic cell
morphology, including stellate shape and/or well defined veils.

Compositions including mature antigen presenting dendritic cells may be
used as vaccines or as adjuvants to elicit or boost immune responses against

15   antigens. For example, activated, antigen-presenting mature dendritic cells can be
used as vaccines to prevent future infection, or may be used to activate the immune
system to treat ongoing diseases, such as cancer.

Mature dendritic cells can also be utilized to produce activated T
lymphocytes. The method includes contacting the dendritic cell with a T

20   lymphocyte *in vitro*, thereby producing an activated T lymphocyte.

Mature dendritic cells generated by the methods disclosed herein can be
administered to a subject. Mature dendritic cells generated by contacting a dendrtic
cell precursor with a D ODN *in vitro* can be administered to a subject to
preferentially stimulate immune responses which block allergic responses (e.g.

25   interferon production). Thus, the mature dendritic cells generated by D ODN
treatment may be administered to a subject for treating an allergic condition in that
individual. The treatment of allergic conditions is based on the discovery that D
ODN can stimulate dendritic cells to produce anti-allergic agents, such as IFN-α,
which in turn increased the production of IFN-γ by natural killer (NK) cells and T

30   cells (See Example 1).

The mature dendritic cells generated by the methods disclosed herein can
also be used for tumor immunotherapy. In one embodiment, mature antigen

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presenting cells are generated to present a tumor antigen. These dendritic cells are then administered to a subject with a tumor that expresses the tumor antigen. In another embodiment, the mature dendritic cells are administered in conjunction with a chemotherapeutic agent.

5   In another embodiment, mature dendritic cells are administered to boost an immune response against another antigen. In one specific, non-limiting example, the antigen is from an infectious agent, including but not limited to, an antigen from a bacterium, virus, or fungus. The dendritic cells can be from the same subject (autologous) or can be from a different individual (heterologous).

10   A method is also disclosed herein for inducing the differentiation of dendritic cell precursors *in vivo*. The method includes administering a therapeutically effective amount of a D type ODN to a subject, thereby inducing differentiation of dendritic cell precursors into differentiated dendritic cells in the subject. The subject can be any mammal, such as a primate. In one specific, non-limiting example, the

15   subject is a human, but veterinary use is contemplated.

As discussed above, in one embodiment, an agent that enhances dendritic cell maturation is administered in conjunction with the D type ODN. Specific, non-limiting examples of agents of use are IL-4 and GM-CSF, or flt-3L. In another embodiment, a therapeutically effective amount of a D type ODN is administered in

20   the absence of another agent that enhances dendritic cell maturation. In another embodiment, a therapeutically effective amount of a D type ODN is administered to a subject in conjunction with a therapeutically effective amount of an antigen to produce an antigen-presenting differentiated dendritic cells in the subject. In a further embodiment, antigen can be co-administered with D ODN, for example, in a

25   liposome, to trigger antigen uptake and maturation of dendritic cells *in vivo* and enhance antigen presentation by the dendritic cells to T cells *in vivo*. Thus, antigen presentation and immunity can be significantly enhanced using the methods described herein.

In one embodiment, compositions comprising D ODN and antigen may be

30   administered directly to the patient to trigger antigen uptake and maturation of dendritic cells *in vivo*, thereby enhancing antigen presentation at a specific site *in vivo*. For example, D ODN and antigen may be co-administered in solution, or in a

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delivery vehicle, such as a liposome, which would facilitate delivery and uptake of the D ODN and antigen by the monocytes or other dendritic cell precursors.

In another embodiment, compositions comprising D ODN and mature dendritic cells may be used to treat a subject having cancer. As discussed above, cancer treatments may be based upon the development of anti-tumor vaccines comprising D ODN and tumor antigen, or D ODN and mature, tumor antigen-presenting dendritic cells. Without being bound by theory, such vaccines not only elicit anti-tumor antibody production, but also activate natural killer cell lytic activity and antibody dependent cellular cytotoxicity (ADCC). Thus, in the latter case, administration of compositions comprising D ODN also stimulate production of tumor specific cytotoxic immune cells *in vivo* which actively target and kill cancer cells.

In a further embodiment, compositions comprising activated T cells can be produced *in vitro* by, for example, co-culturing the mature, antigen-presenting dendritic cells prepared according to the invention with T cells *in vitro*. Such compositions are useful in adoptive immunotherapy, such as for the production of antigen-specific cytotoxic T lymphocytes or for generating antigen-specific T helper cells.

As disclosed herein, D ODN can be used to generate mature dendritic cells, such as antigen presenting cells, *in vivo*. Thus, in one embodiment, a therapeutically effective amount of a D type ODN is administered locally, such as to a specific site in a subject in order to trigger maturation of dendritic cells at that site. In another embodiment, a therapuetically effective amount of a D type ODN is administered systemically, such as by intravenous, subcutaneous, intramusclar, intradermal, intraarterial, pareternal, or subcutaneous injection, or by oral administration or inhalation, to induce the maturation of dendritic cells.

In one embodiment, a D type oligodeoxynucleotide is administered in a delivery complex. The delivery complex can include the D ODN and a targeting means. Any suitable targeting means can be used. For example, a D ODN can be associated with (e.g., ionically or covalently bound to, or encapsulated within) a targeting means (e.g., a molecule that results in higher affinity binding to a target cell, such as a B cell). A variety of coupling or cross-linking agents can be used to

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form the delivery complex, such as protein A, carbodiamide, and N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP). Examples of an oligodeoxynucleotide delivery complexes include a D ODN associated with a sterol (e.g., cholesterol), a lipid (e.g., a cationic lipid, virosome or liposome), and a target cell specific binding agent (e.g., a ligand recognized by target cell specific receptor). Without being bound by theory, the complex is sufficiently stable *in vivo* to prevent significant uncoupling prior to delivery to the target cell. In one embodiment, the delivery complex is cleavable such that the oligodeoxynucleotide is released in a functional form at the target cells.

In another embodiment, the D ODN is administered in conjunction with a pharmacologically acceptable carrier. Pharmacologically acceptable carriers (e.g., physiologically or pharmaceutically acceptable carriers) are well known in the art. A suitable pharmacological composition can be formulated to facilitate the use of a D ODN *in vivo* and/or *ex vivo*. Such a composition can be suitable for delivery of the active ingredient to any suitable host, such as a patient for medical application, and can be manufactured in a manner that is itself known, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes.

Pharmacological compositions for use can be formulated in a conventional manner using one or more pharmacologically (e.g., physiologically or pharmaceutically) acceptable carriers comprising excipients, as well as optional auxiliaries that facilitate processing of the active compounds into preparations which can be used pharmaceutically. Proper formulation is dependent upon the route of administration chosen, and whether use will be an *in vivo* or an *ex vivo* use. For use *in vivo*, administration can be either systemic or local. In addition, one of skill in the art can readily select a suitable route of administration, including, but not limited to intravenous, intramuscular, intraperitioneal, transmucosal, subcutaneous, transdermal, transnasal, inhalation, and oral administration.

Thus, for injection, the active ingredient can be formulated in aqueous solutions, preferably in physiologically compatible buffers. For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the

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formulation. Such penetrants are generally known in the art. For oral administration, the active ingredient can be combined with carriers suitable for inclusion into tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions and the like. D ODN can also be formuated for use in inhalation

5   therapy. For administration by inhalation, the active ingredient is conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebuliser, with the use of a suitable propellant. The active ingredient can be formulated for parenteral administration by injection, e.g., by bolus injection or continuous infusion. Similarly, D ODN can be formulated for intratracheal or for

10   inhalation. Such compositions can take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and can contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Other pharmacological excipients are known in the art.

The invention is illustrated by the following non-limiting Examples.

15

EXAMPLES

Example 1

Materials and methods

20   *ODN:* Endotoxin free ODN were synthesized by the CBER core facility. Multiple D ODN were studied with similar results. D35, the most commonly used ODN, had the sequence 5'-Ggtgcat_c_gatgcaggggGG (SEQ ID NO: 1) (phosphodiester bases are in lower case while phosphorothioate bases are in upper case). Additional ODN studied were

25   D19; GgtgcatcgatgcagGGGGG (SEQ ID NO:1),

D29; GgtgcaccggtgcagGGGGG (SEQ ID NO:2) and

control ODN; GgtgcatctatgcaggggGG (SEQ ID NO: 12).

Additional D ODN are shown in the table (Fig. 8). All antibodies were purchased from Pharmingen (San Jose, CA).

30   *Cells and culture conditions*: Normal PBMC and elutriated monocytes (>95% pure) were provided by the NIH Dept. of Transfusion Medicine. 2×10^6 cells/ml were cultured for 24 - 96 h with 3 µM of ODN. Equivalent levels of

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monocyte maturation were obtained by culture in either serum-free X-VIVO 15 medium (BioWittaker, Walkersville, MD, USA) or RPMI 1640 supplemented with 5% FCS, 50 U/ml penicillin, 50 μg/ml streptomycin, 0.3 mg/ml L-glutamine, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 10 mM HEPES and 10^{-5} M
5   2-mercaptoethanol.

Analysis of monocyte differentiation: Cells cultured for various periods with D ODN were washed, fixed, and stained for surface expression of CD83, CD86, CD40, CD80 and/or CD14 using phenotype-specific MAb. Samples were analyzed (40,000 events) by FACScan (Becton Dickinson, San Jose, CA) after gating on live
10   cells with proper electronic compensation. Cytospin slides were prepared by centrifuging 50,000 cultured cells onto glass slides. Following drying and fixation in methanol, slides were stained with Giemsa stain and visualized by light microscopy under X600 magnification.

Analysis of DC function: PBMC cultured with 3 μM of D ODN for 48 h
15   were pulsed with 0.02 LF U/ml of tetanus toxoid for 4 h and 10 μg/ml brefeldin A for 12 h. Cells were washed, fixed, permeabilized and stained with 3 μg/mL PE-conjugated anti-IFNγ plus FITC-conjugated anti-CD3 (as per instructions, Caltag, CA) and analyzed by FACS.

Elutriated monocytes were treated for 48 h with 3 μM of ODN. Increasing
20   numbers of monocytes were added to 10^5 monocyte-depleted allogeneic peripheral blood lymphocytes in 96-well round bottom plates. Proliferation was measured 5 days later after the addition of 1 μCi/well of ^{3}H-thymidine for the last 12 h of culture. IFNγ production by cultured cells was analyzed by ELISA as previously described [24].

25   *Animals and Immunization protocol:* Three year old female rhesus monkeys were immunized subcutaneously on day 0 and day 21 with 4 μg of OVA plus 250 μg of an equimolar mixture of D19 and D29 ODN that were mixed with 12 μg of alum. Animals were bled 10 days after immunization and 2 weeks after boosting. Serum IgG anti-OVA titers were determined by ELISA (Klinman et a., *Vaccine* 17: 19-25,
30   1999).

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Example 2

D ODN Induce Monocytes to Differentiate into DC

PBMC and purified elutriated monocytes were cultured *in vitro* with a variety of D or control (non-CpG) ODN at the optimized concentration of 3 μM. Phenotypic
5   changes associated with DC maturation, including increased expression of costimulatory molecules, were manifested by 10% of monocytes within 24 h of D ODN treatment. By 48 hours in culture, a large fraction of monocytes (~ 40%) matured into DC, as characterized by increased surface expression of CD83 and CD86 but low levels of CD14 (Fig. 1). Cell yield remained constant while cell
10   viability was ≥80% after 4 days of culture.

While D type ODN induced monocytes from all donors to differentiate into DC (N=35), neither non-CpG ODN nor conventional phosphorothioate CpG ODN (referred to in the literature as D or K type ODN) had this effect (Fig 1). Maturation into DC was observed when monocytes were cultured in either RPMI 1640
15   supplemented with fetal calf serum or serum-free X-VIVO 15 medium. The latter medium is preferred for clinical use, since it reduces exposure to non-self proteins and adventitious agents.

GM-CSF and IL-4 in synergize with D ODN in inducing monocyte differentiation in to dendritic cells (Fig. 7).
20

Example 3

Contribution of pDC to monocyte differentiation

The maturation of monocytes into DC was critically dependent upon plasmacytoid DC being present during culture. D ODN directly activate pDC to
25   produce IFNα (Kadowaki et al, *J.Immunol.* 166: 2291-2295, 2001; Bauer et al., *J.Immunol.*, 166: 5000-5007, 2001; Krug et al., *Eur.J.Immunol.* 31: 2154-2163, 2001;Krug et al., . *Eur.J.Immunol.* 31: 3026-3037, 2001; Wagner, *Adv.Immunol.* 73: 329-368, 1999; and Fig 2D), a cytokine that supports the differentiation of monocytes into DC (e.g. see Blanco et al., *Science* 294: 1540-1543, 2001; Santini et
30   al., *J.Exp.Med.* 191: 1777-1788, 2000). Depleting pDC abrogates monocyte maturation (Fig 2 A/B), while supplementing elutriated monocytes with pDC restored their ability to differentiate when treated with D ODN (Fig 2B). Although

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recombinant IFNα alone did not trigger the differentiation of elutriated monocytes (data not shown), anti-IFNα Ab significantly inhibited DC differentiation (Fig 2C), suggesting that IFNα derived from pDC (Fig 2D)is necessary, but not sufficient, to support the generation of DC.

5

Example 4

In vitro Activity of DC Produced by Treating Monocytes with D ODN

Mixed lymphocyte reactions (MLR) are commonly used to assess the functional activity of DC (Hajdinjak, *Pflugers Arch.*440, R49-50, 2000). MLRs

10   were established by treating elutriated monocytes with ODN for 2 days, and then mixing them with monocyte-depleted allogeneic PBMC. Monocytes that had been cultured with D ODN induced 10-fold more proliferation (p <.05) and 3-fold more IFNγ production than monocytes treated with control ODN (p. <.05, Fig 3). These MLRs selectively produced IFNγ (no IL-4 was detected) suggesting that the mature

15   DC generated by D ODN treatment preferentially support the induction of Th1-biased immune responses.

To assess their functional activity, PBMC from tetanus toxoid (TT) immune donors were cultured with D ODN for 48 h, and then pulsed with TT. The resultant DC efficiently presented antigen to autologous Th1 cells, stimulating 3% of $CD3^+$ T

20   cells to secrete IFN-γ (Fig 4). This significantly exceeded the 0.5% of T cells stimulated to produce IFN-γ by monocytes cultured with control ODN plus TT.

DC are typically generated by culturing monocytes in GM-CSF/IL-4 followed by conditioned medium. The antigen presenting function of DC generated after 2 days of treatment with CpG ODN was compared to those from the same

25   donor generated after a week of culture in GM-CSF/IL-4 in a SCID mouse model. DC generated by each technique were loaded with antigen (ovalbumin) and transferred into SCID mice carrying PBL from the same donor. DC generated by both D ODN and GM-CSF/IL-4 treatment induced comparable IgG anti-OVA responses that significantly exceeded the response of control mice (Fig. 5).

30   Antigen presentation is the hallmark of DC function (Banchereau et al., *Ann.Rev.Immunol.* 18: 767-811, 2000). To determine whether D ODN are effective under physiologic conditions in vivo, rhesus macaques were immunized and boosted

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with OVA with or without D ODN. This model was selected on the basis of recent

work showing that PBMC from macaques respond to D ODN that stimulate human

PBMC (Verthelyi et al., *J.Immunol.* 168: 1659-1663, 2002). Macaques immunized

and boosted with OVA plus D ODN developed significantly higher antigen-specific

5   IgG serum titers than macaques treated with OVA alone (p< 0.01, Fig 6). This

finding is consistent with CpG ODN triggering the maturation of DC *in vivo* and

thus improving antigen presentation (Fig 1).

The findings disclosed herein document that mature DC can be rapidly and

reproducibly generated by culturing PBMC or elutriated monocytes with D ODN in

10   serum-free or conventional medium. These DC efficiently present antigen to

autologous T cells *in vitro* and *in vivo*, and support the induction of Th1 biased

immune responses.

Without being bound by theory, the effect of D ODN on DC differentiation

is dependent upon IFNα -secreting pDC being present during culture. Current

15   results indicate that IFNα is necessary but not sufficient by itself to induce the

differentiation of human monocytes into functionally active DC (Fig 2C).

It is likely that functional differences between D and K type CpG ODN

reflects differences in their recognition, uptake and/or processing by immune cells.

Accumulating evidence indicates that Toll-like receptor 9, plays a critical role in

20   mediating the immune activation induced by conventional phosphorothioate ODN

(Hemmi et al., *Nature* 408: 740-745, 2000; Kaisho and Akira, *Trends Immunol.* 22:

78-83, 2001). Additional studies suggest that TLR 9 may not mediate the

recognition of D ODN. Specifically, D ODN do not compete with "K" ODN for

uptake, do not activate TLR 9 transfected cells, and traffic to different intracellular

25   locations than conventional K ODN (Gursel et al., *J.Leuko.Biol.* 71: 813-820, 2002).

These differences in uptake and activity have a structural basis. Whereas the

immunostimulatory motif of a conventional ODN consists of a phosphorothioate

T<u>CG</u>TT/A, the relevant motif in a D ODN consists of a phosphodiester

purine/pyrimidine/<u>CG</u>/purine/pyrimidine hexamer (Verthelyi et al., *J Immunol.*

30   166: 2372-2377, 2001, which is incorporated herein by reference). In addition, the

hexamer of a D ODN is flanked by complementary bases that form a hairpin loop

with the CpG dinucleotide at its apex - secondary structure that is absent from

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conventional CpG ODN. Finally, D but not K ODN are capped at the 3' end with a poly-G tail. This poly-G tail may interact with scavenger receptors on immune cells.

D ODN reproducibly stimulated approximately 60% of monocytes to differentiate into DC, as determined phenotypically, histologically and functionally.

5   The production of IFNα by pDC present in culture contributed to this maturation (Fig 1A and 2B). D ODN also stimulate pDC to secrete other cytokines and chemokines, including GM-CSF, TNFα, IL-6, IL-8 and IP-10 (Bauer et al., *J.Immunol.*, 166: 5000-5007, 2001; Krug et al., *Eur.J.Immunol.* 31: 3026-3037, 2001). which could contribute to and/or support monocyte differentiation. The DC

10   generated by D ODN are functionally active, as they promote antigen-specific immune responses *in vitro* and *in vivo* (Fig 3, 4, 5 and 6). Without being bound by theory, these findings suggest that D ODN act directly on pDC to trigger an immunomodulatory cascade that includes rapidly inducing the differentiation of potent DCs from monocyte precursors. Thus, D ODN can also be used as vaccine

15   adjuvants, facilitating the maturation of DC and the presentation of co-administered antigen *in vivo*.

Example 4
D ODN are Active *In Vivo*

20   Antigen presentation is the hallmark of DC function. To determine whether D ODN are active under physiologic conditions, their ability to improve antigen presentation *in vivo* was analyzed. Rhesus macaques were chosen for this pre-clinical study since they respond to the same D ODN that are active in humans. Animals were immunized and boosted with ovalbumin (OVA) or OVA plus D

25   ODN. Macaques immunized and boosted with antigen plus D ODN developed significantly higher IgG anti-OVA serum titers than macaques treated with OVA alone (p< 0.01, Fig 6). This finding suggests that the CpG ODN triggered the maturation of DC *in vivo* and improved antigen presentation (Fig 4).

DC are traditionally generated by culturing monocytes in GM-CSF/IL-4

30   followed by conditioned medium. The antigen presenting function of DC generated after 2 days of treatment with CpG ODN was compared to those from the same donor generated after a week of culture in GM-CSF/IL-4 in a SCID mouse model.

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DC generated by each technique were loaded with antigen (ovalbumin) and transferred into SCID mice carrying PBL from the same donor. DC generated by both D ODN and GM-CSF/IL-4 treatment induced comparable IgG anti-OVA responses that significantly exceeded the response of control mice (Fig 5).

5

It will be apparent that the precise details of the methods or compositions described may be varied or modified without departing from the spirit of the described invention. We claim all such modifications and variations that fall within the scope and spirit of the claims below.

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CLAIMS

1. A method for generating a mature dendritic cell, comprising

contacting a dendritic cell precursor with an effective amount of an

5   oligodeoxynucleotide of at least about 16 nucleotides in length comprising a

sequence represented by the following formula:

$$5' \ X_1X_2X_3 \ Pu_1 \ Py_2 \ CpG \ Pu_3 \ Py_4 \ X_4X_5X_6(W)_M \ (G)_N\text{-}3'$$

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,

10   and N is any integer from 4 to 10, thereby generating a mature dendritic cell.

2. The method of claim 1, wherein N is about 6.

3. The method of claim 1 wherein Pu Py CpG_Pu Py comprises

15   phosphodiester bases.

4. The method of claim 3 wherein $Pu_1 \ Py_2 \ CpG \ Pu_3 \ Py_4$ are phosphodiester

bases.

5. The method of claim 3, wherein $X_1X_2X_3$ and $X_4X_5X_6(W)_M \ (G)_N$ comprise

20   phosphodiester bases.

6. The method of claim 3, wherein $X_1X_2X_3$ comprises one or more

phosphothioate bases.

25   7. The method of claim 3, wherein $X_4X_5X_6(W)_M \ (G)_N$ comprises one or

more phosphothioate bases.

8. The method of claim 1, wherein $X_1X_2X_3 \ Pu \ Py$ and $Pu \ Py \ X_4X_5X_6$ are

self complementary.

30

9. The method of claim 1, wherein $X_1X_2X_3$ AND $X_4X_5X_6$ are self

complementary.

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10. The method of claim 1, wherein Pu Py and Pu Py are self complementary.

5   11. The method of claim 1, wherein the oligodeoxynucleotide comprises the sequence

GGTGCATCGATGCAGGGGGG (SEQ ID NO: 1); or

GGTGCACCGGTGCAGGGGGG (SEQ ID NO:2).

10

12. The method of claim 1, further comprising contacting the dendritic cell precursor with an antigen.

13.   The method of any of claim 1, wherein the oligodeoxynucleotide is
15   modified to prevent degradation.

14.   The method of claim 1, wherein the oligodeoxynucleotide has a phosphate backbone modification.

20   15.   The method of claim 14, wherein the phosphate backbone modificatioı is a phosphorothioate backbone modification.

16.   The method of claim 1, wherein the oligodeoxynucleotide comprises about 100 nucleotides or less.

25

17.   The method claim 16, wherein the oligodeoxynucleotide comprises about 50 nucleotides or less.

18.   The method of claim 9, wherein the oligodeoxynucleotide comprises
30   about 18 to about 30 nucleotides.

-45-

19. The method according to claim 1, wherein the dendritic cell precursor cell is a monocyte.

20. The method of claim 1, further comprising contacting the dendritic cell precursor with an agent that enhances dendritic cell maturation.
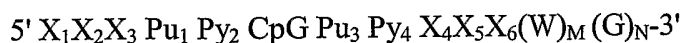
21. The method according to claim 4, wherein the factor is GM-CSF, IL-4, flt-3L or a combination thereof.

22. The method of claim 1, wherein the dendritic cell precursor is *in vivo*.

23. The method of claim 1, wherein the dendritic cell precursor is *in vitro*.

24. A method for producing a mature, antigen-presenting dendritic cell comprising
    contacting a dendritic cell precursor with an effective amount of an oligodeoxynucleotide of at least about 16 nucleotides in length comprising a sequence represented by the following formula:
    $$5' \; X_1X_2X_3 \; Pu_1 \; Py_2 \; CpG \; Pu_3 \; Py_4 \; X_4X_5X_6(W)_M \; (G)_N\text{-}3'$$
    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 to generate a mature dendritic cell and
    contacting the dendritic cell precursor with an antigen for a time sufficient to allow the antigen to be presented on the mature dendritic cell;
    thereby producing a mature antigen-presenting dendritic cell.

25. The method of claim 24, wherein the antigen is a protein, a polypeptide, a polysaccharide, a DNA molecule, a RNA molecule, a whole cell lysate, an apoptotic cell, or any combination thereof.

26. Them method of claim 24, wherein the dendritic cell precursor is *in vivo*.

-46-

27. The method of claim 24, wherein the dendritic cell precursor is *in vitro*.

28. The method of claim 24, further comprising contacting the dendritic cell precursor with an agent that enhances dendritic cell maturation.

5

29. The method according to claim 28 wherein the agent is GM-CSF, IL-4, flt-3 ligand, or a combination thereof.

30. The method of claim 28, wherein the dendritic cell precursor is contacted

10   with the oliogodeoxynucleotide and the antigen sequentially.

31. The method of claim 28, wherein the dendritic cell precursor is contacted with the oliogodeoxynucleotide and the antigen simultaneously.

15   32. The method of claim 30, wherein the dendritic cell precursor is *in vivo*.

33.   The method of claim 30, wherein the dendritic cell precursor is *in vitro*.

34. A method for generating an activated T lymphocyte, comprising:

20   producing a mature antigen presenting dendritic cell according to the method of claim 24; and
        contacting the mature antigen presenting dendritic cell with a T lymphocyte *in vitro*,
            thereby producing an activated T lymphocyte.

25

35. A method of producing an immune response against an antigen in a subject, comprising
        producing mature antigen presenting dendritic cells according the method of claim 24;

30   contacting the mature antigen presenting dendritic cell with a T lymphocyte in vitro; and

-47-

administering a therapeutically effective amount of the activated lymphocytes to the subject,

thereby producing the immune response against the antigen in the subject.

36. A single step method for differentiating a dendritic precursor cell into a mature antigen presenting cell; comprising

contacting a dendritic cell precursor with an effective amount of an antigen and an oligodeoxynucleotide of at least about 16 nucleotides in length comprising a sequence represented by the following formula:

$$5' \; X_1X_2X_3 \; Pu_1 \; Py_2 \; CpG \; Pu_3 \; Py_4 \; X_4X_5X_6(W)_M \; (G)_N\text{-}3'$$
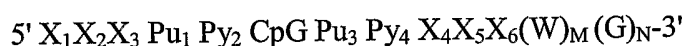
wherein the central CpG motif is unmethylated, Pu is a purine nucleotide, Py is a pyrimidine nucleotide, X and W are any nucleotide, M is any integer from 0 to 10, and N is any integer from 4 to 10, thereby differentiating a mature antigen presenting cell.

37. The method of claim 36, wherein the dendritic cell precursor is not contacted with another mobilization agent.

38. The method of claim 36, wherein the monocyte and the plasmacytoid dendritic cell are *in vivo*.

39. The method of claim 36, wherein the monocyte and the plasmacytoid dendritic cell are *in vitro*.

40. A method of inducing differentiation of a monocyte; comprising

contacting a monocyte and a plasmacytoid dendritic cell with an effective amount of an antigen and an oligodeoxynucleotide of at least about 16 nucleotides in length comprising a sequence represented by the following formula:

$$5' \; X_1X_2X_3 \; Pu_1 \; Py_2 \; CpG \; Pu_3 \; Py_4 \; X_4X_5X_6(W)_M \; (G)_N\text{-}3'$$
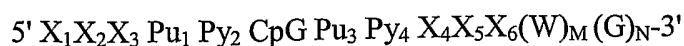
wherein the central CpG motif is unmethylated, Pu is a purine nucleotide, Py is a pyrimidine nucleotide, X and W are any nucleotide, M is any integer from 0 to

-48-

10, and N is any integer from 4 to 10, thereby inducing the plasmacytoid dendritic cell to producing interferon-alpha inducing the differentiation of the monocyte into a dendritic cell.

5   41. Use of an oligodeoxynucleotide of at least about 16 nucleotides in length comprising a sequence represented by the following formula:

5' $X_1X_2X_3$ Pu_1 Py_2 CpG Pu_3 Py_4 $X_4X_5X_6(W)_M$ $(G)_N$-3'
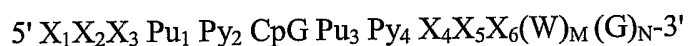
wherein the central CpG motif is unmethylated, Pu is a purine nucleotide, Py is a pyrimidine nucleotide, X and W are any nucleotide, M is any integer from 0 to 10,

10   and N is any integer from 4 to 10, to induce the differentiation of the monocyte into a dendritic cell.

FIG. 1

FIG. 2

FIG. 3

FIG. 4

FIG. 5
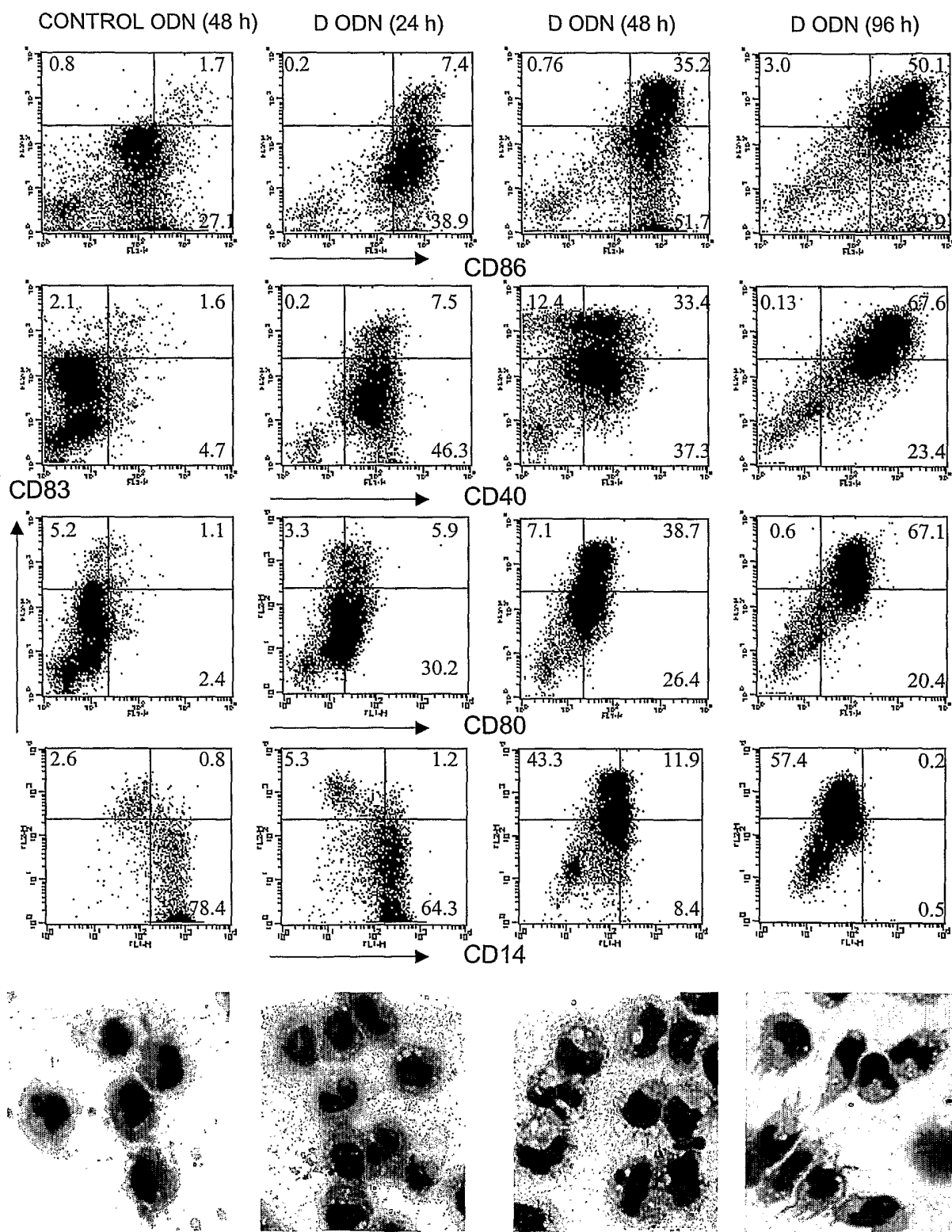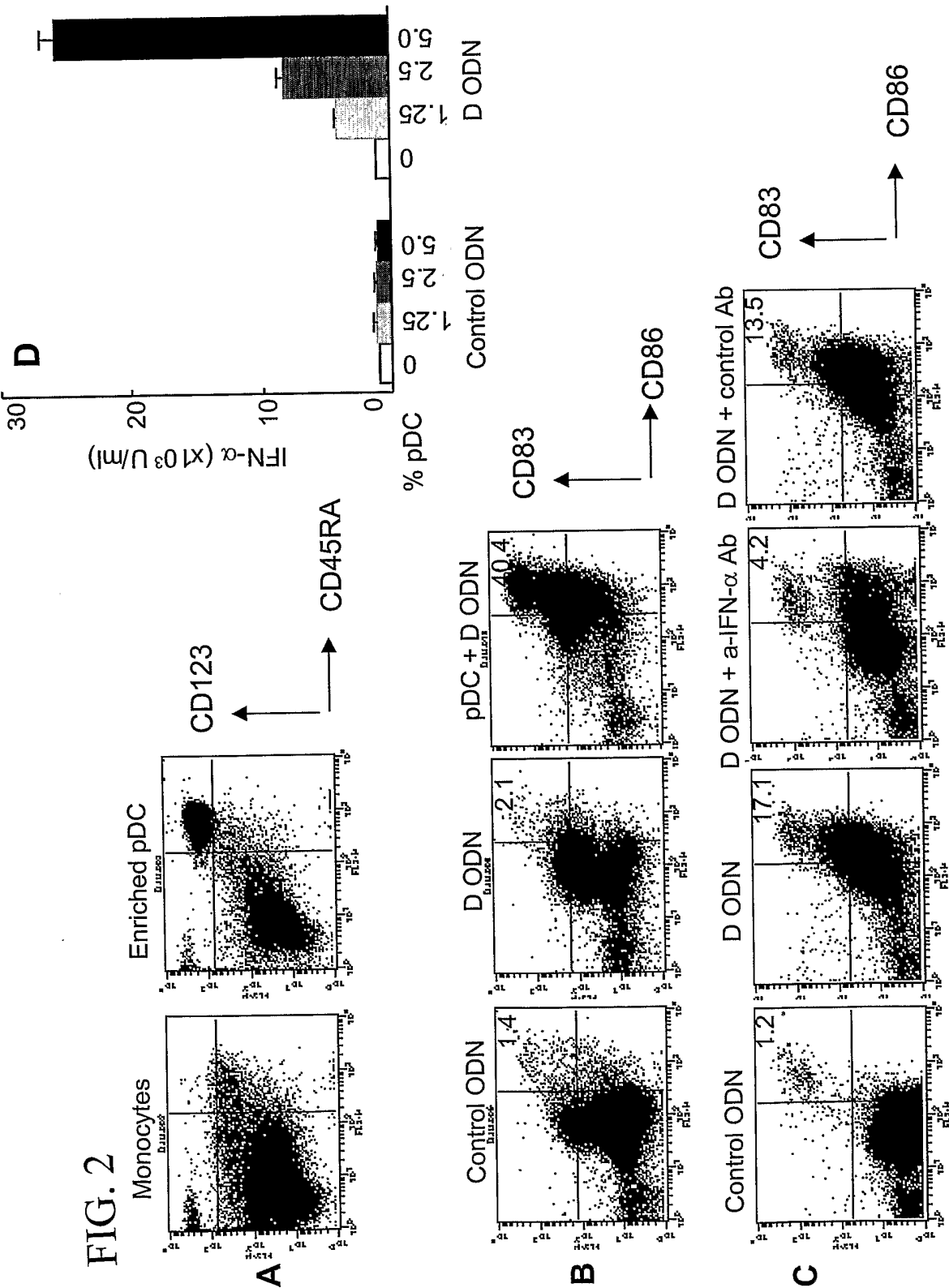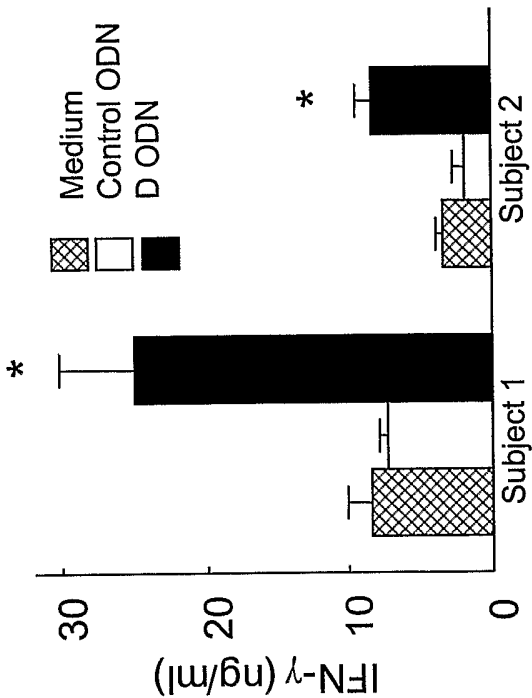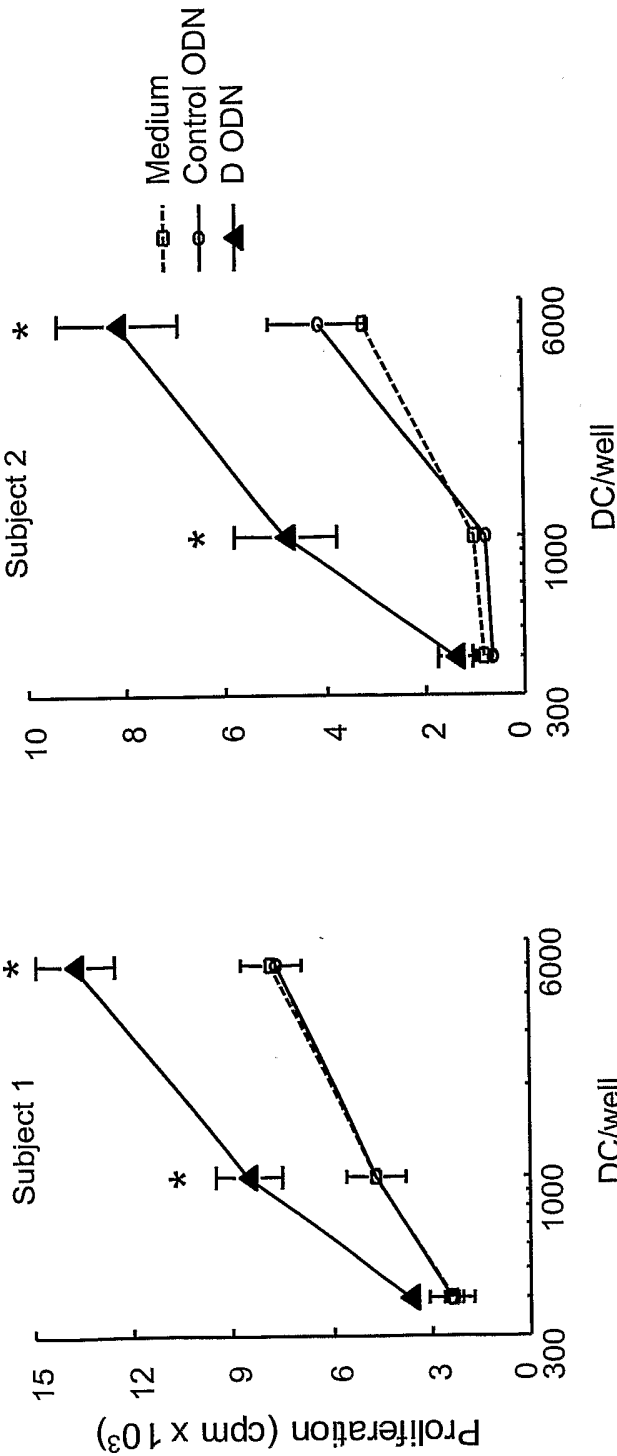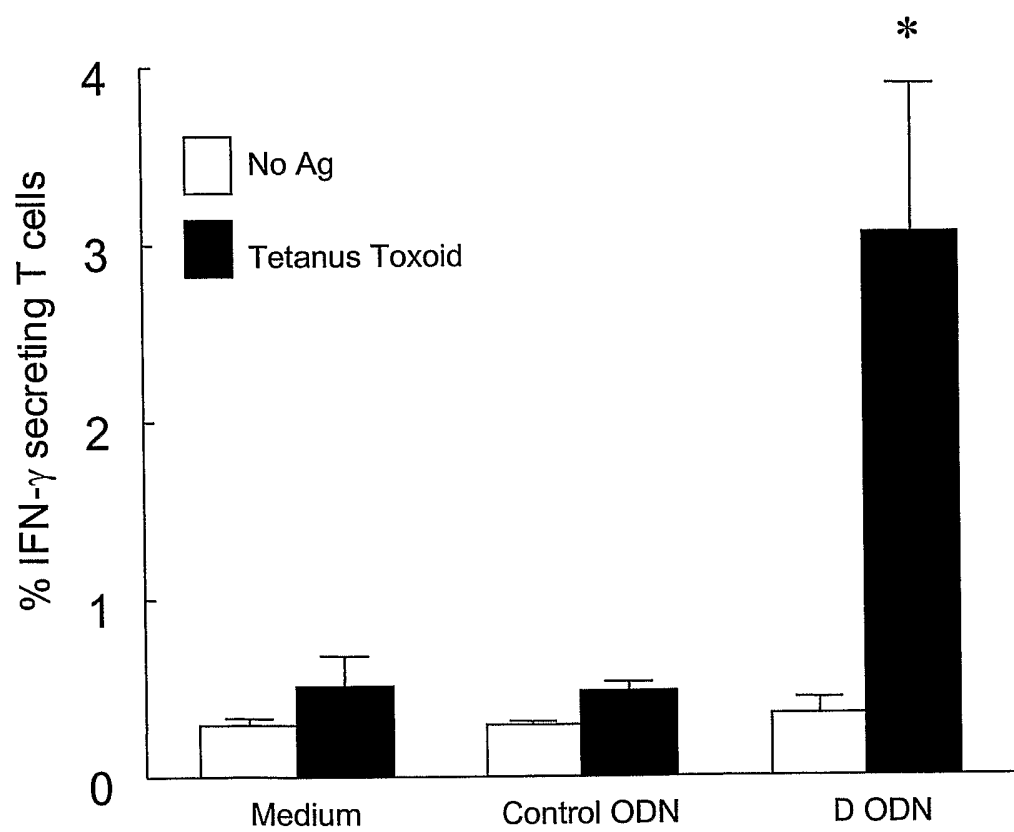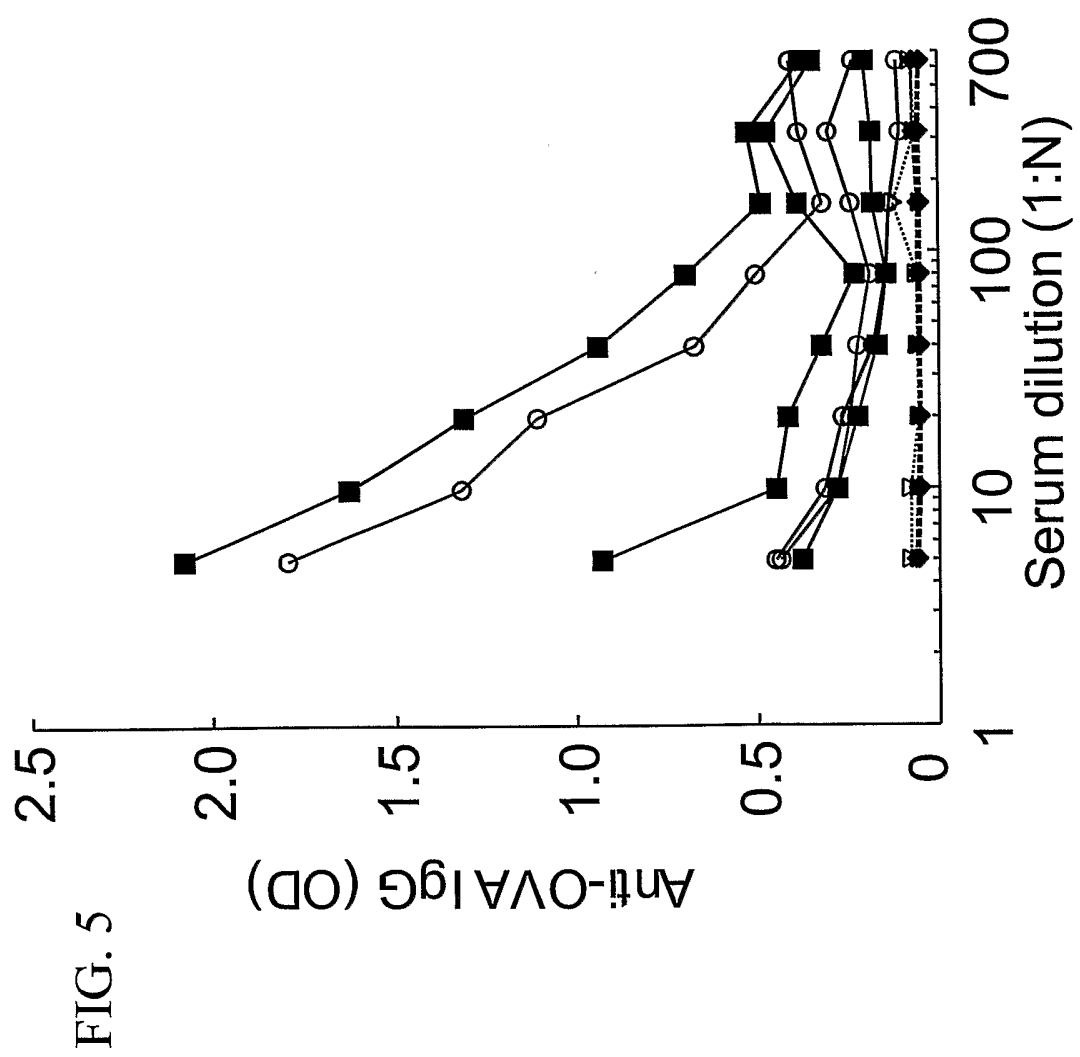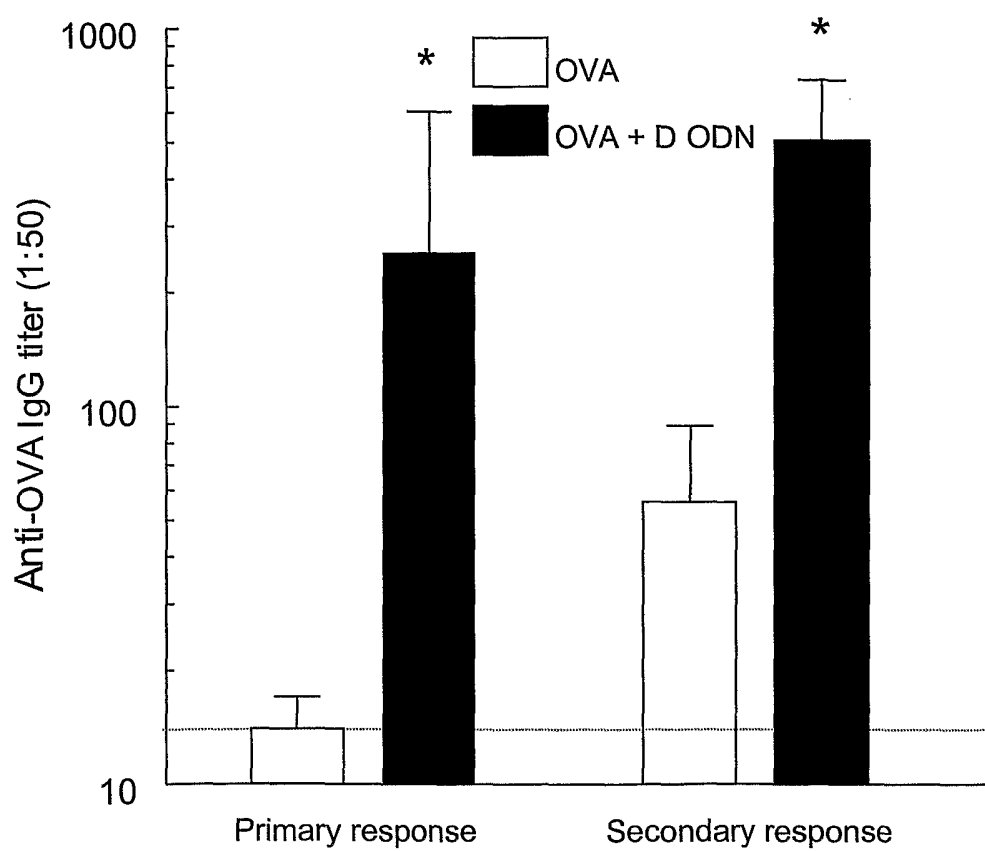
FIG. 6

FIG. 7

Comparison of DC generation induced by GM-CSF+IL-4 to that of D ODN alone or D ODN in the presence of GM-CSF+IL-4 (48 h)

FIG. 8

Table I D class ODN

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DV27
DV28
DV29
DV30
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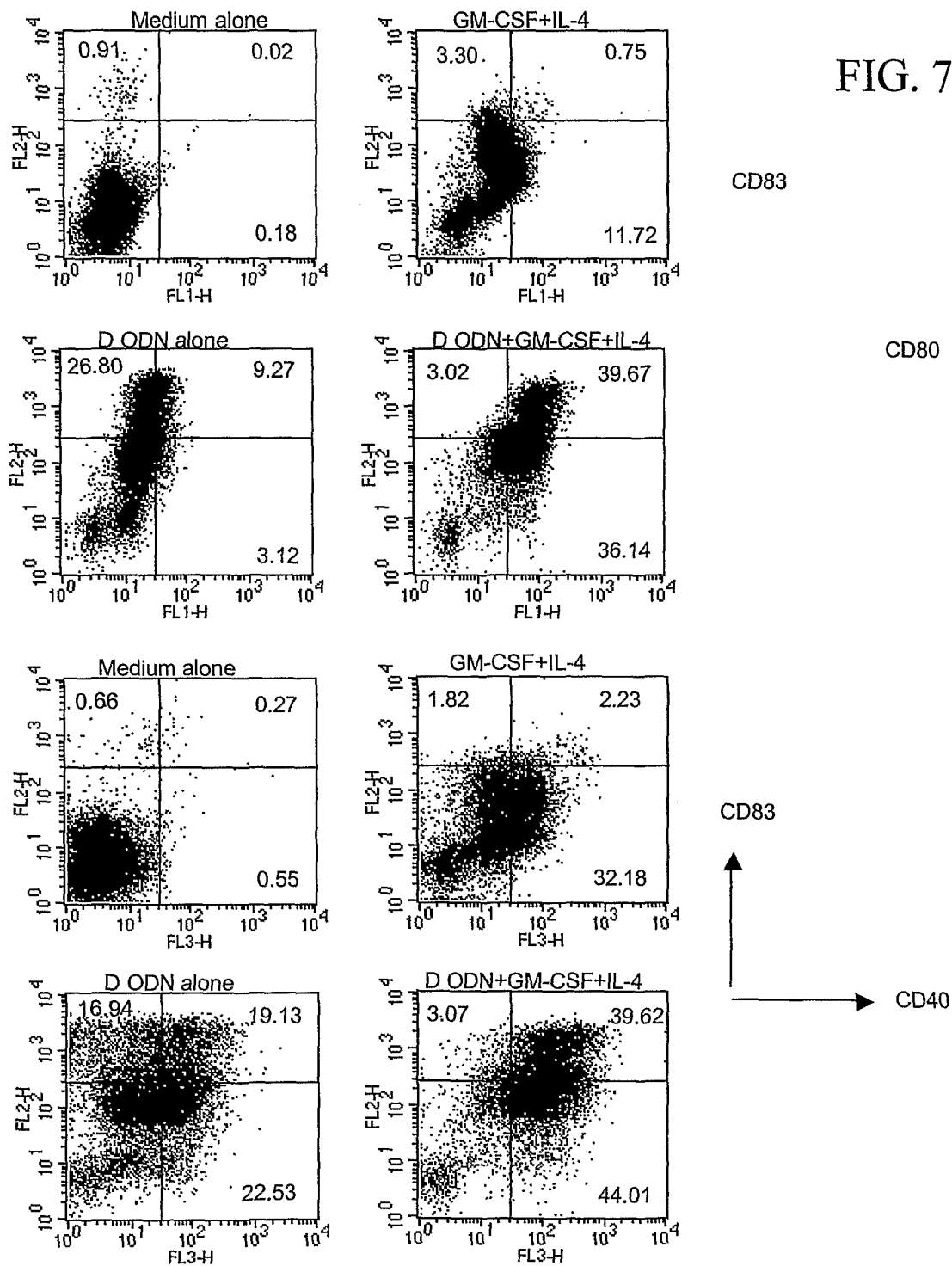
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       Verhelyi, Daniela

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