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(54) **Title:**
COMBINATION MOTIF IMMUNE STIMULATORY OLIGONUCLEOTIDES WITH IMPROVED ACTIVITY

(57) **Abstract:**
Abstract COMBINATION MOTIF IMMUNE STIMULATORY OLIGONUCLEOTIDES WITH IMPROVED ACTIVITY A class of immunostimulatory nucleic acids having at least two functionally and structurally defined domains is provided. This class of combination motif immunostimulatory nucleic acids activates an immune response and is useful for treating a variety of immune realted disorders such as cancer, infectious disease, and allergic disorders. The nucleic acids also stimulate activation of natural killer cells and production of type 1 interferon. Fig. 1

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Abstract

COMBINATION MOTIF IMMUNE STIMULATORY OLIGONUCLEOTIDES WITH IMPROVED ACTIVITY

A class of immunostimulatory nucleic acids having at least two functionally and structurally defined domains is provided. This class of combination motif immunostimulatory nucleic acids activates an immune response and is useful for treating a variety of immune realted disorders such as cancer, infectious disease, and allergic disorders. The nucleic acids also stimulate activation of natural killer cells and production of type 1 interferon.

Fig. 1

COMBINATION MOTIF IMMUNE STIMULATORY OLIGONUCLEOTIDES WITH IMPROVED ACTIVITY

Field of the Invention

The present invention relates generally to immunostimulatory nucleic acids, compositions thereof, and methods of using the immunostimulatory nucleic acids.

Background

Two main classes of immune stimulatory sequences are known in the art which have differing profiles of immune stimulatory activity. Krieg AM (2001) *Trends Microbiol* 9:249-52. These are so-called class B CpG oligodeoxynucleotides (ODN), which are strong activators of B cells, and class A CpG ODN, which are strong activators of natural killer (NK) cells. In addition to these immune stimulatory sequences, at least two classes of neutralizing sequences are known, including CpG sequences in which the CG is preceded by a C or followed by a G (Krieg AM et al. (1998) *Proc Natl Acad Sci USA* 95:12631-12636), and DNA sequences in which the CG is methylated. A neutralizing motif is a motif which has some degree of immunostimulatory capability when present in an otherwise non-stimulatory motif, but, which when present in the context of other immunostimulatory motifs serves to reduce the immunostimulatory potential of the other motifs.

Summary of the Invention

A new class of immune stimulatory nucleic acids is provided herein. In some instances these nucleic acids have a CG-rich palindrome or CG-rich neutralizing motif. Applicants previously recognized and described oligodeoxynucleotides (ODN) containing neutralizing motifs consisting of repeats of the sequence CG such as CGCGCG or a CG dinucleotide preceded by a C (i.e., CCG) and/or followed by a G (i.e., CGG, CCGG). These neutralizing motifs were believed cause some reduction in stimulatory effects of CpG containing ODN on multiple readouts, such as secretion of IL-6, IL-12, IFN-γ, TNF-α, and induction of an antigen-specific immune response. Krieg AM et al. (1998) *Proc Natl Acad Sci USA* 95:12631-6.

The present invention is based in part on the surprising discovery by the Applicants that certain ODN containing a combination of a stimulating motif and a neutralizing motif are highly immunostimulatory. The present invention is also based in part on the surprising discovery by the Applicants that ODN having certain CG-rich palindromic sequences, including palindromic sequences containing neutralizing motifs, are highly

immunostimulatory. The neutralizing motif thus, may, but need not occur within the context of a palindromic sequence to be highly immunostimulatory.

Furthermore, the immunostimulatory ODN of the instant invention have immunostimulatory effects previously associated with both of two distinct classes of CpG ODN, those that characteristically activate B cells (class B CpG ODN) and those that characteristically activate NK cells and induce production of interferon (IFN)-α (class A CpG ODN). The novel immunostimulatory ODN of the instant invention thus have a spectrum of immunostimulatory effects distinct from either class A CpG ODN or class B CpG ODN. The new class of immunostimulatory ODN of the instant invention is referred to as type C CpG ODN. As described in greater detail below, in certain embodiments the ODN of the present invention involve a combination of motifs wherein one motif is a CG-rich palindrome or a neutralizing motif, and another motif is a stimulatory motif, e.g., a CpG motif or the sequence TCGTCG.

In some aspects an immunostimulatory nucleic acid of 14-100 nucleotides in length is provided. The nucleic acid has the formula: 5' X_1DCGHX_2 3'. X_1 and X_2 are independently any sequence 0 to 10 nucleotides long. D is a nucleotide other than C. C is cytosine. G is guanine. H is a nucleotide other than G. The nucleic acid sequence also includes a nucleic acid sequence selected from the group consisting of P and N positioned immediately 5' to X_1 or immediately 3' to X_2. N is a B-cell neutralizing sequence which begins with a CGG trinucleotide and is at least 10 nucleotides long. P is a GC-rich palindrome containing sequence at least 10 nucleotides long.

In some embodiments the immunostimulatory nucleic acid is 5' NX_1DCGHX_2 3', 5' X_1DCGHX_2N 3', 5' PX_1DCGHX_2 3', 5' X_1DCGHX_2P 3', 5' X_1DCGHX_2PX_3 3', 5' X_1DCGHPX_3 3', 5' DCGHX_2PX_3 3', 5' TCGHX_2PX_3 3', or 5' DCGHPX_3 3'. X_3 is any sequence 0 to 10 nucleotides long. In other embodiments the immunostimulatory nucleic acid is 5' DCGHP 3'.

Optionally D and/or H are thymine (T).

In other embodiments H is T and X_2 is CG, CGT, CGTT, CGTTT, or CGTTTT.

H is T and X_2 is CG or CGTTTT according to other embodiments.

According to other embodiments C is unmethylated.

N includes at least four CG dinucleotides and no more than two CCG trinucleotides in some embodiments.

Optionally P includes at least one Inosine.

The nucleic acid may also include a poly-T sequence at the 5' end or the 3' end.

An immunostimulatory nucleic acid of 13-100 nucleotides in length is provided according to other aspects of the invention. The nucleic acid has the formula: 5' N_1PyGN_2P 3'. G is guanine.

N_1 is any sequence 1 to 6 nucleotides long. In some embodiments N_1 is at least 50% pyrimidines and preferably at least 50% T. In other embodiments N_1 includes at least one CG motif, at least one TCG motif, at least one CI motif, at least one TCI motif, at least one IG motif, or at least one TIG motif. N_1 is TCGG or TCGH in other embodiments. H is a nucleotide other than G.

Py is a pyrimidine. In some embodiments Py is an unmethylated C.

N_2 is any sequence 0 to 30 nucleotides long. In some embodiments N_2 is at least 50% pyrimidines or is at least 50% T. In other embodiments N_2 does not includes any poly G or poly A motifs.

P is a GC-rich palindrome containing sequence at least 10 nucleotides long. In some embodiments P is completely palindromic. In other embodiments P is a palindrome having between 1 and 3 consecutive intervening nucleotides. Optionally the intervening nucleotides may be TG. In other embodiments P includes at least 3, 4, or 5 C and at least 3, 4, or 5 G nucleotides. According to other embodiments P includes at least one Inosine.

In one embodiment the GC-rich palindrome has a base content of at least two-thirds G and C. In another embodiment the GC-rich palindrome has a base content of at least 81 percent G and C. In some embodiments the GC-rich palindrome is at least 12 nucleotides long. The GC-rich palindrome may be made up exclusively of C and G. In some embodiments the GC-rich palindrome can include at least one nucleotide that is neither C nor G.

In some embodiments the GC-rich palindrome includes at least one CGG trimer, at least one CCG trimer, or at least one CGCG tetramer. In some embodiments the GC-rich palindrome includes at least four CG dinucleotides. In certain preferred embodiments the GC-rich palindrome has a central CG dinucleotide.

In certain embodiments the GC-rich palindrome is CGGCGCGCGCCG (SEQ ID NO: 23), CGGCGGCCGCCG (SEQ ID NO: 28), CGACGATCGTCG (SEQ ID NO: 68) or CGACGTACGTCG (SEQ ID NO: 69).

In certain embodiments the GC-rich palindrome is not CGCGCGCGCGCG (SEQ ID NO: 29), GCGCGCGCGCGC (SEQ ID NO: 30), CCCCCCGGGGGG (SEQ ID NO: 31), GGGGGGCCCCCC (SEQ ID NO: 32), CCCCCGGGGG (SEQ ID NO: 33) or GGGGGCCCCC (SEQ ID NO: 34).

In some embodiments N_1PyGN_2 is a sequence selected from the group consisting of TTTTTCG, TCG, TTCG, TTTCG, TTTTCG, TCGT, TTCGT, TTTCGT, and TCGTCGT.

An immunostimulatory nucleic acid of 13-100 nucleotides in length is provided according to other aspects of the invention. The nucleic acid has the formula: 5' N_1PyG/IN_2P 3'. G/I refers to single nucleotide which is either a G or an I. G is guanine and I is Inosine.

N_1 is any sequence 1 to 6 nucleotides long. Py is a pyrimidine. N_2 is any sequence 0 to 30 nucleotides long.

P is a palindrome containing sequence at least 10 nucleotides long. In some embodiments P is a GC-rich palindrome. In other embodiments P is an IC-rich palindrome.

N_1PyIN_2 in some embodiments is TCITCITTTT (SEQ ID NO: 47).

The nucleic acid molecules described herein may have any type of backbone composition. In some embodiments the immunostimulatory nucleic acid has a completely nuclease-resistant backbone. The nuclease-resistant backbone may be composed of phosphorothioate linkages. In other embodiments the immunostimulatory nucleic acid has a completely phosphodiester backbone. In yet other embodiments the immunostimulatory nucleic acid has a chimeric backbone. In one embodiment the immunostimulatory nucleic acid has at least one phosphodiester linkage between a CG, CI or a IG motif. Alternatively, the ODN of the instant invention are formulated with microparticles, emulsions, or other means to avoid rapid digestion *in vivo*.

The immunostimulatory nucleic acid molecules described herein have a variety of lengths. In some embodiments the immunostimulatory nucleic acid is 13-100, 13-40, 13-30, 14-100, 14-40, or 14-30 nucleotides in length or any integer therebetween.

An immunostimulatory nucleic acid having one of the following sequences is also provided: TCGTCGTTTTCGGCGCGCGCCG (SEQ ID NO: 1), TCGTCGTTTTCGGCGGCCGCCG (SEQ ID NO: 4), TCGTCGTTTTCGGCGCGCCGCG (SEQ ID NO: 5), TCGTCGTTTTCGGCGCCGGCCG (SEQ ID NO: 6), TCGTCGTTTTCGGCCCGCGCGG (SEQ ID NO: 7), TCGTCGTTTTCGGCGCGCGCCGTTTTT (SEQ ID NO: 12),

TCCTGACGTTCGGCGCGCGCCG (SEQ ID NO: 13), TZGTZGTTTTZGGZGZGZGZZG (SEQ ID NO: 14), wherein Z is 5-methylcytosine, TCCTGACGTTCGGCGCGCGCCC (SEQ ID NO: 19), TCGGCGCGCGCCGTCGTCGTTT (SEQ ID NO: 11), TCCTGACGTTCGGCGCGCGCCC (ODN 2136, SEQ ID NO: 19), TCGTCGTTTTCGGCGGCCGACG (ODN 5513, SEQ ID NO: 64), TCGTCGTTTTCGTCGGCCGCCG (ODN 5514, SEQ ID NO: 65), TCGTCGTTTTCGACGGCCGCCG (ODN 5515, SEQ ID NO: 66), and TCGTCGTTTTCGGCGGCCGTCG (ODN 5516, SEQ ID NO: 67).

Further according to other embodiments of the invention the immunostimulatory nucleic acid is one of the following sequences: TCGTCGTTTTCGGCGCGCGCCG (ODN 2395), TCGTCGTTTTCGGCGGCCGCCG (ODN 2429), TCGTCGTTTTCGGCGCGCCGCG (ODN 2430), TCGTCGTTTTCGGCGCCGGCCG (ODN 2431), TCGTCGTTTTCGGCCCGCGCGG (ODN 2432), TCGTCGTTTTCGGCGCGCGCCGTTTTT (ODN 2452), TCCTGACGTTCGGCGCGCGCCG (ODN 5315), TZGTZGTTTTZGGZGZGZGZZG (ODN 5327, wherein Z is 5-methylcytosine), TCCTGACGTTCGGCGCGCGCCC (ODN 2136), TCGTCGTTTTCGGCGGCCGACG (ODN 5513), TCGTCGTTTTCGTCGGCCGCCG (ODN 5514), TCGTCGTTTTCGACGGCCGCCG (ODN 5515), TCGTCGTTTTCGGCGGCCGTCG (ODN 5516), TCGTCGTTTTCGGCGCGCGCCG (ODN 2395), TCGTCGTTTTCGGCGGCCGCCG (ODN 2429), TCGTCGTTTTCGGCGCGCCGCG (ODN 2430), TCGTCGTTTTCGGCGCCGGCCG (ODN 2431), TCGTCGTTTTCGGCCCGCGCGG (ODN 2432), TCGTCGTTTTCGGCGCGCGCCGTTTTT (ODN 2452), TCCTGACGTTCGGCGCGCGCCG (ODN 5315), TZGTZGTTTTZGGZGZGZGZZG (ODN 5327, wherein Z is 5-methylcytosine), TCCTGACGTTCGGCGCGCGCCC (ODN 2136), TCGTCGTTTTCGGCGGCCGACG (ODN 5513), TCGTCGTTTTCGTCGGCCGCCG (ODN 5514), TCGTCGTTTTCGACGGCCGCCG (ODN 5515), TCGTCGTTTTCGGCGGCCGTCG (ODN 5516), TCGGCGCGCGCCGTCGTCGTTT (ODN 2451), TCGTCGTTTCGACGGCCGTCG (ODN 20173, SEQ ID NO: 71), TCGTCGTTTCGACGATCGTCG (ODN 20176, SEQ ID NO: 72), TCGTCGTTTCGACGTACGTCG (ODN 20177, SEQ ID NO: 73), TCGTCGCGACGGCCGTCG (ODN 20178, SEQ ID NO: 74),

TCGTCGCGACGATCGTCG (ODN 20179, SEQ ID NO: 75),
TCGTCGCGACGTACGTCG (ODN 20180, SEQ ID NO: 76),
TCGTTTTTTTCGACGGCCGTCG (ODN 20184, SEQ ID NO: 77),
TCGTTTTTTTCGACGATCGTCG (ODN 20185, SEQ ID NO: 78), and
TCGTTTTTTTCGACGTACGTCG (ODN 20186, SEQ ID NO: 79).

According to certain embodiments the immunostimulatory nucleic acid includes the sequence TCGGCGCGCGCCGTCGTCGTTT (ODN 2451, SEQ ID NO: 11). In certain embodiments the immunostimulatory nucleic acid is the sequence TCGGCGCGCGCCGTCGTCGTTT (ODN 2451).

A pharmaceutical composition, comprising the immunostimulatory nucleic acids described herein and a pharmaceutically acceptable carrier is provided according to other aspects of the invention.

In other aspects of the invention a method for inducing type 1 interferon (IFN) expression is provided. The method involves contacting a cell capable of expressing type 1 IFN with an effective amount of an immunostimulatory nucleic acid described herein to induce expression of type 1 IFN.

The invention in other aspects is a method for activating a natural killer (NK) cell. The method involves contacting an NK cell with an effective amount of an immunostimulatory nucleic acid described herein to activate the NK cell.

In yet other aspects the invention is a method for treating infection by administering to a subject having or at risk of developing an infection an effective amount of an immunostimulatory nucleic acid described herein, to treat or prevent the infection. In some embodiments the subject has or is at risk of developing an infection selected from the group consisting of a viral, bacterial, fungal and parasitic infection.

In certain embodiments the method involves administering an immunostimulatory nucleic acid of the invention alone to treat or prevent the infection. In certain embodiments the method according to this aspect of the invention further includes administering to the subject an antibiotic agent, which may be an antibacterial agent, an antiviral agent, an antifungal agent, or an antiparasitic agent.

In other aspects the invention is a method for treating an allergic condition by administering to a subject having or at risk of developing an allergic condition an effective

amount of an immunostimulatory nucleic acid described herein, to treat or prevent the allergic condition. In some embodiments the allergic condition is allergic asthma. In one embodiment the allergic condition is asthma. In certain embodiments the method involves administering an immunostimulatory nucleic acid of the invention alone to treat or prevent the allergic condition. In certain embodiments the method according to this aspect of the invention further includes administering to the subject an asthma/allergy medicament e.g., steroids, antihistamines, and prostaglandin inducers.

A method for treating cancer is provided according to other aspects of the invention. The method involves administering to a subject having or at risk of developing a cancer an effective amount of an immunostimulatory nucleic acid described herein, to treat or prevent the cancer. In some embodiments the cancer is selected from the group consisting of basal cell carcinoma, biliary tract cancer; bladder cancer; bone cancer; brain and CNS cancer; breast cancer; cervical cancer; choriocarcinoma; colon and rectum cancer; connective tissue cancer; cancer of the digestive system; endometrial cancer; esophageal cancer; eye cancer; cancer of the head and neck; gastric cancer; intra-epithelial neoplasm; kidney cancer; larynx cancer; leukemia; liver cancer; lung cancer (e.g. small cell and non-small cell); lymphoma including Hodgkin's and Non-Hodgkin's lymphoma; melanoma; myeloma; neuroblastoma; oral cavity cancer (e.g., lip, tongue, mouth, and pharynx); ovarian cancer; pancreatic cancer; prostate cancer; retinoblastoma; rhabdomyosarcoma; rectal cancer; renal cancer; cancer of the respiratory system; sarcoma; skin cancer; stomach cancer; testicular cancer; thyroid cancer; uterine cancer; cancer of the urinary system, and other carcinomas and sarcomas

In certain embodiments the method involves administering an immunostimulatory nucleic acid of the invention alone to treat the cancer. In certain embodiments the method according to this aspect of the invention further includes administering to the subject an anti-cancer medicament or treatment e.g., chemotherapeutic agents, radiation.

Each of the limitations of the invention can encompass various embodiments of the invention. It is, therefore, anticipated that each of the limitations of the invention involving any one element or combinations of elements can be included in each aspect of the invention.

Brief Description of the Drawings

The following figures are provided for illustrative purposes only and are not required for understanding or practicing the invention.

Figure 1 is a bar graph depicting amounts of IFN-α (pg/ml) induced in human PBMCs after 24 hours of culture alone, with IL-2, or in the presence of the indicated ODN at the indicated concentrations.

Figure 2 is a bar graph depicting amounts of MCP-1 (pg/ml) induced in human PBMCs after 24 hours of culture alone, with IL-2, or in the presence of the indicated ODN at the indicated concentrations.

Figure 3 is a bar graph depicting amounts of IP-10 (pg/ml) induced in human PBMCs after 24 hours of culture alone, with IL-2, or in the presence of the indicated ODN at the indicated concentrations.

Figure 4 is a bar graph depicting amounts of IFN-α (pg/ml) induced in human PBMCs after 48 hours of culture alone (N/A) or in the presence of the indicated ODN at 1.0 μg/ml.

Figure 5 is a pair of bar graphs depicting surface staining on B cells for CD86 (MFI) after 48 hours of culture alone (N/A) or in the presence of the indicated ODN at 0.25 μg/ml (panel A) or 1.0 μg/ml (panel B).

Figure 6 is a pair of bar graphs depicting results of a 72 hour B cell proliferation assay (cpm ^{3}H-thymidine incorporation) alone (N/A) or in the presence of the indicated ODN at 0.25 μg/ml (panel A) or 1.0 μg/ml (panel B).

Figure 7 is a pair of bar graphs depicting amounts of IL-10 (pg/ml) induced in human PBMCs after 24 hours of culture either alone (N/A) or in the presence of the indicated ODN at 0.25 μg/ml (panel A) or 1.0 μg/ml (panel B).

Figure 8 is a bar graph depicting amounts of IFN-α (pg/ml) induced in PBMC from two donors (D127, solid bars, and D124, open bars) following 24 hours of culture alone (w/o) or in the presence of the indicated ODN at the indicated concentrations (1 or 6 μg/ml).

Figure 9 is a bar graph depicting B cell activation as measured by percent CD86-positive cells in human PBMC cultured for 24 hours alone (w/o) or in the presence of the indicated ODN at the indicated concentrations (0.4, 1.0, or 10.0 μg/ml).

Figure 10 is a bar graph depicting the amount of IFN-α (pg/ml) secreted by PBMC from two donors (D141, open bars, and D142, solid bars) following 24 hours of culture alone (w/o) or in the presence of the indicated ODN at the indicated concentrations (1 or 6 μg/ml).

Figure 11 is a bar graph depicting the amount of IFN-α (pg/ml) secreted by PBMC from two donors (D141, open bars, and D142, solid bars) following 48 hours of culture alone (w/o) or in the presence of the indicated ODN at the indicated concentrations (1 or 6 μg/ml).

Figure 12 is a bar graph depicting the amount of IFN-α (pg/ml) secreted by PBMC from two donors (D141, shaded bars, and D142, open bars) following 24 hours of culture alone (w/o) or in the presence of the indicated ODN at 6 μg/ml.

Figure 13 is a series of three bar graphs depicting the amount of IFN-γ (pg/ml) secreted by PBMC following 24 hours of culture alone (n/a) or in the presence of the indicated ODN at the indicated concentrations (1, 3 or 10 μg/ml in panels A, B, and C, respectively).

Figure 14 is a bar graph depicting the percentage of CD3+ cells staining positive for IFN-γ following 48 hours of culture alone (NA) or in the presence of the indicated ODN.

Figure 15 is a bar graph depicting the mean fluorescence intensity (MFI) of IFN-γ staining in T cells following 48 hours of culture alone (NA) or in the presence of the indicated ODN.

Figure 16 is a bar graph depicting the amount of IFN-α (pg/ml) secreted by human PBMC following 24 hours of culture alone (N/A) or in the presence of the indicated ODN at 1.0 μg/ml.

Figure 17 is a pair of bar graphs depicting the amount of IFN-α (pg/ml) secreted by human PBMC following 24 or 48 hour culture alone (w/o) or in the presence of the indicated ODN at the indicated concentration (1 or 6 μg/ml). Panel A depicts results for PBMC pooled from two donors. Panel B depicts results for PBMC obtained from two donors (D141 and D142).

Figure 18 is a bar graph depicting the percent CD86-positive B cells following 24 hours of culture alone (w/o) or in the presence of the indicated ODN at the indicated concentrations (0.4 and 1.0 μg/ml).

Figure 19 is a series of three bar graphs depicting the concentration of IFN-γ (pg/ml) in culture supernatants of human PBMC after incubation alone (w/o), with LPS, or with the indicated ODN at the indicated concentrations (0.2 to 1.0 μg/ml) for 6 hours (panel A), 24 hours (panel B), or 48 hours (panel C).

Figure 20 is a bar graph depicting the amount IFN-γ (pg/ml) generated in a two-way mixed lymphocyte reaction (MLR) in which lymphocytes obtained from two donors were cultured for 24 hours alone (w/o) or in the presence of the indicated ODN at 6 μg/ml and then mixed.

Figure 21 is a series of three bar graphs depicting the concentration of IL-10 (pg/ml) in culture supernatants of human PBMC after incubation alone (w/o), with LPS, or with the indicated ODN at the indicated concentrations (0.2 to 1.0 μg/ml) for 6 hours (panel A), 24 hours (panel B), or 48 hours (panel C).

Figure 22 is a bar graph depicting the amounts of IP-10 (pg/ml) in PBMC supernatants after 24 hours of incubation alone (n/a) or in the presence of controls (IL-2, ODN 1585 (GGGGTCAACGTTGAGGGGGG, SEQ ID NO: 35) and ODN 2118 (GGGGTCAAGCTTGAGGGGGG, SEQ ID NO: 36)) or various indicated ODN at either 0.6 μg/ml (open bars) or 3.0 μg/ml (solid bars).

Figure 23 is a pair of bar graphs depicting the amounts of IFN-α (pg/ml) in PBMC supernatants after 24 hours of incubation alone (n/a) or in the presence of controls (IL-2, ODN 1585, and ODN 2118) or various indicated ODN at either 0.6 μg/ml (panel A) or 3.0 μg/ml (panel B).

Figure 24 is a bar graph depicting the amounts of IFN-γ (pg/ml) in PBMC supernatants after 24 hours of incubation alone (n/a) or in the presence of controls (IL-2, ODN 1585, and ODN 2118) or various indicated ODN at either 0.6 μg/ml (open bars) or 3.0 μg/ml (filled bars).

Figure 25 is a bar graph depicting the amounts of IL-6 (pg/ml) in PBMC supernatants after 24 hours of incubation alone (n/a) or in the presence of controls (IL-2, ODN 1585, and ODN 2118) or various indicated ODN at either 0.6 μg/ml (open bars) or 3.0 μg/ml (filled bars).

Figure 26 is a bar graph depicting amounts of IFN-α secretion (pg/ml) by PBMC following 24 hours of culture alone (w/o) or in the presence of the indicated ODN at the indicated concentrations (3.0 and 6.0 μg/ml).

Detailed Description of the Invention

It has been discovered that certain oligodeoxynucleotides (ODN), which contain at least two distinct motifs have unique and desirable stimulatory effects on cells of the immune system. Some of these ODN have both a traditional "stimulatory" CpG sequence and a

"GC-rich" or "B-cell neutralizing" motif. These combination motif nucleic acids have immune stimulating effects that fall somewhere between those effects associated with traditional "class B" CpG ODN, which are strong inducers of B cell activation and dendritic cell (DC) activation, and those effects associated with a more recently described class of immune stimulatory nucleic acids ("class A" CpG ODN) which are strong inducers of IFN-α and natural killer (NK) cell activation but relatively poor inducers of B-cell and DC activation. Krieg AM et al. (1995) *Nature* 374:546-9; Ballas ZK et al. (1996) *J Immunol* 157:1840-5; Yamamoto S et al. (1992) *J Immunol* 148:4072-6. While preferred class B CpG ODN often have phosphorothioate backbones and preferred class A CpG ODN have mixed or chimeric backbones, the new class of combination motif immune stimulatory nucleic acids may have either stabilized, e.g., phosphorothioate, chimeric, or phosphodiester backbones.

In one aspect the invention provides immune stimulatory nucleic acids belonging to this new class of combination motif immune-stimulatory nucleic acids. The B cell stimulatory domain is defined by a formula: 5' X_1DCGHX_2 3'. D is a nucleotide other than C. C is cytosine. G is guanine. H is a nucleotide other than G.

X_1 and X_2 are any nucleic acid sequence 0 to 10 nucleotides long. X_1 may include a CG, in which case there is preferably a T immediately preceding this CG. In some embodiments DCG is TCG. X_1 is preferably from 0 to 6 nucleotides in length. In some embodiments X_2 does not contain any poly G or poly A motifs. In other embodiments the immunostimulatory nucleic acid has a poly-T sequence at the 5' end or at the 3' end. As used herein, "poly-A" or "poly-T" shall refer to a stretch of four or more consecutive A's or T's respectively, e.g., 5' AAAA 3' or 5' TTTT 3'.

As used herein, "poly-G end" shall refer to a stretch of four or more consecutive G's, e.g., 5' GGGG 3', occurring at the 5' end or the 3' end of a nucleic acid. As used herein, "poly-G nucleic acid" shall refer to a nucleic acid having the formula 5' X_1X_2GGGX_3X_4 3' wherein X_1, X_2, X_3, and X_4 are nucleotides and preferably at least one of X_3 and X_4 is a G.

Some preferred designs for the B cell stimulatory domain under this formula comprise TTTTTCG, TCG, TTCG, TTTCG, TTTTCG, TCGT, TTCGT, TTTCGT, TCGTCGT.

The second motif of the nucleic acid is referred to as either P or N and is positioned immediately 5' to X_1 or immediately 3' to X_2.

N is a B-cell neutralizing sequence that begins with a CGG trinucleotide and is at least 10 nucleotides long. A B-cell neutralizing motif includes at least one CpG sequence in

which the CG is preceded by a C or followed by a G (Krieg AM et al. (1998) *Proc Natl Acad Sci USA* 95:12631-12636) or is a CG containing DNA sequence in which the C of the CG is methylated. As used herein, "CpG" shall refer to a 5' cytosine (C) followed by a 3' guanine (G) and linked by a phosphate bond. At least the C of the 5' CG 3' must be unmethylated. Neutralizing motifs are motifs which has some degree of immunostimulatory capability when present in an otherwise non-stimulatory motif, but, which when present in the context of other immunostimulatory motifs serve to reduce the immunostimulatory potential of the other motifs.

P is a GC-rich palindrome containing sequence at least 10 nucleotides long. As used herein, "palindrome" and, equivalently, "palindromic sequence" shall refer to an inverted repeat, i.e., a sequence such as ABCDEE'D'C'B'A' in which A and A', B and B', etc., are bases capable of forming the usual Watson-Crick base pairs.

As used herein, "GC-rich palindrome" shall refer to a palindrome having a base composition of at least two-thirds G's and C's. In some embodiments the GC-rich domain is preferably 3' to the "B cell stimulatory domain". In the case of a 10-base long GC-rich palindrome, the palindrome thus contains at least 8 G's and C's. In the case of a 12-base long GC-rich palindrome, the palindrome also contains at least 8 G's and C's. In the case of a 14-mer GC-rich palindrome, at least ten bases of the palindrome are G's and C's. In some embodiments the GC-rich palindrome is made up exclusively of G's and C's.

In some embodiments the GC-rich palindrome has a base composition of at least 81 percent G's and C's. In the case of such a 10-base long GC-rich palindrome, the palindrome thus is made exclusively of G's and C's. In the case of such a 12-base long GC-rich palindrome, it is preferred that at least ten bases (83 percent) of the palindrome are G's and C's. In some preferred embodiments, a 12-base long GC-rich palindrome is made exclusively of G's and C's. In the case of a 14-mer GC-rich palindrome, at least twelve bases (86 percent) of the palindrome are G's and C's. In some preferred embodiments, a 14-base long GC-rich palindrome is made exclusively of G's and C's. The C's of a GC-rich palindrome can be unmethylated or they can be methylated.

In general this domain has at least 3 Cs and Gs, more preferably 4 of each, and most preferably 5 or more of each. The number of Cs and Gs in this domain need not be identical. It is preferred that the Cs and Gs are arranged so that they are able to form a self-complementary duplex, or palindrome, such as CCGCGCGG. This may be interrupted by As

or Ts, but it is preferred that the self-complementarity is at least partially preserved as for example in the motifs CGACGTTCGTCG (SEQ ID NO: 80) or CGGCGCCGTGCCG (SEQ ID NO: 81). When complementarity is not preserved, it is preferred that the non-complementary base pairs be TG. In a preferred embodiment there are no more than 3 consecutive bases that are not part of the palindrome, preferably no more than 2, and most preferably only 1. In some embodiments the GC-rich palindrome includes at least one CGG trimer, at least one CCG trimer, or at least one CGCG tetramer. In other embodiments the GC-rich palindrome is not CCCCCCGGGGGG (SEQ ID NO: 31) or GGGGGGCCCCCC (SEQ ID NO: 32), CCCCCGGGGG (SEQ ID NO: 33) or GGGGGCCCCC (SEQ ID NO: 34).

At least one of the G's of the GC rich region may be substituted with an inosine (I). In some embodiments P includes more than one I.

In certain embodiments the immunostimulatory nucleic acid has one of the following formulas 5' NX_1DCGHX_2 3', 5' X_1DCGHX_2N 3', 5' PX_1DCGHX_2 3', 5' X_1DCGHX_2P 3', 5' $X_1DCGHX_2PX_3$ 3', 5' $X_1DCGHPX_3$ 3', 5' $DCGHX_2PX_3$ 3', 5' $TCGHX_2PX_3$ 3', 5' $DCGHPX_3$ 3', or 5' DCGHP 3'.

In other aspects the invention provides immune stimulatory nucleic acids which are defined by a formula: 5' N_1PyGN_2P 3'. N_1 is any sequence 1 to 6 nucleotides long. Py is a pyrimidine. G is guanine. N_2 is any sequence 0 to 30 nucleotides long. P is a GC-rich palindrome containing sequence at least 10 nucleotides long.

N_1 and N_2 may contain more than 50% pyrimidines, and more preferably more than 50% T. N_1 may include a CG, in which case there is preferably a T immediately preceding this CG. In some embodiments N_1PyG is TCG (such as ODN 5376, which has a 5' TCGG), and most preferably a $TCGN_2$, where N_2 is not G.

N_1PyGN_2P may include one or more inosine (I) nucleotides. Either the C or the G in N1 may be replaced by inosine, but the CpI is preferred to the IpG. For inosine substitutions such as IpG, the optimal activity may be achieved with the use of a "semi-soft" or chimeric backbone, where the linkage between the IG or the CI is phosphodiester. N_1 may include at least one CI, TCI, IG or TIG motif.

In certain embodiments N_1PyGN_2 is a sequence selected from the group consisting of TTTTTCG, TCG, TTCG, TTTCG, TTTTCG, TCGT, TTCGT, TTTCGT, and TCGTCGT.

In other aspects the invention provides immune stimulatory nucleic acids which are defined by a formula: 5' N_1PyG/IN_2P 3'. N_1 is any sequence 1 to 6 nucleotides long. Py is a pyrimidine, G/I refers to single nucleotide which is either a G or an I. G is guanine and I is inosine. N_2 is any sequence 0 to 30 nucleotides long. P is a GC or IC rich palindrome containing sequence at least 10 nucleotides long. In some embodiments N_1PyIN_2 is TCITCITTTT (SEQ ID NO: 47).

Some non-limiting examples of combination motif immune stimulatory nucleic acids, which are described by the formulas above, include the following: TCGTCGTTTTCGGCGCGCGCCG (ODN 2395), TCGTCGTTTTCGGCGGCCGCCG (ODN 2429), TCGTCGTTTTCGGCGCGCCGCG (ODN 2430), TCGTCGTTTTCGGCGCCGGCCG (ODN 2431), TCGTCGTTTTCGGCCCGCGCGG (ODN 2432), TCGTCGTTTTCGGCGCGCGCCGTTTTT (ODN 2452), TCCTGACGTTCGGCGCGCGCCG (ODN 5315), TZGTZGTTTTZGGZGZGZGZZG (ODN 5327, wherein Z is 5-methylcytosine), TCCTGACGTTCGGCGCGCGCCC (ODN 2136), TCGTCGTTTTCGGCGGCCGACG (ODN 5513), TCGTCGTTTTCGTCGGCCGCCG (ODN 5514), TCGTCGTTTTCGACGGCCGCCG (ODN 5515), TCGTCGTTTTCGGCGGCCGTCG (ODN 5516), TCGGCGCGCGCCGTCGTCGTTT (ODN 2451), TCGTCGTTTCGACGGCCGTCG (ODN 20173), TCGTCGTTTCGACGATCGTCG (ODN 20176), TCGTCGTTTCGACGTACGTCG (ODN 20177), TCGTCGCGACGGCCGTCG (ODN 20178), TCGTCGCGACGATCGTCG (ODN 20179), TCGTCGCGACGTACGTCG (ODN 20180), TCGTTTTTTTCGACGGCCGTCG (ODN 20184), TCGTTTTTTTCGACGATCGTCG (ODN 20185), TCGTTTTTTTCGACGTACGTCG (ODN 20186), TIGTIGTTTTCGGCGGCCGCCG (ODN 5569, SEQ ID NO: 63), and TCITCITTTTCGGCGGCCGCCG (ODN 5570, SEQ ID NO: 70).

As used herein, "nucleic acid" and "oligonucleotide" are used interchangeably and shall refer to mean 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 (e.g., cytosine (C), thymine (T) or uracil (U)) or a substituted purine (e.g., adenine (A) or guanine (G)). As used herein, the terms refer to oligoribonucleotides as well as oligodeoxyribonucleotides (ODN). The terms shall also include polynucleosides (i.e., a polynucleotide minus the phosphate) and any other organic

base containing polymer. Nucleic acid molecules can be obtained from existing nucleic acid sources (e.g., genomic or cDNA), but are preferably synthetic (e.g., produced by nucleic acid synthesis).

The terms nucleic acid and oligonucleotide also encompass nucleic acids or oligonucleotides with substitutions or modifications, such as in the bases and/or sugars. For example, they include nucleic acids having backbone sugars which are covalently attached to low molecular weight organic groups other than a hydroxyl group at the 3' position and other than a phosphate group at the 5' position. Thus modified nucleic acids may include a 2'-O-alkylated ribose group. In addition, modified nucleic acids may include sugars such as arabinose instead of ribose. Thus the nucleic acids may be heterogeneous in backbone composition thereby containing any possible combination of polymer units linked together such as peptide- nucleic acids (which have amino acid backbone with nucleic acid bases). In some embodiments, the nucleic acids are homogeneous in backbone composition. Nucleic acids also include substituted purines and pyrimidines such as C-5 propyne modified bases. Wagner RW et al. (1996) *Nat Biotechnol* 14:840-4. Purines and pyrimidines include but are not limited to adenine, cytosine, guanine, thymidine, 5-methylcytosine, 2-aminopurine, 2-amino-6-chloropurine, 2,6-diaminopurine, hypoxanthine, and other naturally and non-naturally occurring nucleobases, substituted and unsubstituted aromatic moieties. Other such modifications are well known to those of skill in the art.

The immunostimulatory oligonucleotides of the instant invention can encompass various chemical modifications and substitutions, in comparison to natural RNA and DNA, involving a phosphodiester internucleoside bridge, a β-D-ribose unit and/or a natural nucleoside base (adenine, guanine, cytosine, thymine, uracil). Examples of chemical modifications are known to the skilled person and are described, for example, in Uhlmann E et al. (1990) *Chem Rev* 90:543; "Protocols for Oligonucleotides and Analogs" Synthesis and Properties & Synthesis and Analytical Techniques, S. Agrawal, Ed, Humana Press, Totowa, USA 1993; Crooke ST et al. (1996) *Annu Rev Pharmacol Toxicol* 36:107-129; and Hunziker J et al. (1995) *Mod Synth Methods* 7:331-417. An oligonucleotide according to the invention can have one or more modifications, wherein each modification is located at the a particular phosphodiester internucleoside bridge and/or at a particular β-D-ribose unit and/or at a particular natural nucleoside base position in comparison to an oligonucleotide of the same sequence which is composed of natural DNA or RNA.

For example, the invention relates to an oligonucleotide which may comprise one or more modifications and wherein each modification is independently selected from:

a) the replacement of a sugar phosphate unit from the sugar phosphate backbone by another unit,

b) the replacement of a β-D-ribose unit by a modified sugar unit, and

c) the replacement of a natural nucleoside base by a modified nucleoside base.

More detailed examples for the chemical modification of an oligonucleotide are as follows.

A sugar phosphate unit (i.e., a β-D-ribose and phosphodiester internucleoside bridge together forming a sugar phosphate unit) from the sugar phosphate backbone (i.e., a sugar phosphate backbone is composed of sugar phosphate units) can be replaced by another unit, wherein the other unit is for example suitable to build up a "morpholino-derivative" oligomer (as described, for example, in Stirchak EP et al. (1989) *Nucleic Acids Res* 17:6129-41), that is, e.g., the replacement by a morpholino-derivative unit; or to build up a polyamide nucleic acid ("PNA"; as described for example, in Nielsen PE et al. (1994) *Bioconjug Chem* 5:3-7), that is, e.g., the replacement by a PNA backbone unit, e.g., by 2-aminoethylglycine.

A β-ribose unit or a β-D-2'-deoxyribose unit can be replaced by a modified sugar unit, wherein the modified sugar unit is for example selected from β-D-ribose, α-D-2'-deoxyribose, L-2'-deoxyribose, 2'-F-2'-deoxyribose, 2'-O-(C_1-C_6)alkyl-ribose, preferably 2'-O-(C_1-C_6)alkyl-ribose is 2'-O-methylribose, 2'-O-(C_2-C_6)alkenyl-ribose, 2'-[O-(C_1-C_6)alkyl-O-(C_1-C_6)alkyl]-ribose, 2'-NH_2-2'-deoxyribose, β-D-xylo-furanose, α-arabinofuranose, 2,4-dideoxy-β-D-erythro-hexo-pyranose, and carbocyclic (described, for example, in Froehler J (1992) *Am Chem Soc* 114:8320) and/or open-chain sugar analogs (described, for example, in Vandendriessche et al. (1993) *Tetrahedron* 49:7223) and/or bicyclosugar analogs (described, for example, in Tarkov M et al. (1993) *Helv Chim Acta* 76:481).

A natural nucleoside base can be replaced by a modified nucleoside base, wherein the modified nucleoside base is for example selected from hypoxanthine, uracil, dihydrouracil, pseudouracil, 2-thiouracil, 4-thiouracil, 5-aminouracil, 5-(C_1-C_6)-alkyluracil, 5-(C_2-C_6)-alkenyluracil, 5-(C_2-C_6)-alkynyluracil, 5-(hydroxymethyl)uracil, 5-chlorouracil, 5-fluorouracil, 5-bromouracil, 5-hydroxycytosine, 5-(C_1-C_6)-alkylcytosine, 5-(C_2-C_6)-alkenylcytosine, 5-(C_2-C_6)-alkynylcytosine, 5-chlorocytosine, 5-fluorocytosine, 5-bromocytosine, N^2-dimethylguanosine, 2,4-diamino-purine, 8-azapurine, a substituted

7-deazapurine, preferably 7-deaza-7-substituted and/or 7-deaza-8-substituted purine or other modifications of a natural nucleoside bases. This list is meant to be exemplary and is not to be interpreted to be limiting.

As used herein, "immune stimulatory nucleic acid" and, equivalently, "immunostimulatory nucleic acid" shall refer to a ribonucleic acid or deoxyribonucleic acid molecule, derivative or analog thereof, characterized by its capacity to induce a functional aspect of a cell of the immune system. Such functional aspect of a cell of the immune system can include, for example, elaboration of a cytokine or chemokine, expression of a cell surface marker, secretion of an antibody, proliferation, or other activity in response to or directed against an antigen or antigen-bearing membrane-bound target.

For use in the instant invention, the nucleic acids of the invention can be synthesized *de novo* using any of a number of procedures well known in the art, for example, the β-cyanoethyl phosphoramidite method (Beaucage SL and Caruthers MH (1981) *Tetrahedron Lett* 22:1859); and the nucleoside H-phosphonate method (Garegg et al. (1986) *Tetrahedron Lett* 27:4051-4; Froehler et al. (1986) *Nucl Acid Res* 14:5399-407; Garegg et al. (1986) *Tetrahedron Lett* 27:4055-8; Gaffney et al. (1988) *Tetrahedron Lett* 29:2619-22). These chemistries can be performed by a variety of automated nucleic acid synthesizers available in the market. These nucleic acids are referred to as synthetic nucleic acids. Alternatively, nucleic acids of the invention can be produced on a large scale in plasmids, (see Sambrook T et al., "Molecular Cloning: A Laboratory Manual", Cold Spring Harbor Laboratory Press, New York, 1989) and separated into smaller pieces or administered whole. Nucleic acids can be prepared from existing nucleic acid sequences (e.g., genomic or cDNA) using known techniques, such as those employing restriction enzymes, exonucleases or endonucleases. Nucleic acids prepared in this manner are referred to as isolated nucleic acids. An isolated nucleic acid generally refers to a nucleic acid which is separated from components which it is normally associated with in nature. As an example, an isolated nucleic acid may be one which is separated from a cell, from a nucleus, from mitochondria or from chromatin. The combination motif nucleic acids of the instant invention encompass both synthetic and isolated combination motif nucleic acids.

For use *in vivo*, the combination motif immunostimulatory nucleic acids may optionally be relatively resistant to degradation (e.g., are stabilized). A "stabilized nucleic acid molecule" shall mean a nucleic acid molecule that is relatively resistant to *in vivo*

degradation (e.g., via an exonuclease or endonuclease). Nucleic acid stabilization can be accomplished via phosphate backbone modifications. Preferred stabilized nucleic acids of the instant invention have a modified backbone. It has been demonstrated that modification of the nucleic acid backbone provides enhanced activity of the combination motif immunostimulatory nucleic acids when administered *in vivo*. Combination motif immunostimulatory nucleic acids having phosphorothioate linkages in some instances provide maximal activity and protect the nucleic acid from degradation by intracellular exonucleases and endonucleases. Other modified nucleic acids include modified phosphodiester nucleic acids, combinations of phosphodiester and phosphorothioate nucleic acids (i.e., chimeric), methylphosphonate, methylphosphorothioate, phosphorodithioate, p-ethoxy, and combinations thereof.

Modified backbones such as phosphorothioates may be synthesized using automated techniques employing either phosphoramidate or H-phosphonate chemistries. Aryl-and alkyl-phosphonates can be made, e.g., as described in U.S. Patent No. 4,469,863; and alkylphosphotriesters (in which the charged oxygen moiety is alkylated as described in U.S. Patent No. 5,023,243 and European Patent No. 092,574) can be prepared by automated solid phase synthesis using commercially available reagents. Methods for making other DNA backbone modifications and substitutions have been described. Uhlmann E and Peyman A (1990) *Chem Rev* 90:544; Goodchild J (1990) *Bioconjugate Chem* 1:165.

Other stabilized nucleic acids include: nonionic DNA analogs, such as alkyl- and aryl-phosphates (in which the charged phosphonate oxygen is replaced by an alkyl or aryl group), phosphodiester and alkylphosphotriesters, in which the charged oxygen moiety is alkylated. Nucleic acids which contain diol, such as tetraethyleneglycol or hexaethyleneglycol, at either or both termini have also been shown to be substantially resistant to nuclease degradation.

In other embodiments the immunostimulatory nucleic acids may have phosphodiester or chimeric e.g., soft or semi-soft backbones. A chimeric backbone includes a combination of phosphodiester and modified backbone linkages. A chimeric oligonucleotide, for instance, may be a soft oligonucleotide or a semi-soft oligonucleotide.

A soft oligonucleotide is an immunostimulatory oligonucleotide having a partially stabilized backbone, in which phosphodiester or phosphodiester-like internucleoside linkages occur only within and immediately adjacent to at least one internal pyrimidine nucleoside-guanosine (YG) dinucleotide. The at least one internal YG dinucleotide itself has a

phosphodiester or phosphodiester-like internucleoside linkage. A phosphodiester or phosphodiester-like internucleoside linkage occurring immediately adjacent to the at least one internal YG dinucleotide can be 5', 3', or both 5' and 3' to the at least one internal YG dinucleotide. Preferably a phosphodiester or phosphodiester-like internucleoside linkage occurring immediately adjacent to the at least one internal YG dinucleotide is itself an internal internucleoside linkage. Thus for a sequence N_1YGN_2, wherein N_1 and N_2 are each, independent of the other, any single nucleotide, the YG dinucleotide has a phosphodiester or phosphodiester-like internucleoside linkage, and in addition (a) N_1 and Y are linked by a phosphodiester or phosphodiester-like internucleoside linkage when N_1 is an internal nucleotide, (b) G and N_2 are linked by a phosphodiester or phosphodiester-like internucleoside linkage when N_2 is an internal nucleotide, or (c) N_1 and Y are linked by a phosphodiester or phosphodiester-like internucleoside linkage when N_1 is an internal nucleotide and G and N_2 are linked by a phosphodiester or phosphodiester-like internucleoside linkage when N_2 is an internal nucleotide.

A semi-soft oligonucleotide is an immunostimulatory oligonucleotide having a partially stabilized backbone, in which phosphodiester or phosphodiester-like internucleoside linkages occur only within at least one internal pyrimidine nucleoside-guanosine (YG) dinucleotide. Semi-soft oligonucleotides can have a number of advantages over immunostimulatory oligonucleotides with fully stabilized backbones. For instance, semi-soft oligonucleotides may possess increased immunostimulatory potency relative to corresponding fully stabilized immunostimulatory oligonucleotides.

The immunostimulatory nucleic acids may be used to treat a subject to induce an immune response or treat an immune related disease such as, for example, infectious disease, cancer, and allergic disorders. As used herein, "subject" shall refer to a human or vertebrate animal including, but not limited to, a dog, cat, horse, cow, pig, sheep, goat, chicken, monkey, rabbit, rat, mouse, etc.

As used herein, the terms "treat", "treating" and "treated" shall refer to a prophylactic treatment which increases the resistance of a subject to developing a disease or, in other words, decreases the likelihood that the subject will develop a disease or slows the development of the disease, as well as to a treatment after the subject has developed the disease in order to fight the disease, e.g., reduce or eliminate it altogether or prevent it from becoming worse. For example, when used with respect to the treatment of an infectious

disease the terms refer to a prophylactic treatment which increases the resistance of a subject to a microorganism or, in other words, decreases the likelihood that the subject will develop an infectious disease to the microorganism, as well as to a treatment after the subject has been infected in order to fight the infectious disease, e.g., reduce or eliminate it altogether or prevent it from becoming worse. When used with respect to a disease such as cancer the terms refer to the prevention or delay of the development of a cancer, reducing the symptoms of cancer, and/or inhibiting or slowing the growth of an established cancer.

Thus, the nucleic acids are useful as prophylactics for the induction of immunity of a subject at risk of developing an infection with an infectious organism or a subject at risk of developing an allergic disorder or cancer. A "subject at risk" as used herein is a subject who has any risk of exposure to an infection-causing infectious pathogen, exposure to an allergen, or developing cancer. For instance, a subject at risk may be a subject who is planning to travel to an area where a particular type of infectious agent or allergen is found or it may be a subject who through lifestyle or medical procedures is exposed to bodily fluids which may contain infectious organisms or even any subject living in an area that an infectious organism or an allergen has been identified and is exposed directly to the infectious agent or allergen. It also may be a subject at risk of biowarfare such as military personnel or those living in areas at risk of terrorist attack. Subjects at risk of developing infection also include general populations to which a medical agency recommends vaccination with a particular infectious organism antigen. If the antigen is an allergen and the subject develops allergic responses to that particular antigen and the subject is exposed to the antigen, i.e., during pollen season, then that subject is at risk of exposure to the antigen. Subjects at risk of developing cancer include those with a genetic predisposition or previously treated for cancer, and those exposed to carcinogens such as tobacco, asbestos, and other chemical toxins or excessive sunlight and other types of radiation. The nucleic acids are also useful as therapeutics in the treatment of infectious disease, cancer and allergic disorders.

A "subject having an infection" is a subject that has been exposed to an infectious pathogen and has acute or chronic detectable levels of the pathogen in the body. The nucleic acids can be used alone, or in conjunction with other therapeutic agents such as an antigen or an antimicrobial medicament to mount an immune response that is capable of reducing the level of or eradicating the infectious pathogen. The method entails administering to a subject having or at risk of developing an infection an effective amount of a combination motif

immune stimulatory nucleic acid of the invention to treat the infection. The method can be used to treat viral, bacterial, fungal, and parasitic infections in human and non-human vertebrate subjects.

As used herein, "infection" and, equivalently, "infectious disease" shall refer to a disease arising from the presence of a foreign microorganism in the body of a subject. A foreign microorganism may be a virus, a bacterium, a fungus, or a parasite.

Examples of infectious viruses include: *Retroviridae* (e.g., human immunodeficiency viruses, such as HIV-1 (also referred to as HTLV-III, LAV or HTLV-III/LAV, or HIV-III; and other isolates, such as HIV-LP; *Picornaviridae* (e.g., polio viruses, hepatitis A virus; enteroviruses, human coxsackie viruses, rhinoviruses, echoviruses); *Calciviridae* (e.g., strains that cause gastroenteritis); *Togaviridae* (e.g., equine encephalitis viruses, rubella viruses); *Flaviridae* (e.g., dengue viruses, encephalitis viruses, yellow fever viruses); *Coronaviridae* (e.g., coronaviruses); *Rhabdoviridae* (e.g., vesicular stomatitis viruses, rabies viruses); *Filoviridae* (e.g., ebola viruses); *Paramyxoviridae* (e.g., parainfluenza viruses, mumps virus, measles virus, respiratory syncytial virus); *Orthomyxoviridae* (e.g., influenza viruses); *Bungaviridae* (e.g., Hantaan viruses, bunga viruses, phleboviruses and Nairo viruses); *Arena* viridae (hemorrhagic fever viruses); *Reoviridae* (e.g., 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 2, varicella zoster virus, cytomegalovirus (CMV), herpes viruses); *Poxviridae* (variola viruses, vaccinia viruses, pox viruses); and *Iridoviridae* (e.g., African swine fever virus); and unclassified viruses (e.g., 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).

Examples of infectious bacteria include: *Actinomyces israelii, Bacillus anthracis, Bacteroides* spp., *Borrelia burgdorferi, Chlamydia trachomatis, Clostridium perfringens, Clostridium tetani, Corynebacterium diphtheriae, Corynebacterium* spp., *Enterobacter aerogenes, Enterococcus sp., Erysipelothrix rhusiopathiae, Fusobacterium nucleatum, Haemophilus influenzae, Helicobacter pyloris, Klebsiella pneumoniae, Legionella pneumophilia, Leptospira, Listeria monocytogenes, Mycobacteria* spp. (e.g., *M. tuberculosis,*

M. avium, *M. intracellulare*, *M. kansasii*, *M. gordonae*), *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Pasturella multocida*, pathogenic *Campylobacter sp.*, *Staphylococcus aureus*, *Streptobacillus moniliformis*, *Streptococcus* (anaerobic spp.), *Streptococcus* (viridans group), *Streptococcus agalactiae* (Group B Streptococcus), *Streptococcus bovis*, *Streptococcus faecalis*, *Streptococcus pneumoniae*, *Streptococcus pyogenes* (Group A Streptococcus), *Treponema pallidium*, and *Treponema pertenue*.

Examples of infectious fungi include: *Candida albicans*, *Cryptococcus neoformans*, *Histoplasma capsulatum*, *Coccidioides immitis*, and *Blastomyces dermatitidis*.

Other infectious organisms (i.e., protists) include *Plasmodium* spp. such as *Plasmodium falciparum*, *Plasmodium malariae*, *Plasmodium ovale*, and *Plasmodium vivax*, and *Toxoplasma gondii*. Blood-borne and/or tissue parasites include *Plasmodium* spp., *Babesia microti*, *Babesia divergens*, *Leishmania tropica*, *Leishmania* spp., *Leishmania braziliensis*, *Leishmania donovani*, *Trypanosoma gambiense* and *Trypanosoma rhodesiense* (African sleeping sickness), *Trypanosoma cruzi* (Chagas' disease), and *Toxoplasma gondii*.

The foregoing lists of viruses, bacteria, fungi, and other infectious microorganisms is understood to be representative and not limiting. Other medically relevant microorganisms have been described extensively in the literature, e.g., see C.G.A Thomas, *Medical Microbiology*, Bailliere Tindall, Great Britain 1983, the entire contents of which is hereby incorporated by reference.

Although many of the microbial agents described above relate to human disorders, the invention is also useful for treating non-human vertebrates. Non-human vertebrates are also capable of developing infections which can be prevented or treated with the immunostimulatory nucleic acids disclosed herein. For instance, in addition to the treatment of infectious human diseases, the methods of the invention are useful for treating infections of animals.

Infectious viruses of both human and non-human vertebrates include retroviruses, RNA viruses and DNA viruses. This group of retroviruses includes both simple retroviruses and complex retroviruses. The simple retroviruses include the subgroups of B-type retroviruses, C-type retroviruses and D-type retroviruses. An example of a B-type retrovirus is mouse mammary tumor virus (MMTV). The C-type retroviruses include subgroups C-type group A (including Rous sarcoma virus (RSV), avian leukemia virus (ALV), and avian myeloblastosis virus (AMV)) and C-type group B (including feline leukemia virus (FeLV),

gibbon ape leukemia virus (GALV), spleen necrosis virus (SNV), reticuloendotheliosis virus (RV) and simian sarcoma virus (SSV)). The D-type retroviruses include Mason-Pfizer monkey virus (MPMV) and simian retrovirus type 1 (SRV-1). The complex retroviruses include the subgroups of lentiviruses, T-cell leukemia viruses and the foamy viruses. Lentiviruses include HIV-1, but also include HIV-2, SIV, Visna virus, feline immunodeficiency virus (FIV), and equine infectious anemia virus (EIAV). The T-cell leukemia viruses include HTLV-1, HTLV-II, simian T-cell leukemia virus (STLV), and bovine leukemia virus (BLV). The foamy viruses include human foamy virus (HFV), simian foamy virus (SFV) and bovine foamy virus (BFV).

Examples of other RNA viruses that are infectious agents in vertebrate animals include, but are not limited to, members of the family Reoviridae, including the genus *Orthoreovirus* (multiple serotypes of both mammalian and avian retroviruses), the genus *Orbivirus* (Bluetongue virus, Eugenangee virus, Kemerovo virus, African horse sickness virus, and Colorado Tick Fever virus), the genus *Rotavirus* (human rotavirus, Nebraska calf diarrhea virus, simian rotavirus, bovine or ovine rotavirus, avian rotavirus); the family *Picornaviridae*, including the genus Enterovirus (poliovirus, Coxsackie virus A and B, enteric cytopathic human orphan (ECHO) viruses, hepatitis A virus, Simian enteroviruses, Murine encephalomyelitis (ME) viruses, Poliovirus muris, Bovine enteroviruses, Porcine enteroviruses, the genus *Cardiovirus* (Encephalomyocarditis virus (EMC), Mengovirus), the genus *Rhinovirus* (Human rhinoviruses including at least 113 subtypes; other rhinoviruses), the genus *Apthovirus* (Foot and Mouth disease virus (FMDV); the family *Calciviridae*, including Vesicular exanthema of swine virus, San Miguel sea lion virus, Feline picornavirus and Norwalk virus; the family *Togaviridae*, including the genus *Alphavirus* (Eastern equine encephalitis virus, Semliki forest virus, Sindbis virus, Chikungunya virus, O'Nyong-Nyong virus, Ross river virus, Venezuelan equine encephalitis virus, Western equine encephalitis virus), the genus *Flavivirus* (Mosquito-borne yellow fever virus, Dengue virus, Japanese encephalitis virus, St. Louis encephalitis virus, Murray Valley encephalitis virus, West Nile virus, Kunjin virus, Central European tick borne virus, Far Eastern tick borne virus, Kyasanur forest virus, Louping III virus, Powassan virus, Omsk hemorrhagic fever virus), the genus *Rubivirus* (Rubella virus), the genus *Pestivirus* (Mucosal disease virus, Hog cholera virus, Border disease virus); the family *Bunyaviridae*, including the genus *Bunyvirus* (Bunyamwera and related viruses, California encephalitis group viruses), the genus *Phlebovirus* (Sandfly

fever Sicilian virus, Rift Valley fever virus), the genus *Nairovirus* (Crimean-Congo hemorrhagic fever virus, Nairobi sheep disease virus), and the genus *Uukuvirus* (Uukuniemi and related viruses); the family *Orthomyxoviridae*, including the genus *Influenza* virus (Influenza virus type A, many human subtypes); Swine influenza virus, and Avian and Equine Influenza viruses; influenza type B (many human subtypes), and influenza type C (possible separate genus); the family *paramyxoviridae*, including the genus *Paramyxovirus* (Parainfluenza virus type 1, Sendai virus, Hemadsorption virus, Parainfluenza viruses types 2 to 5, Newcastle Disease Virus, Mumps virus), the genus *Morbillivirus* (Measles virus, subacute sclerosing panencephalitis virus, distemper virus, Rinderpest virus), the genus *Pneumovirus* (respiratory syncytial virus (RSV), Bovine respiratory syncytial virus and Pneumonia virus); the family *Rhabdoviridae*, including the genus *Vesiculovirus* (VSV), Chandipura virus, Flanders-Hart Park virus), the genus *Lyssavirus* (Rabies virus), fish Rhabdoviruses, and two probable Rhabdoviruses (Marburg virus and Ebola virus); the family *Arenaviridae*, including Lymphocytic choriomeningitis virus (LCM), Tacaribe virus complex, and Lassa virus; the family *Coronoaviridae*, including Infectious Bronchitis Virus (IBV), Hepatitis virus, Human enteric corona virus, and Feline infectious peritonitis (Feline coronavirus).

Illustrative DNA viruses that are infectious agents in vertebrate animals include, but are not limited to, the family *Poxviridae*, including the genus *Orthopoxvirus* (Variola major, Variola minor, Monkey pox Vaccinia, Cowpox, Buffalopox, Rabbitpox, Ectromelia), the genus *Leporipoxvirus* (Myxoma, Fibroma), the genus *Avipoxvirus* (Fowlpox, other avian poxvirus), the genus *Capripoxvirus* (sheep-pox, goatpox), the genus *Suipoxvirus* (Swinepox), the genus *Parapoxvirus* (contagious pustular dermatitis virus, pseudocowpox, bovine papular stomatitis virus); the family *Iridoviridae* (African swine fever virus, Frog viruses 2 and 3, Lymphocystis virus of fish); the family *Herpesviridae*, including the alpha-Herpesviruses (Herpes Simplex Types 1 and 2, Varicella-Zoster, Equine abortion virus, Equine herpes virus 2 and 3, pseudorabies virus, infectious bovine keratoconjunctivitis virus, infectious bovine rhinotracheitis virus, feline rhinotracheitis virus, infectious laryngotracheitis virus), the Beta-herpesviruses (Human cytomegalovirus and cytomegaloviruses of swine and monkeys); the gamma-herpesviruses (Epstein-Barr virus (EBV), Marek's disease virus, Herpes saimiri, Herpesvirus ateles, Herpesvirus sylvilagus, guinea pig herpes virus, Lucke tumor virus); the family *Adenoviridae*, including the genus *Mastadenovirus* (Human subgroups A, B, C, D, E

and ungrouped; simian adenoviruses (at least 23 serotypes), infectious canine hepatitis, and adenoviruses of cattle, pigs, sheep, frogs and many other species, the genus *Aviadenovirus* (Avian adenoviruses); and non-cultivatable adenoviruses; the family *Papoviridae*, including the genus *Papillomavirus* (Human papilloma viruses, bovine papilloma viruses, Shope rabbit papilloma virus, and various pathogenic papilloma viruses of other species), the genus *Polyomavirus* (polyomavirus, Simian vacuolating agent (SV-40), Rabbit vacuolating agent (RKV), K virus, BK virus, JC virus, and other primate polyoma viruses such as Lymphotrophic papilloma virus); the family *Parvoviridae* including the genus Adeno-associated viruses, the genus *Parvovirus* (Feline panleukopenia virus, bovine parvovirus, canine parvovirus, Aleutian mink disease virus, etc). Finally, DNA viruses may include viruses which do not fit into the above families, such as Kuru and Creutzfeldt-Jacob disease viruses and chronic infectious neuropathic agents (CHINA virus).

The nucleic acids may be administered to a subject with an anti-microbial agent. An anti-microbial agent, as used herein, refers to a naturally-occurring, synthetic, or semi-synthetic compound which is capable of killing or inhibiting infectious microorganisms. The type of anti-microbial agent useful according to the invention will depend upon the type of microorganism with which the subject is infected or at risk of becoming infected. Anti-microbial agents include but are not limited to anti-bacterial agents, anti-viral agents, anti-fungal agents and anti-parasitic agents. Phrases such as "anti-infective agent", "anti-bacterial agent", "anti-viral agent", "anti-fungal agent", "anti-parasitic agent" and "parasiticide" have well-established meanings to those of ordinary skill in the art and are defined in standard medical texts. Briefly, anti-bacterial agents kill or inhibit bacteria, and include antibiotics as well as other synthetic or natural compounds having similar functions. Antibiotics are low molecular weight molecules which are produced as secondary metabolites by cells, such as microorganisms. In general, antibiotics interfere with one or more bacterial functions or structures which are specific for the microorganism and which are not present in host cells. Anti-viral agents can be isolated from natural sources or synthesized and are useful for killing or inhibiting viruses. Anti-fungal agents are used to treat superficial fungal infections as well as opportunistic and primary systemic fungal infections. Anti-parasite agents kill or inhibit parasites.

Antibacterial agents kill or inhibit the growth or function of bacteria. A large class of antibacterial agents is antibiotics. Antibiotics, which are effective for killing or inhibiting a

wide range of bacteria, are referred to as broad spectrum antibiotics. Other types of antibiotics are predominantly effective against the bacteria of the class gram-positive or gram-negative. These types of antibiotics are referred to as narrow spectrum antibiotics. Other antibiotics which are effective against a single organism or disease and not against other types of bacteria, are referred to as limited spectrum antibiotics. Antibacterial agents are sometimes classified based on their primary mode of action. In general, antibacterial agents are cell wall synthesis inhibitors, cell membrane inhibitors, protein synthesis inhibitors, nucleic acid synthesis or functional inhibitors, and competitive inhibitors.

Antiviral agents are compounds which prevent infection of cells by viruses or replication of the virus within the cell. There are many fewer antiviral drugs than antibacterial drugs because the process of viral replication is so closely related to DNA replication within the host cell, that non-specific antiviral agents would often be toxic to the host. There are several stages within the process of viral infection which can be blocked or inhibited by antiviral agents. These stages include, attachment of the virus to the host cell (immunoglobulin or binding peptides), uncoating of the virus (e.g., amantadine), synthesis or translation of viral mRNA (e.g., interferon), replication of viral RNA or DNA (e.g., nucleoside analogues), maturation of new virus proteins (e.g., protease inhibitors), and budding and release of the virus.

Nucleotide analogues are synthetic compounds which are similar to nucleotides, but which have an incomplete or abnormal deoxyribose or ribose group. Once the nucleotide analogues are in the cell, they are phosphorylated, producing the triphosphate formed which competes with normal nucleotides for incorporation into the viral DNA or RNA. Once the triphosphate form of the nucleotide analogue is incorporated into the growing nucleic acid chain, it causes irreversible association with the viral polymerase and thus chain termination. Nucleotide analogues include, but are not limited to, acyclovir (used for the treatment of herpes simplex virus and varicella-zoster virus), gancyclovir (useful for the treatment of cytomegalovirus), idoxuridine, ribavirin (useful for the treatment of respiratory syncitial virus), dideoxyinosine, dideoxycytidine, and zidovudine (azidothymidine).

Anti-fungal agents are useful for the treatment and prevention of infective fungi. Anti-fungal agents are sometimes classified by their mechanism of action. Some anti-fungal agents function as cell wall inhibitors by inhibiting glucose synthase. These include, but are not limited to, basiungin/ECB. Other anti-fungal agents function by destabilizing membrane

integrity. These include, but are not limited to, immidazoles, such as clotrimazole, sertaconzole, fluconazole, itraconazole, ketoconazole, miconazole, and voriconacole, as well as FK 463, amphotericin B, BAY 38-9502, MK 991, pradimicin, UK 292, butenafine, and terbinafine. Other anti-fungal agents function by breaking down chitin (e.g., chitinase) or immunosuppression (501 cream).

The immunostimulatory nucleic acids may be used, either alone or in combination with an anti-cancer therapy, for the treatment of cancer. The method entails administering to a subject having or at risk of developing cancer an effective amount of a combination motif immune stimulatory nucleic acid of the invention to treat cancer.

A "subject having a cancer" is a subject that has detectable cancerous cells. The cancer may be a malignant or non-malignant cancer. Cancers or tumors include but are not limited to biliary tract cancer; brain cancer; breast cancer; cervical cancer; choriocarcinoma; colon cancer; endometrial cancer; esophageal cancer; gastric cancer; intraepithelial neoplasms; lymphomas; liver cancer; lung cancer (e.g., small cell and non-small cell); melanoma; neuroblastomas; oral cancer; ovarian cancer; pancreas cancer; prostate cancer; rectal cancer; sarcomas; skin cancer; testicular cancer; thyroid cancer; and renal cancer, as well as other carcinomas and sarcomas. In one embodiment the cancer is hairy cell leukemia, chronic myelogenous leukemia, cutaneous T-cell leukemia, multiple myeloma, follicular lymphoma, malignant melanoma, squamous cell carcinoma, renal cell carcinoma, prostate carcinoma, bladder cell carcinoma, or colon carcinoma.

Cancer is one of the leading causes of death in companion animals (i.e., cats and dogs). Malignant disorders commonly diagnosed in dogs and cats include but are not limited to lymphosarcoma, osteosarcoma, mammary tumors, mastocytoma, brain tumor, melanoma, adenosquamous carcinoma, carcinoid lung tumor, bronchial gland tumor, bronchiolar adenocarcinoma, fibroma, myxochondroma, pulmonary sarcoma, neurosarcoma, osteoma, papilloma, retinoblastoma, Ewing's sarcoma, Wilms' tumor, Burkitt's lymphoma, microglioma, neuroblastoma, osteoclastoma, oral neoplasia, fibrosarcoma, osteosarcoma and rhabdomyosarcoma. Other neoplasms in dogs include genital squamous cell carcinoma, transmissible venereal tumor, testicular tumor, seminoma, Sertoli cell tumor, hemangiopericytoma, histiocytoma, chloroma (granulocytic sarcoma), corneal papilloma, corneal squamous cell carcinoma, hemangiosarcoma, pleural mesothelioma, basal cell tumor, thymoma, stomach tumor, adrenal gland carcinoma, oral papillomatosis,

hemangioendothelioma and cystadenoma. Additional malignancies diagnosed in cats include follicular lymphoma, intestinal lymphosarcoma, fibrosarcoma and pulmonary squamous cell carcinoma. The ferret, an ever-more popular house pet, is known to develop insulinoma, lymphoma, sarcoma, neuroma, pancreatic islet cell tumor, gastric MALT lymphoma and gastric adenocarcinoma.

The immunostimulatory nucleic acids may also be administered in conjunction with an anti-cancer therapy. Anti-cancer therapies include cancer medicaments, radiation and surgical procedures. As used herein, a "cancer medicament" refers to an agent which is administered to a subject for the purpose of treating a cancer. Various types of medicaments for the treatment of cancer are described herein. For the purpose of this specification, cancer medicaments are classified as chemotherapeutic agents, immunotherapeutic agents, cancer vaccines, hormone therapy, and biological response modifiers.

The use of immunostimulatory nucleic acids in conjunction with immunotherapeutic agents such as monoclonal antibodies is able to increase long-term survival through a number of mechanisms including significant enhancement of antibody-dependent cellular cytotoxicity (ADCC), activation of NK cells and an increase in IFN-α levels. ADCC can be performed using an immunostimulatory nucleic acid in combination with an antibody specific for a cellular target, such as a cancer cell. When the immunostimulatory nucleic acid is administered to a subject in conjunction with the antibody the subject's immune system is induced to kill the tumor cell. The antibodies useful in the ADCC procedure include antibodies which interact with a cell in the body. Many such antibodies specific for cellular targets have been described in the art and many are commercially available. The nucleic acids when used in combination with monoclonal antibodies serve to reduce the dose of the antibody required to achieve a biological result.

Other types of chemotherapeutic agents which can be used according to the invention include Aminoglutethimide, Asparaginase, Busulfan, Carboplatin, Chlorombucil, Cytarabine HCl, Dactinomycin, Daunorubicin HCl, Estramustine phosphate sodium, Etoposide (VP16-213), Floxuridine, Fluorouracil (5-FU), Flutamide, Hydroxyurea (hydroxycarbamide), Ifosfamide, Interferon Alfa-2a, Alfa-2b, Leuprolide acetate (LHRH-releasing factor analogue), Lomustine (CCNU), Mechlorethamine HCl (nitrogen mustard), Mercaptopurine, Mesna, Mitotane (o.p'-DDD), Mitoxantrone HCl, Octreotide, Plicamycin, Procarbazine HCl, Streptozocin, Tamoxifen citrate, Thioguanine, Thiotepa, Vinblastine sulfate, Amsacrine (m-

AMSA), Azacitidine, Erythropoietin, Hexamethylmelamine (HMM), Interleukin 2, Mitoguazone (methyl-GAG; methyl glyoxal bis-guanylhydrazone; MGBG), Pentostatin (2'deoxycoformycin), Semustine (methyl-CCNU), Teniposide (VM-26) and Vindesine sulfate.

Cancer vaccines are medicaments which are intended to stimulate an endogenous immune response against cancer cells. Currently produced vaccines predominantly activate the humoral immune system (i.e., the antibody dependent immune response). Other vaccines currently in development are focused on activating the cell-mediated immune system including cytotoxic T lymphocytes which are capable of killing tumor cells. Cancer vaccines generally enhance the presentation of cancer antigens to both antigen presenting cells (e.g., macrophages and dendritic cells) and/or to other immune cells such as T cells, B cells, and NK cells. In some instances, cancer vaccines may be used along with adjuvants, such as those described above.

Some cancer cells are antigenic and thus can be targeted by the immune system. In one aspect, the combined administration of immunostimulatory nucleic acids and cancer medicaments, particularly those which are classified as cancer immunotherapies, is useful for stimulating a specific immune response against a cancer antigen. used herein, the terms "cancer antigen" and "tumor antigen" are used interchangeably to refer to antigens which are differentially expressed by cancer cells and can thereby be exploited in order to target cancer cells. Cancer antigens are antigens which can potentially stimulate apparently tumor-specific immune responses. Some of these antigens are encoded, although not necessarily expressed, by normal cells. These antigens can be characterized as those which are normally silent (i.e., not expressed) in normal cells, those that are expressed only at certain stages of differentiation and those that are temporally expressed such as embryonic and fetal antigens. Other cancer antigens are encoded by mutant cellular genes, such as oncogenes (e.g., activated ras oncogene), suppressor genes (e.g., mutant p53), fusion proteins resulting from internal deletions or chromosomal translocations. Still other cancer antigens can be encoded by viral genes such as those carried on RNA and DNA tumor viruses. "Tumor-associated" antigens are present in both tumor cells and normal cells but are present in a different quantity or a different form in tumor cells. Examples of such antigens are oncofetal antigens (e.g., carcinoembryonic antigen), differentiation antigens (e.g., T and Tn antigens), and oncogene products (e.g., HER/neu).

Cancer antigens, such as those present in cancer vaccines or those used to prepare cancer immunotherapies, can be prepared from crude cancer cell extracts, as described in Cohen PA et al. (1994) *Cancer Res* 54:1055-8, or by partially purifying the antigens, using recombinant technology, or de novo synthesis of known antigens. Cancer antigens can be used in the form of immunogenic portions of a particular antigen or in some instances a whole cell or a tumor mass can be used as the antigen. Such antigens can be isolated or prepared recombinantly or by any other means known in the art.

Other vaccines take the form of dendritic cells which have been exposed to cancer antigens in vitro, have processed the antigens and are able to express the cancer antigens at their cell surface in the context of MHC molecules for effective antigen presentation to other immune system cells. Dendritic cells form the link between the innate and the acquired immune system by presenting antigens and through their expression of pattern recognition receptors which detect microbial molecules like LPS in their local environment.

The combination motif immunostimulatory nucleic acids are useful for the treatment of allergy, including asthma. The combination motif immune stimulatory nucleic acids can be used, either alone or in combination with an allergy/asthma medicament, to treat allergy. The method entails administering to a subject having or at risk of developing an allergic or asthmatic condition an effective amount of a combination motif immune stimulatory nucleic acid of the invention to treat the allergic or asthmatic condition.

As used herein, "allergy" shall refer to acquired hypersensitivity to a substance (allergen). Allergic conditions include eczema, allergic rhinitis or coryza, hay fever, bronchial asthma, urticaria (hives) and food allergies, and other atopic conditions. A "subject having an allergy" is a subject that has or is at risk of developing an allergic reaction in response to an allergen. An "allergen" refers to a substance that can induce an allergic or asthmatic response in a susceptible subject. The list of allergens is enormous and can include pollens, insect venoms, animal dander, dust, fungal spores and drugs (e.g., penicillin).

Examples of natural animal and plant allergens include proteins specific to the following genuses: *Canine* (*Canis familiaris*); *Dermatophagoides* (e.g., *Dermatophagoides farinae*); *Felis* (*Felis domesticus*); *Ambrosia* (*Ambrosia artemiisfolia*; *Lolium* (e.g., *Lolium perenne* or *Lolium multiflorum*); *Cryptomeria* (*Cryptomeria japonica*); *Alternaria* (*Alternaria alternata*); *Alder*; *Alnus* (*Alnus gultinosa*); *Betula* (*Betula verrucosa*); *Quercus* (*Quercus alba*); *Olea* (*Olea europa*); *Artemisia* (*Artemisia vulgaris*); *Plantago* (e.g., *Plantago*

lanceolata); *Parietaria* (e.g., *Parietaria officinalis* or *Parietaria judaica*); *Blattella* (e.g., *Blattella germanica*); *Apis* (e.g., *Apis multiflorum*); *Cupressus* (e.g., *Cupressus sempervirens*, *Cupressus arizonica* and *Cupressus macrocarpa*); *Juniperus* (e.g., *Juniperus sabinoides*, *Juniperus virginiana*, *Juniperus communis* and *Juniperus ashei*); *Thuya* (e.g., *Thuya orientalis*); *Chamaecyparis* (e.g., *Chamaecyparis obtusa*); *Periplaneta* (e.g., *Periplaneta americana*); *Agropyron* (e.g., *Agropyron repens*); *Secale* (e.g., *Secale cereale*); *Triticum* (e.g., *Triticum aestivum*); *Dactylis* (e.g., *Dactylis glomerata*); *Festuca* (e.g., *Festuca elatior*); *Poa* (e.g., *Poa pratensis* or *Poa compressa*); *Avena* (e.g., *Avena sativa*); *Holcus* (e.g., *Holcus lanatus*); *Anthoxanthum* (e.g., *Anthoxanthum odoratum*); *Arrhenatherum* (e.g., *Arrhenatherum elatius*); *Agrostis* (e.g., *Agrostis alba*); *Phleum* (e.g., *Phleum pratense*); *Phalaris* (e.g., *Phalaris arundinacea*); *Paspalum* (e.g., *Paspalum notatum*); *Sorghum* (e.g., *Sorghum halepensis*); and *Bromus* (e.g., *Bromus inermis*).

As used herein, "asthma" shall refer to a disorder of the respiratory system characterized by inflammation, narrowing of the airways and increased reactivity of the airways to inhaled agents. Asthma is frequently, although not exclusively, associated with atopic or allergic symptoms.

An "asthma/allergy medicament" as used herein is a composition of matter which reduces the symptoms, inhibits the asthmatic or allergic reaction, or prevents the development of an allergic or asthmatic reaction. Various types of medicaments for the treatment of asthma and allergy are described in the Guidelines For The Diagnosis and Management of Asthma, Expert Panel Report 2, NIH Publication No. 97/4051, July 19, 1997, the entire contents of which are incorporated herein by reference. The summary of the medicaments as described in the NIH publication is presented below.

In most embodiments the asthma/allergy medicament is useful to some degree for treating both asthma and allergy. Some asthma/allergy medicaments are preferably used in combination with the immunostimulatory nucleic acids to treat asthma. These are referred to as asthma medicaments. Asthma medicaments include, but are not limited, PDE-4 inhibitors, bronchodilator/beta-2 agonists, K+ channel openers, VLA-4 antagonists, neurokin antagonists, TXA2 synthesis inhibitors, xanthanines, arachidonic acid antagonists, 5 lipoxygenase inhibitors, thromboxin A2 receptor antagonists, thromboxane A2 antagonists, inhibitor of 5-lipoxygenase activation proteins, and protease inhibitors.

Other asthma/allergy medicaments are preferably used in combination with the immunostimulatory nucleic acids to treat allergy. These are referred to as allergy medicaments. Allergy medicaments include, but are not limited to, anti-histamines, steroids, immunomodulators, and prostaglandin inducers. Anti-histamines are compounds which counteract histamine released by mast cells or basophils. These compounds are well known in the art and commonly used for the treatment of allergy. Anti-histamines include, but are not limited to, loratidine, cetirizine, buclizine, ceterizine analogues, fexofenadine, terfenadine, desloratadine, norastemizole, epinastine, ebastine, ebastine, astemizole, levocabastine, azelastine, tranilast, terfenadine, mizolastine, betatastine, CS 560, and HSR 609. Prostaglandin inducers are compounds which induce prostaglandin activity. Prostaglandins function by regulating smooth muscle relaxation. Prostaglandin inducers include, but are not limited to, S-5751.

The steroids include, but are not limited to, beclomethasone, fluticasone, tramcinolone, budesonide, corticosteroids and budesonide. The combination of immunostimulatory nucleic acids and steroids are particularly well suited to the treatment of young subjects (e.g., children). To date, the use of steroids in children has been limited by the observation that some steroid treatments have been reportedly associated with growth retardation. Thus, according to the present invention, the immunostimulatory nucleic acids can be used in combination with growth retarding steroids, and can thereby provide a "steroid sparing effect." The combination of the two agents can result in lower required doses of steroids.

The immunomodulators include, but are not limited to, the group consisting of anti-inflammatory agents, leukotriene antagonists, IL-4 muteins, soluble IL-4 receptors, immunosuppressants (such as tolerizing peptide vaccine), anti-IL-4 antibodies, IL-4 antagonists, anti-IL-5 antibodies, soluble IL-13 receptor-Fc fusion proteins, anti-IL-9 antibodies, CCR3 antagonists, CCR5 antagonists, VLA-4 inhibitors, and , and downregulators of IgE.

The immunostimulatory nucleic acids of the invention can be used to induce type 1 IFN, i.e., IFN-α and IFN-β. The method involves contacting a cell capable of expressing a type 1 IFN with an effective amount of a combination motif immune stimulatory nucleic acid of the invention to induce type 1 IFN expression by the cell. It has recently been appreciated that the major producer cell type of IFN-α in humans is the plasmacytoid dendritic cell

(pDC). This type of cell occurs at very low frequency (0.2-0.4 percent) in PBMC and is characterized by a phenotype that is lineage negative (i.e., does not stain for CD3, CD14, CD19, or CD56) and CD11c negative, while positive for CD4, CD123 (IL-3Rα), and class II major histocompatibility complex (MHC class II). Grouard G et al. (1997) *J Exp Med* 185:1101-11; Rissoan M-C et al. (1999) *Science* 283:1183-6; Siegal FP et al. (1999) *Science* 284:1835-7; Cella M et al. (1999) *Nat Med* 5:919-23. Methods of measuring type 1 IFN are well known by those skilled in the art, and they include, for example, enzyme-linked immunosorbent assay (ELISA), bioassay, and fluorescence-activated cell sorting (FACS). Assays of this sort can be performed using readily available commercial reagents and kits.

The immunostimulatory nucleic acids of the invention may be used to activate NK cells. The method involves contacting an NK cell with an effective amount of a combination motif immune stimulatory nucleic acid of the invention to activate the NK cell. The activation of the NK cells may be direct activation or indirect activation. Indirect activation refers to the induction of cytokines or other factors which cause the subsequent activation of the NK cells. NK cell activation can be assessed by various methods, including measurement of lytic activity, measurement of induction of activation markers such as CD69, and measurement of induction of certain cytokines. In addition to their characteristic ability to kill certain tumor targets spontaneously, NK cells participate in ADCC and are major producers of IFN-γ, TNF-α, GM-CSF and IL-3.

The prototypical NK-sensitive cell target for mouse NK cells is yeast artificial chromosome (YAC)-1, a thymoma derived from Moloney virus-infected A strain mice. For human NK cells, a standard target is K562, a cell line derived from an erythroleukemic lineage. In microtiter plates, a constant number of radiolabeled targets (e.g., ^{51}Cr-labeled K562) is incubated either alone (spontaneous), with detergent (maximum), or with varying numbers of effector cells (experimental). The ratio of effector to target cells is referred to as the E:T ratio. Enriched, activated NK cells typically are effective at E:T ratios of less than 10:1, while unfractionated PBMCs or splenocytes require E:T ratios of 100:1 or more.

The immunostimulatory nucleic acids also are useful as adjuvants for inducing a systemic and/or mucosal immune response. The combination motif immune stimulatory nucleic acids of the invention can be delivered to a subject exposed to an antigen to produce an enhanced immune response to the antigen. Thus for example combination motif immune stimulatory nucleic acids are useful as vaccine adjuvants.

The immunostimulatory nucleic acids may be administered in combination with non-nucleic acid adjuvants. A non-nucleic acid adjuvant is any molecule or compound except for the immunostimulatory nucleic acids described herein which can stimulate the humoral and/or cellular immune response. Non-nucleic acid adjuvants include, for instance, adjuvants that create a depot effect, immune stimulating adjuvants, and adjuvants that create a depo effect and stimulate the immune system. A non-nucleic acid mucosal adjuvant as used herein is an adjuvant other than a immunostimulatory nucleic acid that is capable of inducing a mucosal immune response in a subject when administered to a mucosal surface in conjunction with an antigen.

The immunostimulatory nucleic acids of the invention may be formulated as pharmaceutical compositions in a pharmaceutically acceptable carrier. The immunostimulatory nucleic acids may be directly administered to the subject or may be administered in conjunction with a nucleic acid delivery complex. A nucleic acid delivery complex shall mean a nucleic acid molecule 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 target cell (e.g., B-cell surfaces) and/or increased cellular uptake by target cells). Examples of nucleic acid delivery complexes include nucleic acids associated with a sterol (e.g., cholesterol), a lipid (e.g., a cationic lipid, virosome or liposome), or a target cell specific binding agent (e.g., a ligand recognized by target cell specific receptor). Preferred complexes may be sufficiently stable *in vivo* to prevent significant uncoupling prior to internalization by the target cell. However, the complex can be cleavable under appropriate conditions within the cell so that the nucleic acid is released in a functional form.

The immunostimulatory nucleic acid and/or the antigen and/or other therapeutics may be administered alone (e.g., in saline or buffer) or using any delivery vehicles known in the art. For instance the following delivery vehicles have been described: Cochleates (Gould-Fogerite et al., 1994, 1996); Emulsomes (Vancott et al., 1998, Lowell et al., 1997); ISCOMs (Mowat et al., 1993, Carlsson et al., 1991, Hu et., 1998, Morein et al., 1999); Liposomes (Childers et al., 1999, Michalek et al., 1989, 1992, de Haan 1995a, 1995b); Microspheres (Gupta et al., 1998, Jones et al., 1996, Maloy et al., 1994, Moore et al., 1995, O'Hagan et al., 1994, Eldridge et al., 1989); Polymers (e.g., carboxymethylcellulose, chitosan) (Hamajima et al., 1998, Jabbal-Gill et al., 1998); Polymer rings (Wyatt et al., 1998); Proteosomes (Vancott et al., 1998, Lowell et al., 1988, 1996, 1997); Virosomes (Gluck et al., 1992, Mengiardi et al.,

1995, Cryz et al., 1998); Virus-like particles (Jiang et al., 1999, Leibl et al., 1998). Other delivery vehicles are known in the art.

Subject doses of the compounds described herein for mucosal or local delivery typically range from about 0.1 μg to 10 mg per administration, which depending on the application could be given daily, weekly, or monthly and any other amount of time therebetween. More typically mucosal or local doses range from about 10 μg to 5 mg per administration, and most typically from about 100 μg to 1 mg, with 2 - 4 administrations being spaced days or weeks apart. More typically, immune stimulant doses range from 1 μg to 10 mg per administration, and most typically 10 μg to 1 mg, with daily or weekly administrations. Subject doses of the compounds described herein for parenteral delivery for the purpose of inducing an antigen-specific immune response, wherein the compounds are delivered with an antigen but not another therapeutic agent are typically 5 to 10,000 times higher than the effective mucosal dose for vaccine adjuvant or immune stimulant applications, and more typically 10 to 1,000 times higher, and most typically 20 to 100 times higher. Doses of the compounds described herein for parenteral delivery for the purpose of inducing an innate immune response or for increasing ADCC or for inducing an antigen specific immune response when the immunostimulatory nucleic acids are administered in combination with other therapeutic agents or in specialized delivery vehicles typically range from about 0.1 μg to 10 mg per administration, which depending on the application could be given daily, weekly, or monthly and any other amount of time therebetween. More typically parenteral doses for these purposes range from about 10 μg to 5 mg per administration, and most typically from about 100 μg to 1 mg, with 2 - 4 administrations being spaced days or weeks apart. In some embodiments, however, parenteral doses for these purposes may be used in a range of 5 to 10,000 times higher than the typical doses described above.

As used herein, "effective amount" shall refer to the amount necessary or sufficient to realize a desired biological effect. For example, an effective amount of an immunostimulatory nucleic acid for treating an infection is that amount necessary to treat the infection. Combined with the teachings provided herein, by choosing among the various active compounds and weighing factors such as potency, relative bioavailability, patient body weight, severity of adverse side-effects and preferred mode of administration, an effective prophylactic or therapeutic treatment regimen can be planned which does not cause substantial toxicity and yet is entirely effective to treat the particular subject. The effective

amount for any particular application can vary depending on such factors as the disease or condition being treated, the particular immunostimulatory nucleic acid being administered, the antigen, the size of the subject, or the severity of the disease or condition. One of ordinary skill in the art can empirically determine the effective amount of a particular immunostimulatory nucleic acid and/or antigen and/or other therapeutic agent without necessitating undue experimentation.

For any compound described herein the therapeutically effective amount can be initially determined from animal models. A therapeutically effective dose can also be determined from human data for CpG oligonucleotides which have been tested in humans (human clinical trials have been initiated) and for compounds which are known to exhibit similar pharmacological activities, such as other mucosal adjuvants, e.g., LT and other antigens for vaccination purposes, for the mucosal or local administration. Higher doses are required for parenteral administration. The applied dose can be adjusted based on the relative bioavailability and potency of the administered compound. Adjusting the dose to achieve maximal efficacy based on the methods described above and other methods as are well-known in the art is well within the capabilities of the ordinarily skilled artisan.

The formulations of the invention are administered in pharmaceutically acceptable solutions, which may routinely contain pharmaceutically acceptable concentrations of salt, buffering agents, preservatives, compatible carriers, adjuvants, and optionally other therapeutic ingredients.

For use in therapy, an effective amount of the immunostimulatory nucleic acid can be administered to a subject by any mode that delivers the nucleic acid to the desired surface, e.g., mucosal, systemic. Administering the pharmaceutical composition of the present invention may be accomplished by any means known to the skilled artisan. Preferred routes of administration include but are not limited to oral, parenteral, intramuscular, intranasal, intratracheal, inhalation, ocular, sublingual, vaginal, and rectal.

For oral administration, the compounds (i.e., immunostimulatory nucleic acids, antigens and other therapeutic agents) can be formulated readily by combining the active compound(s) with pharmaceutically acceptable carriers well known in the art. Such carriers enable the compounds of the invention to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a subject to be treated. Pharmaceutical preparations for oral use can be obtained as solid excipient,

optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate. Optionally the oral formulations may also be formulated in saline or buffers for neutralizing internal acid conditions or may be administered without any carriers.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules can contain the active ingredients in admixture with filler such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added. Microspheres formulated for oral administration may also be used. Such microspheres have been well defined in the art. All formulations for oral administration should be in dosages suitable for such administration.

For buccal administration, the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by inhalation, the compounds for use according to the present invention may be conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined

by providing a valve to deliver a metered amount. Capsules and cartridges of e.g., gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The compounds, when it is desirable to deliver them systemically, may be formulated for parenteral administration by injection, e.g., by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents.

Pharmaceutical formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

Alternatively, the active compounds may be in powder form for constitution with a suitable vehicle, e.g., sterile pyrogen-free water, before use.

The compounds may also be formulated in rectal or vaginal compositions such as suppositories or retention enemas, e.g., containing conventional suppository bases such as cocoa butter or other glycerides.

In addition to the formulations described previously, the compounds may also be formulated as a depot preparation. Such long acting formulations may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

The pharmaceutical compositions also may comprise suitable solid or gel phase carriers or excipients. Examples of such carriers or excipients include but are not limited to calcium carbonate, calcium phosphate, various sugars, starches, cellulose derivatives, gelatin, and polymers such as polyethylene glycols.

Suitable liquid or solid pharmaceutical preparation forms are, for example, aqueous or saline solutions for inhalation, microencapsulated, encochleated, coated onto microscopic gold particles, contained in liposomes, nebulized, aerosols, pellets for implantation into the skin, or dried onto a sharp object to be scratched into the skin. The pharmaceutical compositions also include granules, powders, tablets, coated tablets, (micro)capsules, suppositories, syrups, emulsions, suspensions, creams, drops or preparations with protracted release of active compounds, in whose preparation excipients and additives and/or auxiliaries such as disintegrants, binders, coating agents, swelling agents, lubricants, flavorings, sweeteners or solubilizers are customarily used as described above. The pharmaceutical compositions are suitable for use in a variety of drug delivery systems. For a brief review of methods for drug delivery, see Langer(1990) *Science* 249:1527-33, which is incorporated herein by reference.

The immunostimulatory nucleic acids and optionally other therapeutics and/or antigens may be administered *per se* (neat) or in the form of a pharmaceutically acceptable salt. When used in medicine the salts should be pharmaceutically acceptable, but non-pharmaceutically acceptable salts may conveniently be used to prepare pharmaceutically acceptable salts thereof. Such salts include, but are not limited to, those prepared from the following acids: hydrochloric, hydrobromic, sulfuric, nitric, phosphoric, maleic, acetic, salicylic, p-toluene sulphonic, tartaric, citric, methane sulphonic, formic, malonic, succinic, naphthalene-2-sulphonic, and benzene sulphonic. Also, such salts can be prepared as alkaline metal or alkaline earth salts, such as sodium, potassium or calcium salts of the carboxylic acid group.

Suitable buffering agents include: acetic acid and a salt (1-2% w/v); citric acid and a salt (1-3% w/v); boric acid and a salt (0.5-2.5% w/v); and phosphoric acid and a salt (0.8-2% w/v). Suitable preservatives include benzalkonium chloride (0.003-0.03% w/v); chlorobutanol (0.3-0.9% w/v); parabens (0.01-0.25% w/v) and thimerosal (0.004-0.02% w/v).

The pharmaceutical compositions of the invention contain an effective amount of an immunostimulatory nucleic acid and optionally antigens and/or other therapeutic agents optionally included in a pharmaceutically acceptable carrier. The term "pharmaceutically acceptable carrier" means one or more compatible solid or liquid filler, diluents or encapsulating substances which are suitable for administration to a human or other vertebrate animal. The term "carrier" denotes an organic or inorganic ingredient, natural or synthetic,

with which the active ingredient is combined to facilitate the application. The components of the pharmaceutical compositions also are capable of being commingled with the compounds of the present invention, and with each other, in a manner such that there is no interaction

For treatment of a subject, depending on activity of the compound, manner of administration, purpose of the immunization (i.e., prophylactic or therapeutic), nature and severity of the disorder, age and body weight of the patient, different doses may be necessary. The administration of a given dose can be carried out both by single administration in the form of an individual dose unit or else several smaller dose units. Multiple administration of doses at specific intervals of weeks or months apart is usual for boosting the antigen-specific responses.

Other delivery systems can include time-release, delayed release or sustained release delivery systems. Such systems can avoid repeated administrations of the compounds, increasing convenience to the subject and the physician. Many types of release delivery systems are available and known to those of ordinary skill in the art. They include polymer base systems such as poly(lactide-glycolide), copolyoxalates, polycaprolactones, polyesteramides, polyorthoesters, polyhydroxybutyric acid, and polyanhydrides. Microcapsules of the foregoing polymers containing drugs are described in, for example, U.S. Patent 5,075,109. Delivery systems also include non-polymer systems that are: lipids including sterols such as cholesterol, cholesterol esters and fatty acids or neutral fats such as mono-, di-, and tri-glycerides; hydrogel release systems; silastic systems; peptide based systems; wax coatings; compressed tablets using conventional binders and excipients; partially fused implants; and the like. Specific examples include, but are not limited to: (a) erosional systems in which an agent of the invention is contained in a form within a matrix such as those described in U.S. Patent Nos. 4,452,775, 4,675,189, and 5,736,152, and (b) diffusional systems in which an active component permeates at a controlled rate from a polymer such as described in U.S. Patent Nos. 3,854,480, 5,133,974 and 5,407,686. In addition, pump-based hardware delivery systems can be used, some of which are adapted for implantation.

The present invention is further illustrated by the following Examples, which in no way should be construed as further limiting. The entire contents of all of the references (including literature references, issued patents, published patent applications, and co-pending

patent applications) cited throughout this application are hereby expressly incorporated by reference.

Examples

Example 1. ODN 2395 is a remarkably strong activator of NK cells and IFN-α production.

We previously recognized and described oligodeoxynucleotides (ODN) containing neutralizing motifs consisting of repeats of the sequence CG such as CGCGCG or where the CG is preceded by a C and/or followed by a G. These neutralizing motifs were believed to reduce the stimulatory effects of ODN on multiple readouts, such as secretion of IL-6, IL-12, IFN-γ, TNF-α, and induction of an antigen-specific immune response. Krieg AM et al. (1998) *Proc Natl Acad Sci USA* 95:12631-6.

In many cases, the presence of a neutralizing motif in an oligonucleotide together with a stimulatory motif was believed to prevent immune activation. One such ODN containing both stimulatory and neutralizing motifs is ODN 2136, which has the sequence TCCTGACGTTCGGCGCGCGCCC (SEQ ID NO: 19). The 3' end of this ODN contains a fairly typical neutralizing motif, CGGCGCGCGCCC (SEQ ID NO: 37), derived from the 3' end of the inhibitory ODN 2010 (GCGGCGGGCGGCGCGCGCCC, SEQ ID NO: 38). Surprisingly, ODN 2136 had strong activity for inducing NK cell lytic activity (lytic units, L.U.). As shown in **Table 1**, ODN 2136 at a concentration of 3 μg/ml was actually stronger than our standard B-cell and NK cell stimulatory phosphorothioate ODN 2006 (TCGTCGTTTTGTCGTTTTGTCGTT, SEQ ID NO: 39) for induction of L.U. More strikingly, whereas ODN 2006 only induced the production of 2,396 pg/ml of IFN-α, ODN 2136 induced the production of 14,278 pg/ml (**Figure 1**). This indicated that, surprisingly, the presence of this neutralizing sequence was not necessarily to be avoided.

Table 1. Human PBL Cultured Overnight With Various ODN.

ODN	E:T RATIO						L.U.
	3.1	6.3	12.5	25.0	50.0	100.0	
ALONE	0.86	1.47	4.15	7.25	11.66	18.57	0.13
IL-2 (100 U/ml)	12.21	29.21	46.63	67.88	78.28	76.65	33.26
1585 (3 μg/ml)	6.47	12.61	24.65	36.82	49.30	53.00	11.69
1585 (10 μg/ml)	8.52	18.17	33.20	51.26	72.13	73.89	20.94
1585 (30 μg/ml)	5.75	13.05	20.00	34.34	45.02	56.49	10.66
2118 (10 μg/ml)	0.62	2.08	3.90	8.53	12.79	15.93	0.09

ODN	E:T RATIO						L.U.
	3.1	6.3	12.5	25.0	50.0	100.0	
2006 (0.6 μg/ml)	1.62	2.88	8.24	14.10	21.85	31.91	1.73
2006 (3 μg/ml)	7.07	17.02	30.28	50.66	69.13	74.27	19.41
2169(0.6 μg/ml)	3.65	3.81	6.67	13.45	24.48	32.42	1.84
2169 (3 μg/ml)	11.20	21.47	38.15	59.66	78.96	77.72	25.76
1760 (0.6 μg/ml)	0.35	2.70	6.85	8.59	16.09	20.63	0.33
1760 (3 μg/ml)	7.57	12.94	27.50	46.63	62.43	66.97	16.60
1758 (0.6 μg/ml)	2.07	6.05	12.80	23.25	34.57	44.93	5.43
1758 (3 μg/ml)	8.40	17.84	33.41	52.20	69.52	74.46	20.78
2398 (0.6 μg/ml)	1.83	1.92	6.21	11.21	20.38	26.71	0.98
2398 (3 μg/ml)	4.36	12.90	24.10	42.37	60.51	70.03	15.02
2397 (0.6 μg/ml)	2.14	3.15	8.79	17.37	28.71	42.45	3.80
2397 (3 μg/ml)	10.09	22.52	38.96	61.85	77.69	74.87	26.12
2396 (0.6 μg/ml)	2.93	5.80	13.22	25.32	36.83	46.77	6.13
2396 (3 μg/ml)	9.03	18.65	32.71	54.62	72.62	73.67	21.64
2395 (0.6 μg/ml)	5.10	9.22	17.21	31.67	49.53	60.53	10.59
2395 (3 μg/ml)	10.91	24.55	40.42	61.23	71.11	75.52	26.94
2136 (0.6 μg/ml)	0.39	2.89	7.12	12.70	18.88	24.11	0.78
2136 (3 μg/ml)	11.94	23.57	39.11	55.16	70.84	71.99	25.62

ODN sequences for Table 1

1585	GGGGTCAACGTTGAGGGGGG	(SEQ ID NO: 35)
1758	TCTCCCAGCGTGCGCCAT	(SEQ ID NO: 40)
1760	ATAATCGACGTTCAAGCAAG	(SEQ ID NO: 41)
2006	TCGTCGTTTTGTCGTTTTGTCGTT	(SEQ ID NO: 39)
2118	GGGGTCAAGCTTGAGGGGGG	(SEQ ID NO: 36)
2136	TCCTGACGTTCGGCGCGCGCCC	(SEQ ID NO: 19)
2169	TCTATCGACGTTCAAGCAAG	(SEQ ID NO: 42)
2395	TCGTCGTTTTCGGCGCGCGCCG	(SEQ ID NO: 1)
2396	TCGTCGTTTTTGTCGTTTTTGTCGTT	(SEQ ID NO: 43)
2397	TCGTCGTTTTGTCGTTTTTGTCGTTT	(SEQ ID NO: 44)
2398	TTCGTGTTTTCGTGTTTTCGTCGT	(SEQ ID NO: 45)

However, in an effort to understand this observation, an even stronger NK activator and IFN-α inducer was created by combining the 3' end of ODN 2136 with the 5' end of ODN 2006. The resulting ODN 2395 (TCGTCGTTTTCGGCGCGCGCCG, SEQ ID NO: 1) serendipitously incorporated a change of the last base on the 3' end from a C to a G. This single base change has the effect of creating a perfect 12-base-long palindrome at the 3' end of ODN 2395 where in ODN 2136 the palindrome is only 10 bases long.

Table 2 shows another example of data where ODN 2395 is remarkably potent at inducing NK cell L.U. compared to most other all-phosphorothioate backbone ODN. In this

assay ODN 2395 is weaker than the positive control ODN 1585, which has a chimeric phosphorothioate/phosphodiester (SOS) backbone. ODN 1585 (ggGGTCAACGTTGAgggggG, SEQ ID NO: 35), is described in published PCT Application WO 01/22990. At the low concentration of 0.6 μg/ml tested in this experiment, ODN 2136 induced no L.U. above the background of 0.03 in the no-ODN control. **Figure 2** and **Figure 3** show the level of monocyte chemotactic protein (MCP)-1 and IFN-inducible protein (IP)-10, respectively, in the supernatants from the NK cell cultures in **Table 2**. MCP-1 is a chemokine that is a ligand for CCR2 and is associated with both Th1 and Th2-type immune responses. IP-10 is a CXC chemokine that is a ligand for CXCR3 and is associated with Th1 responses. Loetscher P et al. (2001) *J Biol Chem* 276:2986-91. These data show that ODN 2395 is a relatively strong inducer of IP-10 production, but induces only average levels of MCP-1.

Table 2. Human PBL Cultured Overnight With Various ODN.

ODN	E:T RATIO						L.U.
	3.1	6.3	12.5	25.0	50.0	100.0	
ALONE	1.73	3.10	4.25	7.72	12.07	14.56	0.03
IL-2 (100 U/ml)	16.68	29.41	49.42	74.78	87.64	92.63	37.17
1585 (10 μg/ml)	9.60	17.25	35.63	55.76	77.53	87.14	22.94
2118 (10 μg/ml)	2.99	2.88	3.41	6.72	9.26	14.18	0.01
2183 (0.6 μg/ml)	2.13	2.28	3.29	8.17	10.47	17.87	0.07
2186 (0.6 μg/ml)	1.23	2.18	3.50	6.26	9.58	14.51	0.02
2133 (0.6 μg/ml)	2.13	3.45	9.69	18.85	32.72	44.67	4.63
2135 (0.6 μg/ml)	2.07	4.06	7.70	12.63	21.90	34.58	1.92
2139 (0.6 μg/ml)	2.94	5.15	9.63	15.15	24.90	38.71	2.83
2117 (0.6 μg/ml)	1.21	2.32	4.08	7.61	10.09	16.27	0.05
2137 (0.6 μg/ml)	1.66	2.79	4.43	7.92	10.64	16.91	0.06
2006 (0.6 μg/ml)	1.92	3.38	5.06	11.57	16.82	25.30	0.65
2006 (0.6 μg/ml)	0.91	2.19	4.52	7.39	13.86	21.57	0.28
2006 (0.6 μg/ml)	1.92	3.59	7.67	12.51	18.99	28.03	1.04
2395 (0.6 μg/ml)	2.88	7.20	10.80	23.96	37.97	54.38	7.02
2396 (0.6 μg/ml)	0.92	2.18	4.07	5.78	10.18	14.95	0.03
2397 (0.6 μg/ml)	3.05	5.24	10.51	17.50	33.51	46.50	4.92
2398 (0.6 μg/ml)	1.37	2.82	5.16	8.48	15.72	21.72	0.34
2012 (0.6 μg/ml)	0.88	1.71	4.41	7.07	10.97	16.47	0.06
2102 (0.6 μg/ml)	2.36	5.82	10.59	17.88	30.96	39.79	3.81
2103 (0.6 μg/ml)	2.12	4.32	8.83	13.49	25.23	35.47	2.37
2013 (0.6 μg/ml)	1.11	2.42	4.42	6.01	9.15	13.44	0.01

ODN	E:T RATIO						L.U.
	3.1	6.3	12.5	25.0	50.0	100.0	
2142 (0.6 μg/ml)	0.94	1.55	4.38	7.44	11.45	16.84	0.08
2180 (0.6 μg/ml)	2.06	4.08	6.91	11.54	16.82	25.76	0.67
2007 (0.6 μg/ml)	1.83	3.30	6.68	12.34	20.74	29.10	1.25
2136 (0.6 μg/ml)							0.01

ODN sequences for Table 2

1585	GGGGTCAACGTTGAGGGGGG	(SEQ ID NO: 35)
2006	TCGTCGTTTTGTCGTTTTGTCGTT	(SEQ ID NO: 39)
2007	TCGTCGTTGTCGTTTTGTCGTT	(SEQ ID NO: 46)
2013	TGTCGTTGTCGTTGTCGTTGTCGTT	(SEQ ID NO: 48)
2102	TCGTCGTTTTGACGTTTTGTCGTT	(SEQ ID NO: 49)
2103	TCGTCGTTTTGACGTTTTGACGTT	(SEQ ID NO: 50)
2117	TZGTZGTTTTGTZGTTTTGTZGTT	(SEQ ID NO: 51)
2118	GGGGTCAAGCTTGAGGGGGG	(SEQ ID NO: 36)
2133	TCGTCGTTGGTTGTCGTTTTGGTT	(SEQ ID NO: 17)
2135	ACCATGGACGAGCTGTTTCCCCTC	(SEQ ID NO: 18)
2136	TCCTGACGTTCGGCGCGCGCCC	(SEQ ID NO: 19)
2137	TGCTGCTTTTGTGCTTTTGTGCTT	(SEQ ID NO: 20)
2139	TCGTCGTTTCGTCGTTTTGACGTT	(SEQ ID NO: 21)
2142	TCGCGTGCGTTTTGTCGTTTTGACGTT	(SEQ ID NO: 22)
2180	TCGTCGTTTTTTGTCGTTTTTTGTCGTT	(SEQ ID NO: 52)
2183	TTTTTTTTTTTTTTTTTTTTTTTT	(SEQ ID NO: 53)
2186	TCGTCGCTGTCTCCGCTTCTTCTTGCC	(SEQ ID NO: 54)
2395	TCGTCGTTTTCGGCGCGCGCCG	(SEQ ID NO: 1)
2396	TCGTCGTTTTTGTCGTTTTTGTCGTT	(SEQ ID NO: 43)
2397	TCGTCGTTTTGTCGTTTTTGTCGTTT	(SEQ ID NO: 44)
2398	TTCGTGTTTTCGTGTTTTCGTCGT	(SEQ ID NO: 45)

Based on these and other data, we concluded that the ODN 2395 sequence was a remarkably strong activator of NK cells and IFN-α production.

Example 2. ODN related to ODN 2395 are also strong activators of NK cells and IFN-α production.

Additional ODN 2427-2433 (SEQ ID NOs: 2 - 8) were designed and synthesized to test the possibility that the palindrome at the 3' end of ODN 2395 may be important in its immune stimulatory activity. **Table 3** compares the ability of these different ODN to activate NK L.U. As is evident from these data, the strongest ODN at the concentration of 1 μg/ml is ODN 2429 (TCGTCGTTTTCGGCGGCCGCCG, SEQ ID NO: 4) which induced 2.85 L.U. of NK activity. ODN 2006 was very weak in the experiment, and all of the other oligos that were tested except for the control ODN 2118 (GGGGTCAAGCTTGAGGGGGG, SEQ ID NO: 36) that has no CG were stronger than 2006. ODN 2429 is notable because it is the only

one that maintains a 12-base palindrome, although this is a different palindrome from the one that was present in 2395. ODN 2430 (TCGTCGTTTTCGGCGCGCCGCG, SEQ ID NO: 5), which is the second strongest ODN at the 1 μg/ml concentration, is similar; but the palindrome has been slightly shortened to 10 bases long. The remainder of the ODN have either no or shorter palindromic sequences, and induce less NK activity.

Table 3. Human PBL Cultured Overnight With Various ODN.

ODN	E:T RATIO						L.U.
	3.1	6.3	12.5	25.0	50.0	100.0	
ALONE	0.37	0.64	0.25	1.02	2.15	3.23	0.00
IL-2 (100 U/ml)	3.01	4.20	9.01	18.92	27.37	38.17	3.22
1585 (10 μg/ml)	1.35	2.30	4.38	8.07	13.96	22.31	0.31
2118 (10 μg/ml)	-0.31	-0.21	0.22	1.57	1.24	2.41	0.00
2395 (1 μg/ml)	1.01	2.61	5.73	11.39	18.92	28.16	1.04
2395 (3 μg/ml)	1.59	2.55	5.96	12.09	20.46	33.87	1.71
2006 (1 μg/ml)	-0.08	0.73	1.45	3.03	7.11	12.49	0.01
2006 (3 μg/ml)	0.16	0.76	2.98	4.98	9.79	20.58	0.15
2427 (1 μg/ml)	0.85	1.80	4.03	6.37	12.53	24.12	0.34
2427 (3 μg/ml)	0.96	2.24	4.40	8.00	15.01	21.85	0.33
2428 (1 μg/ml)	1.19	1.97	3.64	7.72	16.27	24.74	0.53
2428 (3 μg/ml)	1.42	2.36	5.67	11.06	19.11	28.17	1.03
2429 (1 μg/ml)	1.47	3.84	7.83	14.17	25.47	38.99	2.85
2429 (3 μg/ml)	0.57	2.38	4.21	8.98	16.88	26.36	0.72
2430 (1 μg/ml)	1.49	3.55	6.25	12.76	20.51	31.67	1.51
2430 (3 μg/ml)	1.23	1.52	3.89	8.78	15.28	25.56	0.57
2431 (1 μg/ml)	0.96	2.90	3.58	8.29	15.23	25.29	0.53
2431 (3 μg/ml)	1.82	3.25	5.53	9.67	21.04	32.78	1.51
2432 (1 μg/ml)	1.67	2.97	4.87	8.54	19.26	27.10	0.84
2432 (3 μg/ml)	1.03	2.39	5.22	9.41	18.48	25.74	0.76
2433 (1 μg/ml)	0.74	1.84	2.30	6.97	12.43	18.94	0.15
2433 (3 μg/ml)	1.25	3.13	4.47	9.85	14.77	22.75	0.38

ODN sequences for Table 3

1585	GGGGTCAACGTTGAGGGGGG	(SEQ ID NO: 35)
2006	TCGTCGTTTTGTCGTTTTGTCGTT	(SEQ ID NO: 39)
2118	GGGGTCAAGCTTGAGGGGGG	(SEQ ID NO: 36)
2395	TCGTCGTTTTCGGCGCGCGCCG	(SEQ ID NO: 1)
2427	TCGTCGTTTTCGTCGCGCGCCG	(SEQ ID NO: 2)
2428	TCGTCGTTTTCGTCGCGCGGCG	(SEQ ID NO: 3)
2429	TCGTCGTTTTCGGCGGCCGCCG	(SEQ ID NO: 4)
2430	TCGTCGTTTTCGGCGCGCCGCG	(SEQ ID NO: 5)
2431	TCGTCGTTTTCGGCGCCGGCCG	(SEQ ID NO: 6)

2432 TCGTCGTTTTCGGCCCGCGCGG (SEQ ID NO: 7)
2433 TCGTCGTTTTCCGCCGCCGGGG (SEQ ID NO: 8)

Figure 4 shows the ability of these oligos to induce IFN-α production compared to the positive control SOS ODN 2216 (GGGGGACGATCGTCGGGGG, SEQ ID NO: 55), 2334 (GGGGTCGACGTCGACGTCGAGGGGGGG, SEQ ID NO: 56), and 2336 (GGGGACGACGTCGTGGGGGGG, SEQ ID NO: 57). All of the 2395-related ODN induce a higher level of IFN-α production than ODN 2006, although the levels are below the levels induced by the chimeric SOS ODN. The rank order of induction of IFN-α expression is roughly similar to that of NK L.U., with the strongest effects seen by ODN 2395 and 2429.

Example 3. The strong stimulatory effects on NK cells and IFN-α production do not correspond to B-cell effects.

As shown in **Figure 5A**, ODN 2395 and its relatives were significantly weaker at a 0.25 μg/ml concentration than ODN 2006 or its relative 2397, in terms of their ability to induce B-cell expression of CD86 at 48 hours. As we have noticed previously, at higher ODN concentrations such as 1 μg/ml, less difference was seen between the various ODN (**Figure 5B**). In the same experiment, we also measured B-cell activation by a proliferation assay (^{3}H-thymidine incorporation; **Figure 6**). Again, at the 0.25 μg/ml concentration ODN 2006 and ODN 2397 (SEQ ID NO: 44) were by far the strongest (**Figure 6A**). However, at higher concentrations, the 2395-related ODN were similar in their efficacy (**Figure 6B**).

Example 4. ODN 2395 and related ODN are weak inducers of IL-10.

Our previous studies have suggested that most of the IL-10 production that is induced by CpG is derived from B cells. As shown in **Figure 7**, IL-10 expression correlated well with B-cell proliferation. Again, ODN 2006 and its relative ODN 2397 were the strongest at the low concentration of 0.25 μg/ml. ODN 2395 and its relatives induced less IL-10 production at this concentration.

Example 5. Concentration dependence of immune stimulatory effect.

Additional studies on this class of oligonucleotides and the derivatives involved ODN numbers 2427-2433 (SEQ ID NOs: 2 - 8). Data for these ODN are shown in **Figure 8**. This demonstrates again that ODN 2006 was very weak at inducing IFN-α production at a concentration of either 1 or 6 μg/ml. However, ODN 2395 induced substantial amounts of IFN-α, especially at the lower concentration of 1 μg/ml. We have occasionally seen ODN where the stimulatory activity was reduced at higher concentrations, such as 6 μg/ml, in

comparison to the effects seen at lower concentrations such as 1 μg/ml. In the experiments shown in **Figure 8**, ODN 2395 was more potent at the lower concentration than at the higher concentration, but ODN 2429 was more potent at the higher concentration. In contrast to the common inverted dose-response curve of phosphorothioate ODN, chimeric ODN such as ODN 2336 in this experiment typically showed increased immune stimulatory effects at higher concentrations. The stimulatory effect of ODN 2432 in this experiment shown in **Figure 8** was interesting considering that this ODN has no good palindrome. This system with the relatively weak B cell stimulatory activity is shown in **Figure 5** and **Figure 6**.

Example 6. Reciprocal relationship between B-cell stimulation and NK stimulation and IFN-α secretion.

Figure 9 shows another experiment, where ODN 2395 at a low concentration of 0.4 μg/ml was significantly weaker than ODN 2006 at inducing B cell expression of CD86. The other relatives of 2395 show a less marked loss of B cell stimulation. Interestingly, there is the suggestion of the same rank order for loss of B cell stimulation that had previously been seen for gain of NK stimulation: ODN 2429, followed by ODN 2430, are the weakest B cell stimulators among the 2395 relatives. This raises the possibility that the loss of B cell stimulation by the 2395-like ODN is closely related to the gain of NK stimulation and IFN-α secretion. **Figure 10** and **Figure 11** show the IFN-α induction is seen with ODN 2395 and ODN 2429, followed by ODN 2430. **Table 4** and **Figure 12**, from a separate experiment, also show the strong ability of ODN 2395 and ODN 2429 to induce IFN-α secretion in two different human donors (D141 and D142).

Table 4. IFN-α Secretion by Variants of ODN 2395[1]

ODN, 6 μg/ml	**IFN-α, pg/ml**	
	D141	**D142**
2006	10 ± 10	7 ± 0.5
2336	83,297 ± 1876.5	53530.5 ± 4840
2395	6214 ± 84.5	2031 ± 96
2429	7215 ± 68	1117.5 ± 495
5293	10 ± 0.5	27 ± 27
5294	2.5 ± 0.1	23 ± 23
5295	5 ± 0.5	0 ± 0
5296	10 ± 0	10 ± 0
5297	10 ± 0.5	26.5 ± 1
without (w/o)	110 ± 77.5	12 ± 12

[1]Data expressed in units of pg/ml as mean ± standard deviation.

ODN sequences for Table 4

2006	TCGTCGTTTTGTCGTTTTGTCGTT	(SEQ ID NO: 39)
2336	GGGGACGACGTCGTGGGGGGG	(SEQ ID NO: 57)
2395	TCGTCGTTTTCGGCGCGCGCCG	(SEQ ID NO: 1)
2429	TCGTCGTTTTCGGCGGCCGCCG	(SEQ ID NO: 4)
5293	TCGTCGTTTTCGGCGGCCGCC	(SEQ ID NO: 58)
5294	TCGTCGTTTTCGGCCGCCGCC	(SEQ ID NO: 59)
5295	TCGTCGTTTTCGGCCGCCGCCG	(SEQ ID NO: 60)
5296	TCGTCGTTTTCGCCGCCGCCG	(SEQ ID NO: 61)
5297	TGCTGCTTTTCGGCGGCCGCCG	(SEQ ID NO: 62)

Example 7. Characteristics of the GC-rich domain.

Surprisingly, none of the ODN 5293-5297 demonstrated strong immune stimulatory responses. ODN 5293 contains a 10-base palindrome, but the palindrome differs from that in 2395 in that the central CG is inverted to a GC. However, it is believed that this change by itself cannot explain the loss of activity since ODN 2429 also has such an inversion. Rather, greater levels of activity may occur with a 12-base palindrome unless there is a central CG in the palindrome. However, ODN 2430 also has only a 10-base palindrome with a central GC dinucleotide. The immune stimulatory activity of ODN 2430 may be enhanced by the fact that it contains five CpG dinucleotides in the 3' terminus, whereas ODN 5293 contains only three.

ODN 5294 contains only a 6-base palindrome, which could possibly be related to its low activity. ODN 5295 likewise has no good palindrome. The low activity of ODN 5296 suggests that simple repeats of CCG are not sufficient to confer the immune stimulatory effects of ODN 2395. ODN 2397 has a perfect 12-base palindrome at the 3' end, but has no CpG motifs at the 5' end. Since the 12-base palindrome in ODN 5297 is the same at that in ODN 2429, it can be concluded that the 5' TCGTCG motif of ODN 2429 is important for its immune stimulatory activity. That is, it is believed that the presence of the neutralizing palindrome of ODN 2429 at one end of an oligonucleotide will be insufficient to provide immune stimulatory activity in the absence of at least one stimulatory motif at the other end.

Example 8. Effects on IFN-γ production.

Several additional types of assays have been performed to better understand the range of immune stimulatory effects of this new class of immune stimulatory nucleic acid. **Figure 13** shows some of the effects of these ODN on IFN-γ production from the supernatants of human PBMCs. These cells were the same as those used in the experiments shown in **Table 3**, but the supernatants from the cultures were assayed for their IFN-γ levels. Panel C in **Figure 13** shows that SOS CpG ODNs such as ODN 1585 induce some IFN-γ production,

whereas ODNs without the CpG motif (e.g., control ODN 2118) do not. Panels A and B of **Figure 13** show that ODN 2006 is relatively weak at inducing IFN-γ production, while ODN 2395 and its cousins are somewhat stronger.

Another set of studies was performed to examine the effects of these different ODN on dendritic cells. The plasmacytoid DC (pDC) is the source of the IFN-α that is produced in response to ODN 2395 and its relatives. The effects of the various ODN on myeloid DC (mDC) are relatively similar in that all of the ODN induce partially purified mDC to activate $CD4^+$ T cells to produce IFN-γ (**Figure 14** and **Figure 15**). Myeloid DC were isolated from a buffy coat and incubated with GM-CSF (4.4 ng/ml) and various ODN for 2 days. CD4+ naïve T cells were then isolated from a different donor and mixed with the DC at selected effector to target (E:T) ratios and incubated for 6 more days. Cells were then stained and analyzed by fluorescence activated cell sorting (FACS). Results were measured in terms of the percentage of CD3+ cells that stained for IFN-γ. **Figure 14** shows the percentage of T cells that stained positive for IFN-γ and **Figure 15** shows the mean fluorescence intensity (MFI) of IFN-γ staining in these T cells.

Example 9. Not all GC-rich palindromes are effective.

Several additional ODN were synthesized in order better to understand the structural requirements for this new class of ODN. Since we noted that potent immune stimulatory ODN contained GC-rich palindromes, ODN 2449 (TCGTCGTTTTCGGGGGGCCCCC, SEQ ID NO: 9) and 2450 (TCGTCGTTTTCCCCCCGGGGGG, SEQ ID NO: 10) were synthesized to have GC-rich palindromes which were simply straight Gs followed by straight Cs, or straight Cs followed by straight Gs. As shown in **Figure 16**, neither of these ODN induced IFN-α production.

Example 10. Effect of orientation of immune stimulatory sequence and neutralizing motif.

ODN 2451 (TCGGCGCGCGCCGTCGTCGTTT, SEQ ID NO: 11) was synthesized to test the possibility that the 5' and 3' orientation of the "stimulatory" TCGTCG motif and the "neutralizing" CGGCGCGCGCCG (SEQ ID NO: 23) palindrome could be inverted without losing immune stimulatory activity. Indeed, ODN 2451 was highly stimulatory (**Figure 16**). ODN 2452 (TCGTCGTTTTCGGCGCGCGCCGTTTTT, SEQ ID NO: 12) was synthesized to determine whether additional sequence could be added to the 3' end of the CGGCGCGCGCCG (SEQ ID NO: 23) palindrome without reducing the immune stimulatory

activity, provided the stimulatory TCGTCG motif was on the 5' end. Indeed, this ODN was also highly immune stimulatory (**Figure 16**).

Example 11. Variants of ODN 2395 and their induction of IFN-α.

To study in more detail the structural requirements of this new class of ODN to induce IFN-α secretion, variants of ODN 2395 were synthesized and tested for their immunostimulatory activity. **Table 5** summarizes the data concerning IFN-α induction.

Table 5. Variants of ODN 2395 and their induction of IFN-α[1,2]

ODN	SEQ ID NO:	Sequence	Palindrome	Description	IFN-α Induction
2006	39	tcgtcgttttgtcgttttgtcgtt	/	ODN class B	-
2336	57	ggGGA*CGACGTCCTG*ggggggG	+	ODN class A	+++++
2395	1	tcgtcgtttt*cggcgcgcgccg*	+	2006 - 2136	++
2427	2	tcgtcgtttt<u>c</u>g<u>t</u>cgcgcgccg	-		-
2428	3	tcgtcgtttt<u>c</u>g<u>t</u>cgcgcggcg	-		-
2429	4	tcgtcgtttt*cggcggccgccg*	+	cg → gc by preserving palindrome	+++
2430	5	tcgtcgtttt<u>c</u>ggcgcgc<u>c</u>gcg	-		+
2431	6	tcgtcgtttt<u>c</u>ggcgcgc<u>c</u>gcg	-		+/-
2432	7	tcgtcgtttt<u>c</u>ggc<u>c</u>cgcgcgg	-		+
2433	8	tcgtcgtttt<u>c</u>cgc<u>c</u>gcc<u>g</u>ggg	-		-
5293	58	tcgtcgtttt*cggcggccgcc*	(+)	2429 w/o 3' g	-
5294	59	tcgtcgtttt<u>c</u>ggc<u>c</u>gc<u>c</u>gcc	-	3xgcc w/o 3' g	-
5295	60	tcgtcgtttt<u>c</u>ggc<u>c</u>gc<u>c</u>gc<u>c</u>g	-	5295 w/3' g	-
5296	61	tcgtcgtttt<u>c</u>gc<u>c</u>gc<u>c</u>gc<u>c</u>g	-		-
5297	62	tgctgctttt*cggcggccgccg*	+	gc of 2429	-
5327	14	tgctzgtttt*zggzgzgzgzzg*	+	2395 w/methyl-c (z)	+
5328	15	tgctgctttt*cggcgcgcgccg*	+	gc of 2395	-
2136	19	tcctgacgtt*cggcgcgcgccc*	(+)		+/-
5315	13	tcctgacgtt*cggcgcgcgccg*	+	2136 w/3' g longer palindrome	+?
5329	16	tcgtcgtttt*cgcgcgcgcgcg*	+	2006 + 1631	(-)

[1]Underlined are nucleotides that differ from 2395; palindromic sequences are in italics.

[2]All except ODN 2336, that represents a chimeric backbone ODN (capitals indicate phosphodiester linkage and lower case represent phosphorothioate linkage), are completely phosphorothioate ODNs.

From the first set of experiments using the phosphorothioate ODNs 2395 and 2427-2433 it became clear that the palindromic sequence at the 3' end of the ODN has an

important role for induction of IFN-α secretion by dendritic cells that are the main producers of IFN-α (see 2395 and 2429), although some ODN without such a palindrome at the 3' end (e.g., ODN 2430 and ODN 2432) also induced IFN-α in somewhat lower amounts (example in **Figure 17A**). ODN 2395 and ODN 2429 induced the highest amounts of IFN-α, whereas 2006 (class B ODN) induced none to minimal amounts, and ODN 2336 (class A ODN) induced large amounts of this cytokine. Most experiments demonstrated that ODN 2429 induced even higher amounts of this cytokine (**Figure 17B**). An introduction of an additional TCG motif (e.g., ODN 2427 and ODN 2428) appeared to have negative effects on IFN-α secretion. Based on data from these and other studies of ODN 2186, the gcc at the 3' end seemed to play a possible role in the observed effects.

Therefore, we tested another set of ODNs all having GCC sequences at the 3' end. None of these ODN were observed to induce IFN-α. Therefore, only GCC itself in a palindrome seems not to be sufficient for the observed effects.

In addition, ODN 5297 with a TGC at the 5' end did not induce any IFN-α despite bearing the palindromic 3' sequence. This led to the conclusion that not only the 3' palindromic sequence but also the 5' TCG motif is important for the activity of these ODNs.

This was confirmed by using ODN 5328 (2395 with 5' TGC motif). In contrast to methylation of class A ODNs, methylation at least of the 5' motif decreased, but did not abrogate, IFN-α secretion. This finding is in accordance with results obtained with class B ODNs. Nevertheless, an ODN with part of the 3' palindrome but a different sequence at the 5' end with only one CpG dinucleotide (ODN 2136) also induced IFN-α. In preliminary results using this ODN and an ODN with the full 3' palindrome (ODN 5315), ODN 5315 was better than ODN 2136 but not as good as ODN 2395.

The fact that ODN 5329 seems to induce no or only very low amounts of IFN-α although having a full CG palindrome at the 3' end indicates that specific palindromic sequences are preferred for IFN-α activity.

Example 12. Reciprocal relationship between B-cell activation and induction of IFN-α.

An additional B-cell activation experiment was performed with a panel of some of the ODNs of Example 11 (**Figure 18**). The results indicated that the better is an ODN for induction of IFN-α, the less active it is on B cells (compare especially ODNs 2006, 2336, 2395 and 2429). Nevertheless, it also demonstrated that all of these ODNs were superior to 2336 (class A ODN) in stimulating B cells.

Example 13. Effect on secretion of IFN-γ.

We also determined secretion of IFN-γ upon incubation of PBMCs with different concentrations of ODN at different time points (**Figure 19 A – C**). The ODNs tested induced IFN-γ secretion with the rank order 2336 > 2395, 2429 > 2006. Nevertheless, the difference between the ODNs was not as clear as by using IFN-α as a read-out.

Example 14. Effect on IFN-γ in MLR.

We also determined the effect of these ODN on the induction of IFN-γ in a mixed lymphocyte reaction (MLR). In this setting lymphocytes of one donor respond to antigens expressed on cells of another donor. The results demonstrated that ODNs 2006, 2336, as well as 2395 were able to enhance IFN-γ secretion during such an antigen-specific response (**Figure 20**). This indicated that all these ODN were able to enhance the reactivity to specific antigen(s).

Example 15. ODN 2395 induces less IL-10 than ODN 2006.

A further set of experiments focused on the induction of the pro-inflammatory cytokine IL-10. Again, as before for IFN-γ, PBMCs were incubated for different times and with different concentrations of ODNs (**Figure 21 A - C**). The results demonstrate that, as shown before, ODN 2006 induces relatively high amounts of IL-10 in contrast to ODN 2336 that induces only minimal to low amounts. In contrast, ODNs 2395 as well as ODN 2429 induce more IL-10 than ODN 2336 but less than ODN 2006. This again confirms that ODN of this new class of immune stimulatory ODN have stimulatory activities that place them between those described for ODNs of class A and class B.

Example 16. ODN 2395 induces less TNF-α than ODN 2006 but more than ODN 8954.

Human PBMCs were cultured for 6 hours with 1.6 μg/ml of ODN 2006, 8954, 2395, 2429, or LPS, and supernatants were then harvested and TNF-α measured by specific ELISA. Results are shown in **Table 6.**

Table 6. Induction of TNF-α by representative ODN of different classes

ODN	TNF-α, pg/ml
(LPS)	>120
2006	40
2429	35
2395	21
8954	14
none	16

Additional experiments indicated that cytokines IL-5 as well as IL-15 could not be detected in our experimental settings upon incubation of PBMCs with these ODNs.

Example 17. Induction of IP-10

Human PBMCs were cultured either alone, in the presence of IL-2, in the presence of control ODN 1585 or control ODN 2118 at 10 μg/ml, or in the presence of various ODN at 0.6 or 3.0 μg/ml. Supernatants were harvested after 24 hours and IP-10 was measured by specific enzyme-linked immunosorbant assay (ELISA). Results are shown in **Figure 22.** ODNs 2395, 2429, 2430, 2432, and 2451 at 3.0 μg/ml, and ODN 2452 at 0.6 μg/ml, all induced large amounts of IP-10.

Example 18. Induction of IFN-α

Human PBMCs were cultured either alone, in the presence of IL-2, in the presence of control ODN 1585 or control ODN 2118 at 10 μg/ml, or in the presence of various ODN at 0.6 or 3.0 μg/ml. Supernatants were harvested after 24 hours and IFN-α was measured by specific ELISA. Results are shown in **Figure 23A** (ODN at 0.6 μg/ml) and **Figure 23B** (ODN at 3.0 μg/ml). ODNs 2395, 2427, 2429, 2430, 2431, 2432, and 2451 at 3.0 μg/ml, and ODN 2452 at 0.6 μg/ml, all induced large amounts of IFN-α.

Example 19. Induction of IFN-γ.

Human PBMCs were cultured either alone, in the presence of IL-2, in the presence of control ODN 1585 or control ODN 2118 at 10 μg/ml, or in the presence of various ODN at 0.6 or 3.0 μg/ml. Supernatants were harvested after 24 hours and IFN-γ was measured by specific ELISA. Results are shown in **Figure 24.** ODNs 2395, 2427, 2429, 2430, 2431, 2432, 2451 and 2452 at 3.0 μg/ml, and ODN 2352 at 0.6 μg/ml, all induced large amounts of IFN-γ.

Example 20. Induction of IL-6.

Human PBMCs were cultured either alone, in the presence of IL-2, in the presence of control ODN 1585 or control ODN 2118 at 10 μg/ml, or in the presence of various ODN at 0.6 or 3.0 μg/ml. Supernatants were harvested after 24 hours and IL-6 was measured by specific ELISA. Results are shown in **Figure 25.** ODNs 2395, 2430, 2432, 2433, 2136, 2449, 2450, 2451 and 2452 at 0.6 μg/ml, and ODN 2449 and ODN 2451 at 3.0 μg/ml, all induced large amounts of IL-6.

Example 21. Induction of IFN-α.

Human PBMCs were cultured either alone or in the presence of various ODN at 3.0 or 6.0 μg/ml. ODNs included 2006, 8954, 2395, 2449, 2450, 2451, 2452, 5373 (CGGCGCGCGCCG, SEQ ID NO: 23), 5374 (CGGCGCGCGCCGCGGCGCGCCG, SEQ ID NO: 24), 5375 (CGGCGCGCGCCGTCGTCGTTT, SEQ ID NO: 25), 5376 (TCGGCGCGCGCCGTGCTGCTTT, SEQ ID NO: 26), and 5377 (CCGCCGTTTTCGGCGCGCGCCG, SEQ ID NO: 27). Supernatants were harvested after 24 hours and IFN-α was measured by specific ELISA. Results are shown in **Figure 26**. ODNs 2395, 2451, 2452, and 5376 all induced IFN-α.

Example 22. Induction of IFN-α by ODN 5515 and ODN 5516.

Human PBMCs obtained from two donors (D346 and D240) were cultured either alone or in the presence of ODN 2006, ODN 5515, or ODN 5516 at 0.8, 2.4, or 6.0 μg/ml. Supernatants were harvested after 24 hours and IFN-α was measured by specific ELISA. Results are shown in **Table 7**. ODN 5515 and ODN 5516 induced IFN-α more effectively than ODN 2006, particularly at ODN concentrations of 2.4 and 6.0 μg/ml.

Example 23. Induction of IFN-α by ODN 20184, 20185, and 20186.

Human PBMCs obtained from three donors (D445, D446, and D448) were cultured either alone or in the presence of ODN 2006, ODN 20184, ODN 20185, or ODN 20186 at 0.05, 0.1, 0.2, 0.5, or 1.0 μg/ml. Supernatants were harvested after 24 hours and IFN-α was measured by specific ELISA. Results are shown in **Table 8**. ODN 20184, ODN 20185, and ODN 20186 induced IFN-α more effectively than ODN 2006, particularly at 0.2-0.5 μg/ml.

Table 7. Induction of IFN-α (pg/ml) by ODN 5515 and ODN 5516

ODN	Conc. μg/ml	D346 Mean ± SD	D240 Mean ± SD
2006	0.8	18.5±13.8	36±3.3
	2.4	0±0	19.7±6.4
	6	2.7±0	2.8±0
5515	0.8	34.1±6.9	16.5±2.8
	2.4	36.6±2.1	106.7±17.3
	6	39.2±26.5	127.3±7.7
5516	0.8	4.3±0	22.3±0.1
	2.4	31.9±0	172.5±82.3
	6	26.6±19	90.4±15.4
none	--	0±0	20.9±6.5

Table 8. Induction of IFN-α (pg/ml) by ODN 20184, 20185, and 20186

ODN	Conc. μg/ml	D445 Mean ± SD	D446 Mean ± SD	D448 Mean ± SD
2006	0.05	5.2±0.0	58.8±1.9	0.9±0.0
	0.1	27.7±14.4	283.5±16.1	23.5±3.8
	0.2	54.9±17.6	503.7±9.7	39.1±5.0
	0.5	61.1±14.6	227.8±12.7	49.8±0.4
	1.0	26.4±15.5	142.6±23.1	48.7±29.8
20184	0.05	25.6±2.1	88.0±12.2	0.0±0.0
	0.1	32.9±7.3	691.2±32.3	129.1±24.8
	0.2	256.2±8.2	2155.1±35.1	314.0±22.2
	0.5	757.2±5.7	2171.8±95.9	268.7±15.9
	1.0	194.3±5.7	1181.9±15.1	5.8±3.4
20185	0.05	65.0±10.8	217.9±28.4	54.3±14.2
	0.1	63.6±1.3	467.4±23.7	150.9±5.9
	0.2	79.3±2.4	1420.5±83.7	160.2±5.5
	0.5	281.3±0.2	1965.7±72.3	162.4±3.8
	1.0	176.9±12.5	1710.3±19.7	181.1±0.1
20186	0.05	21.9±1.7	223.1±1.2	79.8±1.6
	0.1	58.3±7.6	812.2±28.1	111.3±6.8
	0.2	153.6±1.5	1302.5±56.2	193.5±10.5
	0.5	267.7±7.9	1744.1±54.7	227.4±6.9
	1.0	153.0±0.3	1113.6±6.4	13.7±15.4
Medium	--	0.0±0.0	12.8±2.0	64.8±32.7
	--	0.0±0.0	45.3±12.9	36.4±2.6

Example 24. Induction of IFN-α by ODN 8954, 5569, and 5570.

Human PBMCs obtained from three donors (D521, D525, and D526) were cultured either alone or in the presence of ODN 2006 (SEQ ID NO: 39), ODN 8954, ODN 5569 (TIGTIGTTTTCGGCGGCCGCCG SEQ ID NO: 63), or ODN 5570 (TCITCITTTTCGGCGGCCGCCG SEQ ID NO: 70) at 0.03, 0.06, 0.125, 0.25, or 1.0 μg/ml. Supernatants were harvested after 24 hours and IFN-α and IL-10 were measured by specific ELISA. Results are shown in **Table 9 and 10**.

Table 9. Induction of IFN-α (pg/ml) by ODN 8954, 5569, and 5570

ODN	Conc. μg/ml	D521 Mean ± SD	D525 Mean ± SD	D526 Mean ± SD
2006	0.03	238.674	239.286	216.393
	0.06	2405.63	385.161	126.516
	0.125	3826.53	549.612	86.173
	0.25	2248.94	532.67	74.493
	1.0	362.74	161.892	57.087
8954	0.03	305.626	309.581	599.971
	0.06	6039.51	2028.52	4707.01
	0.125	7322.45	4669.31	5340.21
	0.25	7651.13	4641.1	5324.55
	1.0	7078.59	4679.59	5474.94

5569	0.03	112.784	121.422	87.751
	0.06	110.723	65.753	47.888
	0.125	104.547	49.365	41.046
	0.25	111.755	62.383	43.216
	1.0	2247.97	115.77	1101.58
5570	0.03	822.648	427.535	250.196
	0.06	1858.16	1021.18	218.201
	0.125	3470.67	1657.3	477.938
	0.25	5612.53	3369.99	669.706
	1.0	6798.3	3501.59	2560.93
Medium	--	145.436	214.212	66.853
	--	245.121	218.622	0

Table 10. Induction of IL10 (pg/ml) by ODN 8954, 10101-2, 5569, and 5570

ODN	Conc. µg/ml	D521 Mean ± SD	D525 Mean ± SD	D526 Mean ± SD
2006	0.03	151.976	112.414	485.823
	0.06	384.377	218.651	898.299
	0.125	404.352	242.289	991.614
	0.25	357.657	247.405	1150.94
	1.0	255.344	162.444	1171.72
8954	0.03	7.456	6.617	6.919
	0.06	5.34	5.721	19.787
	0.125	10.723	2.986	35.892
	0.25	15.308	13.056	67.18
	1.0	48.904	30.892	230.725
5569	0.03	0	1.287	1.348
	0.06	0	0.127	4.592
	0.125	18.815	3.615	62.963
	0.25	105.32	30.094	350.529
	1.0	256.785	136.833	1156.07
5570	0.03	0	0.31	5.867
	0.06	6.599	7.027	29.879
	0.125	98.553	38.528	455.145
	0.25	259.812	107.164	1169.46
	1.0	312.189	206.126	1595.63
Medium	--	1.755	10.543	0
	--	0.29	11.192	0

The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. The present invention is not to be limited in scope by examples provided, since the examples are intended as a single illustration of one aspect of the invention and other functionally equivalent embodiments are within the scope of the invention. Various modifications of the invention in addition to those shown and described

herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims. The advantages and objects of the invention are not necessarily encompassed by each embodiment of the invention.

All references, patents and patent publications that are recited in this application are incorporated in their entirety herein by reference.

We claim:

Claims

1. An immunostimulatory nucleic acid of 14-100 nucleotides in length comprising the formula:

5' X_1DCGHX_2 3'

wherein X_1 and X_2 are independently any sequence 0 to 10 nucleotides long, D is a nucleotide other than C, C is cytosine, G is guanine, H is a nucleotide other than G, further comprising a nucleic acid sequence selected from the group consisting of P and N positioned immediately 5' to X_1 or immediately 3' to X_2, and N is a B-cell neutralizing sequence, wherein N begins with a CGG trinucleotide and is at least 10 nucleotides long, and P is a GC-rich palindrome containing sequence at least 10 nucleotides long.

2. The nucleic acid of claim 1, wherein the immunostimulatory nucleic acid comprises 5' NX_1DCGHX_2 3'.

3. The nucleic acid of claim 1, wherein the immunostimulatory nucleic acid comprises 5' X_1DCGHX_2N 3'.

4. The nucleic acid of claim 1, wherein the immunostimulatory nucleic acid comprises 5' PX_1DCGHX_2 3'.

5. The nucleic acid of claim 1, wherein the immunostimulatory nucleic acid comprises 5' X_1DCGHX_2P 3'.

6. The nucleic acid of claim 1, wherein the immunostimulatory nucleic acid comprises 5' X_1DCGHX_2PX_3 3', wherein X_3 is any sequence 0 to 10 nucleotides long.

7. The nucleic acid of claim 1, wherein the immunostimulatory nucleic acid comprises 5' X_1DCGHPX_3 3', wherein X_3 is any sequence 0 to 10 nucleotides long.

8. The nucleic acid of claim 1, wherein the immunostimulatory nucleic acid comprises 5' DCGHX_2PX_3 3', wherein X_3 is any sequence 0 to 10 nucleotides long.

9. The nucleic acid of claim 1, wherein the immunostimulatory nucleic acid comprises 5' TCGHX_2PX_3 3', wherein X_3 is any sequence 0 to 10 nucleotides long.

10. The nucleic acid of claim 1, wherein the immunostimulatory nucleic acid comprises 5' DCGHPX_3 3', wherein X_3 is any sequence 0 to 10 nucleotides long.

11. The nucleic acid of claim 1, wherein the immunostimulatory nucleic acid comprises 5' DCGHP 3'.

12. The nucleic acid of claim 1, wherein D is thymine (T).

13. The nucleic acid of claim 1, wherein H is T.

14. The nucleic acid of claim 1, wherein H is T and X_2 is selected from the group consisting of CG, CGT, CGTT, CGTTT, and CGTTTT.

15. The nucleic acid of claim 1, wherein H is T and X_2 is CG.

16. The nucleic acid of claim 1, wherein H is T and X_2 is CGTTTT.

17. The nucleic acid of claim 1, wherein C is unmethylated.

18. The nucleic acid of claim 1, wherein N comprises at least four CG dinucleotides and no more than two CCG trinucleotides.

19. The nucleic acid of claim 1, wherein P includes at least one inosine.

20. The nucleic acid of claim 1, wherein the immunostimulatory nucleic acid has a nuclease-resistant backbone.

21. The nucleic acid of claim 1, wherein the immunostimulatory nucleic acid has a phosphorothioate backbone.

22. The nucleic acid of claim 1, further comprising a poly-T sequence at the 5' end.

23. The nucleic acid of claim 1, further comprising a poly-T sequence at the 3' end.

24. The nucleic acid of claim 1, wherein the immunostimulatory nucleic acid is 14-40 nucleotides in length.

25. The nucleic acid of claim 1, wherein the immunostimulatory nucleic acid is 14-30 nucleotides in length.

26. An immunostimulatory nucleic acid of 13-100 nucleotides in length comprising the formula:

5' N_1PyGN_2P 3'

wherein N_1 is any sequence 1 to 6 nucleotides long, Py is a pyrimidine, G is guanine, N_2 is any sequence 0 to 30 nucleotides long, and P is a GC-rich palindrome containing sequence at least 10 nucleotides long.

27. The nucleic acid of claim 26, wherein N_1 is at least 50% pyrimidines.

28. The nucleic acid of claim 26, wherein N_1 is at least 50% T.

29. The nucleic acid of claim 26, wherein N_1 includes at least one CG motif.

30. The nucleic acid of claim 26, wherein N_1 includes at least one TCG motif.

31. The nucleic acid of claim 26, wherein N_1 includes at least one CI motif.

32. The nucleic acid of claim 26, wherein N_1 includes at least one TCI motif.

33. The nucleic acid of claim 26, wherein N_1 includes at least one IG motif.

34. The nucleic acid of claim 26, wherein N_1 includes at least one TIG motif.

35. The nucleic acid of claim 26, wherein N_1 is TCGG.

36. The nucleic acid of claim 26, wherein N_1 is TCGH, wherein H is a nucleotide other than G.

37. The nucleic acid of claim 26, wherein Py is an unmethylated C.

38. The nucleic acid of claim 26, wherein N_2 is at least 50% pyrimidines.

39. The nucleic acid of claim 26, wherein N_2 is at least 50% T.

40. The nucleic acid of claim 26, wherein N_2 does not includes any poly G or poly A motifs.

41. The nucleic acid of claim 26, wherein N_1PyGN_2 is a sequence selected from the group consisting of TTTTTCG, TCG, TTCG, TTTCG, TTTTCG, TCGT, TTCGT, TTTCGT, and TCGTCGT.

42. The nucleic acid of claim 26, wherein the immunostimulatory nucleic acid has a completely nuclease-resistant backbone.

43. The nucleic acid of claim 26, wherein the nuclease-resistant backbone is composed of phosphorothioate linkages.

44. The nucleic acid of claim 26, wherein the immunostimulatory nucleic acid has a completely phosphodiester backbone.

45. The nucleic acid of claim 26, wherein the immunostimulatory nucleic acid has a chimeric backbone.

46. The nucleic acid of claim 45, wherein the immunostimulatory nucleic acid has at least one phosphodiester linkage between a CG, CI or IG motif.

47. The nucleic acid of claim 26, wherein P is completely palindromic.

48. The nucleic acid of claim 26, wherein P is a palindrome having between 1 and 3 consecutive intervening nucleotides.

49. The nucleic acid of claim 48, wherein the intervening nucleotides are TG.

50. The nucleic acid of claim 26, wherein P includes at least 3 C and at least 3 G nucleotides.

51. The nucleic acid of claim 26, wherein P includes at least 4 C and at least 4 G nucleotides.

52. The nucleic acid of claim 26, wherein P includes at least 5 C and at least 5 G nucleotides.

53. The nucleic acid of claim 26, wherein P includes at least one inosine.

54. The nucleic acid of claim 26, wherein the immunostimulatory nucleic acid is 13-40 nucleotides in length.

55. The nucleic acid of claim 26, wherein the immunostimulatory nucleic acid is 13-30 nucleotides in length.

56. An immunostimulatory nucleic acid of 13-100 nucleotides in length comprising the formula:

5' N_1PyG/IN_2P 3'

wherein N_1 is any sequence 1 to 6 nucleotides long, Py is a pyrimidine, G/I refers to single nucleotide which is either a G or an I, G is guanine and I is inosine, N_2 is any sequence 0 to 30 nucleotides long, and P is a palindrome containing sequence at least 10 nucleotides long.

57. The nucleic acid of claim 56, wherein N_1PyIN_2 is TCITCITTTT (SEQ ID NO: 47).

58. The nucleic acid of claim 56, wherein P is a GC-rich palindrome.

59. The nucleic acid of claim 56, wherein P is an IC-rich palindrome.

60. The nucleic acid of claim 56, wherein G/I is G.

61. The nucleic acid of claim 56, wherein G/I is I.

62. The nucleic acid of claim 56, wherein the immunostimulatory nucleic acid is 13-30 nucleotides in length.

63. A pharmaceutical composition, comprising an immunostimulatory nucleic acid of any one of claims 1-62, and a pharmaceutically acceptable carrier.

64. A method for inducing type 1 interferon (IFN) expression, comprising contacting a cell capable of expressing type 1 IFN with an effective amount of an immunostimulatory nucleic acid of any one of claims 1 – 62 to induce expression of type 1 IFN.

65. A method for activating a natural killer (NK) cell, comprising contacting an NK cell with an effective amount of an immunostimulatory nucleic acid of any one of claims 1 – 62., to activate the NK cell.

66. A method for treating infection, comprising

administering to a subject having or at risk of developing an infection an effective amount of an immunostimulatory nucleic acid of any one of claims 1 – 62, to treat or prevent the infection.

67. The method of claim 66, wherein the subject has or is at risk of developing an infection selected from the group consisting of a viral, bacterial, fungal and parasitic infection.

68. A method for treating an allergic condition, comprising

administering to a subject having or at risk of developing an allergic condition an effective amount of an immunostimulatory nucleic acid of any one of claims 1 – 62, to treat or prevent the allergic condition.

69. The method of claim 68, wherein the allergic condition is allergic asthma.

70. A method for treating cancer, comprising

administering to a subject having or at risk of developing a cancer an effective amount of an immunostimulatory nucleic acid of any one of claims 1 – 62, to treat or prevent the cancer.

71. The method of claim 70, wherein the cancer is selected from the group consisting of basal cell carcinoma, biliary tract cancer; bladder cancer; bone cancer; brain and CNS cancer; breast cancer; cervical cancer; choriocarcinoma; colon and rectum cancer; connective tissue cancer; cancer of the digestive system; endometrial cancer; esophageal cancer; eye cancer; cancer of the head and neck; gastric cancer; intra-epithelial neoplasm; kidney cancer; larynx cancer; leukemia; liver cancer; lung cancer; lymphoma including Hodgkin's and Non-Hodgkin's lymphoma; melanoma; myeloma; neuroblastoma; oral cavity cancer; ovarian cancer; pancreatic cancer; prostate cancer; retinoblastoma; rhabdomyosarcoma; rectal cancer; renal cancer; cancer of the respiratory system; sarcoma; skin cancer; stomach cancer; testicular cancer; thyroid cancer; uterine cancer; cancer of the urinary system, and other carcinomas and sarcomas

72. An immunostimulatory nucleic acid comprising a sequence selected from the group consisting of:

TCGTCGTTTTCGGCGCGCGCCG (SEQ ID NO: 1),

TCGTCGTTTTCGGCGGCCGCCG (SEQ ID NO: 4),

TCGTCGTTTTCGGCGCGCCGCG (SEQ ID NO: 5),

TCGTCGTTTTCGGCGCCGGCCG (SEQ ID NO: 6),

TCGTCGTTTTCGGCCCGCGCGG (SEQ ID NO: 7),

TCGTCGTTTTCGGCGCGCGCCGTTTTT (SEQ ID NO: 12),

TCCTGACGTTCGGCGCGCGCCG (SEQ ID NO: 13),

TZGTZGTTTTZGGZGZGZGZZG (SEQ ID NO: 14), wherein Z is 5-methylcytosine,

TCCTGACGTTCGGCGCGCGCCC (SEQ ID NO: 19), and

TCGGCGCGCGCCGTCGTCGTTT (SEQ ID NO: 11).

163163

G00002

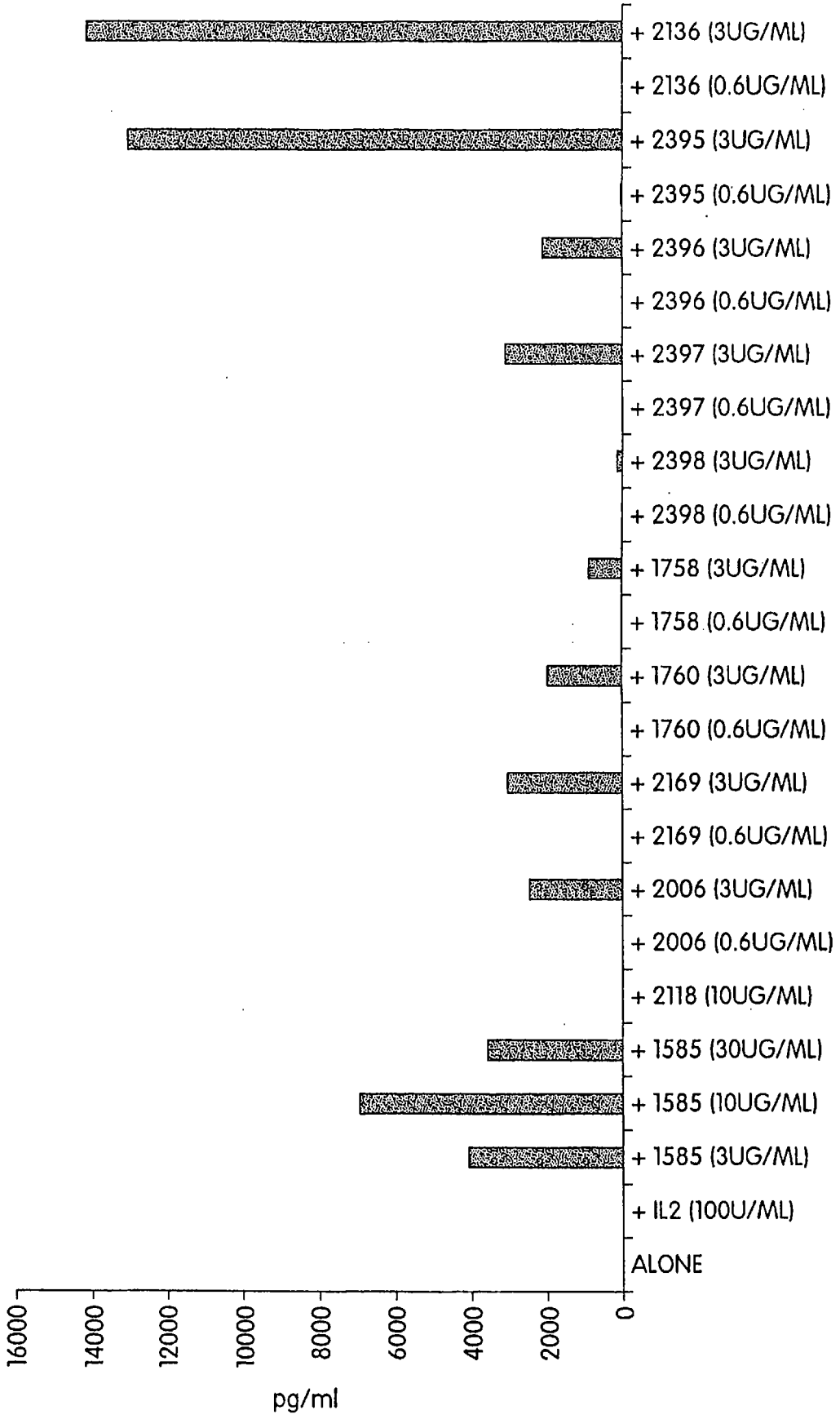


Fig. 1

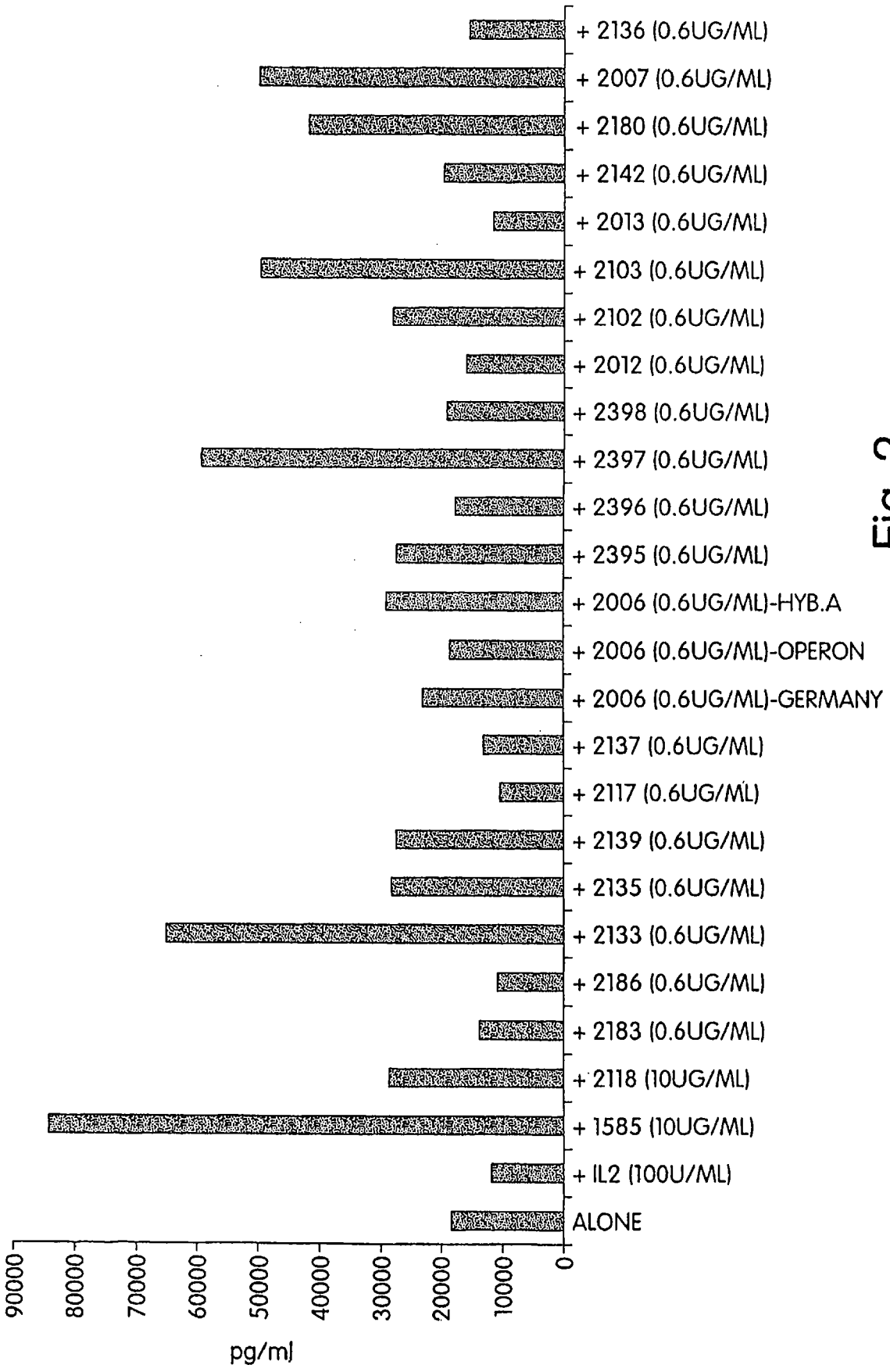

+ 2136 (0.6UG/ML)
+ 2007 (0.6UG/ML)
+ 2180 (0.6UG/ML)
+ 2142 (0.6UG/ML)
+ 2013 (0.6UG/ML)
+ 2103 (0.6UG/ML)
+ 2102 (0.6UG/ML)
+ 2012 (0.6UG/ML)
+ 2398 (0.6UG/ML)
+ 2397 (0.6UG/ML)
+ 2396 (0.6UG/ML)
+ 2395 (0.6UG/ML)
+ 2006 (0.6UG/ML)-HYB.A
+ 2006 (0.6UG/ML)-OPERON
+ 2006 (0.6UG/ML)-GERMANY
+ 2137 (0.6UG/ML)
+ 2117 (0.6UG/ML)
+ 2139 (0.6UG/ML)
+ 2135 (0.6UG/ML)
+ 2133 (0.6UG/ML)
+ 2186 (0.6UG/ML)
+ 2183 (0.6UG/ML)
+ 2118 (10UG/ML)
+ 1585 (10UG/ML)
+ IL2 (100U/ML)
ALONE
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
pg/ml

Fig. 2

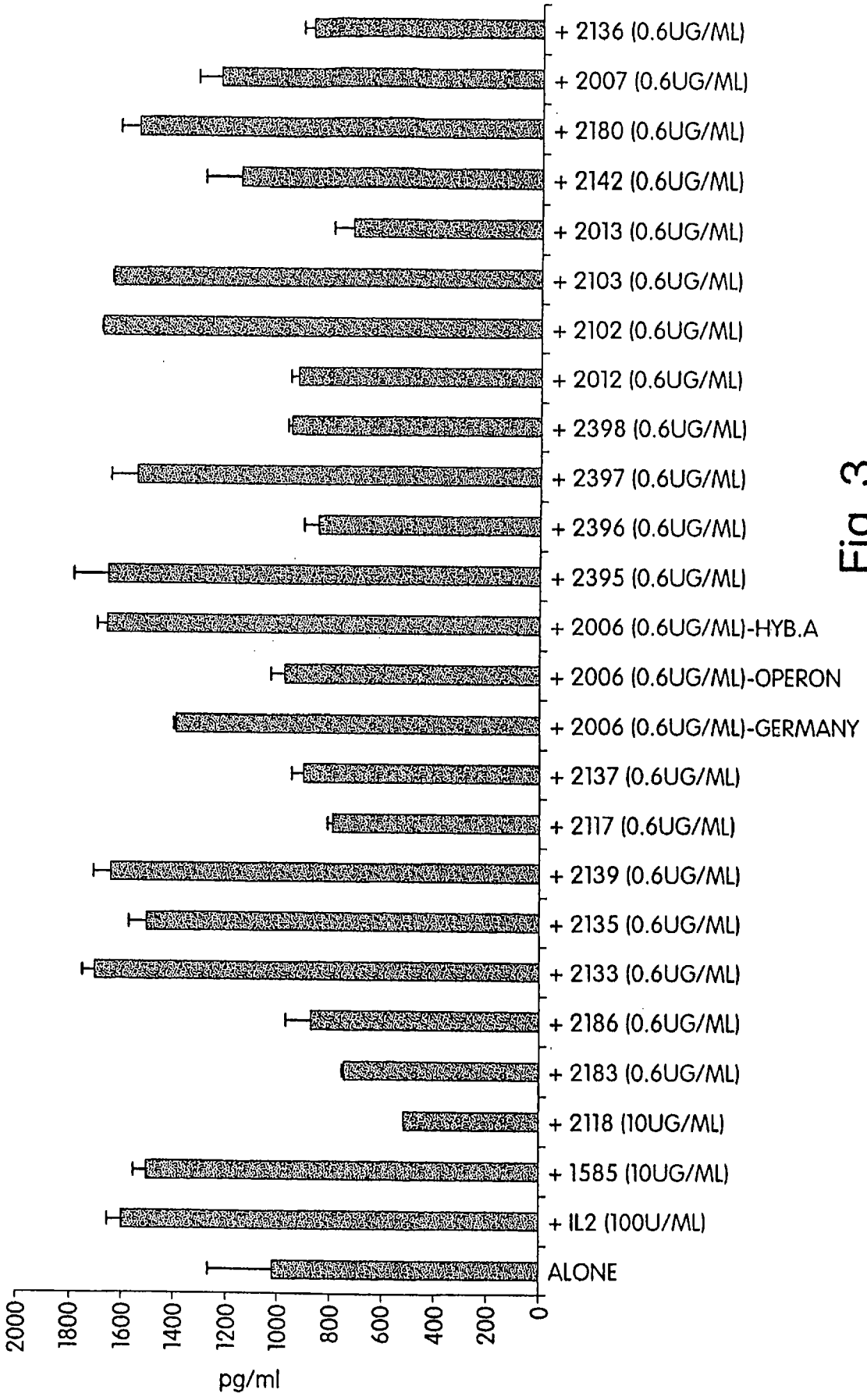

+ 2136 (0.6UG/ML)
+ 2007 (0.6UG/ML)
+ 2180 (0.6UG/ML)
+ 2142 (0.6UG/ML)
+ 2013 (0.6UG/ML)
+ 2103 (0.6UG/ML)
+ 2102 (0.6UG/ML)
+ 2012 (0.6UG/ML)
+ 2398 (0.6UG/ML)
+ 2397 (0.6UG/ML)
+ 2396 (0.6UG/ML)
+ 2395 (0.6UG/ML)
+ 2006 (0.6UG/ML)-HYB.A
+ 2006 (0.6UG/ML)-OPERON
+ 2006 (0.6UG/ML)-GERMANY
+ 2137 (0.6UG/ML)
+ 2117 (0.6UG/ML)
+ 2139 (0.6UG/ML)
+ 2135 (0.6UG/ML)
+ 2133 (0.6UG/ML)
+ 2186 (0.6UG/ML)
+ 2183 (0.6UG/ML)
+ 2118 (10UG/ML)
+ 1585 (10UG/ML)
+ IL2 (100U/ML)
ALONE
2000
1800
1600
1400
1200
1000
800
600
400
200
0
pg/ml

Fig. 3

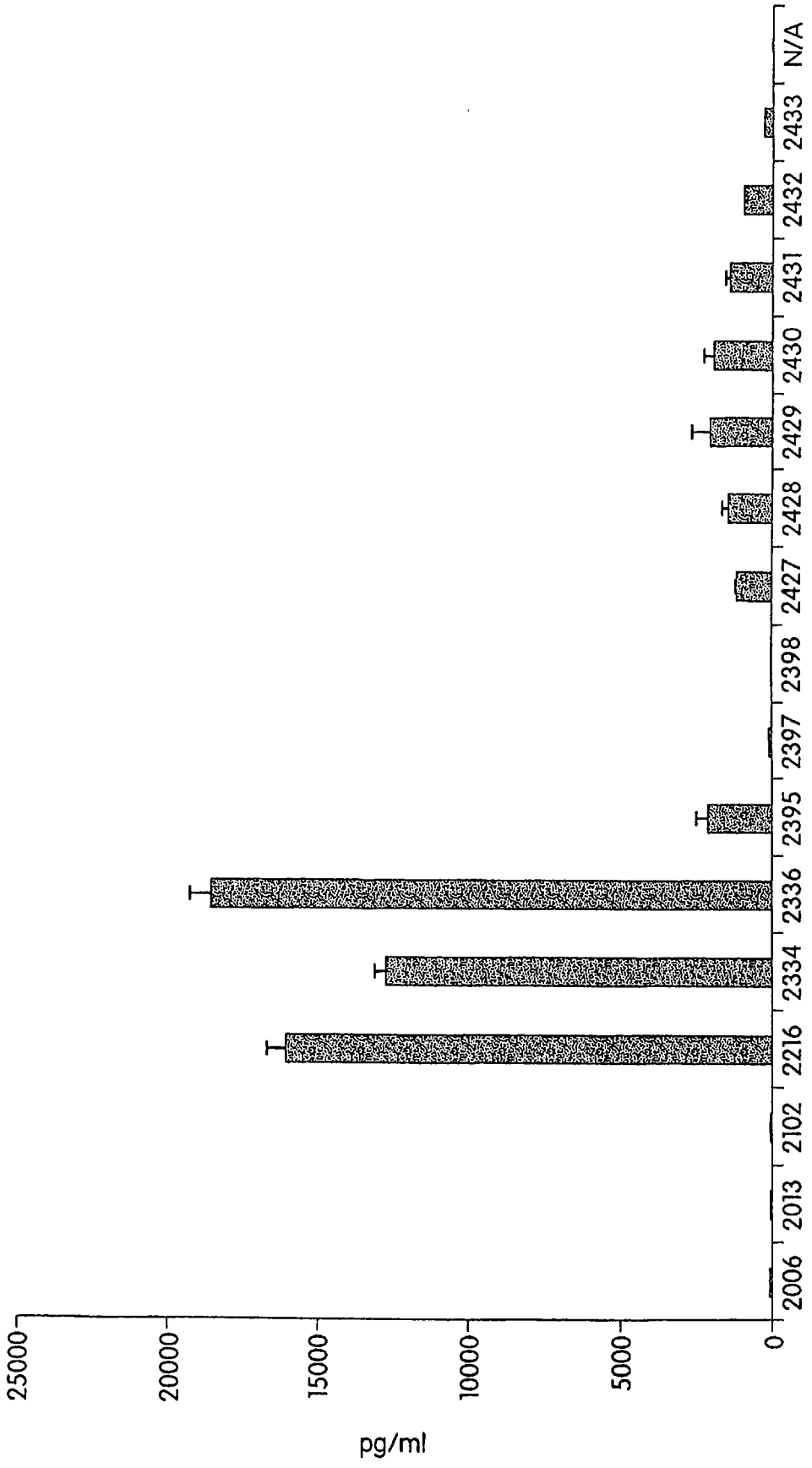

25000
20000
15000
10000
5000
0
pg/ml
2006
2013
2102
2216
2334
2336
2395
2397
2398
2427
2428
2429
2430
2431
2432
2433
N/A
ODN @ 1.0 ug/ml

Fig. 4

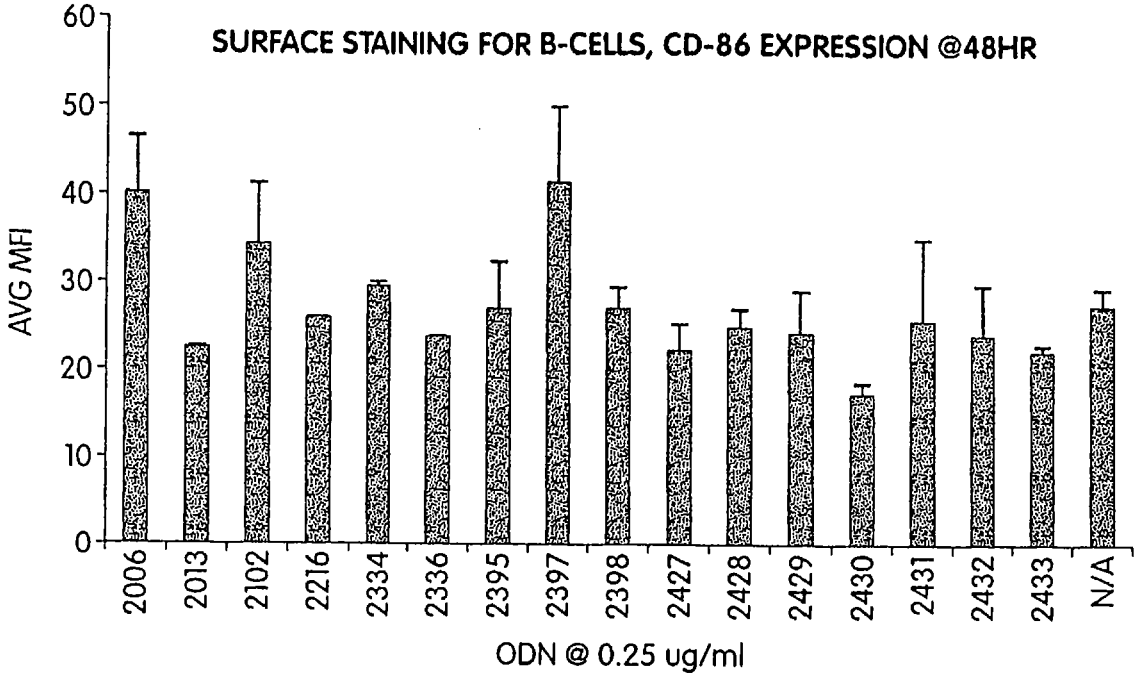

SURFACE STAINING FOR B-CELLS, CD-86 EXPRESSION @48HR
AVG MFI
60
50
40
30
20
10
0
2006
2013
2102
2216
2334
2336
2395
2397
2398
2427
2428
2429
2430
2431
2432
2433
N/A
ODN @ 0.25 ug/ml

Fig. 5A

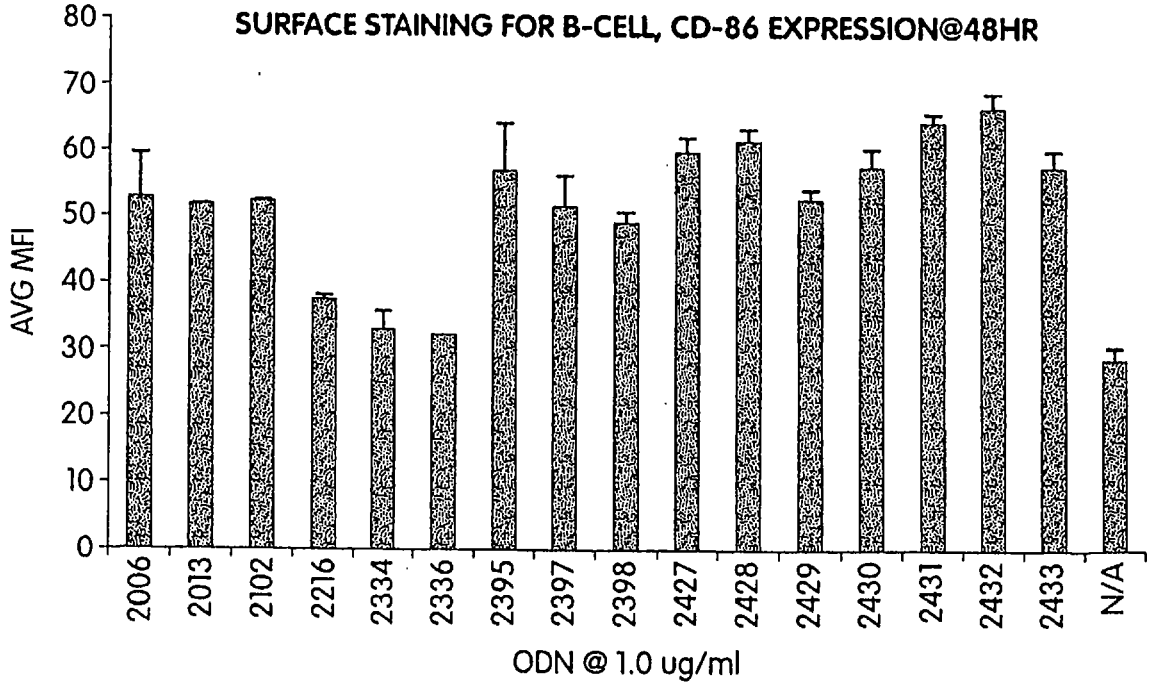

SURFACE STAINING FOR B-CELL, CD-86 EXPRESSION@48HR
AVG MFI
80
70
60
50
40
30
20
10
0
2006
2013
2102
2216
2334
2336
2395
2397
2398
2427
2428
2429
2430
2431
2432
2433
N/A
ODN @ 1.0 ug/ml

Fig. 5B

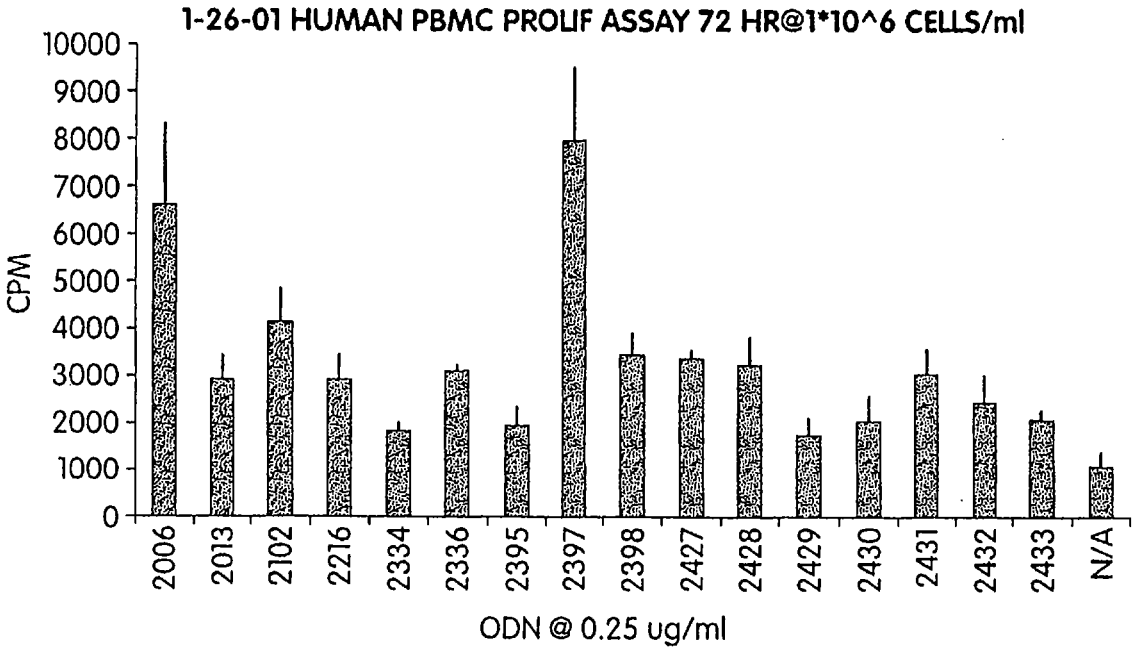

1-26-01 HUMAN PBMC PROLIF ASSAY 72 HR@1*10^6 CELLS/ml
CPM
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
2006
2013
2102
2216
2334
2336
2395
2397
2398
2427
2428
2429
2430
2431
2432
2433
N/A
ODN @ 0.25 ug/ml

Fig. 6A

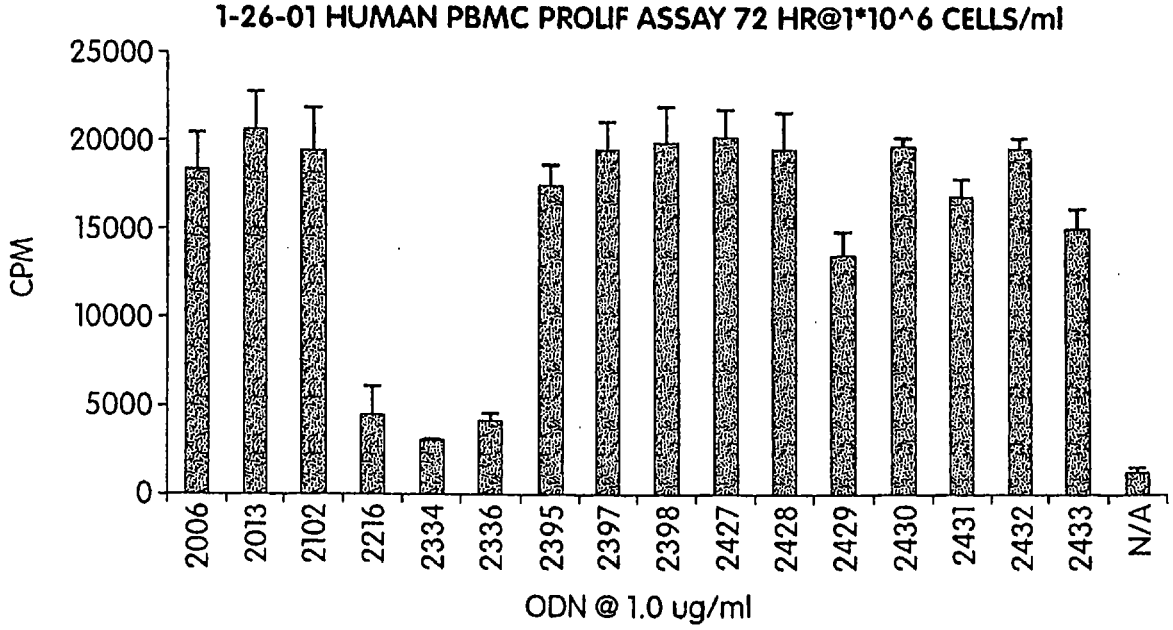

1-26-01 HUMAN PBMC PROLIF ASSAY 72 HR@1*10^6 CELLS/ml
CPM
25000
20000
15000
10000
5000
0
2006
2013
2102
2216
2334
2336
2395
2397
2398
2427
2428
2429
2430
2431
2432
2433
N/A
ODN @ 1.0 ug/ml

Fig. 6B

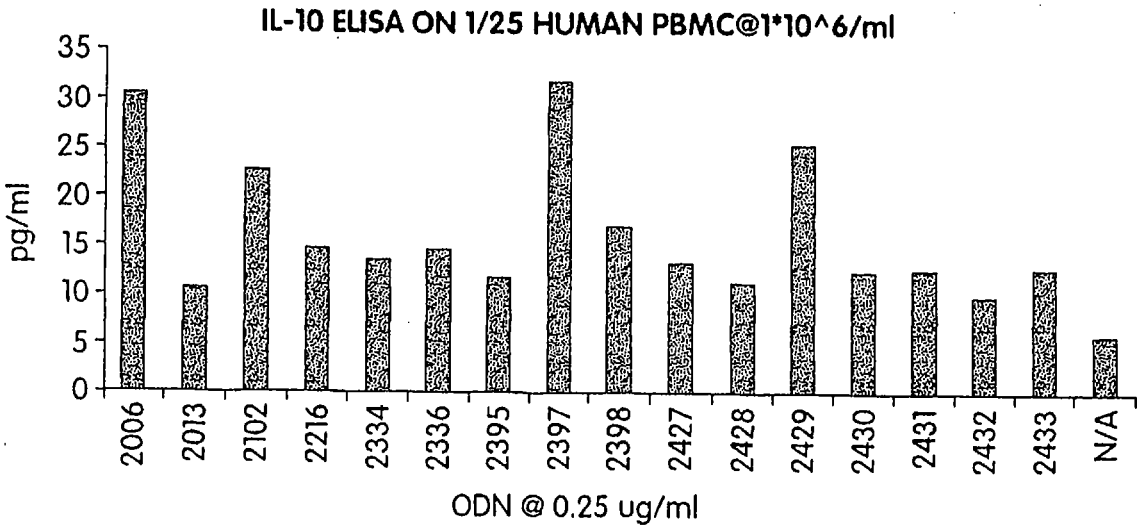

IL-10 ELISA ON 1/25 HUMAN PBMC@1*10^6/ml
pg/ml
35
30
25
20
15
10
5
0
2006
2013
2102
2216
2334
2336
2395
2397
2398
2427
2428
2429
2430
2431
2432
2433
N/A
ODN @ 0.25 ug/ml

Fig. 7A

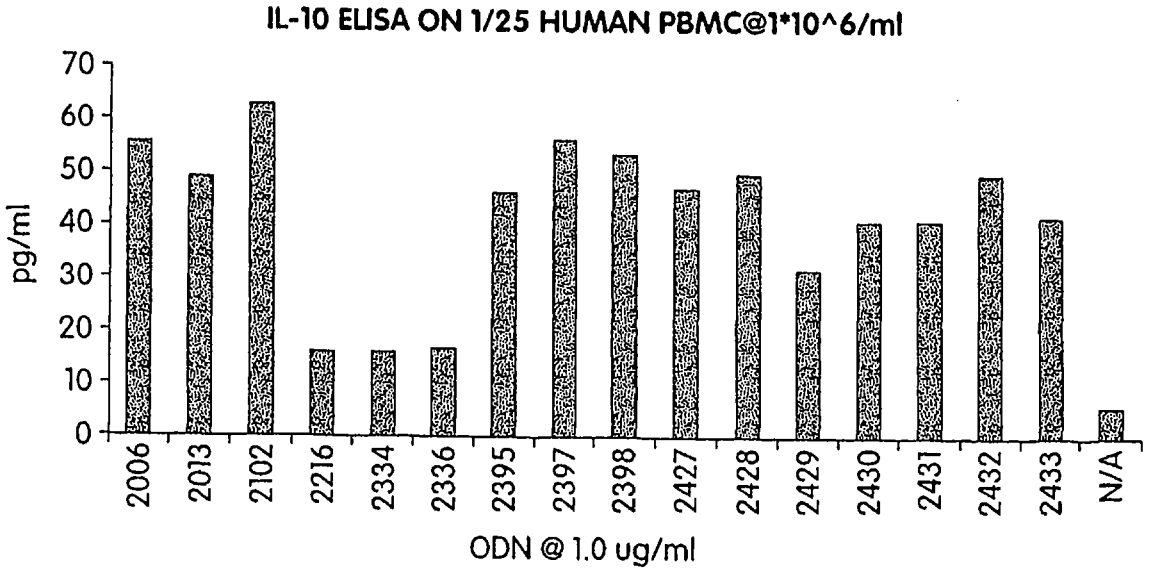

IL-10 ELISA ON 1/25 HUMAN PBMC@1*10^6/ml
pg/ml
70
60
50
40
30
20
10
0
2006
2013
2102
2216
2334
2336
2395
2397
2398
2427
2428
2429
2430
2431
2432
2433
N/A
ODN @ 1.0 ug/ml

Fig. 7B

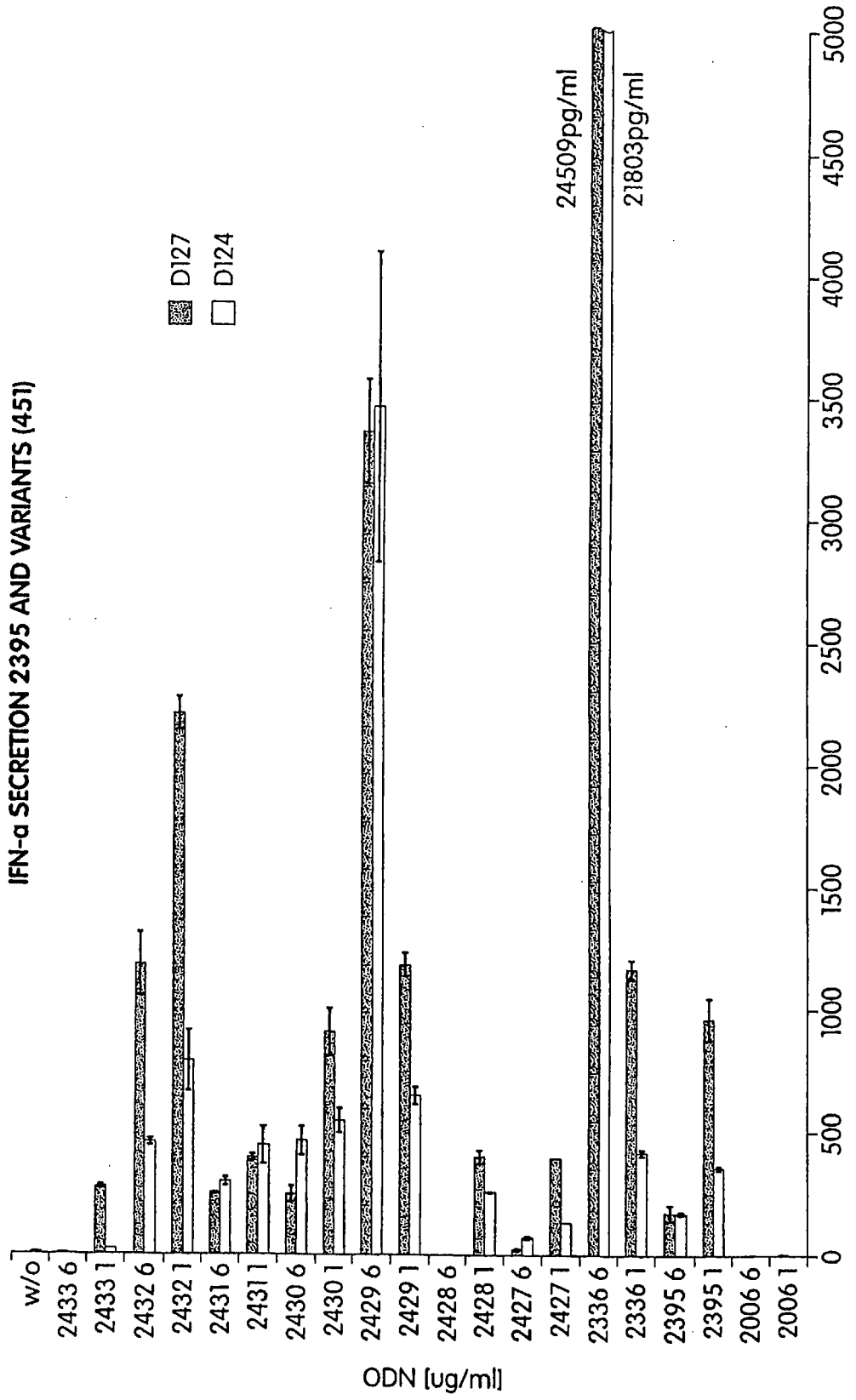

IFN-α SECRETION 2395 AND VARIANTS (451)
D127
D124
24509pg/ml
21803pg/ml
w/o
2433 6
2433 1
2432 6
2432 1
2431 6
2431 1
2430 6
2430 1
2429 6
2429 1
2428 6
2428 1
2427 6
2427 1
2336 6
2336 1
2395 6
2395 1
2006 6
2006 1
ODN [ug/ml]
0
500
1000
1500
2000
2500
3000
3500
4000
4500
5000
IFN-α [pg/ml]

Fig. 8

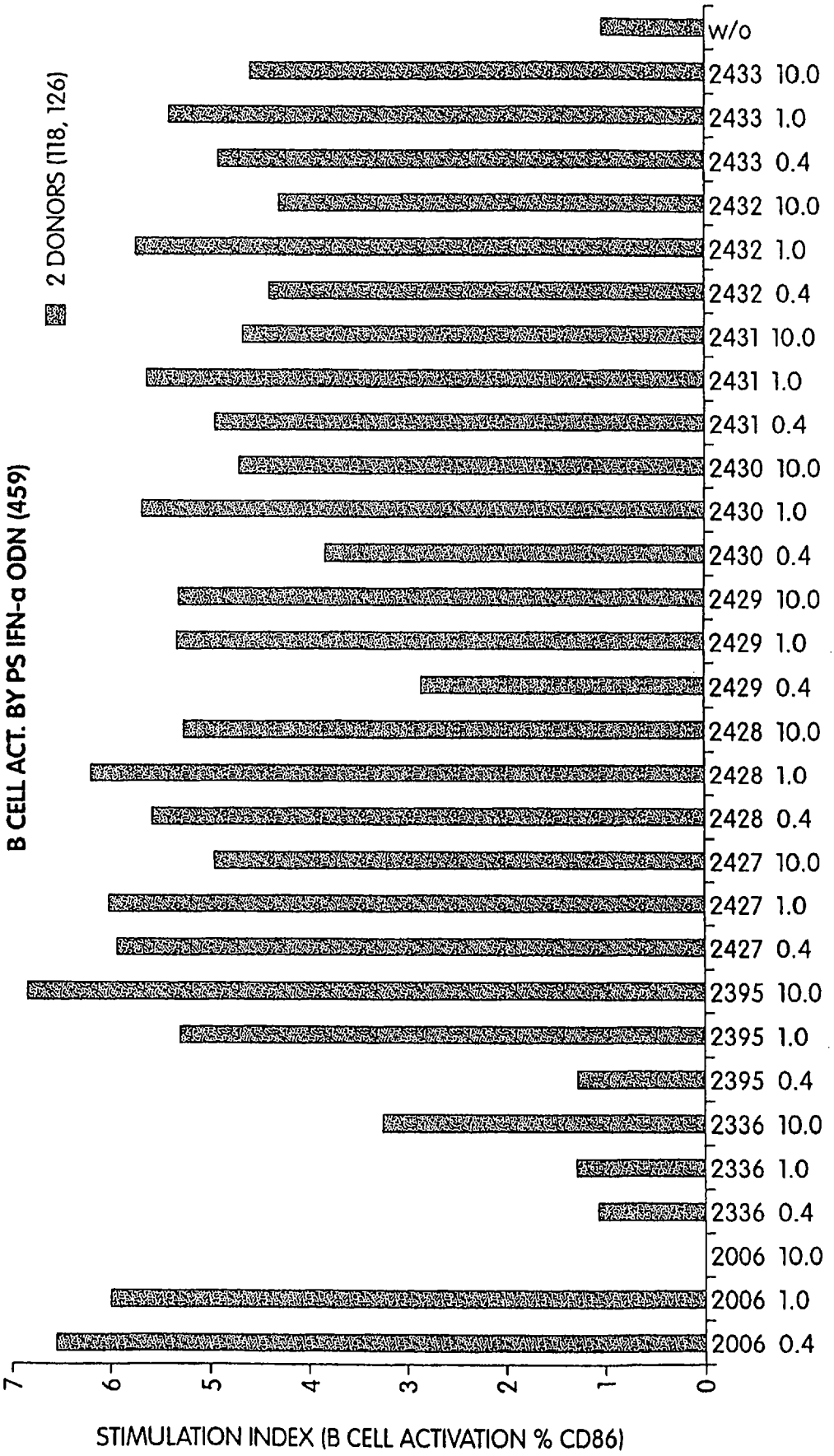

B CELL ACT. BY PS IFN-α ODN (459)
2 DONORS (118, 126)
w/o
2433 10.0
2433 1.0
2433 0.4
2432 10.0
2432 1.0
2432 0.4
2431 10.0
2431 1.0
2431 0.4
2430 10.0
2430 1.0
2430 0.4
2429 10.0
2429 1.0
2429 0.4
2428 10.0
2428 1.0
2428 0.4
2427 10.0
2427 1.0
2427 0.4
2395 10.0
2395 1.0
2395 0.4
2336 10.0
2336 1.0
2336 0.4
2006 10.0
2006 1.0
2006 0.4
ODN [ug/ml]
0
1
2
3
4
5
6
7
STIMULATION INDEX (B CELL ACTIVATION % CD86)

Fig. 9

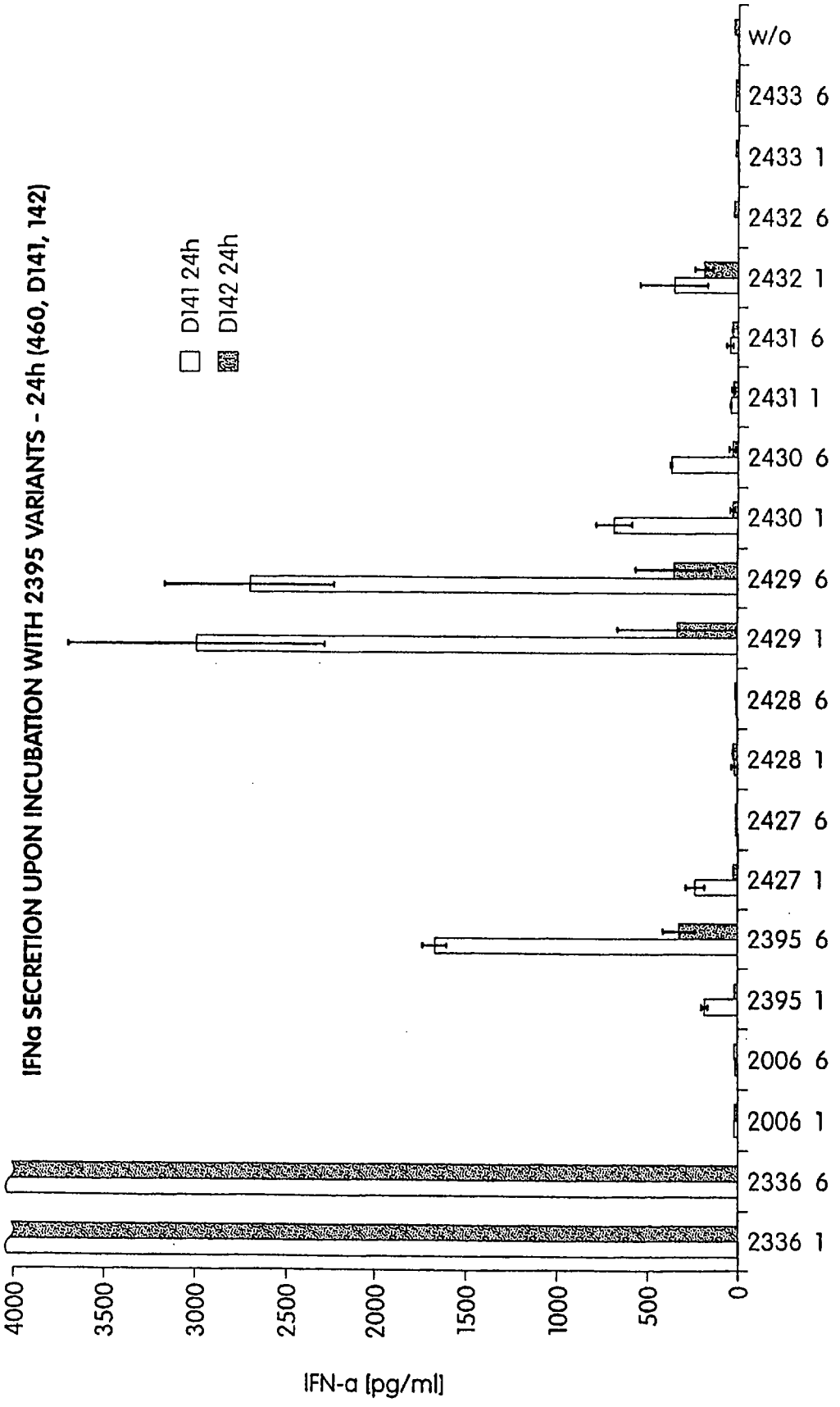

IFNa SECRETION UPON INCUBATION WITH 2395 VARIANTS - 24h (460, D141, 142)
D141 24h
D142 24h
w/o
2433 6
2433 1
2432 6
2432 1
2431 6
2431 1
2430 6
2430 1
2429 6
2429 1
2428 6
2428 1
2427 6
2427 1
2395 6
2395 1
2006 6
2006 1
2336 6
2336 1
4000
3500
3000
2500
2000
1500
1000
500
0
IFN-a [pg/ml]
ODN [ug/ml]

Fig. 10

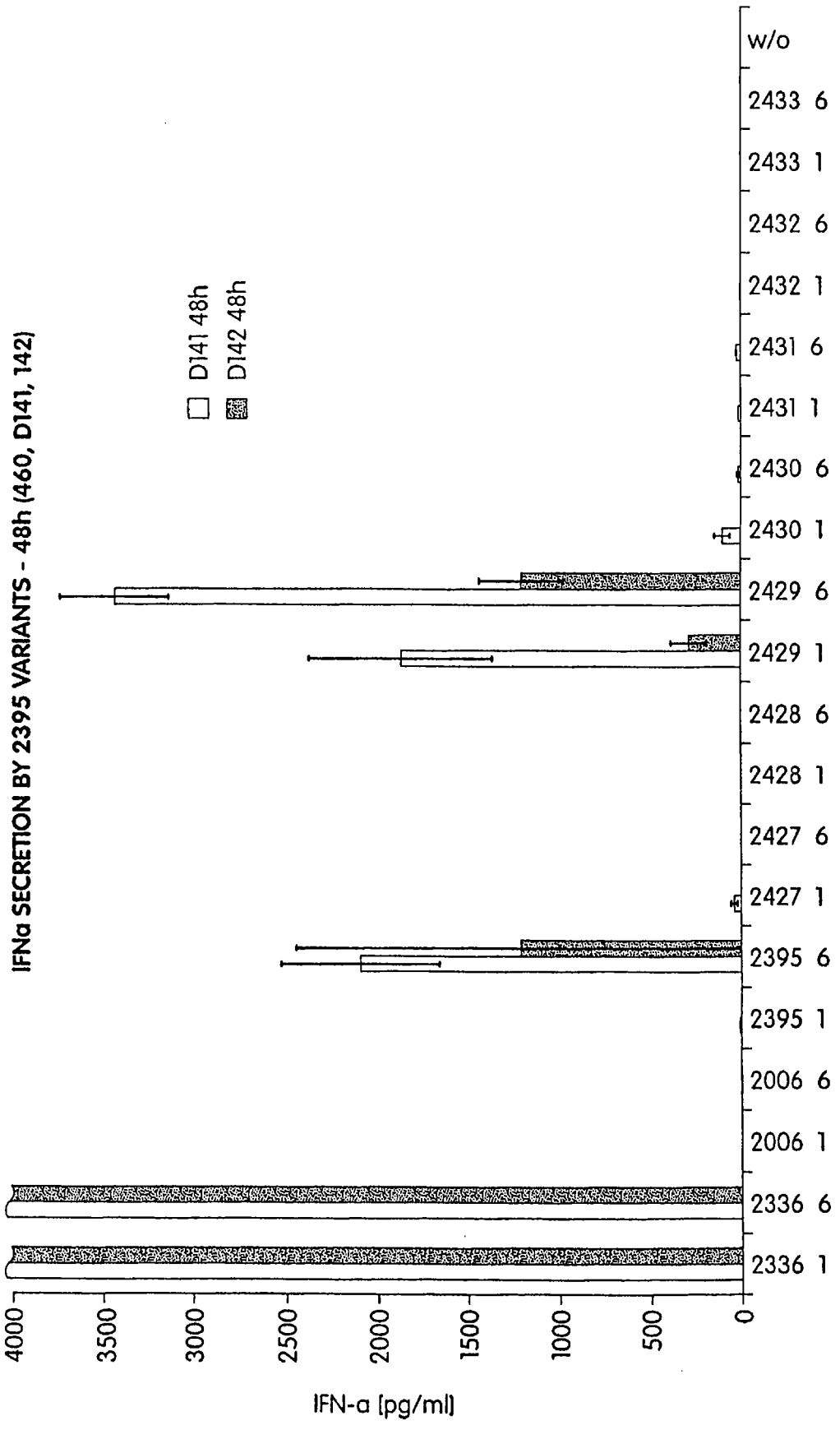

IFNa SECRETION BY 2395 VARIANTS - 48h (460, D141, 142)
D141 48h
D142 48h
w/o
2433 6
2433 1
2432 6
2432 1
2431 6
2431 1
2430 6
2430 1
2429 6
2429 1
2428 6
2428 1
2427 6
2427 1
2395 6
2395 1
2006 6
2006 1
2336 6
2336 1
ODN [ug/ml]
4000
3500
3000
2500
2000
1500
1000
500
0
IFN-a [pg/ml]

Fig. 11

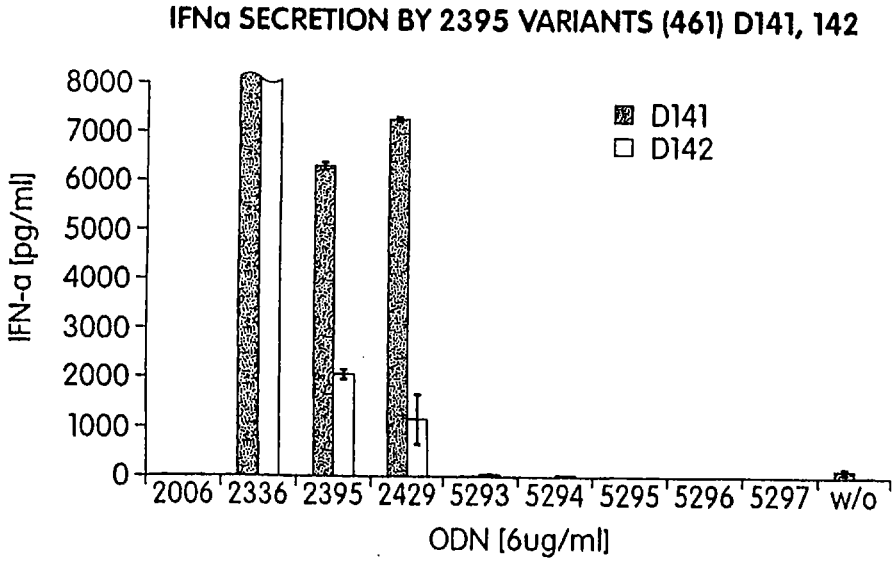

IFNα SECRETION BY 2395 VARIANTS (461) D141, 142
IFN-α [pg/ml]
8000
7000
6000
5000
4000
3000
2000
1000
0
D141
D142
2006
2336
2395
2429
5293
5294
5295
5296
5297
w/o
ODN [6ug/ml]

Fig. 12

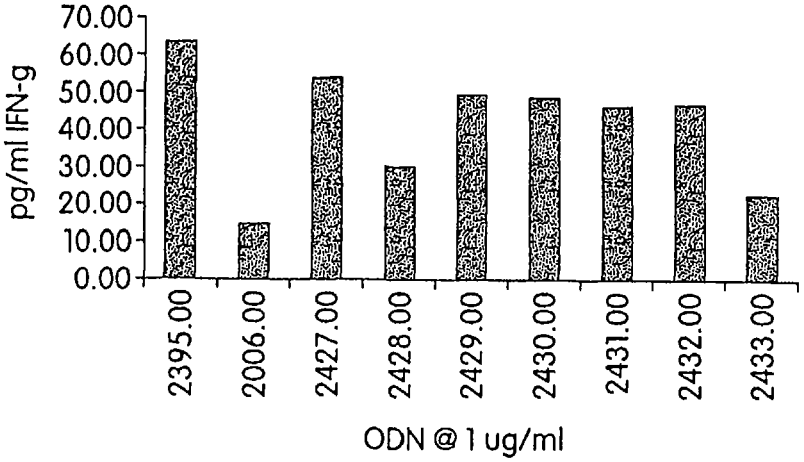


Fig. 13A

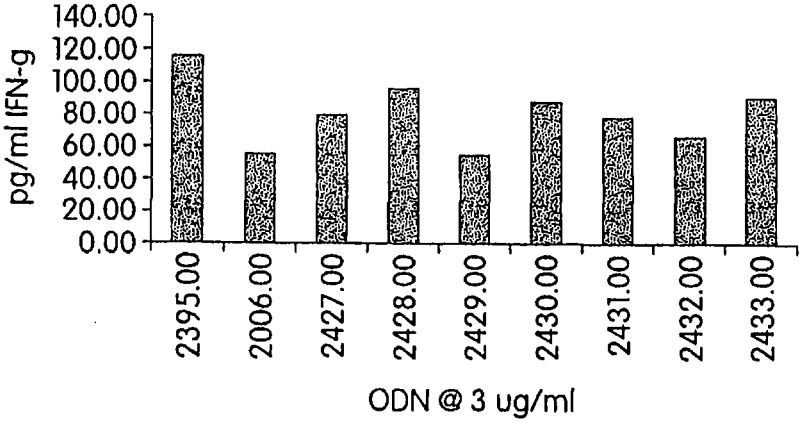


Fig. 13B

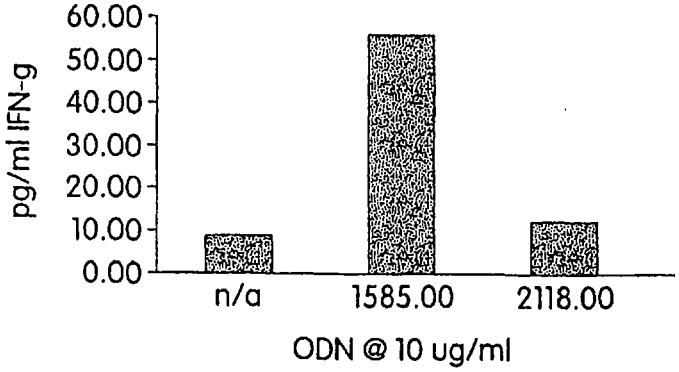


Fig. 13C

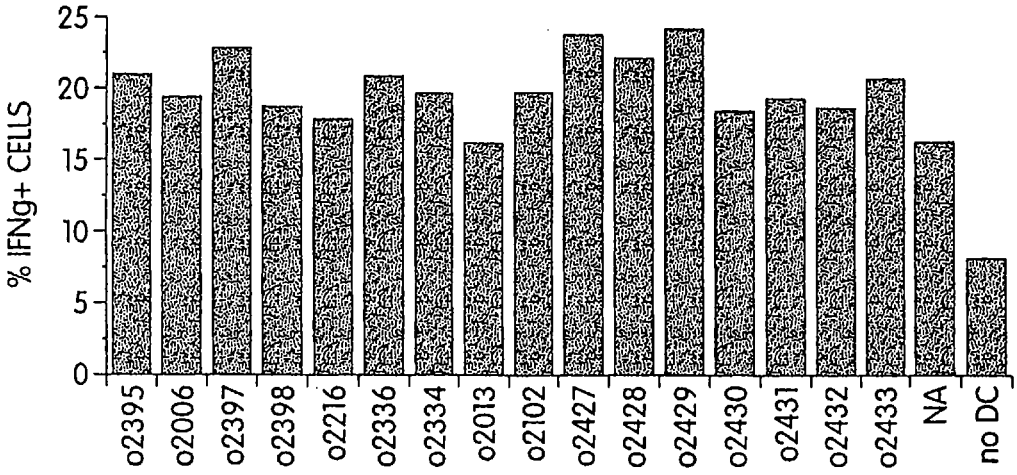

% IFNg+ CELLS
25
20
15
10
5
0
o2395
o2006
o2397
o2398
o2216
o2336
o2334
o2013
o2102
o2427
o2428
o2429
o2430
o2431
o2432
o2433
NA
no DC

Fig. 14

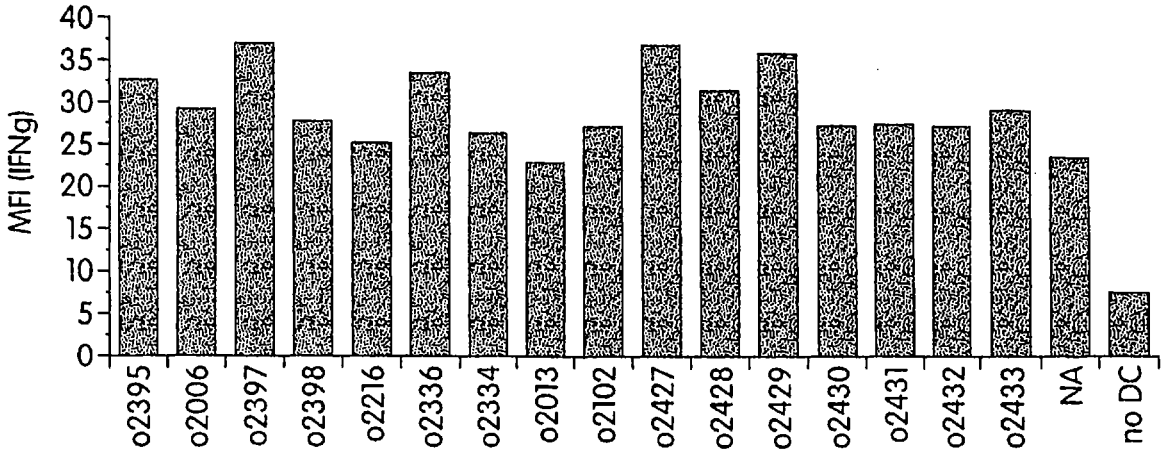

MFI (IFNg)
40
35
30
25
20
15
10
5
0
o2395
o2006
o2397
o2398
o2216
o2336
o2334
o2013
o2102
o2427
o2428
o2429
o2430
o2431
o2432
o2433
NA
no DC

Fig. 15

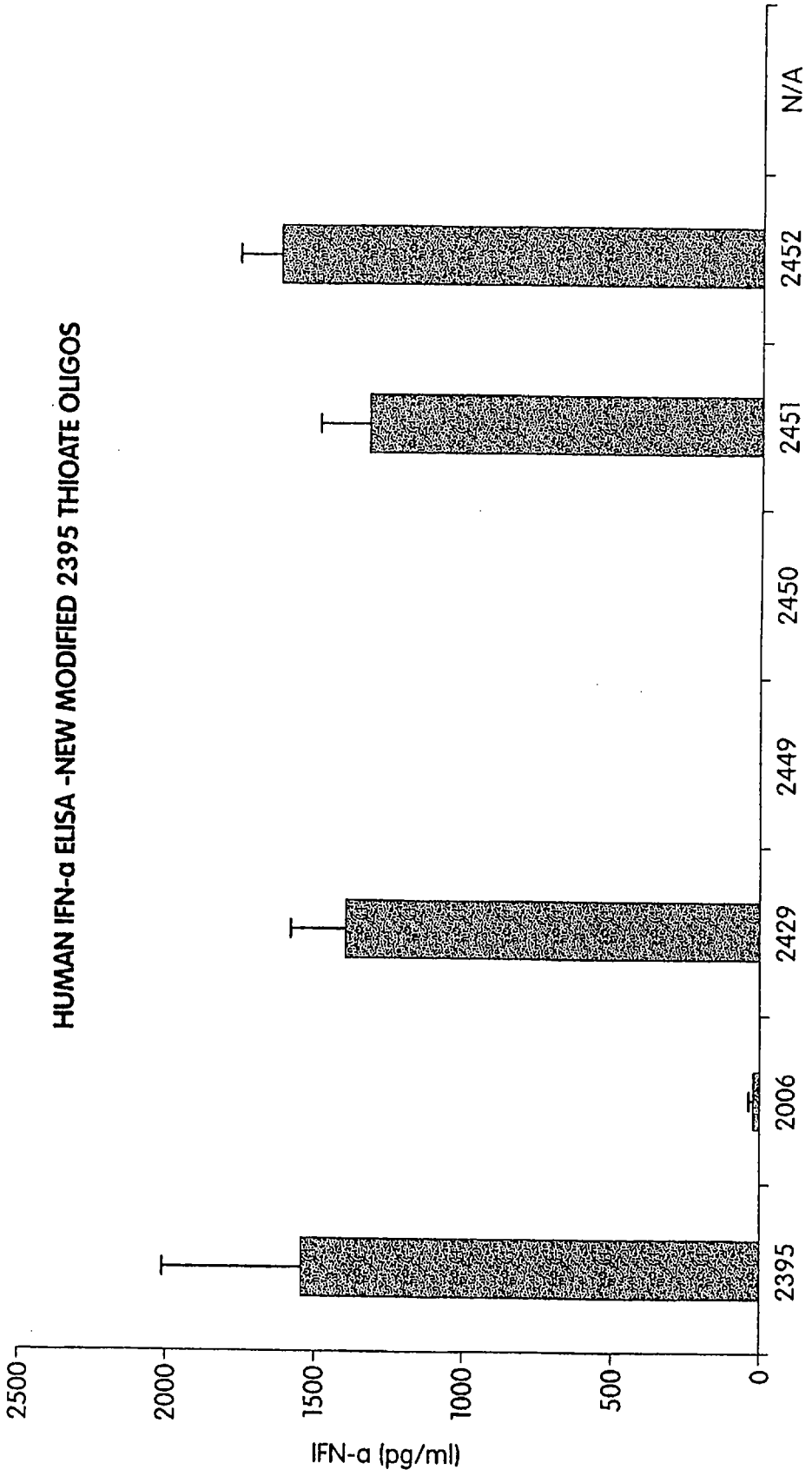

HUMAN IFN-α ELISA -NEW MODIFIED 2395 THIOATE OLIGOS
IFN-α (pg/ml)
2500
2000
1500
1000
500
0
2395
2006
2429
2449
2450
2451
2452
N/A
ODN @ 1.0 ug/ml

Fig. 16

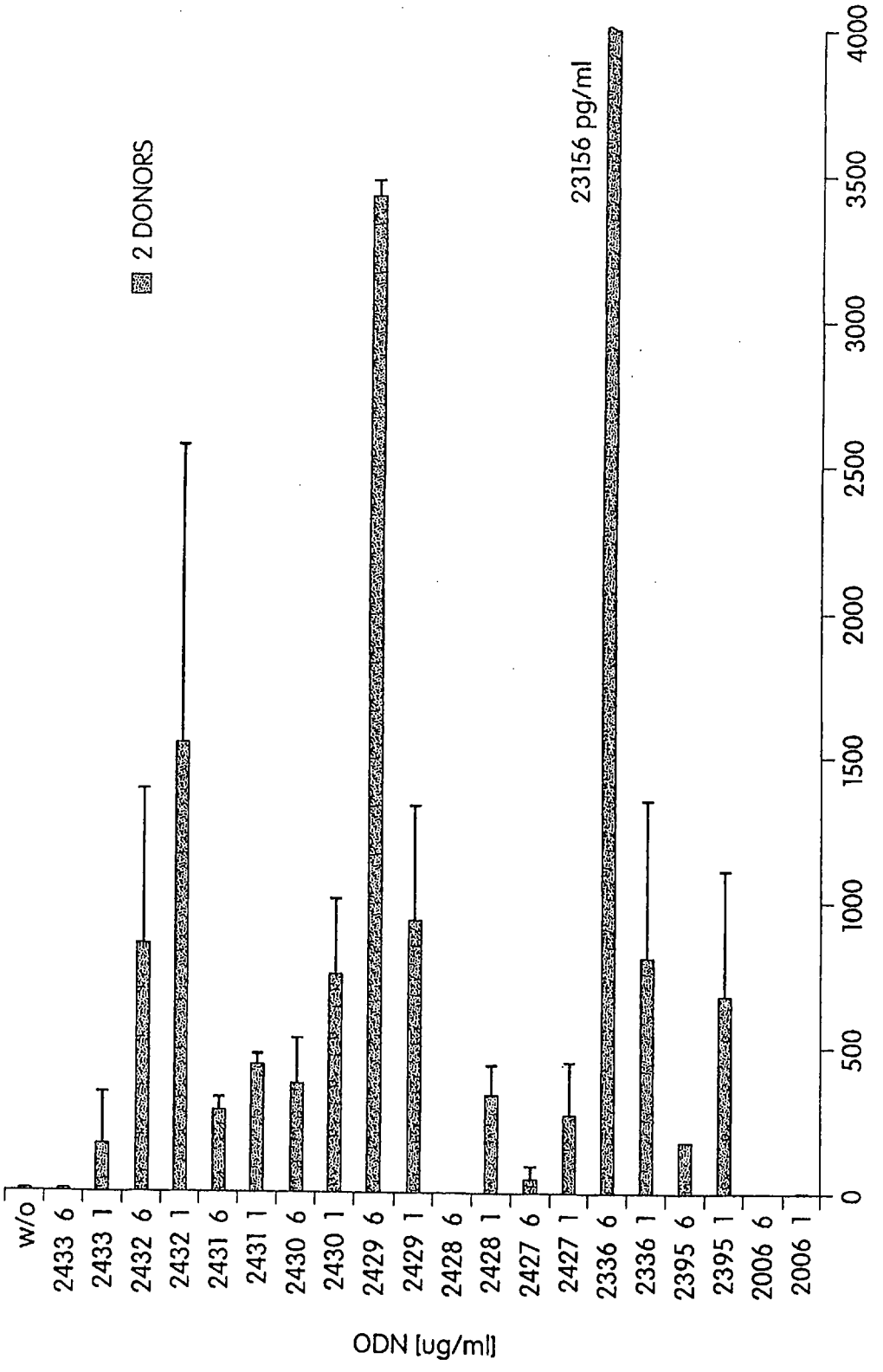

2 DONORS
23156 pg/ml
w/o
2433 6
2433 1
2432 6
2432 1
2431 6
2431 1
2430 6
2430 1
2429 6
2429 1
2428 6
2428 1
2427 6
2427 1
2336 6
2336 1
2395 6
2395 1
2006 6
2006 1
ODN [ug/ml]
0
500
1000
1500
2000
2500
3000
3500
4000
IFN-α [pg/ml]

Fig. 17A

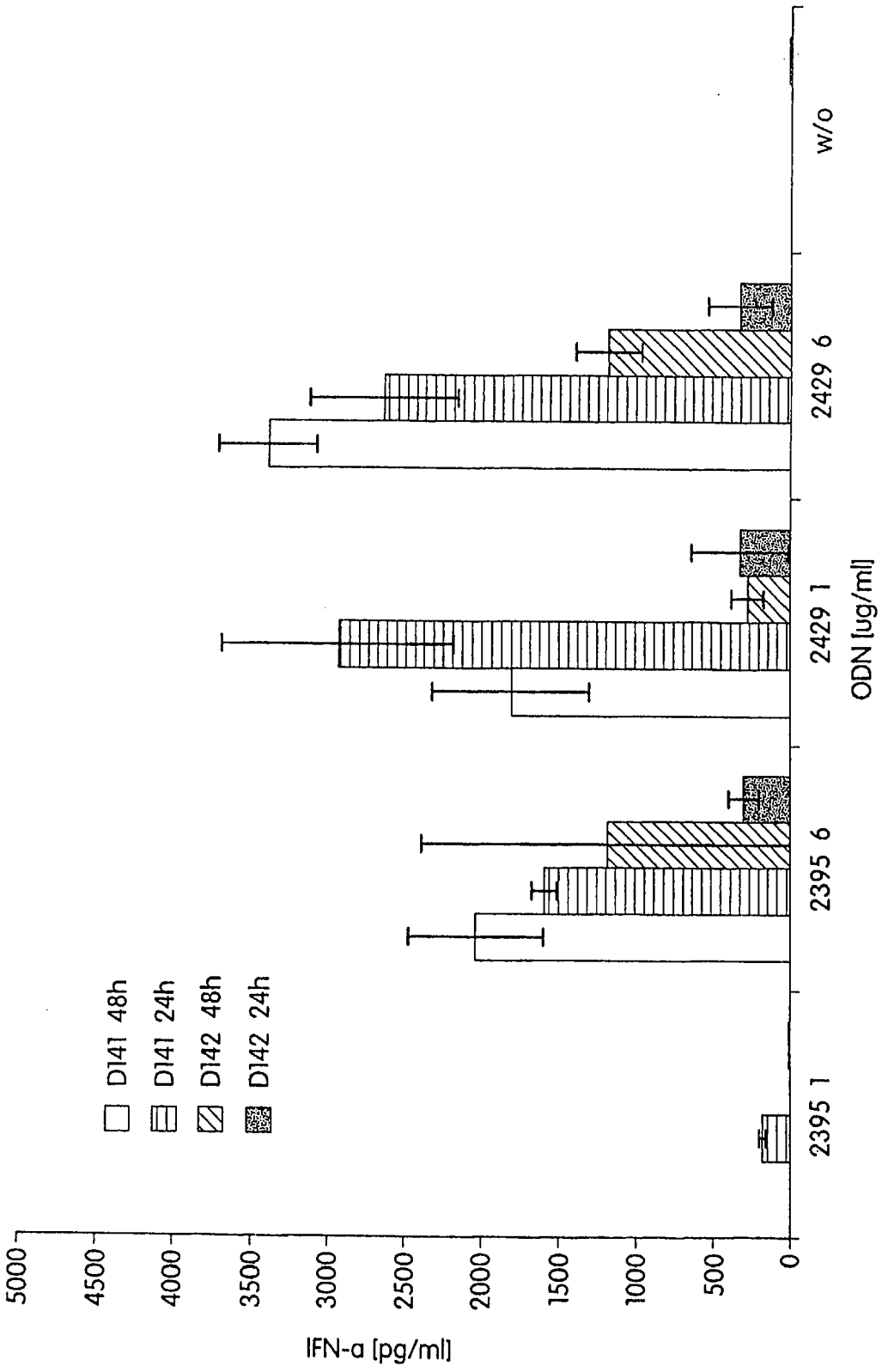

IFN-α [pg/ml]
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
D141 48h
D141 24h
D142 48h
D142 24h
2395 1
2395 6
2429 1
2429 6
w/o
ODN [ug/ml]

Fig. 17B

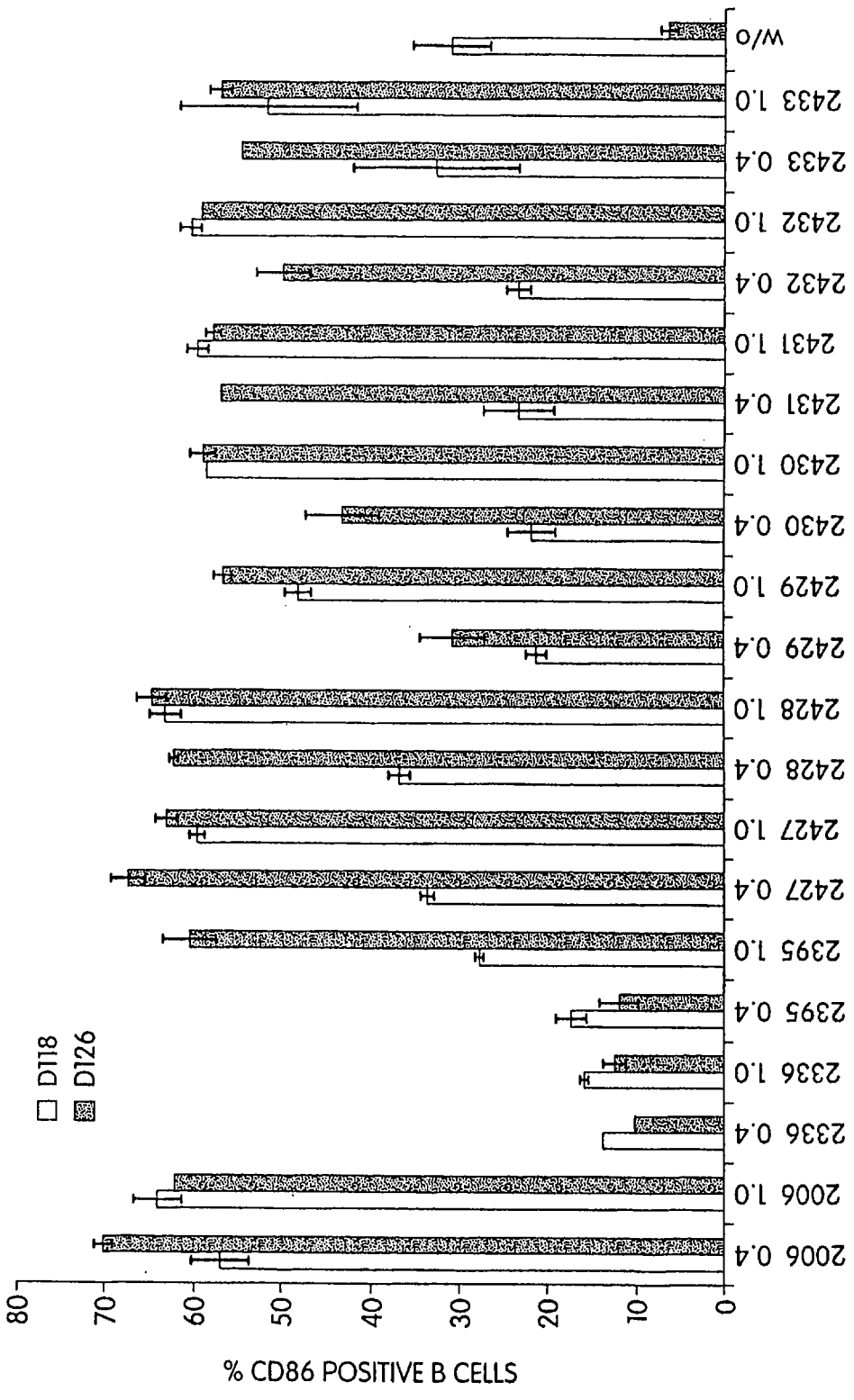

% CD86 POSITIVE B CELLS
0
10
20
30
40
50
60
70
80
D118
D126
2006 0.4
2006 1.0
2336 0.4
2336 1.0
2395 0.4
2395 1.0
2427 0.4
2427 1.0
2428 0.4
2428 1.0
2429 0.4
2429 1.0
2430 0.4
2430 1.0
2431 0.4
2431 1.0
2432 0.4
2432 1.0
2433 0.4
2433 1.0
w/o
ODN [ug/ml]

Fig. 18

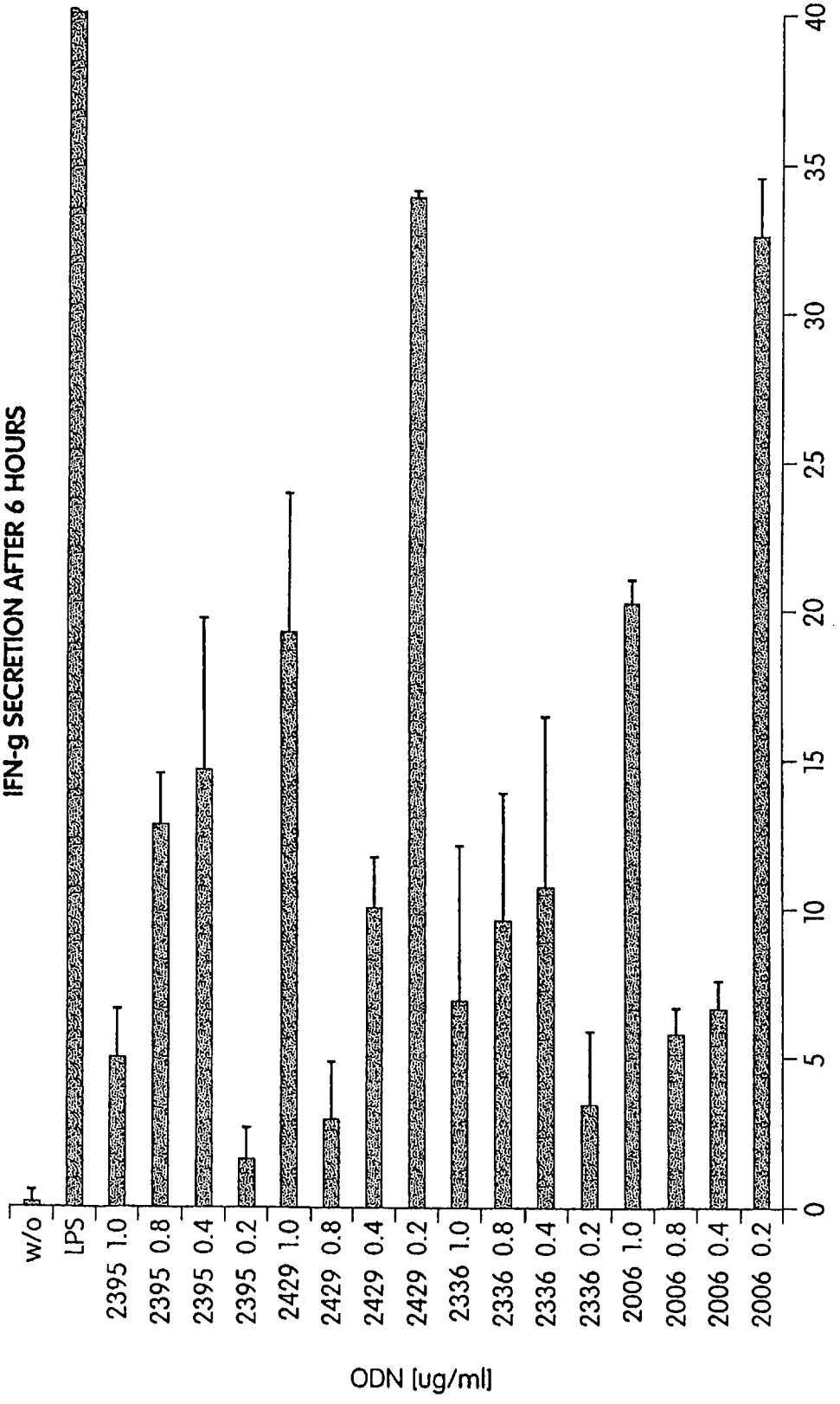

IFN-g SECRETION AFTER 6 HOURS
w/o
LPS
2395 1.0
2395 0.8
2395 0.4
2395 0.2
2429 1.0
2429 0.8
2429 0.4
2429 0.2
2336 1.0
2336 0.8
2336 0.4
2336 0.2
2006 1.0
2006 0.8
2006 0.4
2006 0.2
ODN [ug/ml]
0
5
10
15
20
25
30
35
40
IFN-g [pg/ml]

Fig. 19A

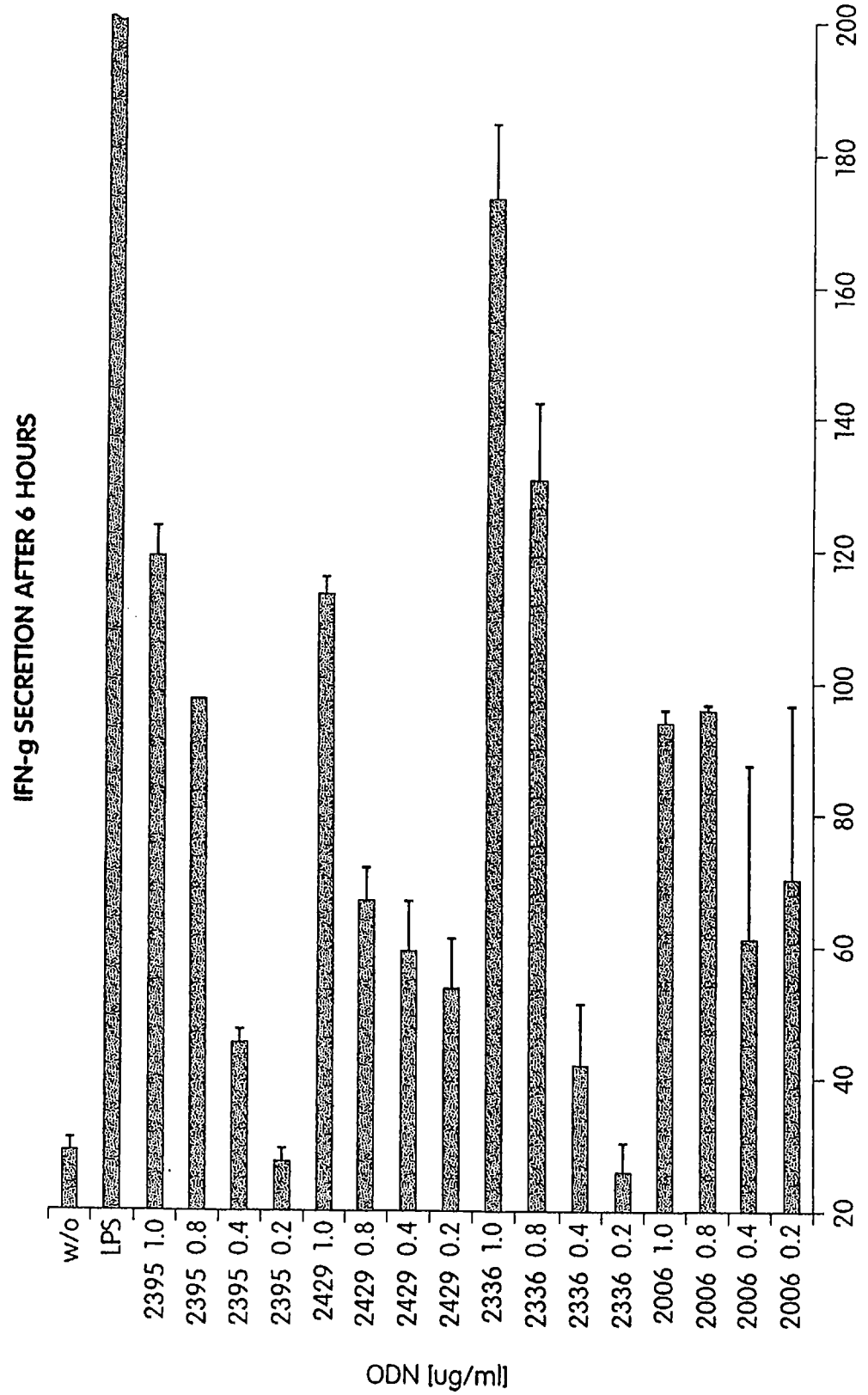

IFN-g SECRETION AFTER 6 HOURS
w/o
LPS
2395 1.0
2395 0.8
2395 0.4
2395 0.2
2429 1.0
2429 0.8
2429 0.4
2429 0.2
2336 1.0
2336 0.8
2336 0.4
2336 0.2
2006 1.0
2006 0.8
2006 0.4
2006 0.2
ODN [ug/ml]
20
40
60
80
100
120
140
160
180
200
IFN-g [pg/ml]

Fig. 19B

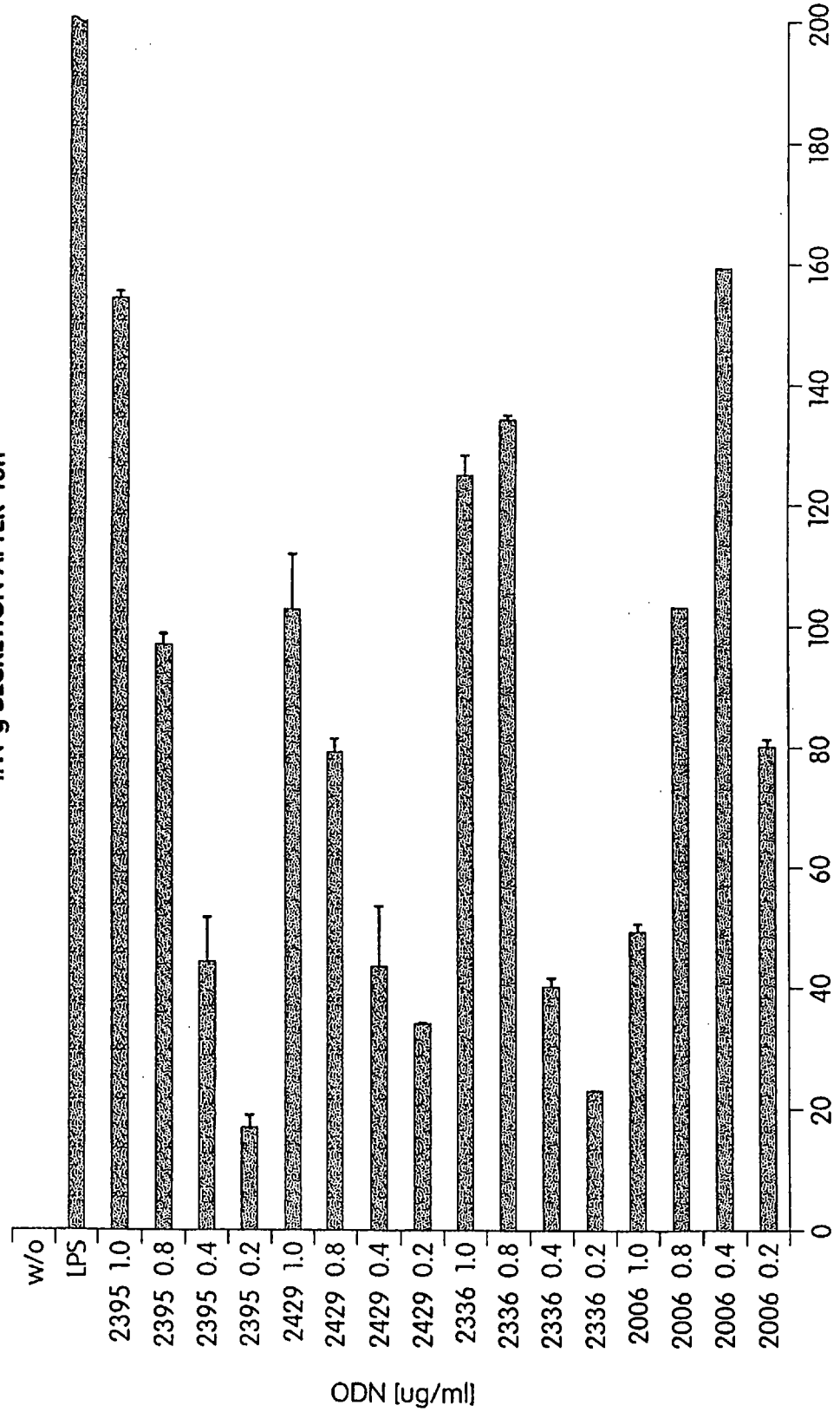

IFN-g SECRETION AFTER 48h
w/o
LPS
2395 1.0
2395 0.8
2395 0.4
2395 0.2
2429 1.0
2429 0.8
2429 0.4
2429 0.2
2336 1.0
2336 0.8
2336 0.4
2336 0.2
2006 1.0
2006 0.8
2006 0.4
2006 0.2
ODN [ug/ml]
0
20
40
60
80
100
120
140
160
180
200
IFN-g [pg/ml]

Fig. 19C

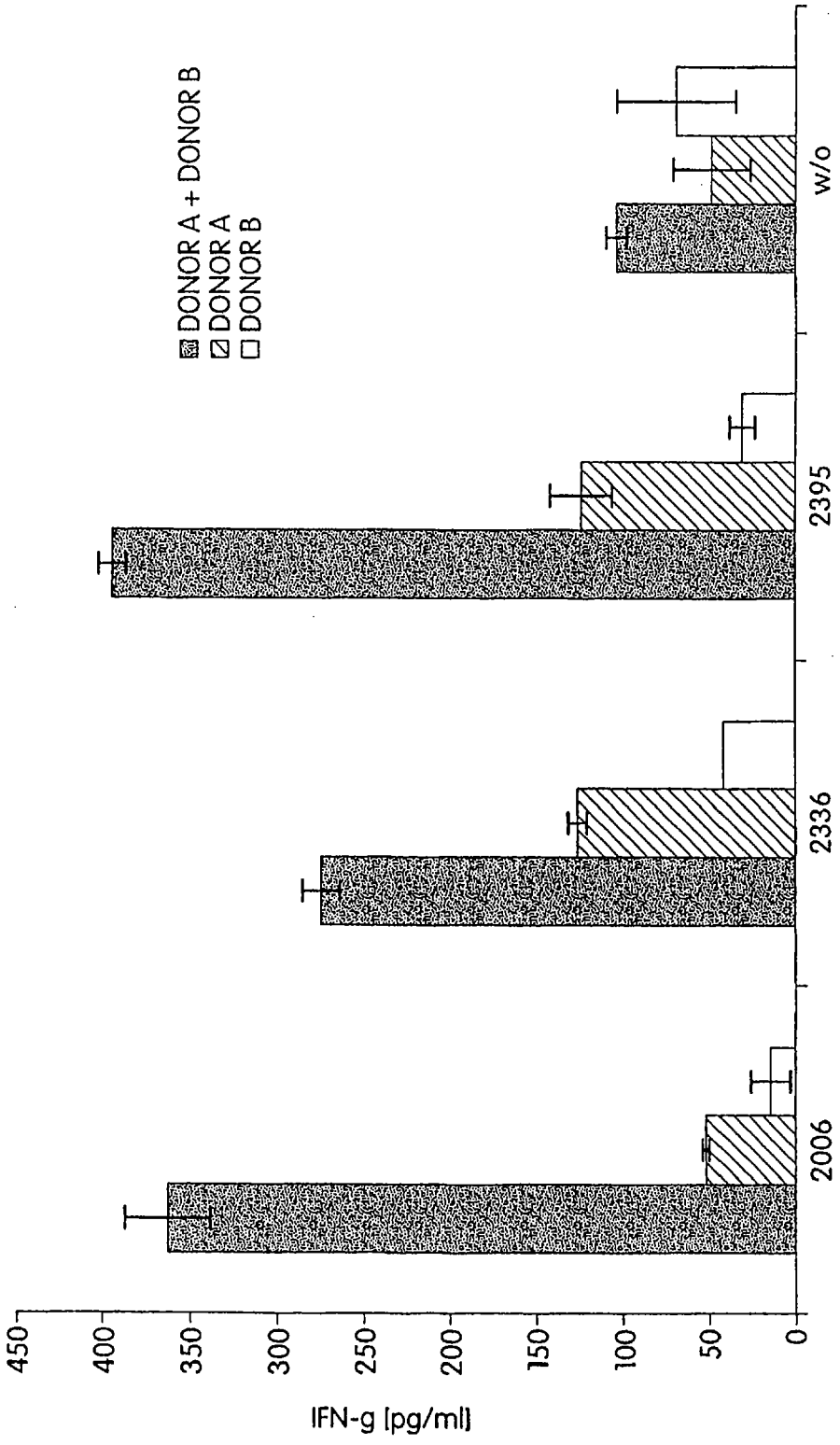

DONOR A + DONOR B
DONOR A
DONOR B
IFN-g [pg/ml]
450
400
350
300
250
200
150
100
50
0
2006
2336
2395
w/o
ODN [6ug/ml]

Fig. 20

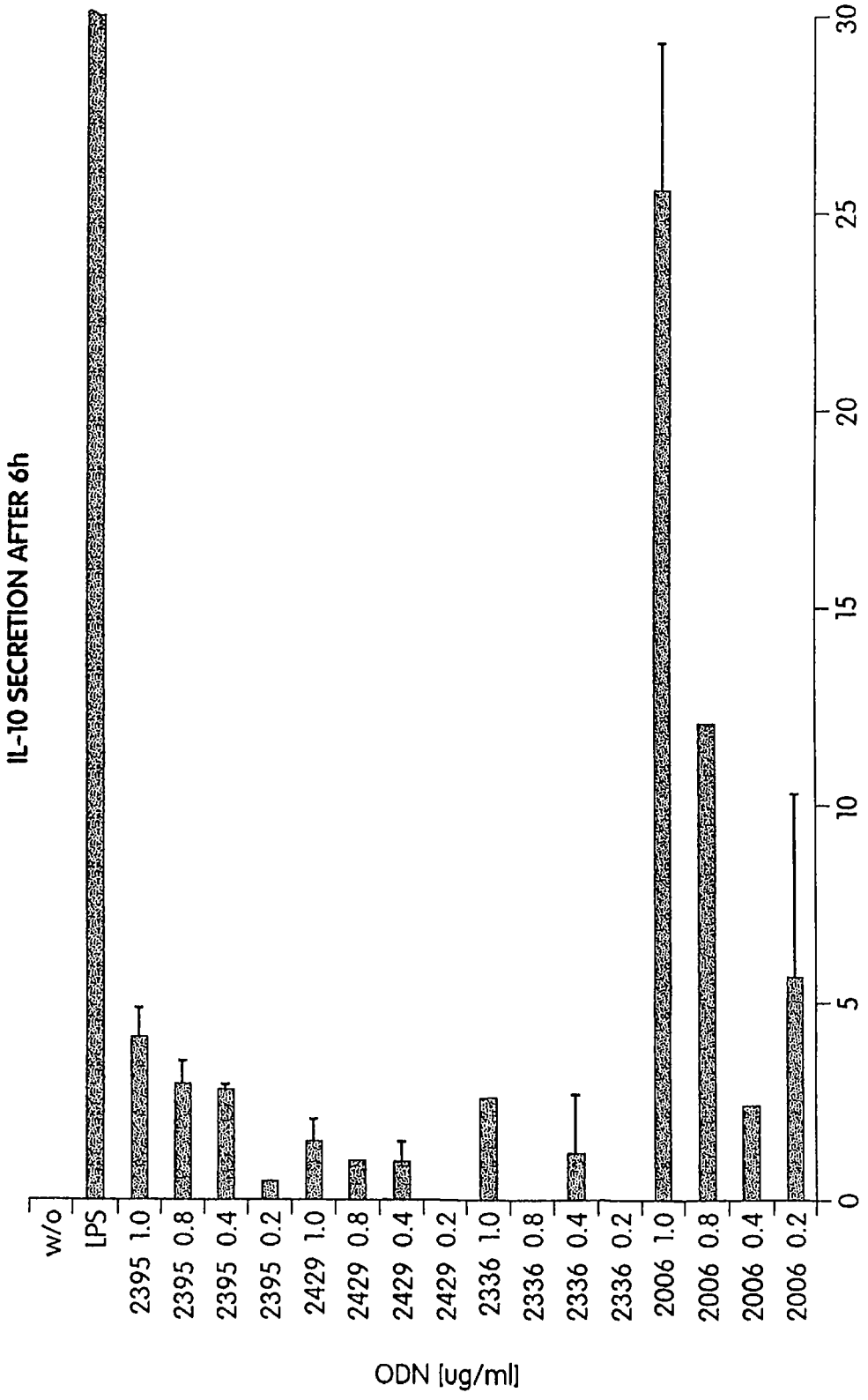

IL-10 SECRETION AFTER 6h
w/o
LPS
2395 1.0
2395 0.8
2395 0.4
2395 0.2
2429 1.0
2429 0.8
2429 0.4
2429 0.2
2336 1.0
2336 0.8
2336 0.4
2336 0.2
2006 1.0
2006 0.8
2006 0.4
2006 0.2
ODN [ug/ml]
0
5
10
15
20
25
30
IL-10 [pg/ml]

Fig. 21A

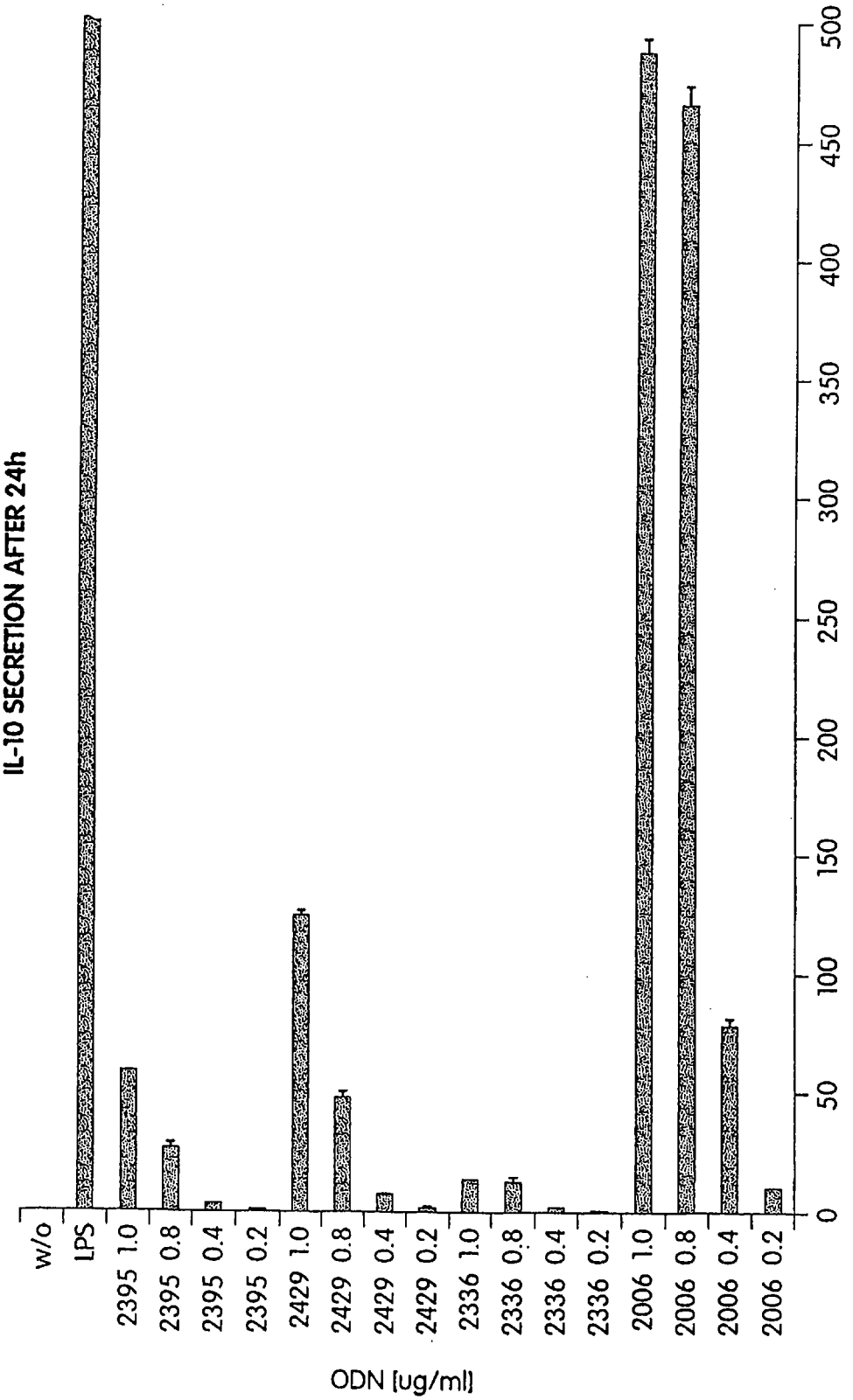

IL-10 SECRETION AFTER 24h
w/o
LPS
2395 1.0
2395 0.8
2395 0.4
2395 0.2
2429 1.0
2429 0.8
2429 0.4
2429 0.2
2336 1.0
2336 0.8
2336 0.4
2336 0.2
2006 1.0
2006 0.8
2006 0.4
2006 0.2
ODN [ug/ml]
0
50
100
150
200
250
300
350
400
450
500
IL-10 [pg/ml]

Fig. 21B

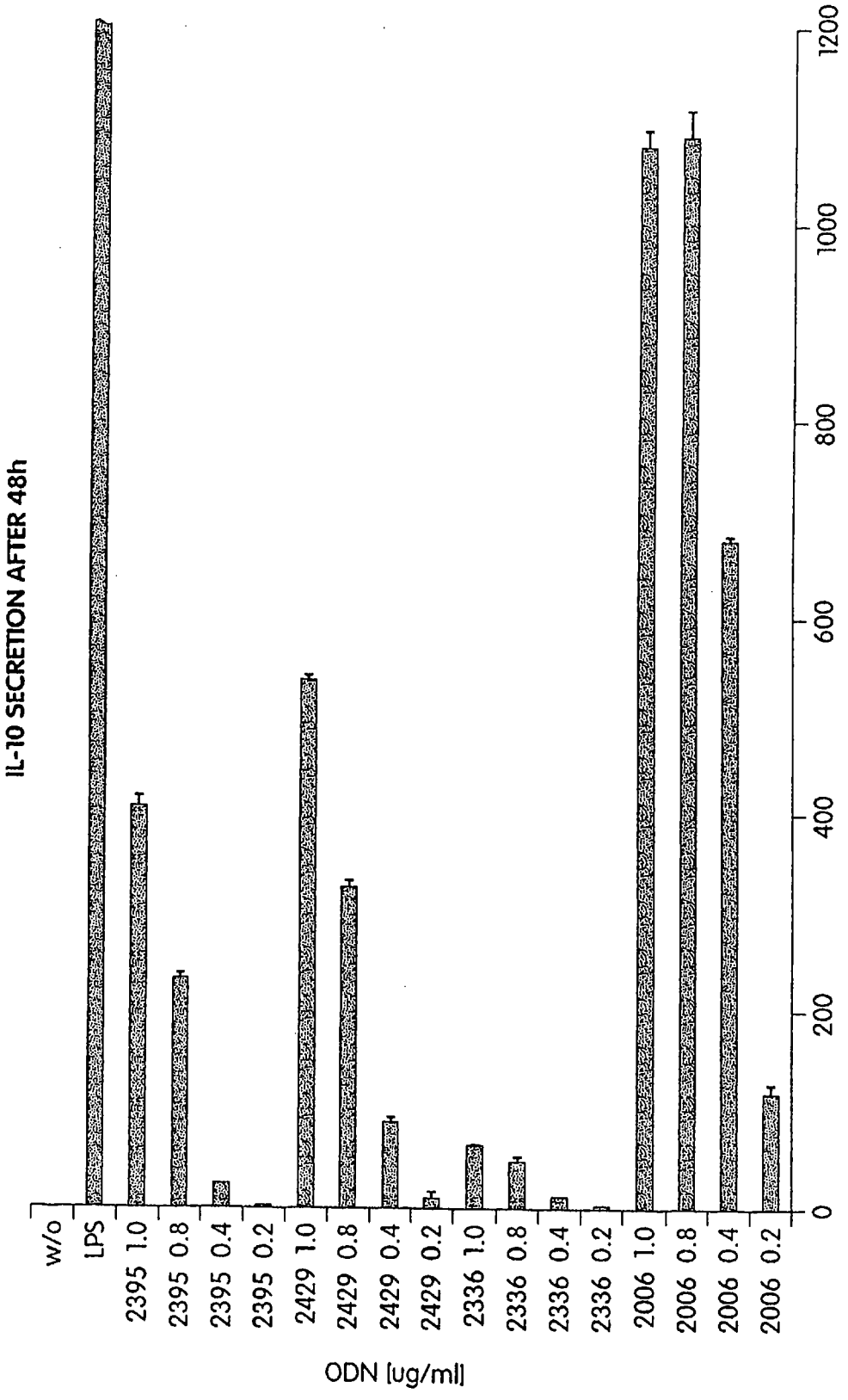

IL-10 SECRETION AFTER 48h
w/o
LPS
2395 1.0
2395 0.8
2395 0.4
2395 0.2
2429 1.0
2429 0.8
2429 0.4
2429 0.2
2336 1.0
2336 0.8
2336 0.4
2336 0.2
2006 1.0
2006 0.8
2006 0.4
2006 0.2
ODN [ug/ml]
0
200
400
600
800
1000
1200
IL-10 [pg/ml]

Fig. 21C

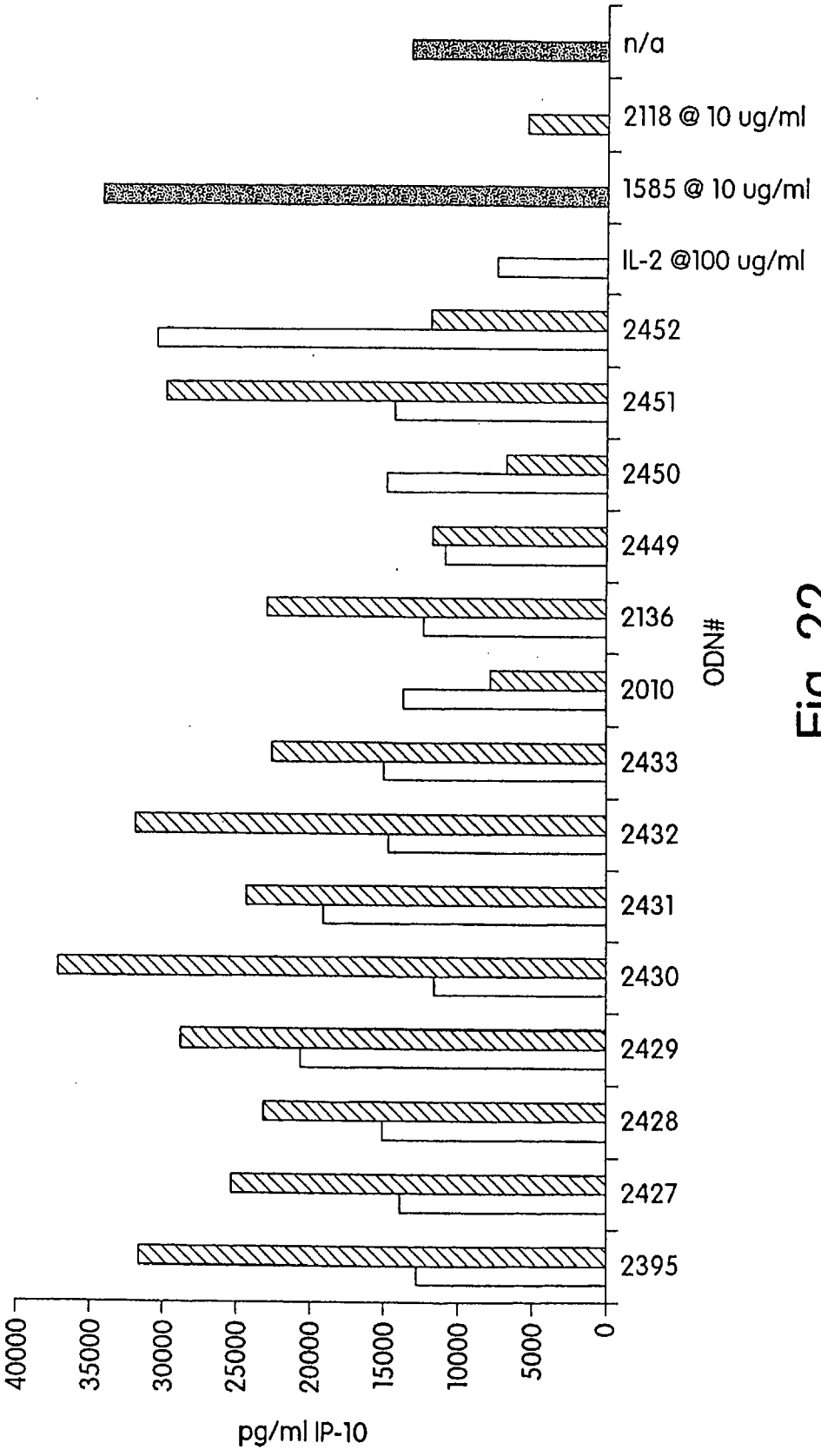

n/a
2118 @ 10 ug/ml
1585 @ 10 ug/ml
IL-2 @100 ug/ml
2452
2451
2450
2449
2136
2010
2433
2432
2431
2430
2429
2428
2427
2395
ODN#
40000
35000
30000
25000
20000
15000
10000
5000
0
pg/ml IP-10

Fig. 22

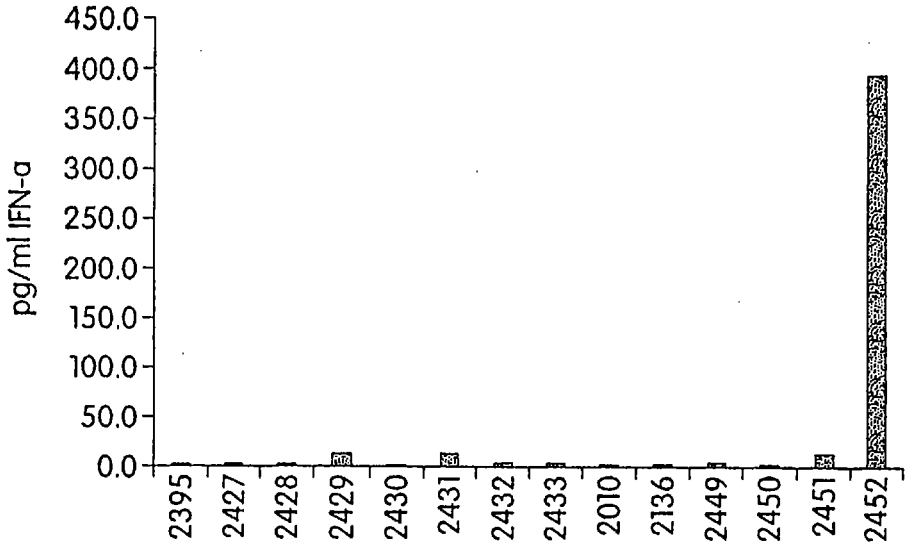


Fig. 23A



Fig. 23B

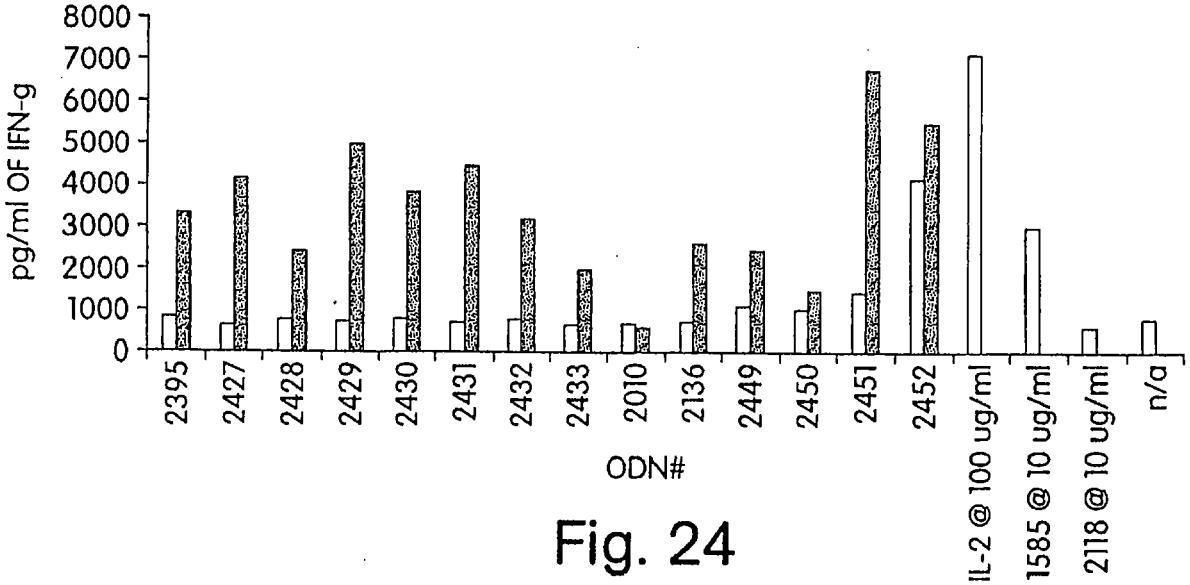

pg/ml OF IFN-g
8000
7000
6000
5000
4000
3000
2000
1000
0
2395
2427
2428
2429
2430
2431
2432
2433
2010
2136
2449
2450
2451
2452
IL-2 @ 100 ug/ml
1585 @ 10 ug/ml
2118 @ 10 ug/ml
n/a
ODN#

Fig. 24

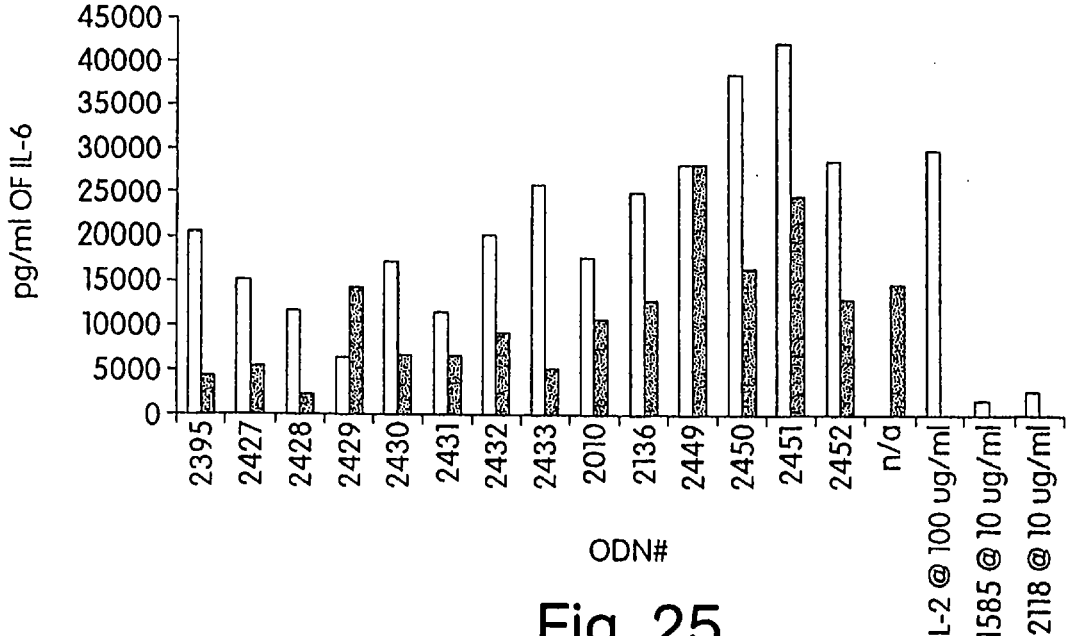

pg/ml OF IL-6
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
2395
2427
2428
2429
2430
2431
2432
2433
2010
2136
2449
2450
2451
2452
n/a
IL-2 @ 100 ug/ml
1585 @ 10 ug/ml
2118 @ 10 ug/ml
ODN#

Fig. 25

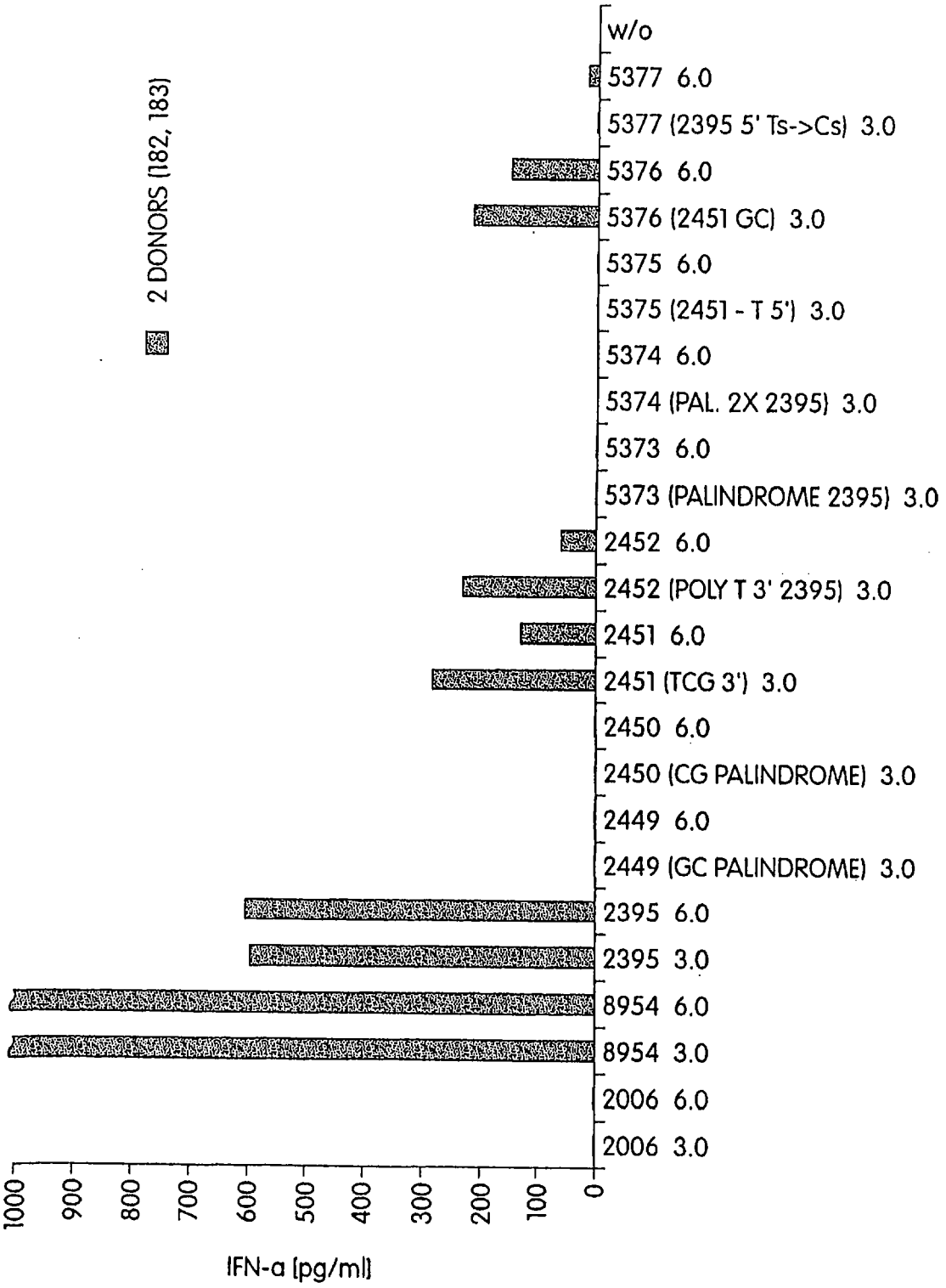

2 DONORS (182, 183)
w/o
5377 6.0
5377 (2395 5' Ts->Cs) 3.0
5376 6.0
5376 (2451 GC) 3.0
5375 6.0
5375 (2451 - T 5') 3.0
5374 6.0
5374 (PAL. 2X 2395) 3.0
5373 6.0
5373 (PALINDROME 2395) 3.0
2452 6.0
2452 (POLY T 3' 2395) 3.0
2451 6.0
2451 (TCG 3') 3.0
2450 6.0
2450 (CG PALINDROME) 3.0
2449 6.0
2449 (GC PALINDROME) 3.0
2395 6.0
2395 3.0
8954 6.0
8954 3.0
2006 6.0
2006 3.0
ODN [ug/ml]
1000
900
800
700
600
500
400
300
200
100
0
IFN-a [pg/ml]

Fig. 26

SEQUENCE LISTING

<110> Coley Pharmaceutical Group Inc.
Coley Pharmaceutical GmbH
University of Iowa Research Foundation

<120> Combination Motif Immune Stimulatory Oligonucleotides with Improved Activity

<130> C01039/70063WO (HCL/AWS)

<150> US 60/313,273
<151> 2001-08-17

<150> US 60/393,952
<151> 2002-07-03

<160> 81

<170> PatentIn version 3.1

<210> 1
<211> 22
<212> DNA
<213> Artificial Sequence

<220>

<223> Synthetic Oligonucleotide

<400> 1
tcgtcgtttt cggcgcgcgc cg 22

<210> 2
<211> 22
<212> DNA
<213> Artificial Sequence

<220>

<223> Synthetic Oligonucleotide

<400> 2
tcgtcgtttt cgtcgcgcgc cg 22

<210> 3
<211> 22
<212> DNA
<213> Artificial Sequence

<220>

<223> Synthetic Oligonucleotide

<400> 3
tcgtcgtttt cgtcgcgcgg cg 22

<210> 4
<211> 22
<212> DNA
<213> Artificial Sequence

<220>

<223> Synthetic Oligonucleotide

<400> 4
tcgtcgtttt cggcggccgc cg 22

<210> 5
<211> 22
<212> DNA
<213> Artificial Sequence

<220>

<223> Synthetic Oligonucleotide

<400> 5
tcgtcgtttt cggcgcgccg cg 22

<210> 6
<211> 22
<212> DNA
<213> Artificial Sequence

<220>

<223> Synthetic Oligonucleotide

<400> 6
tcgtcgtttt cggcgccggc cg 22

<210> 7
<211> 22
<212> DNA
<213> Artificial Sequence

<220>

<223> Synthetic Oligonucleotide

<400> 7
tcgtcgtttt cggcccgcgc gg 22

<210> 8
<211> 22
<212> DNA
<213> Artificial Sequence

<220>

<223> Synthetic Oligonucleotide

<400> 8
tcgtcgtttt ccgccgccgg gg 22

<210> 9
<211> 22
<212> DNA
<213> Artificial Sequence

<220>

<223> Synthetic Oligonucleotide

<400> 9
tcgtcgtttt cgggggggccc cc 22

<210> 10
<211> 22
<212> DNA
<213> Artificial Sequence

<220>

<223> Synthetic Oligonucleotide

<400> 10
tcgtcgtttt ccccccgggg gg 22

<210> 11
<211> 22
<212> DNA
<213> Artificial Sequence

<220>

<223> Synthetic Oligonucleotide

<400> 11
tcggcgcgcg ccgtcgtcgt tt 22

<210> 12
<211> 27
<212> DNA
<213> Artificial Sequence

<220>

<223> Synthetic Oligonucleotide

<400> 12
tcgtcgtttt cggcgcgcgc cgttttt 27

<210> 13
<211> 22
<212> DNA
<213> Artificial Sequence

<220>

<223> Synthetic Oligonucleotide

<400> 13
tcctgacgtt cggcgcgcgc cg 22

<210> 14
<211> 22
<212> DNA
<213> Artificial Sequence

<220>

<223> Synthetic Oligonucleotide

<220>
<221> misc_feature
<222> (2)..(2)
<223> n = 5-methylcytosine

<220>
<221> misc_feature
<222> (5)..(5)
<223> n = 5-methylcytosine

<220>
<221> misc_feature
<222> (11)..(11)
<223> n = 5-methylcytosine

<220>
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<222> (14)..(14)
<223> n = 5-methylcytosine

<220>
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<222> (16)..(16)
<223> n = 5-methylcytosine

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<222> (18)..(18)
<223> n = 5-methylcytosine

<220>
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<222> (20)..(21)
<223> n = 5-methylcytosine

<400> 14
tngtngtttt nggngngngn ng 22

<210> 15
<211> 22
<212> DNA
<213> Artificial Sequence

<220>

<223> Synthetic Oligonucleotide

<400> 15
tgctgctttt cggcgcgcgc cg 22

<210> 16
<211> 22
<212> DNA
<213> Artificial Sequence

<220>

<223> Synthetic Oligonucleotide

<400> 16
tcgtcgtttt cgcgcgcgcg cg 22

<210> 17
<211> 24
<212> DNA
<213> Artificial Sequence

<220>

<223> Synthetic Oligonucleotide

<400> 17
tcgtcgttgg ttgtcgtttt ggtt 24

<210> 18
<211> 24
<212> DNA
<213> Artificial Sequence

<220>

<223> Synthetic Oligonucleotide

<400> 18
accatggacg agctgtttcc cctc 24

<210> 19
<211> 22
<212> DNA
<213> Artificial Sequence

<220>

<223> Synthetic Oligonucleotide

<400> 19
tcctgacgtt cggcgcgcgc cc 22

<210> 20

<211> 24
<212> DNA
<213> Artificial Sequence

<220>

<223> Synthetic Oligonucleotide

<400> 20
tgctgctttt gtgcttttgt gctt 24

<210> 21
<211> 24
<212> DNA
<213> Artificial Sequence

<220>

<223> Synthetic Oligonucleotide

<400> 21
tcgtcgtttc gtcgttttga cgtt 24

<210> 22
<211> 27
<212> DNA
<213> Artificial Sequence

<220>

<223> Synthetic Oligonucleotide

<400> 22
tcgcgtgcgt tttgtcgttt tgacgtt 27

<210> 23
<211> 12
<212> DNA
<213> Artificial Sequence

<220>

<223> Synthetic Oligonucleotide

<400> 23
cggcgcgcgc cg 12

<210> 24
<211> 24
<212> DNA
<213> Artificial Sequence

<220>

<223> Synthetic Oligonucleotide

<400> 24

cggcgcgcgc cgcggcgcgc gccg 24

<210> 25
<211> 21
<212> DNA
<213> Artificial Sequence

<220>

<223> Synthetic Oligonucleotide

<400> 25
cggcgcgcgc cgtcgtcgtt t 21

<210> 26
<211> 22
<212> DNA
<213> Artificial Sequence

<220>

<223> Synthetic Oligonucleotide

<400> 26
tcggcgcgcg ccgtgctgct tt 22

<210> 27
<211> 22
<212> DNA
<213> Artificial Sequence

<220>

<223> Synthetic Oligonucleotide

<400> 27
ccgccgtttt cggcgcgcgc cg 22

<210> 28
<211> 12
<212> DNA
<213> Artificial Sequence

<220>

<223> Synthetic Oligonucleotide

<400> 28
cggcggccgc cg 12

<210> 29
<211> 12
<212> DNA
<213> Artificial Sequence

<220>

<223> Synthetic Oligonucleotide

<400> 29
cgcgcgcgcg cg 12

<210> 30
<211> 12
<212> DNA
<213> Artificial Sequence

<220>

<223> Synthetic Oligonucleotide

<400> 30
gcgcgcgcgc gc 12

<210> 31
<211> 12
<212> DNA
<213> Artificial Sequence

<220>

<223> Synthetic Oligonucleotide

<400> 31
ccccccgggg gg 12

<210> 32
<211> 12
<212> DNA
<213> Artificial Sequence

<220>

<223> Synthetic Oligonucleotide

<400> 32
gggggggcccc cc 12

<210> 33
<211> 10
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<400> 48
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<400> 59
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<210> 66
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<400> 66
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<210> 67
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<210> 68
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<400> 68
cgacgatcgt cg 12

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tcgtcgtttc gacgtacgtc g 21

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<400> 75
tcgtcgcgac gatcgtcg 18

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tcgtcgcgac gtacgtcg 18

<210> 77
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<400> 77
tcgttttttt cgacggccgt cg 22

<210> 78
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<400> 78
tcgttttttt cgacgatcgt cg 22

<210> 79
<211> 22
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tcgttttttt cgacgtacgt cg 22

<210> 80
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<400> 80
cgacgttcgt cg 12

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<400> 81
cggcgccgtg ccg 13