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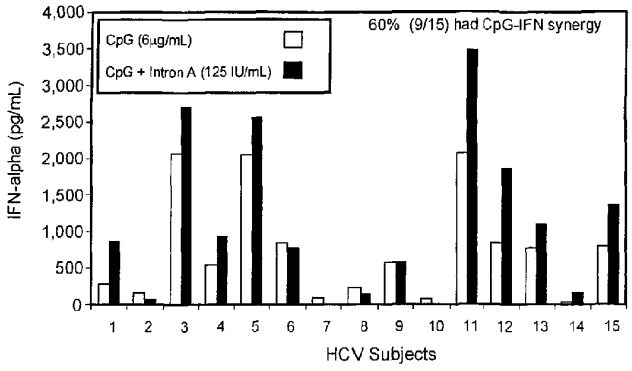

(57) Abstract: The invention provides methods for identifying and treating subjects having hepatitis C infections. In some instances, the subjects are those that are non-responsive to non-CpG therapy. Preferably, the subjects are treated with C class CpG immunostimulatory nucleic acids having a semi-soft backbone.

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METHODS AND PRODUCTS RELATED TO TREATMENT AND PREVENTION OF HEPATITIS C VIRUS INFECTION

Field of the Invention

The invention provides methods and products for the treatment of subjects chronically infected with hepatitis C virus.

Background of the Invention

The hepatitis C virus (HCV) is a positive strand RNA virus of the Flavivirus family that infects hepatocytes of humans and some other primates. First characterized in 1989 (1), HCV has a 9.5 kb genome that encodes for three structural proteins: core and two envelope glycoproteins (E1 and E2), as well as several non-structural (NS) proteins that are involved in the viral replication and interaction with the host cell (2).

HCV is a serious public health concern, causing >90% of parenteral non-A, non-B hepatitis (1). From 0.4 to 1.5% of the world's population is infected (3, 4), including about 300,000 Canadians (Health Canada). Epidemiological statistics are difficult to compile since the vast majority of acute infections are subclinical; however it is estimated that 50-80% of HCV infected individuals fail to clear the virus, and most of these become life-long carriers. About 50% of carriers develop chronic hepatitis and 20% of these will develop liver cirrhosis, many of whom will subsequently develop hepatocellular carcinoma (5-9). Hepatitis C causes an estimated 8,000 to 10,000 deaths annually in the United States (CDC).

In the United States and Canada, there are two different regimens, which have been approved as therapy for hepatitis C: monotherapy with alpha interferon and combination therapy with alpha interferon and Ribavirin. Although more expensive and associated with more side effects, combination therapy consistently yields higher rates of sustained response than monotherapy.

Several forms of alpha interferon are available (alpha-2a, alpha-2b, and consensus interferon (Alfacon)). These interferons are typically given subcutaneously three times weekly. Pegylated interferon, i.e., alpha interferon modified by addition of polyethylene glycol (PEG) in order to increase the duration in the circulation, is another of interferon, and it is given only once weekly. Ribavirin, in contrast, is an oral antiviral agent that is given twice a day in 200 mg capsules.

Side effects of alpha interferon include: fatigue, muscle aches, headaches, nausea and vomiting, skin irritation at the injection site, low-grade fever, weight loss, irritability, depression, suicide, mild bone marrow suppression and hair loss (reversible). For Ribavirin the side effects include; anemia fatigue and irritability, itching, skin rash, nasal stuffiness, sinusitis and cough.

Treatment with interferon alone or in combination with interferon and Ribavirin leads to rapid improvements in serum ALT levels in 50-75% of patients and the disappearance of detectable HCV RNA from the serum in 30-50% of patients. Long-term improvement in liver disease usually occurs only if HCV RNA disappears during therapy and stays undetectable for at least 6 months after therapy is completed. Combination treatment results in both a higher rate of loss of HCV RNA on treatment and a lower rate of relapse when treatment is complete. However, results depend strongly on the genotype of virus, with better results being obtained for genotypes 2 and 3 (about 90% with 1 year of treatment with pegylated IFN-α and Ribavirin), but much poorer results (about 40% sustained response) for genotype 1 HCV. The majority of HCV chronic carriers in North America now are of genotype 1.

The optimal duration of treatment varies depending on whether interferon monotherapy or combination therapy is used, as well as by HCV genotype. Typically, the duration ranges from 6 to 12 months.

There is currently no vaccine against HCV, or highly effective therapy for chronic infection. Thus there is an urgent need for an effective treatment that could be used to treat chronic carriers.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

The reference in this specification to any prior publication (or information derived from it), or to any matter which is known, is not, and should not be taken as an acknowledgment or admission or any form of suggestion that that prior publication (or information derived from it) or known matter forms part of the common general knowledge in the field of endeavour to which this specification relates.

Summary of the Invention

The invention is premised in part on several surprising findings including the observation that CpG immunostimulatory nucleic acids can be used to treat subjects that are chronically infected with hepatitis C virus (HCV) and that are non-responsive to previously administered non-CpG therapies. The invention is further premised in part on the observation that a synergistic response can be had in such subjects from the combined use of CpG immunostimulatory nucleic acids and an anti-viral agent such as IFN-alpha.

In one aspect, the invention provides a method of stimulating an immune response in a subject having an HCV infection that was not successfully treated using a previous non-CpG therapy comprising administering to a subject in need of such stimulation a CpG immunostimulatory nucleic acid in an amount effective to stimulate said immune response.

In one embodiment, the non-CpG therapy includes interferon-alpha. In a related embodiment, the interferon-alpha is interferon-alpha-2b, interferon-alpha-2a or consensus interferon-alpha. In another embodiment, the non-CpG therapy includes interferon-alpha and Ribavirin, or interferon-alpha and Ribavirin and emantidine. In some important embodiments, the non-CpG therapy includes pegylated interferon-alpha and an anti-viral such as Ribavirin.

In one embodiment, the CpG immunostimulatory nucleic acid is an A class CpG immunostimulatory nucleic acid. In another embodiment, the CpG immunostimulatory nucleic acid is a B class CpG immunostimulatory nucleic acid.

In yet a further embodiment, the CpG immunostimulatory nucleic acid is a C class CpG immunostimulatory nucleic acid.

The method may optionally comprise administration of an anti-viral such as interferon-alpha to the subject along with the CpG immunostimulatory nucleic acid. The interferon-alpha may be interferon-alpha-2b, interferon-alpha-2a or consensus interferon

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alpha, but is not so limited. In one embodiment, the anti-viral is administered substantially simultaneously with the CpG immunostimulatory nucleic acid.

In one embodiment, the CpG immunostimulatory nucleic acid comprises a backbone modification. In a related embodiment, the backbone modification is a phosphorothioate backbone modification. In some important embodiments, the CpG immunostimulatory nucleic acid comprises a semi-soft backbone. In other important embodiments, the CpG immunostimulatory nucleic acid is a C class immunostimulatory nucleic acid having a semi-soft backbone.

Thus, in another aspect, a method is provided for stimulating an immune response in a subject having an HCV infection that was not successfully treated using a previous non-CpG therapy comprising administering to a subject in need of such stimulation a C class CpG immunostimulatory nucleic acid having a semi-soft backbone in an amount effective to stimulate said immune response.

In yet another aspect, a method is provided for stimulating an immune response in a subject having an HCV infection that was not successfully treated using a previous non-CpG therapy comprising contacting peripheral blood mononuclear cells from a subject in need of such stimulation, with a CpG immunostimulatory nucleic acid in an amount effective to stimulate an immune response, and re-infusing the cells into the subject.

In one embodiment, the peripheral blood mononuclear cells comprise dendritic cells. In another embodiment, the dendritic cells comprise plasmacytoid dendritic cells. In one embodiment, the CpG immunostimulatory nucleic acid is a C class immunostimulatory nucleic acid. In a related embodiment, the C class immunostimulatory nucleic acid has a semi-soft backbone.

In another aspect, the invention provides a method of treating a subject having an HCV infection and likely to be non-responsive to a non-CpG therapy comprising administering to a subject in need of such treatment a CpG immunostimulatory nucleic acid in an amount effective to treat the infection.

In one embodiment, the method further comprises identifying a subject likely to be non-responsive to a non-CpG therapy. In one embodiment, the subject is identified as likely to be non-responsive based on an assay of interferon-alpha produced per dendritic cell. In another embodiment, the subject is identified as likely to be non-responsive based on HCV genotype.

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In one embodiment, the non-CpG therapy includes IFN-alpha. In a related embodiment, the non-CpG therapy includes interferon-alpha and Ribavirin.

In one embodiment, the method further comprises administering to the subject an anti-viral agent. In important embodiments, the anti-viral agent is interferon-alpha. The interferon-alpha may be interferon-alpha-2b, interferon-alpha-2a or consensus interferon alpha, but it is not so limited. In one embodiment, the interferon-alpha is administered in a sub-therapeutic amount, and optionally the combination of the CpG immunostimulatory nucleic acid and the interferon-alpha is synergistic.

In one embodiment, the CpG immunostimulatory nucleic acid used to treat the subject is an A class CpG immunostimulatory nucleic acid, a B class CpG immunostimulatory nucleic acid, or a C class CpG immunostimulatory nucleic acid.

In one embodiment, the CpG immunostimulatory nucleic acid used to identify whether a subject is likely to be non-responsive to a non-CpG therapy is an A class CpG immunostimulatory nucleic acid, or a C class CpG immunostimulatory nucleic acid.

In one embodiment, the anti-viral agent is administered to the subject substantially simultaneously with the CpG immunostimulatory nucleic acid. In other embodiments, the interferon-alpha is administered for a period prior to treatment with the CpG immunostimulatory nucleic acid.

In certain embodiments, the CpG immunostimulatory nucleic acid comprises a backbone modification. In related embodiments, the backbone modification is a phosphorothioate backbone modification. In some preferred embodiments, the CpG immunostimulatory nucleic acid comprises a semi-soft backbone, and some even more preferred embodiments, the CpG immunostimulatory nucleic acid is a C class CpG immunostimulatory nucleic acid having a semi-soft backbone.

In another aspect, a method is provided for stimulating an immune response in a subject having an HCV infection and likely to be non-responsive to a non-CpG therapy comprising administering to a subject in need of such stimulation a C class CpG immunostimulatory nucleic acid having a semi-soft backbone in an amount effective to stimulate said immune response.

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In yet another aspect, the invention provides a method for screening CpG immunostimulatory nucleic acids useful in the treatment of chronic hepatitis C viral infection. The method involves contacting peripheral blood mononuclear cells from a subject having a chronic hepatitis C viral infection, to a CpG immunostimulatory nucleic acid, and measuring a test response of the blood mononuclear cells after exposure. The subject from which the peripheral blood mononuclear cells wherein the subject was not successfully treated using a previous therapy.

In one embodiment, the test response is selected from the group consisting of B cell stimulation, secretion of IL-6, secretion of IL-10, secretion of IL-12, secretion of interferon-gamma, secretion of type 1 interferons (alpha + beta), secretion of IP-10, NK activity, expression of CD80, expression of CD 86, expression of CD83, and upregulation of class II MHC expression.

In another embodiment, the peripheral blood mononuclear cells comprise dendritic cells. In a related embodiment, the dendritic cells comprise plasmacytoid dendritic cells. In still another embodiment, the cells are dendritic cells and the test response is selected from the group consisting of secretion of IL-12, secretion of type 1 interferons, expression of CD80, expression of CD 86, expression of CD83, and upregulation of class II MHC expression.

In one embodiment, the contacting occurs in vitro. In another embodiment, the peripheral blood mononuclear cells are cultured. In yet another embodiment, the CpG immunostimulatory nucleic acid is added to the cultured peripheral blood mononuclear cells.

In one embodiment, the previous therapy is a non-CpG therapy. In another embodiment, the non-CpG therapy comprises interferon-alpha. In another embodiment, the non-CpG therapy further comprises Ribavirin. In other embodiments, the interferon-alpha is pegylated interferon-alpha. In one embodiment, the previous therapy is therapy with a CpG nucleic acid of a different sequence or class.

In other embodiments, the method further comprises screening the CpG immunostimulatory nucleic acid for the ability to stimulate a control response from peripheral blood mononuclear cells from a normal subject.

The method may further comprise contacting peripheral blood mononuclear cells to interferon-alpha substantially simultaneously with the CpG immunostimulatory nucleic acid.

In one embodiment, the CpG immunostimulatory nucleic acid comprises a backbone modification. In a related embodiment, the backbone modification is a phosphorothioate backbone modification. In important embodiments, the CpG immunostimulatory nucleic acid comprises a semi-soft backbone. The CpG immunostimulatory nucleic acid may be an A class CpG immunostimulatory nucleic acid, a B class CpG immunostimulatory nucleic acid, or a C class CpG immunostimulatory nucleic acid. In some embodiments, the CpG immunostimulatory nucleic acid is a C class immunostimulatory nucleic acid, and in other embodiments, the CpG immunostimulatory nucleic acid is a C class immunostimulatory nucleic acid with a semi-soft backbone.

In another aspect, the invention provides a method for identifying a subject having an HCV infection and likely to be non-responsive to a non-CpG therapy. The method involves exposing peripheral blood mononuclear cells harvested from a subject having a hepatitis C viral infection to a CpG immunostimulatory nucleic acid, measuring interferon-alpha produced from the cells, and determining an amount of interferon-alpha produced per dendritic cell, wherein an amount that is below 1.0 pg/ml is indicative of a subject that is likely to be non-responsive to a non-CpG

therapy. In one embodiment, an amount that is below 0.5 pg/ml is indicative of a subject that is likely to be non-responsive to a non-CpG therapy.

In one embodiment, the non-CpG therapy comprises interferon-alpha. In another embodiment, the non-CpG therapy comprises Ribavirin. In another embodiment, the IFN-alpha is pegylated IFN-alpha.

In some important embodiments, the CpG immunostimulatory nucleic acid is an A class or a C class CpG immunostimulatory nucleic acid.

In still other embodiments, the peripheral blood mononuclear cells are further exposed to an anti-viral agent together with a CpG immunostimulatory nucleic acid. The anti-viral agent may be interferon-alpha, but it is not so limited. In one embodiment, the interferon-alpha is interferon-alpha-2b, interferon-alpha-2a or consensus interferon alpha.

In one embodiment, the peripheral blood mononuclear cells comprise dendritic cells. In another embodiment, the dendritic cells comprise plasmacytoid dendritic cells.

In another embodiment, the hepatitis C viral infection is an acute hepatitis C viral infection.

In another embodiment, the method further comprises determining a genotype of the HCV.

In still a further aspect, a method is provided for identifying a subject having an HCV infection and likely to be non-responsive to a non-CpG therapy comprising exposing peripheral blood mononuclear cells harvested from a subject having a hepatitis C viral infection to an A class or a C class CpG immunostimulatory nucleic acid, measuring interferon-alpha produced from the cells, and determining an amount of interferon-alpha produced per dendritic cell, wherein an amount that is below 1.0 pg/ml is indicative of a subject that is likely to be non-responsive to a non-CpG therapy.

In yet another aspect, the invention provides a method of treating a subject having a hepatitis C viral infection comprising administering to a subject, identified according to the method described above, a CpG immunostimulatory nucleic acid molecule in an amount effective to treat the infection.

In one embodiment, the method further comprises administering to the subject interferon-alpha. In one embodiment, the interferon-alpha is interferon-alpha-2b, interferon-alpha-2a or consensus interferon-alpha.

In one embodiment, the CpG immunostimulatory nucleic acid used to treat the subject is an A class CpG immunostimulatory nucleic acid, a B class CpG immunostimulatory nucleic acid, or a C class CpG immunostimulatory nucleic acid.

In another embodiment, the CpG immunostimulatory nucleic acid comprises a backbone modification. In a related embodiment, the backbone modification is a phosphorothioate backbone modification. In yet another embodiment, the CpG immunostimulatory nucleic acid comprises a semi-soft backbone.

In one embodiment, the hepatitis C viral infection is a chronic hepatitis C viral infection. In another embodiment, the hepatitis C viral infection is an acute hepatitis C viral infection.

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.

These and other aspects of the invention are described in greater detail below.

Brief Description of the Figures

Figure 1 shows the induction of IFN-α secretion from HCV-infected and normal PBMCs following stimulation with 3 classes of CpG. PBMCs from normal or HCV-infected subjects were incubated with different classes of CpG for 48h. Cell supernatants were collected and assayed for IFN-α secretion by commercial ELISA kits. The average IFN-α secretion for 10 normal subjects and 10 HCV-infected subjects are shown by the black bars.

Figure 2 shows the flow cytometric analysis of freshly isolated PBMCs from chronic HCV carriers and normal subjects. PBMCs were isolated from the blood of HCV infected subjects and from normal healthy donors and immunostained with fluorescent-tagged anti-plasmacytoid dendritic cell (pDC) antibodies. Cells were analyzed on a flow cytometer and results were compared to IFN-α secretion data on these same subjects when stimulated with CpG.

Figure 3 shows the IFN-α induction by stimulation of PBMCs with C-class and soft-C oligonucleotides. PBMCs from normal or HCV-infected subjects were incubated with different classes of CpG for 48h. Cell supernatants were collected and assayed for IFN- α secretion by commercial ELISA kits. The average IFN- α secretion for 10 normal subjects and 10 HCV-infected subjects are shown by the black bars.

Figure 4 shows the IFN-α induction following stimulation with a panel of semi-soft C-class CpG. PBMCs isolated from 5 HCV-infected subjects were incubated with a panel of semi-soft C-class oligonucleotides for 48h. Cell supernatants were collected and assayed for IFN-α secretion by commercial ELISA kits. The average IFN-α secretion for 5 HCV-infected subjects are shown by the black bars.

Figure 5 shows the IFN-γ secretion following stimulation with three classes of CpG. PBMCs from normal or HCV-infected subjects were incubated with different classes of CpG for 48h. Cell supernatants were collected and assayed for IFN-γ secretion by commercial ELISA kits. The average IFN-γ secretion for 10 normal subjects and 10 HCV-infected subjects are shown by the black bars.

Figure 6 shows the IFN-γ induction following stimulation with a panel of semi-soft C-class CpG. PBMCs isolated from 5 HCV-infected subjects were incubated with a panel of semi-soft C-class oligonucleotides for 48h. Cell supernatants were collected and assayed for IFN-γ secretion by commercial ELISA kits. The average IFN-γ secretion for 5 HCV subjects are shown by the black bars.

Figure 7 shows the IP-10 secretion following stimulation with three classes of CpG. PBMCs from normal or HCV-infected subjects were incubated with different classes of CpG for 48h. Cell supernatants were collected and assayed for IP-10 secretion by commercial ELISA kits. The average IP-10 secretion for 10 normal subjects and 10 HCV-infected subjects are shown by the black bars.

Figure 8 shows the effect of CpG on B cell proliferation. PBMCs from HCV-infected or normal donors were incubated with class A, B or C CpG for 5 days. Cells were then pulsed with ^{3}H-thymidine for 16 to18 hours before measuring radioactivity. Values are represented as stimulation indices in comparison with media control (SI = cpm incubated with CpG/cpm of cells incubated with media alone).

Figure 9 shows the effect of semi-soft C-class CpG on B cell proliferation. PBMCs from 5 HCV-infected subjects were incubated with A, B ,C and semi-soft-C class CpG for 5 days. Cells were then pulsed with ^{3}H-thymidine for 16 to 18 hours before measuring radioactivity. Values are represented as stimulation indices in comparison with media control (SI = cpm incubated with CpG/cpm of cells incubated with media alone).

Figure 10 shows the IL-10 secretion following stimulation with three classes of CpG. PBMCs from normal or HCV-infected subjects were incubated with different classes of CpG for 48h. Cell supernatants were collected and assayed for IL-10 secretion by commercial ELISA kits. The average IL-10 secretion for 10 normal subjects and 10 HCV-infected subjects are shown by the black bars.

Figure 11 shows IFN-α secretion following stimulation of HCV-infected cells with Ribavirin and CpG alone or in combination with Intron A. PBMCs from 10 HCV-infected subjects and 10 normal healthy donors were incubated with Intron A, Ribavirin or C-class CpG alone and also with and without Intron A (a purified exogenous source of IFN-α) for 48 hours. Cell supernatants were collected and assayed for IFN-α secretion by commercial ELISA kits. The amount of IFN-α measured for Intron A alone for each subject, was considered background and was subtracted from Intron A, Ribavirin + Intron A and C-Class + Intron A for these same subjects before the data was included in the graph. Mean values for normal and HCV subjects are indicated by black and white bars respectively.

Figure 12. Synergistic effect of CpG combined with Intron A on IFN-α secretion by HCV-infected cells. PBMCs from 15 HCV-infected subjects were incubated with C-class CpG alone or together with Intron A (a purified exogenous source of IFN-α) for 48 hours. Cell supernatants were collected and assayed for IFN-α secretion by commercial ELISA kits. The amount of IFN-α measured for Intron A alone for each subject, was subtracted from CpG + Intron A for these same subjects before the data was included in the graph.

It is to be understood that the figures are not required for enablement of the claimed invention.

Detailed Description of the Invention

It has been discovered according to the invention, that CpG oligonucleotides can activate PBMCs from patients chronically infected with HCV, including those

who have failed previous interferon-alpha (IFN-α) therapy, in a manner similar to PBMCs from healthy subjects.

It was discovered that endogenous IFN-α secretion was strongly induced from plasmacytoid dendritic cells (PDC), which are thought to be infected by HCV resulting in their dysfunction and reduced ability to respond to other stimuli. In some instances, the A and C CpG classes, which induce high levels of IFN-α in PBMCs from healthy volunteers, were found to induce the highest levels of IFN-α from pDCs. It was further discovered that the semi-soft C class CpG ODN are also particularly useful for this effect. These ODN may be preferred in some embodiments since they will not accumulate in the kidney with repeat dosing.

It has been further discovered according to the invention that neither exogenous IFN-α (Intron A) nor Ribavirin have any detectable direct immune stimulatory effects on PBMCs from normal subjects or HCV chronic carriers, when used alone or together. However, when Intron A and CpG ODN (e.g., B or C classes) are used together, then a strong synergy for production of endogenous IFN-α is observed.

These results indicate that CpG ODN are an effective treatment alone, or together with IFN-α, to treat chronic HCV infection. The invention provides methods and products for preventing and treating HCV infection, based on these findings.

Chronic infection appears to be due, at least in part, to the rapid mutation rate of HCV, resulting in the production of quasi-species that can escape immune surveillance (10, 11). Both humoral and cell-mediated immune (CMI) responses can be detected in chronically infected individuals. While neutralizing antibodies are critical to protection from infection, cell-mediated immunity (CMI) appears to play the major role in viral clearance once infection is established.

In one aspect, the invention provides a method of treating a subject infected with hepatitis C virus (HCV) who is not successfully treated with a previous non-CpG therapy. The method comprises administering to a non-responsive subject in need of such treatment a CpG immunostimulatory nucleic acid in an amount effective to inhibit the infection.

A non-CpG therapy, as used herein, is a therapy that uses active or inactive compounds that are not CpG immunostimulatory nucleic acids. In various

embodiments, the non-CpG therapy includes interferon-alpha. Pegylated IFN-alpha is commonly administered to HCV subjects (e.g., human HCV patients), preferably in combination with Ribavirin and optionally amantadine. The interferon-alpha can be interferon-alpha-2b, interferon-alpha-2a or consensus interferon-alpha. All of the foregoing interferon-alpha treatments are included in the definition of non-CpG therapy.

A subject who is not successfully treated with a previous non-CpG therapy is a subject who notwithstanding prior treatment still has detectable viral load in their bloodstream 6 months after the cessation of therapy. These subjects include those that may respond to a previous non-CpG therapy, but who fail to control the infection and subsequently relapse as indicated by detectable viral load. As used herein and for the sake of simplicity, these subjects are referred to as "non-responders", however this term is to be understood as defined herein, and not as defined in a clinical setting. In other words, although in a clinical setting a "non-responder" defines only that narrow subset of subjects that fail to show any response to a treatment, the invention is directed to a broader category of subjects that while perhaps responding at some level to a previous treatment, are still not successfully treated. A subject that is successfully treated is one that has no detectable viral load in its bloodstream 6 months after the cessation of treatment. Successful treatment means treatment that leads to an undetectable level of viral load in the bloodstream that is sustained for at least 6 months after cessation of treatment. It is to be understood that a non-responder, as used herein, implicitly is also chronically infected with HCV.

As used herein, when referring to treatment using CpG nucleic acids, the methods are used to achieve a successful treatment of subjects. Successful treatment of subjects using CpG treatment is defined as a reduction of viral load to undetectable levels in the bloodstream 6 months after the cessation of therapy. Interestingly, viral loads may not be observed to decrease during or immediately after CpG treatment, but rather may only decrease with time after treatment, with the ultimate result that there is no detectable virus in the bloodstream of these subjects 6 months following the cessation of treatment. To treat an infection therefore means to reduce viral load to an undetectable level in the bloodstream of a subject and to sustain that level for 6 months following the cessation of treatment. Effective amounts of agents are therefore administered to achieve this end result.

In some embodiments, the potential non-responders may be identified prospectively (i.e., prior to actual in vivo treatment with a non-CpG therapy), and the invention provides methods not only for the identification of such subjects but also for their treatment. Potential non-responders may be identified by assessing their ability to respond to CpG immunostimulatory nucleic acids, particularly A class and C class. The ability to respond to CpG immunostimulatory nucleic acids will be assessed by the amount of interferon-alpha that is produced per pDC in HCV infected subjects. It was discovered, according to the invention, that HCV infected subjects that would be unlikely to respond to non-CpG therapy such as IFN-alpha therapy could be identified prior to receiving such treatment. The ability to identify such subjects prior to in vivo therapy eliminates unnecessary treatment and places the subjects in a therapeutically advantageous position for treatment with the CpG immunostimulatory nucleic acids of the invention either alone or in combination with other anti-HCV therapies including but not limited to IFN-alpha. These subjects would suffer from less cytotoxicity and the time period for viral growth would be reduced by not undergoing a treatment that will be unsuccessful. Subjects having below a reasonable level of IFN-alpha induction per pDC are likely not to be successfully treated with IFN-alpha and thus should be treated using the methods provided herein. Measurement of IFN-alpha induction and pDC numbers are described in more detail in the Examples.

It is to be understood that an HCV infected subject that is successfully treated with any of the therapeutic agents and methods discussed herein will probably still have virus in their body. However, while the subject is not able to completely eradicate the virus, it is able to control viral load (to undetectable levels). Although not intending to be bound by any particular theory, it is expected that the maintenance of undetectable viral loads in such subjects involve an immune system that is able to control viral replication and spread.

One of ordinary skill given the teachings provided herein will be able to determine whether a subject is likely to be a 'non-responder" to IFN-alpha therapy. As an example, if the IFN-alpha induction were performed with an A class nucleic acid such as nucleic acid designated SEQ ID NO 1, under the culture conditions described in the Examples, then a normal response indicative of the ability to respond to IFN-alpha therapy would be at least 1 pg/ml per pDC. An amount less than this is

indicative of some pDC dysfunction. Amounts that are less than 0.5 pg/ml per pDC correlate with a higher probability of non-response to IFN-alpha treatment. One of ordinary skill will be able to determine such cutoffs for the particular type of nucleic acid used in the assay, and will therefore be capable of identifying subjects expected not to be successfully treated with IFN-alpha therapy (at least) prior to actually treating such subjects in that manner.

In still other embodiments, the method of identifying a subject who is likely to be a non-responder to non-CpG therapy (e.g., IFN-alpha therapy) may further include identification of the genotype of HCV he/she is infected with. It is more likely that a subject infected with a genotype 1 HCV will not be successfully treated with IFN-alpha therapy, for example. Therefore, in addition to assessing the production of IFN-alpha per DC in such subjects, their HCV genotype can also be determined (using methods known in the art), and this combination of information can be used to identify a subject that is likely to be non-responsive to IFN-alpha therapy.

It is to be further understood that in some aspects, the invention provides a method for identifying a subject that is unlikely to be successfully treated using a non-CpG therapy (without actually treating the subject with a non-CpG therapy) and then treating the subject using either CpG immunostimulatory nucleic acids alone or in combination with an anti-viral agent such as but not limited to IFN-alpha.

The above methods can also be used to screen subjects for their response to particular CpG immunostimulatory nucleic acids.

In still other embodiments, the methods may involve the additional step of identifying subjects having received previous non-CpG therapy but not successfully treated. Those of ordinary skill, given the teachings provided herein, will be able to identify such subjects. As an example, such subjects would have detectable viral loads in their bloodstream 6 months after the cessation of treatment. In some embodiments, these subjects may also demonstrate a reduction in viral load immediately following treatment, but this reduction is not sustained.

The invention intends to treat subjects not successfully treated with a previous non-CpG therapy using, inter alia, CpG immunostimulatory nucleic acids alone or in combination with other active agents such as those previously described for HCV infection. As broadly defined, CpG immunostimulatory nucleic acids are nucleic acids having at least one CpG dinucleotide motif in which at least the C of the

dinucleotide is unmethylated. CpG immunostimulatory nucleic acids include but are not limited to A class, B class and C class CpG immunostimulatory nucleic acids, as described more fully herein and in the patent and patent applications cited herein and incorporated by reference. These classes of CpG immunostimulatory nucleic acid have differing properties and activation profiles.

In important embodiments, the CpG immunostimulatory nucleic acid is a C class immunostimulatory nucleic acid. It was surprisingly found, according to the invention, that C class immunostimulatory nucleic acids were preferred in some embodiments, even though these nucleic acids possessed properties intermediate to those of A class and B class. The Examples provided herein demonstrate that, even though pDC of chronically infected subjects not successfully treated with a previous non-CpG therapy are themselves infected with HCV and thereby dysfunctional in some aspects, exposure of such cells to CpG immunostimulatory nucleic acids, and in particular C class immunostimulatory nucleic acids, restores their function. In some embodiments, it is also preferred that the C class immunostimulatory nucleic acids be either of a "soft" or "semi-soft" variety, as described in greater detail herein. In some preferred embodiments, the CpG immunostimulatory is a semi-soft C class nucleic acid.

In other aspects, the CpG immunostimulatory nucleic acids are used in combination with active agents which preferably include those previously described for HCV treatment. Of particular importance is the use of CpG immunostimulatory nucleic acids with interferon-α (e.g., Intron A). The interferons that can be used in combination with the CpG immunostimulatory nucleic acids of the invention include but are not limited to interferon-alpha-2b, interferon-alpha-2a or consensus interferon alpha. Other anti-virals are described herein. Any of the CpG classes can be used in these combinations. As an example, it was unexpectedly found, according to the invention, that although exogenously administered interferon-α fails to treat these subjects successfully, when combined with CpG immunostimulatory nucleic acids it is therapeutically efficacious. In some embodiments, the CpG immunostimulatory nucleic acid is a C class immunostimulatory nucleic acid. In come preferred embodiments, it is a semi-soft C class nucleic acid.

The timing of administration of the CpG nucleic acid and anti-viral agent (e.g., interferon-alpha) may vary depending upon the subject and the severity of

infection. The CpG nucleic acid may be administered substantially simultaneously with the CpG immunostimulatory nucleic acid. This means that the two agents may be combined prior to administration, or may be combined in the process of administration (e.g., with both feeding into an intravenous line in a subject), or they may be administered separately but within a period of time that it would take someone to perform two administrations (e.g., the time to inject a subject twice). Regardless of whether the agents are administered substantially simultaneously or in staggered fashion, the order may vary. Accordingly, in some embodiments, the CpG immunostimulatory nucleic acid may be administered prior to an anti-viral agent such as IFN-alpha while in others it may be administered following the anti-viral agent.

When CpG nucleic acids are used together with other anti-virals (e.g., IFN-alpha), these compounds may be administered in a combined amount that is therapeutically efficacious. The amount of either compound may therefore be sub-therapeutic or supra-therapeutic (i.e., below or above the amount that would be therapeutically efficacious when administered alone). Alternatively, the compounds each may be administered in a therapeutic amount, but the combination of those agents creates a therapeutic benefit such as a reduction of side effects. In preferred embodiments, if the anti-viral is IFN-alpha, it is administered in a therapeutic amount. Regardless of the actual amounts administered, the combination of agents may be synergistic. A synergistic response is one that is greater than the additive response expected by the combination of the agents.

In still other aspects and in keeping with the description provided above, the invention provides methods for screening CpG nucleic acids for the ability to stimulate immune cells isolated from a subject chronically infected with HCV and not successfully treated with a non-CpG therapy or likely to be non-responders to non-CpG therapy. These screening methods are generally performed in vitro by contacting peripheral blood mononuclear cells (PBMCs) with a CpG immunostimulatory nucleic acid in an effective amount sufficient to stimulate an immune response. The immune response can be measured by any number of markers, including IFN-alpha production, B cell stimulation, secretion of cytokines such as Il-6, IL-10, IL-12, interferon-gamma, type 1 interferons (alpha + beta), chemokine secretion such as IP-10, NK activity, expression of costimulatory

molecules (e.g., CD80, CD 86) and maturation molecules (e.g., CD83) and upregulation of class II MHC expression.

In some important embodiments, the immune cells are dendritic cells, and preferably plasmacytoid dendritic cells (pDCs) and the immune response markers are specific to this cell type. These include but are not limited to expression of costimulatory molecules (e.g., CD80 and CD86) expression of maturation molecules (e.g., CD83), expression and/or secretion of IL-12 and type 1 interferons (alpha + beta), and upregulation of class II MHC expression. It is to be understood that these in vitro assays are not dependent upon isolation of dendritic cells such as pDCs from the remainder of PBMCs. Rather the assays can be carried out in homogeneous populations of PBMCs.

In still another aspect, the invention provides a method for identifying a subject having a chronic hepatitis C viral infection to be treated with a CpG immunostimulatory nucleic acid. The method involves exposing peripheral blood mononuclear cells harvested from a subject having a chronic hepatitis C viral infection to i) a CpG immunostimulatory nucleic acid, and ii) a CpG immunostimulatory nucleic acid and an anti-viral (e.g., interferon-alpha), and measuring response of the peripheral blood mononuclear cells after exposure. A response to a CpG immunostimulatory nucleic acid is indicative of a subject to be treated with a CpG immunostimulatory nucleic acid either following or in place of a non-CpG therapy (as described above, but only after identifying a subject that is unlikely to respond to a non-CpG therapy). A response to a CpG immunostimulatory nucleic acid together with an anti-viral agent (e.g., interferon-alpha) that is greater than the response to CpG immunostimulatory nucleic acid alone is indicative of a subject to be treated with the combination. As described herein, the anti-viral agent can be an interferon-alpha including but not limited to interferon-alpha-2b, interferon-alpha-2a or consensus interferon-alpha. Preferably, the peripheral blood mononuclear cells comprise dendritic cells such as plasmacytoid dendritic cells. The invention further includes treatment of subjects identified as just described using either CpG immunostimulatory nucleic acids alone or in combination with an anti-viral agent (e.g., IFN-alpha), depending upon the outcome of the screening assay.

Clinical strategies comprise local and systemic *in vivo* administration of such nucleic acids, as well as *ex vivo* strategies in which pDCs isolated from non-responsive HCV

infected subjects are activated in vitro with immunostimulatory nucleic acids and then reinfused into the patient locally or systemically. These therapeutic strategies may include the combination with other growth factors (IL-3, GM-CSF, flt3-ligand, etc.) as well as with other stimuli (superantigens, viral products). Since natural IFN-α is a family of more than a dozen separate gene products, the individual products of which have unique activity profiles, the clinical use of natural interferon may be preferable compared to recombinant IFN-α derived from a single recombinant IFN-α gene.

The invention further provides a method activating pDCs from an Hepatitis C infected subject. The method involves isolating pDCs from the subject in need of such treatment, culturing the isolated pDCs *in vitro*, contacting the pDCs *in vitro* with an effective amount of an isolated immunostimulatory nucleic acid, and returning the contacted cells to the subject. The cells can also be contacted *in vitro* with a growth factor or with a cytokine. The immunostimulatory nucleic acids and conditions calling for treatment with IFN-α according to this aspect of the invention are as described above.

IFN-alpha itself represents a family of more than a dozen related, homologous proteins (isoforms, see Table 1 below), each encoded by a unique gene and each exhibiting a unique activity profile. The activities of the different alpha-interferon species on viruses can vary as much as twenty-fold or more. IFN-alpha products in clinical use are recombinant proteins or highly purified natural proteins of a single isoform. In the United States IFN-α is available as recombinant human IFN-α2a (ROFERON-A), recombinant human IFN-α2b (INTRON A), and as purified natural IFN-αn3 (ALFERON N). Outside the United States, IFN-α is also available as purified natural IFN-αn1 (WELLFERON).

Table 1. Family of Human IFN-α

IFN-αA	(IFN-α2a)
IFN-α2	(IFN-α2b)
IFN-α4b	(IFN-α4)
IFN-αB2	(IFN-α8)
IFN-αC	(IFN-α10)
IFN-αD	(IFN-α1)
IFN-αF	(IFN-α21)
IFN-αG	(IFN-α5)
IFN-αH2	(IFN-α14)
IFN-αI	(IFN-α17)
IFN-αJ1	(IFN-α7)
IFN-αK	(IFN-α6)

IFN-αM1	
IFN-αN	
IFN-αWA	(IFN-α16)

Some of the methods of the invention require measurement of immune responses including detecting the presence of IFN-α. Assays for IFN-α are well known in the art. These include direct tests, e.g., enzyme-linked immunosorbent assay (ELISA) specific for at least one IFN-α, and indirect tests, e.g., functional tests including NK cell activation/cytotoxicity (Trinchieri G *Adv Immunol* 47:187-376 (1989) and phenotyping by fluorescence-activated cell sorting (FACS) analysis for class I MHC. Additional specific assay methods well known in the art can be particularly useful in settings where local concentration or local presence of IFN-α is of interest. These methods include, for example, immunohistochemistry, nucleic acid hybridization (e.g., Northern blotting), Western blotting, reverse transcriptase/polymerase chain reaction (RT/PCR), and in situ RT/PCR. Intracellular IFN-α can also be detected using flow cytometry.

The invention in some aspects involves measuring pDC activation. pDC activation can be assayed in a number of ways. These include IFN-α production, expression of costimulatory molecules (e.g., CD80 and CD86), expression of maturation molecules (e.g., CD83), expression of IL-12, and upregulation of class II MHC expression. Unlike administration of exogenous IFN-α, activation of pDC leads to the production of various if not all the forms of IFN-α, as well as other type I IFN such as IFN-β. In some embodiments, therefore, the pDC are activated as measured by their ability to produce type I interferons including IFN-α.

The invention provides various methods that involve immunostimulatory nucleic acids. An immunostimulatory nucleic acid is a nucleic acid molecule which, upon contacting cells of the immune system, is itself capable of inducing contacted cells of the immune system to proliferate and/or to become activated. The contacting can be direct or indirect, e.g., the immunostimulatory nucleic acid may directly stimulate a first type of immune cell to express a product which may in turn stimulate a second type of immune cell which has not been exposed to, or is not responsive to, the immunostimulatory nucleic acid. The immunostimulatory effect of the immunostimulatory nucleic acid is separate from any product that might happen to be encoded by the sequence of the immunostimulatory nucleic acid. Similarly, the

immunostimulatory effect of an immunostimulatory nucleic acid is distinct from and does not rely upon any antisense mechanism.

Only certain nucleic acids are immunostimulatory nucleic acids. Originally it was believed that certain palindromic sequences were immunostimulatory. Tokunaga T et al. *Microbiol Immunol* 36:55-66 (1992); Yamamoto T et al. *Antisense Res Dev* 4:119-22 (1994). Further work demonstrated that non-palindromic sequences are also immunostimulatory provided they contained CpG dinucleotides within particular sequence contexts (CpG motifs). Krieg AM et al. *Nature* 374:546-9 (1995).

The immunostimulatory nucleic acids can be single-stranded or double-stranded. Generally, double-stranded nucleic acid molecules are more stable *in vivo*, while single-stranded nucleic acid molecules have increased immune activity. Thus in some aspects of the invention it is preferred that the immunostimulatory nucleic acid be single-stranded and in other aspects it is preferred that the immunostimulatory nucleic acid be double-stranded.

The methods and products provided in accordance with the invention relate to the use of CpG oligonucleotides. CpG ODN trigger most (>95%) B-cells to proliferate, secrete immunoglobulin (Ig), IL-6 and IL-12, and to be protected from apoptosis. In addition, CpG ODN cause DC maturation and also directly activate DCs, monocytes, and macrophages to secrete IFN-α/β, IL-6, IL-12, GM-CSF, chemokines and TNF-α. These cytokines stimulate natural killer (NK) cells to secrete IFN-γ and have increased lytic activity. Overall, CpG induces a strong Th1-like pattern of cytokine production dominated by IL-12 and IFN-γ with little secretion of Th2 cytokines.

In addition to induction of innate immune responses, CpG DNA also augments antigen-specific responses due to (i) a strong synergy between the B-cell signaling pathways triggered through the B-cell antigen receptor and by CpG, (ii) Th1-like cytokines that replace or augment antigen-specific T-help augmenting both B- and T-cell antigen-specific responses and (iii) up-regulation of co-stimulatory molecules that are required for cellular responses.

CpG ODN has been shown to be a potent adjuvant to HBsAg in BALB/c mice with clear Th1-like responses (predominantly IgG2a antibodies and strong CTL) (49). CpG ODN was found to be superior to other Th1 adjuvants such as monophosphoryl lipid A (MPL, Corixa) or even complete Freund's adjuvant (CFA) which is too toxic

for human use. Similar results have been reported using CpG ODN with a variety of other antigens (47, 50-53). CpG ODN have also been reported to redirect a Th2 response previously established by immunization with a Th2 antigen (i.e., Schistosomiasis surface antigen) (54) or a Th2 adjuvant (i.e., alum).

There are at least three basic classes of CpG ODNs found to be effective at stimulating healthy human PBMCs (Table 1). These have differential effects that are likely associated with the different modes of by which CpG ODNs can stimulate immune cells.

The B class of CpG ODN are synthesized with nuclease resistant phosphorothioate backbones and are generally characterized by good B-cell and DC activation, leading to the production of IL-12 and antibody, but only limited NK cell activation. This class of ODN functions well as a vaccine adjuvant, as has already been demonstrated in a phase I/II clinical trial testing CpG (a member of this class) (SEQ ID NO.: 2) as an adjuvant to a commercial hepatitis B vaccine (60).

The A class of CpG ODNs are synthesized with a chimeric backbone where the 5' and 3' ends are phosphorothioate and the central CpG motif region is phosphodiester. These ODNs are characterized by good NK cell and DC activation leading to greater production of IFN-α but limited B-cell activation.

The C class of CpG ODN are synthesized with a phosphorothioate backbone and have stimulatory properties intermediate to the other two classes of CpG ODNs (e.g., good activation of B-cells as well as activation of NK cells and DCs).

Table 1:
Pattern of in vitro immune activation induced by the three different classes of CpG ODNs

Class	Backbone	B-cells	Natural Killer cells	Dendritic cells	IFN-α
A	SOS [2]	+	++++	++++	++++
B	S [1]	++++	++	++++	+
C	S [1]	+++	+++	+++	+++

[1] S-ODN are made with a phosphorothioate backbone

[2] SOS-ODN are made with a chimeric backbone where the central CpG-containing region has phosphodiester linkages and the 3' and 5' ends of the ODN are made with phosphorothioate linkages

The methods of the invention may embrace the use of A class, B class and C class CpG immunostimulatory nucleic acids. As to CpG nucleic acids, it has recently been described that there are different classes of CpG nucleic acids. One class is potent for activating B cells but is relatively weak in inducing IFN-α and NK cell activation; this class has been termed the B class. The B class CpG nucleic acids typically are fully stabilized and include an unmethylated CpG dinucleotide within certain preferred base contexts. See, e.g., U.S. Patent Nos. 6,194,388; 6,207,646; 6,214,806; 6,218,371; 6,239,116; and 6,339,068. Another class is potent for inducing IFN-α and NK cell activation but is relatively weak at stimulating B cells; this class has been termed the A class. The A class CpG nucleic acids typically have stabilized poly-G sequences at 5' and 3' ends and a palindromic phosphodiester CpG dinucleotide-containing sequence of at least 6 nucleotides. See, for example, published patent application PCT/US00/26527 (WO 01/22990). Yet another class of CpG nucleic acids activates B cells and NK cells and induces IFN-α; this class has been termed the C-class. The C-class CpG nucleic acids, as first characterized, typically are fully stabilized, include a B class-type sequence and a GC-rich palindrome or near-palindrome. This class has been described in U.S. provisional patent application 60/313,273, filed August 17, 2001, US10/224,523 filed on August 19, 2002, and US the entire contents of which are incorporated herein by reference.

"A class" CpG immunostimulatory nucleic acids have been described in U.S. Non-Provisional Patent Application Serial No.: 09/672,126 and published PCT application PCT/US00/26527 (WO 01/22990), both filed on September 27, 2000. These nucleic acids are characterized by the ability to induce high levels of interferon-alpha while having minimal effects on B cell activation. The A class CpG immunostimulatory nucleic acid do not necessarily contain a hexamer palindrome GACGTC, AGCGCT, or AACGTT described by Yamamoto and colleagues. Yamamoto S et al. *J Immunol* 148:4072-6 (1992).

Exemplary sequences of A class immunostimulatory nucleic acids are described in U.S. Non-Provisional Patent Application Serial No.: 09/672,126 and

published PCT application PCT/US00/26527 (WO 01/22990), both filed on September 27, 2000.

B class CpG immunostimulatory nucleic acids strongly activate human B cells but have minimal effects inducing interferon-α. B class CpG immunostimulatory nucleic acids have been described in USPs 6,194,388 B1 and 6,239,116 B1, issued on February 27, 2001 and May 29, 2001 respectively.

The CpG oligonucleotides of the invention are oligonucleotides which include at least one unmethylated CpG dinucleotide. An oligonucleotide containing at least one unmethylated CpG dinucleotide is a nucleic acid molecule which contains an unmethylated cytosine-guanine dinucleotide sequence (i.e., "CpG DNA" or DNA containing a 5' cytosine followed by 3' guanine and linked by a phosphate bond) and activates the immune system. The entire CpG oligonucleotide can be unmethylated or portions may be unmethylated but at least the C of the 5' CG 3' must be unmethylated. The terms CpG oligonucleotide or CpG nucleic acid as used herein refer to an immunostimulatory CpG oligonucleotide or a nucleic acid unless otherwise indicated.

In one embodiment the invention provides a B class CpG oligonucleotide represented by at least the formula:

$$5'\ X_1X_2CGX_3X_4\ 3'$$

wherein X_1, X_2, X_3, and X_4 are nucleotides. In one embodiment X_2 is adenine, guanine, or thymine. In another embodiment X_3 is cytosine, adenine, or thymine.

In another embodiment the invention provides an isolated B class CpG oligonucleotide represented by at least the formula:

$$5'\ N_1X_1X_2CGX_3X_4N_2\ 3'$$

wherein X_1, X_2, X_3, and X_4 are nucleotides and N is any nucleotide and N_1 and N_2 are nucleic acid sequences composed of from about 0-25 N's each. In one embodiment X_1X_2 is a dinucleotide selected from the group consisting of: GpT, GpG, GpA, ApA, ApT, ApG, CpT, CpA, CpG, TpA, TpT, and TpG; and X_3X_4 is a dinucleotide selected from the group consisting of: TpT, ApT, TpG, ApG, CpG, TpC, ApC, CpC, TpA, ApA, and CpA. Preferably X_1X_2 is GpA or GpT and X_3X_4 is TpT. In other embodiments X_1 or X_2 or both are purines and X_3 or X_4 or both are pyrimidines or X_1X_2 is GpA and X_3 or X_4 or both are pyrimidines. In another preferred embodiment X_1X_2 is a dinucleotide selected from the group consisting of: TpA, ApA, ApC, ApG,

and GpG. In yet another embodiment X_3X_4 is a dinucleotide selected from the group consisting of: TpT, TpA, TpG, ApA, ApG, GpA, and CpA. X_1X_2 in another embodiment is a dinucleotide selected from the group consisting of: TpT, TpG, ApT, GpC, CpC, CpT, TpC, GpT and CpG; X_3 is a nucleotide selected from the group consisting of A and T and X_4 is a nucleotide, but wherein when X_1X_2 is TpC, GpT, or CpG, X_3X_4 is not TpC, ApT or ApC.

In another preferred embodiment the CpG oligonucleotide has the sequence 5' $TCN_1TX_1X_2CGX_3X_4$ 3' (SEQ ID NO.:26). The CpG oligonucleotides of the invention in some embodiments include X_1X_2 selected from the group consisting of GpT, GpG, GpA and ApA and X_3X_4 is selected from the group consisting of TpT, CpT and TpC.

The B class CpG nucleic acid sequences of the invention are those broadly described above as well as disclosed in PCT Published Patent Applications PCT/US95/01570 and PCT/US97/19791, and USP 6,194,388 B1 and USP 6,239,116 B1, issued February 27, 2001 and May 29, 2001 respectively. Exemplary sequences include but are not limited to those disclosed in these latter applications and patents.

The C class immunostimulatory nucleic acids 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 C class of combination motif immune stimulatory nucleic acids may have either stabilized, e.g., phosphorothioate, chimeric, or phosphodiester backbones, and in some preferred embodiments, they have semi-soft 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.

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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: 26) or CGGCGCCGTGCCG (SEQ ID NO: 27). 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

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

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. Ni 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.

Some non limiting examples of C-Class nucleic acids include:

SEQ ID NO:	Sequence
17	T*C_G*C_G*T*C_G*T*T*C_G*G*C*G*C_G*C*G*C*C*G
18	T*C_G*T*C_G*A*C_G*T*T*C_G*G*C*G*C_G*C*G*C*C*G
19	T*C_G*G*A*C_G*T*T*C_G*G*C*G*C_G*C*G*C*C*G

20	T*C_G*G*A*C_G*T*T*C_G*G*C*G*C*G*C*C*G
21	T*C_G*C_G*T*C_G*T*T*C_G*G*C*G*C*G*C*C*C
22	T*C_G*A*C_G*T*T*C_G*G*C*G*C_G*C*G*C*C*G
23	T*C_G*A*C_G*T*T*C_G*G*C*G*C*G*C*C*G
24	T*C_G*C_G*T*C_G*T*T*C_G*G*C*G*C*C*G
25	T*C_G*C_G*A*C_G*T*T*C_G*G*C*G*C_G*C*G*C*C*G

For facilitating uptake into cells, immunostimulatory nucleic acids, including CpG-containing oligonucleotides, are preferably in the range of 8 to 100 bases in length. However, nucleic acids of any size greater than 8 nucleotides (even many kb long) are capable of inducing an immune response according to the invention if sufficient immunostimulatory motifs are present, since larger nucleic acids are degraded into oligonucleotides inside of cells. Preferably the immunostimulatory nucleic acid is in the range of between 8 and 100 nucleotides in length. In some preferred embodiments the immunostimulatory nucleic acids is between 12 and 40 nucleotides in length. In more preferred embodiments the immunostimulatory nucleic acids is between 8 and 30 nucleotides in length. In most preferred embodiments the immunostimulatory nucleic acids is between 8 and 24 nucleotides in length.

"Palindromic sequence" shall mean an inverted repeat, i.e., a sequence such as ABCDEE'D'C'B'A' in which A and A', B and B', C and C', D and D', and E and E' are bases capable of forming the usual Watson-Crick base pairs. *In vivo*, such palindromic sequences may form double-stranded structures. In one embodiment the CpG oligonucleotide contains a palindromic sequence. A palindromic sequence used in this context refers to a palindrome in which the CpG is part of the palindrome, and preferably is the center of the palindrome. In another embodiment the CpG oligonucleotide is free of a palindrome. A CpG oligonucleotide that is free of a palindrome is one in which the CpG dinucleotide is not part of a palindrome. Such an oligonucleotide may include a palindrome in which the CpG is not the center of the palindrome.

In some embodiments of the invention the immunostimulatory oligonucleotides include immunostimulatory motifs which are "CpG dinucleotides". A CpG dinucleotide can be methylated or unmethylated. An immunostimulatory

nucleic acid containing at least one unmethylated CpG dinucleotide is a nucleic acid molecule which contains an unmethylated cytosine-guanine dinucleotide sequence (i.e., an unmethylated 5' cytidine followed by 3' guanosine and linked by a phosphate bond) and which activates the immune system; such an immunostimulatory nucleic acid is a CpG nucleic acid. CpG nucleic acids have been described in a number of issued patents, published patent applications, and other publications, including U.S. Patent Nos. 6,194,388; 6,207,646; 6,214,806; 6,218,371; 6,239,116; and 6,339,068. An immunostimulatory nucleic acid containing at least one methylated CpG dinucleotide is a nucleic acid which contains a methylated cytosine-guanine dinucleotide sequence (i.e., a methylated 5' cytidine followed by a 3' guanosine and linked by a phosphate bond) and which activates the immune system. In other embodiments the immunostimulatory oligonucleotides are free of CpG dinucleotides. These oligonucleotides which are free of CpG dinucleotides are referred to as non-CpG oligonucleotides, and they have non-CpG immunostimulatory motifs. The invention, therefore, also encompasses nucleic acids with other types of immunostimulatory motifs, which can be methylated or unmethylated. The immunostimulatory oligonucleotides of the invention, further, can include any combination of methylated and unmethylated CpG and non-CpG immunostimulatory motifs.

The immunostimulatory nucleic acid molecules may have a chimeric backbone. For purposes of the instant invention, a chimeric backbone refers to a partially stabilized backbone, wherein at least one internucleotide linkage is phosphodiester or phosphodiester-like, and wherein at least one other internucleotide linkage is a stabilized internucleotide linkage, wherein the at least one phosphodiester or phosphodiester-like linkage and the at least one stabilized linkage are different. Since boranophosphonate linkages have been reported to be stabilized relative to phosphodiester linkages, for purposes of the chimeric nature of the backbone, boranophosphonate linkages can be classified either as phosphodiester-like or as stabilized, depending on the context. For example, a chimeric backbone according to the instant invention could in one embodiment include at least one phosphodiester (phosphodiester or phosphodiester-like) linkage and at least one boranophosphonate (stabilized) linkage. In another embodiment a chimeric backbone according to the instant invention could include boranophosphonate (phosphodiester or

phosphodiester-like) and phosphorothioate (stabilized) linkages. A "stabilized internucleotide linkage" shall mean an internucleotide linkage that is relatively resistant to *in vivo* degradation (e.g., via an exo- or endo-nuclease), compared to a phosphodiester internucleotide linkage. Preferred stabilized internucleotide linkages include, without limitation, phosphorothioate, phosphorodithioate, methylphosphonate, and methylphosphorothioate. Other stabilized internucleotide linkages include, without limitation: peptide, alkyl, dephospho, and others as described above.

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 et al. (1990) *Chem Rev* 90:544; Goodchild J (1990) *Bioconjugate Chem* 1:165. Methods for preparing chimeric oligonucleotides are also known. For instance patents issued to Uhlmann et al have described such techniques.

Mixed backbone modified ODN may be synthesized using a commercially available DNA synthesizer and standard phosphoramidite chemistry. (F. E. Eckstein, "Oligonucleotides and Analogues - A Practical Approach" IRL Press, Oxford, UK, 1991, and M. D. Matteucci and M. H. Caruthers, Tetrahedron Lett. 21, 719 (1980)) After coupling, PS linkages are introduced by sulfurization using the Beaucage reagent (R. P. Iyer, W. Egan, J. B. Regan and S. L. Beaucage, J. Am. Chem. Soc. 112, 1253 (1990)) (0.075 M in acetonitrile) or phenyl acetyl disulfide (PADS) followed by capping with acetic anhydride, 2,6-lutidine in tetrahydrofurane (1:1:8; v:v:v) and *N*-methylimidazole (16 % in tetrahydrofurane). This capping step is performed after the sulfurization reaction to minimize formation of undesired phosphodiester (PO) linkages at positions where a phosphorothioate linkage should be located. In the case of the introduction of a phosphodiester linkage, e.g. at a CpG dinucleotide, the intermediate phosphorous-III is oxidized by treatment with a

solution of iodine in water/pyridine. After cleavage from the solid support and final deprotection by treatment with concentrated ammonia (15 hrs at 50°C), the ODN are analyzed by HPLC on a Gen-Pak Fax column (Millipore-Waters) using a NaCl-gradient (e.g. buffer A: 10 mM NaH_2PO_4 in acetonitrile/water = 1:4/v:v pH 6.8; buffer B: 10 mM NaH_2PO_4, 1.5 M NaCl in acetonitrile/water = 1:4/v:v; 5 to 60 % B in 30 minutes at 1 ml/min) or by capillary gel electrophoresis. The ODN can be purified by HPLC or by FPLC on a Source High Performance column (Amersham Pharmacia). HPLC-homogeneous fractions are combined and desalted *via* a C18 column or by ultrafiltration. The ODN was analyzed by MALDI-TOF mass spectrometry to confirm the calculated mass.

The nucleic acids of the invention can also include other modifications. These 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 some embodiments the oligonucleotides may be soft or semi-soft oligonucleotides. A soft oligonucleotide is an immunostimulatory oligonucleotide having a partially stabilized backbone, in which phosphodiester or phosphodiester-like internucleotide linkages occur only within and immediately adjacent to at least one internal pyrimidine -purine dinucleotide (YZ). Preferably YZ is YG, a pyrimidine-guanosine (YG) dinucleotide. The at least one internal YZ dinucleotide itself has a phosphodiester or phosphodiester-like internucleotide linkage. A phosphodiester or phosphodiester-like internucleotide linkage occurring immediately adjacent to the at least one internal YZ dinucleotide can be 5', 3', or both 5' and 3' to the at least one internal YZ dinucleotide.

In particular, phosphodiester or phosphodiester-like internucleotide linkages involve "internal dinucleotides". An internal dinucleotide in general shall mean any pair of adjacent nucleotides connected by an internucleotide linkage, in which neither nucleotide in the pair of nucleotides is a terminal nucleotide, i.e., neither nucleotide in the pair of nucleotides is a nucleotide defining the 5' or 3' end of the oligonucleotide.

Thus a linear oligonucleotide that is n nucleotides long has a total of n-1 dinucleotides and only n-3 internal dinucleotides. Each internucleotide linkage in an internal dinucleotide is an internal internucleotide linkage. Thus a linear oligonucleotide that is n nucleotides long has a total of n-1 internucleotide linkages and only n-3 internal internucleotide linkages. The strategically placed phosphodiester or phosphodiester-like internucleotide linkages, therefore, refer to phosphodiester or phosphodiester-like internucleotide linkages positioned between any pair of nucleotides in the nucleic acid sequence. In some embodiments the phosphodiester or phosphodiester-like internucleotide linkages are not positioned between either pair of nucleotides closest to the 5' or 3' end.

Preferably a phosphodiester or phosphodiester-like internucleotide linkage occurring immediately adjacent to the at least one internal YZ dinucleotide is itself an internal internucleotide linkage. Thus for a sequence N_1 YZ N_2, wherein N_1 and N_2 are each, independent of the other, any single nucleotide, the YZ dinucleotide has a phosphodiester or phosphodiester-like internucleotide linkage, and in addition (a) N_1 and Y are linked by a phosphodiester or phosphodiester-like internucleotide linkage when N_1 is an internal nucleotide, (b) Z and N_2 are linked by a phosphodiester or phosphodiester-like internucleotide linkage when N_2 is an internal nucleotide, or (c) N_1 and Y are linked by a phosphodiester or phosphodiester-like internucleotide linkage when N_1 is an internal nucleotide and Z and N_2 are linked by a phosphodiester or phosphodiester-like internucleotide linkage when N_2 is an internal nucleotide.

Soft oligonucleotides according to the instant invention are believed to be relatively susceptible to nuclease cleavage compared to completely stabilized oligonucleotides. Without meaning to be bound to a particular theory or mechanism, it is believed that soft oligonucleotides of the invention are cleavable to fragments with reduced or no immunostimulatory activity relative to full-length soft oligonucleotides. Incorporation of at least one nuclease-sensitive internucleotide linkage, particularly near the middle of the oligonucleotide, is believed to provide an "off switch" which alters the pharmacokinetics of the oligonucleotide so as to reduce the duration of maximal immunostimulatory activity of the oligonucleotide. This can be of particular value in tissues and in clinical applications in which it is desirable to

avoid injury related to chronic local inflammation or immunostimulation, e.g., the kidney.

A semi-soft oligonucleotide is an immunostimulatory oligonucleotide having a partially stabilized backbone, in which phosphodiester or phosphodiester-like internucleotide linkages occur only within at least one internal pyrimidine-purine (YZ) dinucleotide. Semi-soft oligonucleotides generally possess increased immunostimulatory potency relative to corresponding fully stabilized immunostimulatory oligonucleotides. Due to the greater potency of semi-soft oligonucleotides, semi-soft oligonucleotides may be used, in some instances, at lower effective concentations and have lower effective doses than conventional fully stabilized immunostimulatory oligonucleotides in order to achieve a desired biological effect.

It is believed that the foregoing properties of semi-soft oligonucleotides generally increase with increasing "dose" of phosphodiester or phosphodiester-like internucleotide linkages involving internal YZ dinucleotides. Thus it is believed, for example, that generally for a given oligonucleotide sequence with five internal YZ dinucleotides, an oligonucleotide with five internal phosphodiester or phosphodiester-like YZ internucleotide linkages is more immunostimulatory than an oligonucleotide with four internal phosphodiester or phosphodiester-like YG internucleotide linkages, which in turn is more immunostimulatory than an oligonucleotide with three internal phosphodiester or phosphodiester-like YZ internucleotide linkages, which in turn is more immunostimulatory than an oligonucleotide with two internal phosphodiester or phosphodiester-like YZ internucleotide linkages, which in turn is more immunostimulatory than an oligonucleotide with one internal phosphodiester or phosphodiester-like YZ internucleotide linkage. Importantly, inclusion of even one internal phosphodiester or phosphodiester-like YZ internucleotide linkage is believed to be advantageous over no internal phosphodiester or phosphodiester-like YZ internucleotide linkage. In addition to the number of phosphodiester or phosphodiester-like internucleotide linkages, the position along the length of the nucleic acid can also affect potency.

The soft and semi-soft oligonucleotides will generally include, in addition to the phosphodiester or phosphodiester-like internucleotide linkages at preferred

internal positions, 5' and 3' ends that are resistant to degradation. Such degradation-resistant ends can involve any suitable modification that results in an increased resistance against exonuclease digestion over corresponding unmodified ends. For instance, the 5' and 3' ends can be stabilized by the inclusion there of at least one phosphate modification of the backbone. In a preferred embodiment, the at least one phosphate modification of the backbone at each end is independently a phosphorothioate, phosphorodithioate, methylphosphonate, or methylphosphorothioate internucleotide linkage. In another embodiment, the degradation-resistant end includes one or more nucleotide units connected by peptide or amide linkages at the 3' end.

A phosphodiester internucleotide linkage is the type of linkage characteristic of nucleic acids found in nature. As shown in Figure 20, the phosphodiester internucleotide linkage includes a phosphorus atom flanked by two bridging oxygen atoms and bound also by two additional oxygen atoms, one charged and the other uncharged. Phosphodiester internucleotide linkage is particularly preferred when it is important to reduce the tissue half-life of the oligonucleotide.

A phosphodiester-like internucleotide linkage is a phosphorus-containing bridging group that is chemically and/or diastereomerically similar to phosphodiester. Measures of similarity to phosphodiester include susceptibility to nuclease digestion and ability to activate RNAse H. Thus for example phosphodiester, but not phosphorothioate, oligonucleotides are susceptible to nuclease digestion, while both phosphodiester and phosphorothioate oligonucleotides activate RNAse H. In a preferred embodiment the phosphodiester-like internucleotide linkage is boranophosphate (or equivalently, boranophosphonate) linkage. U.S. Patent No. 5,177,198; U.S. Patent No. 5,859,231; U.S. Patent No. 6,160,109; U.S. Patent No. 6,207,819; Sergueev et al., (1998) *J Am Chem Soc* 120:9417-27. In another preferred embodiment the phosphodiester-like internucleotide linkage is diasteromerically pure Rp phosphorothioate. It is believed that diasteromerically pure Rp phosphorothioate is more susceptible to nuclease digestion and is better at activating RNAse H than mixed or diastereomerically pure Sp phosphorothioate. Stereoisomers of CpG oligonucleotides are the subject of co-pending U.S. patent application 09/361,575 filed July 27, 1999, and published PCT application PCT/US99/17100 (WO

00/06588). It is to be noted that for purposes of the instant invention, the term "phosphodiester-like internucleotide linkage" specifically excludes phosphorodithioate and methylphosphonate internucleotide linkages.

As described above the soft and semi-soft oligonucleotides of the invention may have phosphodiester like linkages between C and G. One example of a phosphodiester-like linkage is a phosphorothioate linkage in an Rp conformation. Oligonucleotide p-chirality can have apparently opposite effects on the immune activity of a CpG oligonucleotide, depending upon the time point at which activity is measured. At an early time point of 40 minutes, the R_p but not the S_p stereoisomer of phosphorothioate CpG oligonucleotide induces JNK phosphorylation in mouse spleen cells. In contrast, when assayed at a late time point of 44 hr, the S_p but not the R_p stereoisomer is active in stimulating spleen cell proliferation. This difference in the kinetics and bioactivity of the R_p and S_p stereoisomers does not result from any difference in cell uptake, but rather most likely is due to two opposing biologic roles of the p-chirality. First, the enhanced activity of the Rp stereoisomer compared to the Sp for stimulating immune cells at early time points indicates that the Rp may be more effective at interacting with the CpG receptor, TLR9, or inducing the downstream signalling pathways. On the other hand, the faster degradation of the Rp PS-oligonucleotides compared to the Sp results in a much shorter duration of signalling, so that the Sp PS-oligonucleotides appear to be more biologically active when tested at later time points.

A surprisingly strong effect is achieved by the p-chirality at the CpG dinucleotide itself. In comparison to a stereo-random CpG oligonucleotide the congener in which the single CpG dinucleotide was linked in Rp was slightly more active, while the congener containing an Sp linkage was nearly inactive for inducing spleen cell proliferation.

The size (i.e., the number of nucleotide residues along the length of the nucleic acid) of the immunostimulatory oligonucleotide may also contribute to the stimulatory activity of the oligonucleotide. For facilitating uptake into cells immunostimulatory oligonucleotides preferably have a minimum length of 6 nucleotide residues. Nucleic acids of any size greater than 6 nucleotides (even many kb long) are capable of inducing an immune response according to the invention if

sufficient immunostimulatory motifs are present, since larger nucleic acids are degraded inside of cells. It is believed by the instant inventors that semi-soft oligonucleotides as short as 4 nucleotides can also be immunostimulatory if they can be delivered to the interior of the cell. In certain preferred embodiments according to the instant invention, the immunostimulatory oligonucleotides are between 4 and 100 nucleotides long. In typical embodiments the immunostimulatory oligonucleotides are between 6 and 40 nucleotides long. In certain preferred embodiments according to the instant invention, the immunostimulatory oligonucleotides are between 6 and 19 nucleotides long.

The immunostimulatory oligonucleotides generally have a length in the range of between 4 and 100 and in some embodiments 10 and 40. The length may be in the range of between 16 and 24 nucleotides.

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 that are covalently attached to low molecular weight organic groups other than a hydroxyl group at the 2' position and other than a phosphate group or hydroxy 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 or 2'-fluoroarabinose 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 an amino acid backbone with nucleic acid bases).

Nucleic acids also include substituted purines and pyrimidines such as C-5 propyne pyrimidine and 7-deaza-7-substituted purine modified bases. Wagner RW et al. (1996) *Nat Biotechnol* 14:840-4. Purines and pyrimidines include but are not limited to adenine, cytosine, guanine, thymine, 5-methylcytosine, 5-hydroxycytosine, 5-fluorocytosine, 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 internucleotide bridge, a β-D-ribose unit and/or a natural nucleotide 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 may have one or more modifications, wherein each modification is located at a particular phosphodiester internucleotide bridge and/or at a particular β-D-ribose unit and/or at a particular natural nucleotide 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 phosphodiester internucleotide bridge located at the 3' and/or the 5' end of a nucleotide by a modified internucleotide bridge,

b) the replacement of phosphodiester bridge located at the 3' and/or the 5' end of a nucleotide by a dephospho bridge,

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

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

e) the replacement of a natural nucleotide base by a modified nucleotide base.

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

A phosphodiester internucleotide bridge located at the 3' and/or the 5' end of a nucleotide can be replaced by a modified internucleotide bridge, wherein the modified internucleotide bridge is for example selected from phosphorothioate, phosphorodithioate, NR^1R^2-phosphoramidate, boranophosphate, α-hydroxybenzyl

phosphonate, phosphate-(C_1-C_{21})-O-alkyl ester, phosphate-[(C_6-C_{12})aryl-(C_1-C_{21})-O-alkyl]ester, (C_1-C_8)alkylphosphonate and/or (C_6-C_{12})arylphosphonate bridges, (C_7-C_{12})-α-hydroxymethyl-aryl (e.g., disclosed in WO 95/01363), wherein (C_6-C_{12})aryl, (C_6-C_{20})aryl and (C_5-C_{14})aryl are optionally substituted by halogen, alkyl, alkoxy, nitro, cyano, and where R^1 and R^2 are, independently of each other, hydrogen, (C_1-C_{18})-alkyl, (C_6-C_{20})-aryl, (C_6-C_{14})-aryl-(C_1-C_8)-alkyl, preferably hydrogen, (C_1-C_8)-alkyl, preferably (C_1-C_4)-alkyl and/or methoxyethyl, or R^1 and R^2 form, together with the nitrogen atom carrying them, a 5-6-membered heterocyclic ring which can additionally contain a further heteroatom from the group O, S and N.

The replacement of a phosphodiester bridge located at the 3' and/or the 5' end of a nucleotide by a dephospho bridge (dephospho bridges are described, for example, in Uhlmann E and Peyman A in "Methods in Molecular Biology", Vol. 20, "Protocols for Oligonucleotides and Analogs", S. Agrawal, Ed., Humana Press, Totowa 1993, Chapter 16, pp. 355 ff), wherein a dephospho bridge is for example selected from the dephospho bridges formacetal, 3'-thioformacetal, methylhydroxylamine, oxime, methylenedimethyl-hydrazo, dimethylenesulfone and/or silyl groups.

A sugar phosphate unit (i.e., a β-D-ribose and phosphodiester internucleotide 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'-F-arabinose, 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).

In some preferred embodiments the sugar is 2'-O-methylribose, particularly for one or both nucleotides linked by a phosphodiester or phosphodiester-like internucleotide linkage.

Nucleic acids also include substituted purines and pyrimidines such as C-5 propyne pyrimidine and 7-deaza-7-substituted purine modified bases. Wagner RW et al. (1996) *Nat Biotechnol* 14:840-4. Purines and pyrimidines include but are not limited to adenine, cytosine, guanine, and thymine, and other naturally and non-naturally occurring nucleobases, substituted and unsubstituted aromatic moieties.

A modified base is any base which is chemically distinct from the naturally occurring bases typically found in DNA and RNA such as T, C, G, A, and U, but which share basic chemical structures with these naturally occurring bases. The modified nucleotide base may be, 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-dimethylguanine, 2,4-diamino-purine, 8-azapurine, a substituted 7-deazapurine, preferably 7-deaza-7-substituted and/or 7-deaza-8-substituted purine, 5-hydroxymethylcytosine, N4-alkylcytosine, e.g., N4-ethylcytosine, 5-hydroxydeoxycytidine, 5-hydroxymethyldeoxycytidine, N4-alkyldeoxycytidine, e.g., N4-ethyldeoxycytidine, 6-thiodeoxyguanosine, and deoxyribonucleotides of nitropyrrole, C5-propynylpyrimidine, and diaminopurine e.g., 2,6-diaminopurine, inosine, 5-methylcytosine, 2-aminopurine, 2-amino-6-chloropurine, hypoxanthine or other modifications of a natural nucleotide bases. This list is meant to be exemplary and is not to be interpreted to be limiting.

In particular formulas described herein a set of modified bases is defined. For instance the letter Y is used to refer to a nucleotide containing a cytosine or a modified cytosine. A modified cytosine as used herein is a naturally occurring or non-naturally occurring pyrimidine base analog of cytosine which can replace this base without impairing the immunostimulatory activity of the oligonucleotide. Modified cytosines include but are not limited to 5-substituted cytosines (e.g. 5-methyl-cytosine, 5-fluoro-cytosine, 5-chloro-cytosine, 5-bromo-cytosine, 5-iodo-cytosine, 5-hydroxy-cytosine, 5-hydroxymethyl-cytosine, 5-difluoromethyl-cytosine, and unsubstituted or substituted 5-alkynyl-cytosine), 6-substituted cytosines, N4-substituted cytosines (e.g. N4-ethyl-cytosine), 5-aza-cytosine, 2-mercapto-cytosine, isocytosine, pseudo-isocytosine, cytosine analogs with condensed ring systems (e.g. N,N'-propylene cytosine or phenoxazine), and uracil and its derivatives (e.g. 5-fluoro-uracil, 5-bromo-uracil, 5-bromovinyl-uracil, 4-thio-uracil, 5-hydroxy-uracil, 5-propynyl-uracil). Some of the preferred cytosines include 5-methyl-cytosine, 5-fluoro-cytosine, 5-hydroxy-cytosine, 5-hydroxymethyl-cytosine, and N4-ethyl-cytosine. In another embodiment of the invention, the cytosine base is substituted by a universal base (e.g. 3-nitropyrrole, P-base), an aromatic ring system (e.g. fluorobenzene or difluorobenzene) or a hydrogen atom (dSpacer).

The letter Z is used to refer to guanine or a modified guanine base. A modified guanine as used herein is a naturally occurring or non-naturally occurring purine base analog of guanine which can replace this base without impairing the immunostimulatory activity of the oligonucleotide. Modified guanines include but are not limited to 7-deazaguanine, 7-deaza-7-substituted guanine (such as 7-deaza-7-(C2-C6)alkynylguanine), 7-deaza-8-substituted guanine, hypoxanthine, N2-substituted guanines (e.g. N2-methyl-guanine), 5-amino-3-methyl-3H,6H-thiazolo[4,5-d]pyrimidine-2,7-dione, 2,6-diaminopurine, 2-aminopurine, purine, indole, adenine, substituted adenines (e.g. N6-methyl-adenine, 8-oxo-adenine) 8-substituted guanine (e.g. 8-hydroxyguanine and 8-bromoguanine), and 6-thioguanine. In another embodiment of the invention, the guanine base is substituted by a universal base (e.g. 4-methyl-indole, 5-nitro-indole, and K-base), an aromatic ring system (e.g. benzimidazole or dichloro- benzimidazole, 1-methyl-1H-[1,2,4]triazole-3-carboxylic acid amide) or a hydrogen atom (dSpacer).

The oligonucleotides may have one or more accessible 5' ends. It is possible to create modified oligonucleotides having two such 5' ends. This may be achieved, for instance by attaching two oligonucleotides through a 3'-3' linkage to generate an oligonucleotide having one or two accessible 5' ends. The 3'3'-linkage may be a phosphodiester, phosphorothioate or any other modified internucleotide bridge. Methods for accomplishing such linkages are known in the art. For instance, such linkages have been described in Seliger, H.; et al., Oligonucleotide analogs with terminal 3'-3'- and 5'-5'-internucleotidic linkages as antisense inhibitors of viral gene expression, Nucleotides & Nucleotides (1991), 10(1-3), 469-77 and Jiang, et al., Pseudo-cyclic oligonucleotides: in vitro and in vivo properties, Bioorganic & Medicinal Chemistry (1999), 7(12), 2727-2735.

Additionally, 3'3'-linked nucleic acids where the linkage between the 3'-terminal nucleotides is not a phosphodiester, phosphorothioate or other modified bridge, can be prepared using an additional spacer, such as tri- or tetra-ethylenglycol phosphate moiety (Durand, M. et al, Triple-helix formation by an oligonucleotide containing one (dA)12 and two (dT)12 sequences bridged by two hexaethylene glycol chains, Biochemistry (1992), 31(38), 9197-204, US Patent No. 5658738, and US Patent No. 5668265). Alternatively, the non-nucleotidic linker may be derived from ethanediol, propanediol, or from an abasic deoxyribose (dSpacer) unit (Fontanel, Marie Laurence et al., Sterical recognition by T4 polynucleotide kinase of non-nucleosidic moieties 5'-attached to oligonucleotides; Nucleic Acids Research (1994), 22(11), 2022-7) using standard phosphoramidite chemistry. The non-nucleotidic linkers can be incorporated once or multiple times, or combined with each other allowing for any desirable distance between the 3'-ends of the two ODNs to be linked.

For use in the instant invention, the oligonucleotides of the invention can be synthesized *de novo* using any of a number of procedures well known in the art. For example, the b-cyanoethyl phosphoramidite method (Beaucage, S.L., and Caruthers, M.H., *Tet. Let.* 22:1859, 1981); nucleotide H-phosphonate method (Garegg *et al.*, *Tet. Let.* 27:4051-4054, 1986; Froehler *et al.*, *Nucl. Acid. Res.* 14:5399-5407, 1986, ; Garegg *et al.*, *Tet. Let.* 27:4055-4058, 1986, Gaffney *et al.*, *Tet. Let.* 29:2619-2622, 1988). These chemistries can be performed by a variety of automated nucleic acid synthesizers available in the market. These oligonucleotides are referred to as

synthetic oligonucleotides. An isolated oligonucleotide generally refers to an oligonucleotide which is separated from components which it is normally associated with in nature. As an example, an isolated oligonucleotide may be one which is separated from a cell, from a nucleus, from mitochondria or from chromatin.

The oligonucleotides are partially resistant to degradation (*e.g.*, are stabilized). A "stabilized oligonucleotide molecule" shall mean an oligonucleotide that is relatively resistant to *in vivo* degradation (e.g. via an exo- or endo-nuclease). Nucleic acid stabilization can be accomplished via backbone modifications. Oligonucleotides having phosphorothioate linkages provide maximal activity and protect the oligonucleotide from degradation by intracellular exo- and endo-nucleases. Other modified oligonucleotides include phosphodiester modified nucleic acids, combinations of phosphodiester and phosphorothioate nucleic acid, 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 (e.g., Uhlmann, E. and Peyman, A., *Chem. Rev.* 90:544, 1990; Goodchild, J., *Bioconjugate Chem.* 1:165, 1990).

Other stabilized oligonucleotides 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.

The immunostimulatory oligonucleotides may also contain one or more unusual linkages between the nucleotide or nucleotide-analogous moieties. The usual internucleoside linkage is the 3'5'-linkage. All other linkages are considered as

unusual internucleoside linkages, such as 2'5'-, 5'5'-, 3'3'-, 2'2'-, 2'3'-linkages. Thereby, the nomenclature 2' to 5' is chosen according to the carbon atom of ribose. However, if unnatural sugar moieties are employed, such as ring-expanded sugar analogs (e.g. hexanose, cylohexene or pyranose) or bi- or tricyclic sugar analogs, then this nomenclature changes according to the nomenclature of the monomer. In 3'-deoxy-β-D-ribopyranose analogs (also called *p*-DNA), the mononucleotides are e.g. connected via a 4'2'-linkage.

If the nucleotide contains one 3'3'-linkage, then this oligonucleotide analog will have two unlinked 5'-ends. Similarly, if the nucleotide contains one 5'5'-linkage, then this oligonucleotide analog will have two unlinked 3'-ends. The accessibility of unlinked ends of nucleotides may be better accessible by their receptors. Both types of unusual linkages (3'3'- and 5'5') were described by Ramalho Ortigao et al. (Antisense Research and Development (1992) 2, 129-46), whereby oligonucleotides having a 3'3'-linkage were reported to show enhanced stability towards cleavage by nucleases.

Different types of linkages can also be combined in one molecule which may lead to branching of the oligomer. If one part of the oligonucleotide is connected at the 3'-end via a 3'3'-linkage to a second oligonucleotide part and at the 2'-end via a 2'3'-linkage to a third part of the molecule, this results e.g. in a branched oligonucleotide with three 5'-ends (3'3'-, 2'3'-branched).

In principle, linkages between different parts of an oligonucleotide or between different oligonucleotides, respectively, can occur via all parts of the molecule, as long as this does not negatively interfere with the recognition by its receptor. According to the nature of the nucleic acid, the linkage can involve the sugar moiety (Su), the heterocyclic nucleobase (Ba) or the phosphate backbone (Ph). Thus, linkages of the type Su-Su, Su-Ph, Su-Ba, Ba-Ba, Ba-Su, Ba-Ph, Ph-Ph, Ph-Su, and Ph-Ba are possible. If the oligonucleotides are further modified by certain non-nucleotidic substituents, the linkage can also occur via the modified parts of the oligonucleotides. These modifications include also modified nucleic acids, e.g. PNA, LNA, or Morpholino Oligonucleotide analogs.

The linkages are preferably composed of C, H, N,O , S, B, P, and Halogen, containing 3 to 300 atoms. An example with 3 atoms is an acetal linkage (ODN1-3'-

O-CH_2-O-3'-ODN2; Froehler and Matteucci) connecting e.g. the 3'-hydroxy group of one nucleotide to the 3'-hydroxy group of a second oligonucleotide. An example with about 300 atoms is PEG-40 (tetraconta polyethyleneglycol). Preferred linkages are phosphodiester, phosphorothioate, methylphosphonate, phosphoramidate, boranophosphonate, amide, ether, thioether, acetal , thioacetal, urea, thiourea, sulfonamide, Schiff' Base and disulfide linkages. Another possibility is the use of the Solulink BioConjugation System (www.trilinkbiotech.com).

If the oligonucleotide is composed of two or more sequence parts, these parts can be identical or different. Thus, in an oligonucleotide with a 3'3'-linkage, the sequences can be identical 5'-ODN1-3'3'-ODN1-5' or different 5'-ODN1-3'3'-ODN2-5'. Furthermore, the chemical modification of the various oligonucleotide parts as well as the linker connecting them may be different. Since the uptake of short oligonucleotides appears to be less efficient than that of long oligonucleotides, linking of two or more short sequences results in improved immune stimulation. The length of the short oligonucleotides is preferably 2-20 nucleotides, more preferably 3-16 nucleotides, but most preferably 5-10 nucleotides. Preferred are linked oligonucleotides which have two or more unlinked 5'-ends.

The oligonucleotide partial sequences may also be linked by non-nucleotidic linkers, in particular abasic linkers (dSpacers), trietyhlene glycol units or hexaethylene glycol units. Further preferred linkers are alkylamino linkers, such as C3, C6, C12 aminolinkers, and also alkylthiol linkers, such as C3 or C6 thiol linkers. The oligonucleotides can also be linked by aromatic residues which may be further substituted by alkyl or substituted alkyl groups. The oligonucleotides may also contain a Doubler or Trebler unit (www.glenres.com), in particular those oligonucleotides with a 3'3'-linkage. Branching of the oligonucleotides by multiple doubler, trebler, or other multiplier units leads to dendrimers which are a further embodiment of this invention. The oligonucleotides may also contain linker units resulting from peptide modifying reagents or oligonucleotide modifying reagents (www.glenres.com). Furthermore, it may contain one or more natural or unnatural amino acid residues which are connected by peptide (amide) linkages.

Another possibility for linking oligonucleotides is via crosslinking of the heterocyclic bases (Verma and Eckstein; Annu. Rev. Biochem. (1998) 67: 99-134;

page 124). Yet another possibility is a linkage between the sugar moiety of one sequence part with the heterocyclic base of another sequence part (Iyer et al. Curr. Opin. Mol. Therapeutics (1999) 1: 344-358; page 352).

The different oligonucleotides are synthesized by established methods and can be linked together on-line during solid-phase synthesis. Alternatively, they may be linked together post-synthesis of the individual partial sequences.

A "subject" shall mean a human or vertebrate animal including but not limited to a dog, cat, horse, cow, pig, sheep, goat, chicken, non-human primate (e.g., monkey), fish (aquaculture species, e.g., salmon), rabbit, rat, and mouse.

A "subject having a viral infection" is a subject that has been exposed to a virus and has acute or chronic manifestations or detectable levels of the virus in the body. In preferred embodiments of the invention, the subject is one having a chronic viral infection, more preferably a chronic hepatitis C infection. In important aspects of the invention, the subject is one that is non-responsive to prior therapy for hepatitis C infection. For example, a non-responsive subject includes one that was previously treated for hepatitis C infection with, for example, IFN-α (e.g., Intron A), and but such treatment was not successful, as described herein. The invention intends to treat subjects that are non-responsive, and in some instances to identify subjects that would be non-responsive in order to triage effective treatment.

Immunostimulatory nucleic acids can be effective in any vertebrate. Different immunostimulatory nucleic acids can cause optimal immune stimulation depending on the mammalian species. Thus an immunostimulatory nucleic acid causing optimal stimulation or inhibition in humans may not cause optimal stimulation or inhibition in a mouse, and vice versa. One of skill in the art can identify the most appropriate immunostimulatory nucleic acids useful for a particular mammalian species of interest using routine assays described herein and/or known in the art, using the guidance supplied herein.

The immunostimulatory nucleic acid 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., pDCs or B cells) 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 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; emulsomes; ISCOMs; liposomes; live bacterial vectors (e.g., *Salmonella, Escherichia coli, Bacillus Calmette-Guerin, Shigella, Lactobacillus*); live viral vectors (e.g., Vaccinia, adenovirus, Herpes Simplex); microspheres; nucleic acid vaccines; polymers (e.g., carboxymethylcellulose, chitosan); polymer rings; Proteosomes; sodium fluoride; transgenic plants; virosomes; virus-like particles. Those skilled in the art will recognize that other delivery vehicles that are known in the art may also be used.

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 therapeutic treatment regimen can be planned which does not cause substantial toxicity and yet is entirely effective to treat the particular subject as described above. 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 (e.g., the class of CpG immunostimulatory nucleic acid, the number of unmethylated CpG motifs or their location in the nucleic acid, the degree of chirality to the oligonucleotide, etc.), whether an antigen is also administered and the nature of such 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 acids and/or other therapeutic agent without necessitating undue experimentation.

For adult human subjects, doses of the immunostimulatory nucleic acids compounds described herein typically range from about 50 μg/dose to 20 mg/dose,

more typically from about 80 μg/dose to 8 mg/dose, and most typically from about 800 μg/dose to 4 mg/dose. Stated in terms of subject body weight, typical dosages range from about 0.5 to 500 μg/kg/dose, more typically from about 1 to 100 μg/kg/dose, and most typically from about 10 to 50 μg/kg/dose. Doses will depend on factors including the route of administration, e.g., oral administration may require a substantially larger dose than subcutaneous administration.

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.

The immunostimulatory nucleic acids can be given in conjunction with other agents known in the art to be useful in treating viral infections. Of particular importance is the combination of immunostimulatory nucleic acids with anti-viral agents such as IFN-α, as demonstrated in the Examples section, to provide a synergistic response. Immunostimulatory nucleic acids can be used as a substitute for Ribavirin, which currently is administered together with IFN-α. Examples of such other agents currently used or under investigation for use in combination with IFN-α include amantadine, and cytokines, including IL-2, IL-10, IL-12, and IFN-γ.

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, including translation initiation (e.g., interferon, antisense, and ribozymes), virus enzymes (e.g., nonstructural serine proteases, RNA polymerases, reverse transcriptases and helicases), replication of viral RNA or DNA (e.g., nucleoside analogues), maturation of new virus proteins (e.g., protease inhibitors such as serine protease inhibitor BILN2061ZW from Boehringer Ingelheim), anti-oxidants such as Livfit (USP 6,136,316), and budding and release of the virus. Other anti-viral agents are described in USPs 6,130,326, and 6,440, 985, and published US patent application

20020095033. Ribavirin analogues are also anti-viral agents embraced by the invention.

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.

Immunoglobulin therapy is typically used for the prevention of viral infection, but can also be used to reduce levels of circulating virus and preventing newly formed cells from becoming infected. Immunoglobulin therapy for viral infections is different than bacterial infections, because rather than being antigen-specific, the immunoglobulin therapy functions by binding to extracellular virions and preventing them from attaching to and entering cells which are susceptible to the viral infection. The therapy is useful for the reduction of viremia for the period of time that the antibodies are present in the host. In general there are two types of immunoglobulin therapies, normal immunoglobulin therapy and hyper-immunoglobulin therapy. Normal immune globulin therapy utilizes an antibody product which is prepared from the serum of normal blood donors and pooled. This pooled product contains low titers of antibody to a wide range of human viruses, such as hepatitis A, parvovirus, enterovirus (especially in neonates). To use normal immune globulin therapy for HCV, the serum would have to be obtained from people who were previously infected with HCV and who have successfully cleared the infection, either spontaneously or with some form of therapy. Hyper-immune globulin therapy utilizes antibodies which are prepared from the serum of individuals who have high titers of an antibody to a particular virus. Those antibodies are then used against a specific virus. For HCV, hyper-immune globulins could be produced by vaccinating volunteers with recombinant HCV proteins to produce hepatitis C immune globulin.

Other anti-virals suitable in the methods of the invention are manufactured by Triangle Pharmaceuticals, Inc., Gilead, ICN, Procter and Gamble and ViroPharma Incorporated.

For use in therapy, an effective amount of the immunostimulatory nucleic acid can be administered to a subject by any mode that delivers the immunostimulatory nucleic acids to the desired site, 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, intralesional, topical, transdermal, intramuscular, intranasal, intratracheal, inhalational, ocular, vaginal, and rectal.

For oral administration, the compounds (i.e., immunostimulatory nucleic acids, or other therapeutic agents) can be formulated readily by combining 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, acrosols, 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 *Science* 249:1527 (1990), which is incorporated herein by reference.

The immunostimulatory nucleic acids 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 percent w/v); citric acid and a salt (1-3 percent w/v); boric acid and a salt (0.5-2.5 percent w/v); and phosphoric acid and a salt (0.8-2 percent w/v). Suitable preservatives include benzalkonium chloride (0.003-0.03 percent w/v); chlorobutanol (0.3-0.9 percent w/v); parabens (0.01-0.25 percent w/v) and thimerosal (0.004-0.02 percent w/v).

The pharmaceutical compositions of the invention contain 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 which would substantially impair the desired pharmaceutical efficiency.

A variety of administration routes are available. The particular mode selected will depend, of course, upon the particular adjuvants or antigen selected, the particular condition being treated and the dosage required for therapeutic efficacy. The methods of this invention, generally speaking, may be practiced using any mode of administration that is medically acceptable, meaning any mode that produces effective levels of an immune response without causing clinically unacceptable adverse effects. Preferred modes of administration are discussed above.

The compositions may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy. All methods include the step of bringing the compounds into association with a carrier which constitutes one or more accessory ingredients. In general, the compositions are prepared by uniformly and intimately bringing the compounds into association with a

liquid carrier, a finely divided solid carrier, or both, and then, if necessary, shaping the product. Liquid dose units are vials or ampoules. Solid dose units are tablets, capsules and suppositories. For treatment of a patient, 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.

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-based 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.

In some embodiments, the immunostimulatory nucleic acid is modified. In certain embodiments, the immunostimulatory nucleic acid has a modified backbone with at least one nuclease-resistant internucleotide linkage. A nuclease-resistant internucleotide linkage can be selected from the group which includes a phosphorothioate linkage, a phosphorodithioate linkage, a methylphosphonate linkage, and a peptide linkage. In certain embodiments a modified

immunostimulatory nucleic acid includes at least one nucleotide analog or at least one nucleotide analog. The immunostimulatory nucleic acid is a palindrome in certain embodiments, while in other embodiments, the immunostimulatory nucleic acid is not a palindrome. In some preferred embodiments the immunostimulatory nucleic acid is between 8 and 100 nucleotides in length, while in other preferred embodiments the immunostimulatory nucleic acid is between 12 and 40 nucleotides in length. Preferred sizes, sequences and modifications are described in greater detail below.

The following examples are included for purposes of illustration and are not intended to limit the scope of the invention.

Examples

The purpose of this study was to evaluate the ability of different classes of CpG ODN to stimulate PBMC from HCV chronic carriers. PBMC were isolated from whole blood collected from normal, healthy volunteers and chronic carriers of HCV and the ability of the different classes CpG ODNs as well as soft and semi-soft molecules to stimulate B cell proliferation, cytokine secretion (IFN-g, TNF-α, IL-10 and IFN-α) and chemokine secretion (IP-10) in vitro was evaluated.

Also evaluated were the immune stimulatory effects of exogenous IFN-α-2b (Intron A) and Ribavirin, either alone, in combination with each other, and in combination with CpG ODN (B and C classes).

MATERIALS AND METHODS

Oligonucleotides

All oligonucleotide stocks were resuspended in TE buffer at pH 8.0 (OmniPer®; EM Science, Gibbstown, NJ). Dilutions of various ODNs were made in RPMI 1640 complete media (Gibco BRL, Grand Island, NY) containing 10% heat inactivated, normal human AB serum (Wisent Inc, St. Bruno, QC) and 1% penicillin/streptomycin (Gibco BRL, Grand Island, NY) just prior to their use in cell assays. For the exogenous IFN-α synergy experiments, Intron A (Interferon Alfa-2b, DIN 02223406, Schering Canada Inc., Pointe-Claire, Quebec, Canada) was added to the ODN solutions to give final concentrations of 125 or 1000 IU/ml. Ribavirin (CAS 36791-04-5, Calbiochem, CN Biosciences Inc., La Jolla, CA , USA) was

reconstituted with sterile distilled water to produce a 500 μm stock and diluted in media as described above, to give a final concentration of 5μm in wells. Cells were incubated at 37°C with 5% CO_2. After 48h, cell supernatants were collected from each well and frozen at –80°C.

ODNs used in experiments are shown in the following table:

Table 2: Sequences of oligos used in experiments

SEQ ID NO:	CLASS	SEQUENCE
1	A	G-G-GGACGACGTCGTGG-G-G-G-G-G
2	B	TCG TCG TTT TGT CGT TTT GTC GTT
3	Control for B Class	TGC TGC TTT TTG CTG GCT TTT T
4	C	TCGTCGTTTTCGGCGGCCGCCG
5	B	TCGTCGTTTCGTCGTTTTGTCGTT
6	B	TCG TCG TTT TTC GTG CGT TTT T
7	Soft C	TCGTCGTTT-T-C-G-G-CGGCCGCCG
8	semi-soft B	TC-GTC-GTTTT-GTC-GTTTTGTC-GTT
9	Semi-soft C	TCGTC-GTTTTCGGC-GGCCGCCG
10	Semi-soft C	TCGTCGTTTTC-GGCGGCC-GCCG
11	Semi-soft C	TCGTCG-TTTTC-GGCGCGC-GCCG
12	Semi-soft C	TCGTC-GTTTTC-GGC-GCGC-GCCG
13	Semi-soft C	TCGTCGTTTTAC-GGC-GCC-GTGCCG
14	Semi-soft C	TCGTCG-TTTTAC-GGCGCC-GTGCCG
15	Semi-soft C	TCGTC-GTTTTAC-GGCGCC-GTGCCG
16	Semi-soft C	TCGTC-GTTTTC-GGCGGCC-GCCG

* A phosphodiester bond replacing a phosphorothioate bond within the oligonucleotide backbone is indicated by (-)

Isolation of PBMCs

Whole blood (200 ml) was collected by venous puncture into heparinized green top vacutainers from ten (10) normal, healthy, adult subjects and fifteen (15) adult subjects chronically infected with HCV who had a previous 6 month course of an IFN-α-based therapy and were either a treatment failure or a relapsed responder. Peripheral blood mononuclear cells (PBMCs) were purified by centrifugation over Ficoll-Hypaque (Amersham Pharmacia Biotech, Uppsala, Sweden) at 400 x g for 35 min. Cells were resuspended at a concentration of $10x10^6$/ml in RPMI complete media containing 10% normal human AB serum (heat inactivated) and 1% penicillin/streptomycin.

B-cell Proliferation

Cells were isolated as described above and resuspended at $1x10^6$/ml in complete RPMI media 100 µl of cells were added to each well of round-bottom 96 well plates. ODN solutions (100 µl) were added to wells to give the selected range of final concentrations (1, 3, 6µg/ml). Cells were cultured for 5 days and then pulsed with ^{3}H-Thymidine (1 µCi/well) for 18h, before harvesting onto filter paper for measuring radioactivity. Results are reported as stimulation index (SI) with respect to untreated media control.

Cytokine Assays

Freshly isolated PBMCs were resuspended at $10x10^6$/ml (2x final concentration) and 100 µl of cells were added to each well of a 96 well flat-bottom plate containing an equal volume of ODN solution (2x final desired concentration). A range of concentrations (1, 3, 6 µg/ml) was tested for each ODN. Cells were incubated at 37°C with 5% CO_2. After 48h, cell supernatants were collected from each well and frozen at –80°C until assayed.

IFN-α, IP-10, IL-10 and IFN-. levels in supernatants were measured using commercial ELISA Kits (R&D Systems, Minneapolis, MN, USA; IP-10, Cat# DIP 100, IL-10, Cat# D1000, IFN-g Cat# DIF50 or PBL Biomedical, IFN-α Cat# 4110S). When measured ELISA values were below the detection limit of the kit as specified

by the manufacturer, a value equal to the lowest detectable limit was entered into data tables.

RESULTS

PBMCs isolated from blood collected from 15 chronically infected HCV subjects and 10 normal healthy volunteers were incubated at 37°C with different classes of CpG (e.g., class A, B, C, soft C, semi-soft B and semi-soft C), and cell supernatants were assessed for cytokine presence, indicative of cytokine secretion during the incubation period. Results of these experiments are presented below.

Induction of IFN-α secretion by PBMC

When the three classes of CpG ODN were tested on PBMC from normal volunteers, very high levels of IFN-α were produced by the A class (CpG SEQ ID NO. 1), moderately high levels by the C class (CpG SEQ ID NO. 4) and only low levels were induced by the B class (CpG SEQ ID NO. 2) (Figure 1). The main cellular source of IFN-α is pDC .

With the PBMC obtained from HCV chronic carriers, all three classes of CpG could induce secretion of IFN-α. The levels with the B and C classes were the same as those obtained with the normal PBMCs. In contrast, the A class induced only about 50% of the normal level (Figure 1), suggesting that the dysfunction of the HCV-infected pDC has some impact on the efficacy of A class CpG to induce IFN-α, but not the C class. Thus, either A or C class CpG could be used to treat HCV chronic carriers, but in some instances the C class may be preferred.

The number of pDC was determined by FACS analysis. A linear regression was performed against this compared to the amount of IFN-α secreted with either the A and C class CpG ODN, and a reasonable correlation for the normal subjects (e.g., R=0.43 and 0.58, respectively) was found. It was further discovered that the correlation was slightly better for the C class ODNs. In contrast, no correlation was observed between number of pDC and amount of IFN-α secreted for the HCV infected subjects (R=0.02 and 0.08, respectively) (Figure 3). The HCV-infected DC are nevertheless capable of secreting IFN-α in response to the CpG ODN.

The effect of soft and semi-soft alterations of these ODS was also analyzed. Soft molecules were synthesized that had a row of phosphodiester bonds in the central region of the molecule. Semi-soft molecules were synthesized that had one or more individual phosphodiester bonds that are between the cytosine and guanine nucleotides of the CpG motifs. Both soft (Figure 3) and semi-soft (Figure 4) C class CpG ODN were capable of stimulating IFN-α secretion from normal or HCV PBMC in a manner similar to the original C class CpG ODN. Several of the semi-soft C class CpG ODN were even more potent that the regular C class CpG SEQ ID NO. 4 (Figure 4). This may be because the molecule is still sufficient stable to have maximal immune stimulation and the phosphodiester in the middle of the CpG motif ?mass? increase its activity.

Induction of IFN-γ secretion by PBMC

Figure 5 compares the ability of different classes of CpG to induce the secretion of Th1 cytokine, IFN-γ. Class A induced low levels of IFN-γ while in comparison class B produced moderate amounts and class C CpG stimulated high concentrations of IFN-γ. Both HCV-infected and normal PBMCs displayed a similar Th1 response to all three classes of CpG. Similar results were obtained with semi-soft class C CpG ODN (Figure 6).

Induction of IP-10 secretion by PBMC

IP-10, a chemokine associated with production of type 1 and 2 interferons, is also induced by CpG ODN. Highest levels are induced with A class, next highest with C class and lowest with B class CpG ODN. Regardless of the class of CpG ODN, similar levels of IP-10 were induced with PBMCs from normal subjects and HCV chronic carriers (Figure 7).

B cell stimulation by CpG ODN

The effect of CpG on B cell stimulation was also investigated. As shown in Figures 8 and 9, CpG Class A was a poor stimulator of B cells for both HCV-infected and normal populations. In contrast, classes B, C and semi-soft C CpG strongly activated B cells. There were no differences between PBMCs from normal and HCV-infected subjects.

IL-10 secretion from PBMC after stimulation with CpG ODN

The production of cytokine IL-10 following stimulation with CpG was also assessed and these results are shown in Figure 10. For both HCV-infected and normal subjects, all classes of CpG induced significant secretion of IL-10, and there were no differences between PBMC from normal volunteers and those from HCV chronic carriers. Several cell types can produce IL-10 after incubation with CpG; however since B cells are the major producers of this cytokine, IL-10 production can be used as an indicator of the level of B cell activation.

Effects of Intron A and Ribavirin

The in vitro effect of Ribavirin and exogenous IFN-α Intron A , alone or in combination with CpG was tested on HCV-infected cells. Neither Ribavirin nor Intron A, on their own or together, resulted in the induction of IFN-α secretion by HCV-infected PBMCs (Figure 11).

As has been discussed above, A and C class CpG ODN result in strong induction of IFN-α secretion from pDC from normal and HCV-infected subjects. Furthermore, when CpG and Intron-A were used together, there was a synergistic response for the majority (60%) of subjects (Figure 12).

DISCUSSION

CpG ODN are able to induce DC from patients chronically infected with HCV, to secrete IFN-α, with higher levels using Class A and C CpG and lower levels with Class B CpG ODN. The levels of secreted IFN-α are comparable to those observed with cells from normal healthy volunteers. As well, IP-10 is induced from stimulated HCV PBMC, further indicating a Th1-type immune activation.

HCV antigen-specific immune responses are already present in persons chronically infected with HCV. These are Th2-biased and thus cannot bring about clearance of the HCV-infected cells. Th1 type responses would be required for viral clearance. Augmentation of systemic levels of Th1 cytokines, without additional antigen, allows persons chronically infected with HCV to develop Th1-type HCV-specific immune responses that are instrumental in viral clearance. All classes of CpG (A, B and C) are capable of establishing Th1-type responses. These Th1-type

responses are essential for long-term clearance of HCV chronic infection, yet they are difficult to induce with exogenous IFN-α therapy, which has direct anti-viral effects but not direct effects on the immune system. CpG ODN can therefore be used in combination with exogenous IFN-α to treat HCV chronic carriers.

Alternatively, CpG ODN could also be used alone. Owing to induction of cytokines such as IFN-α and IFN-γ, CpG ODN on its own has direct anti-viral effects, in addition to the induction of Th1-type HCV-specific immune responses. In some instances, the A and C class molecules are preferred since they induce higher levels of IFNs. Depending on their characteristic sequence, CpG ODN can preferentially stimulate pDC functions, maturation and type I IFN production (Krug, A et al., Eur J Immunol, 2001; 31:2154-2163). Although according to the invention two classes of CpG ODN were shown to be superior at stimulating IFN-α production, any CpG ODN, regardless of backbone or CpG sequence, could be used in the treatment of chronic HCV. The controlled release of different type I IFN isoforms by specific CpG ODN in vivo is superior to the systemic administration of recombinant type I IFN that is of a single subtype (e.g., Intron A is only IFN-α 2b). Soft and semi-soft versions of CpG ODN are capable of stimulating similar levels of IFN-α as their parent molecule. Soft or semi-soft versions of the CpG ODN, especially the C class, would preferentially be used for chronic treatment of HCV, as they are more easily degraded and would therefore not be expected to accumulate in the organs, specifically the liver, spleen and kidney.

At least 50% of the HCV subjects failed to respond to exogenous IFN-α therapy, however CpG ODN (especially A and C class) were able to induce IFN-α secretion in vitro at levels comparable to normal healthy volunteers in all subjects. CpG ODN could therefore be used to treat patients who have failed to respond to exogenous IFN-α therapy, whether the IFN is pegylated or not, and whether the treatment also includes Ribavirin or not. Classes of CpG ODN that induce high levels of IFN-α would be preferred, and ever more preferred for long-term treatment would be the semi-soft versions.

Neither commercial Intron-A (IFN-α-2b) nor Ribavirin, alone or in combination, were capable of inducing IFN-α secretion from PBMCs from normal or HCV infected subjects in vitro. However when CpG ODN was used in combination

with Intron- A, a synergistic effect was observed for IFN-α secretion from PBMCs from HCV infected subjects. The C class of ODN were shown to have synergy with exogenous IFN-α; however treatment with any class of CpG ODN with commercial alpha-interferons would be therapeutically effective. As mentioned previously, due to their relative ease of degradation into non-stimulatory metabolites, semi-soft versions of the CpG ODN could be used for chronic treatment without fear of accumulation in end-organs such as the kidney.

Ribavirin is purported to have Th1 effects, but in these studies it had no immunostimulatory activity on human PBMCs. Even in combination with Intron A, Ribavirin did not enhance endogenous IFN-α production. Thus, replacing Ribavirin in combination therapy for HCV with a CpG ODN will increase the proportion of sustained viral responses. When combined with CpG ODN, Ribavirin reduced the efficacy of CpG. CpG ODN should therefore be given in combination with alpha-interferons in the absence of Ribavirin.

CpG ODN have been administered IM, SC and IV to human subjects and were determined to be well tolerated and safe (clinical study, in progress). Any effective route of administration would be acceptable such as SC, IM, IV, inhalation etc. however subcutaneous administration would be the route of choice. CpG ODN were diluted in TE buffer and added to PBMCs however, CpG ODN could also be formulated in delivery systems such as bioadhesive polymers (Sha et al., 1999), cochleates (Gould-Fogerite et al., 1994, 1996), dendrimers (Kukowska-Latallo et al.,1996, Qin et al, 1998), enteric-coated capsules (Czerkinsky et al., 1987, Levine et al., 1987), emulsomes (Vancott et al., 1998, Lowell et al., 1997), ISCOMs (Mowat et al., 1993, Morein et al., 1999, Hu et al., 1998, Carlsson et al., 1991), liposomes (Childers et al., 1999, Michalek et al., 1989, 1992), microspheres (Gupta et al., 1998, Maloy et al., 1994, Eldridge et al., 1989), nanospheres (Roy et al., 1999), polymer rings (Wyatt et al., 1998), proteosomes (Lowell et al., 1988, 1996) and virosomes (Gluck et al., 1992, Mengiardi et al., 1995, Cryz et al., 1998).

For treatment of HCV chronic carriers, CpG ODN could be administered on a repeated basis from once daily to once monthly, but preferably every 3-10 days, and most preferably weekly, for a prolonged period. This period could be from one month to two years, but preferably 3 to 12 months, and most preferably for 6 months. Thus the most optimal therapy would be given twice weekly or weekly for 6 months. It

could also be given more frequently during an inductive phase (daily or every other day or twice weekly or weekly for the first 1-3 months), then less frequently for maintenance (weekly, or every other week, or monthly for several more months).

For combination therapy, CpG and alpha-interferons (pegylated or not) could potentially be (i) mixed together and given at the same time and by the same route (subcutaneous), (ii) given at the same time and same route but not mixed, (iii) given at the same time but by different routes (e.g., the alpha-interferon could be given SC and the CpG could be IV, IM, ID, orally or topically), (iv) given at different times and schedules with same or different routes, or (v) given consecutively. In this latter case, preferably the IFN-α would be given first in order to reduce viral load, then the CpG ODN would be given afterwards to induce and sustain Th1-type adaptive immunity for long term control.

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Equivalents

It will be understood that various modifications may be made to the embodiments disclosed herein. Therefore, the above description should not be construed as limiting, but merely as exemplifications of preferred embodiments. Those skilled in the art will envision other modifications within the scope of the claims appended hereto.

All references, patents and patent applications disclosed herein are incorporated by reference in their entirety.

We claim:

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A method of stimulating an immune response in a subject having an HCV infection that was not successfully treated using a previous non-CpG therapy comprising administering to a subject in need of such stimulation a CpG immunostimulatory nucleic acid in an amount effective to stimulate said immune response.

2. The method of claim 1, wherein the non-CpG therapy includes interferon-alpha.

3. The method of claim 2, wherein the interferon-alpha is interferon-alpha-2b, interferon-alpha-2a or consensus interferon-alpha.

4. The method of claim 2, wherein the non-CpG therapy includes interferon-alpha and Ribavirin.

5. The method of claim 2, wherein the non-CpG therapy includes pegylated interferon-alpha and Ribavirin.

6. The method of any one of claims 1 to 5, wherein the CpG immunostimulatory nucleic acid is an A class CpG immunostimulatory nucleic acid.

7. The method of any one of claims 1 to 5, wherein the CpG immunostimulatory nucleic acid is a B class CpG immunostimulatory nucleic acid.

8. The method of any one of claims 1 to 5, wherein the CpG immunostimulatory nucleic acid is a C class CpG immunostimulatory nucleic acid.

9. The method of any one of claims 1 to 8, further comprising the step of administering an antiviral agent to the subject.

10. The method of claim 9, wherein the antiviral agent is interferon-alpha.

11. The method of claim 9, wherein the antiviral agent is interferon-alpha-2b, interferon-alpha-2a or consensus interferon alpha.

12. The method of claim 9, wherein the antiviral agent is administered substantially simultaneously with the CpG immunostimulatory nucleic acid.

13. The method of claim 1, wherein the CpG immunostimulatory nucleic acid comprises a backbone modification.

14. The method of claim 13, wherein the backbone modification is a phosphorothioate backbone modification.

15. The method of claim 1, wherein the CpG immunostimulatory nucleic acid comprises a semi-soft backbone.

16. A method of stimulating an immune response in a subject having an HCV infection that was not successfully treated using a previous non-CpG therapy comprising administering to a subject in need of such stimulation a C class CpG immunostimulatory nucleic acid having a semi-soft backbone in an amount effective to stimulate said immune response.

17. A method of stimulating an immune response in a subject having an HCV infection and likely to be non-responsive to a non-CpG therapy comprising administering to a subject in need of such stimulation a C class CpG immunostimulatory nucleic acid having a semi-soft backbone in an amount effective to stimulate said immune response.

18. A method of stimulating an immune response in a subject having an HCV infection that was not successfully treated using a previous non-CpG therapy comprising contacting peripheral blood mononuclear cells from a subject in need of such stimulation, with a CpG immunostimulatory nucleic acid in an amount effective to stimulate an immune response, and re-infusing the cells into the subject.

19. The method of claim 18, wherein the peripheral blood mononuclear cells comprise dendritic cells.

20. The method of claim 19, wherein the dendritic cells comprise plasmacytoid dendritic cells.

21. The method of claim 18, wherein the CpG immunostimulatory nucleic acid is a C class immunostimulatory nucleic acid.

22. The method of claim 21, wherein the C class immunostimulatory nucleic acid has a semi-soft backbone.

23. The method of claim 1 or any one of claims 16 to 18, substantially as hereinbefore described.

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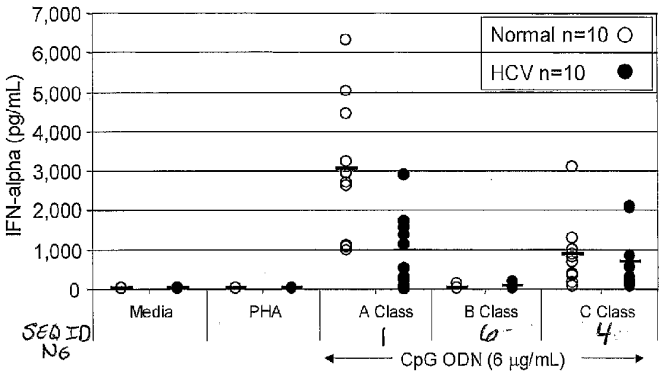

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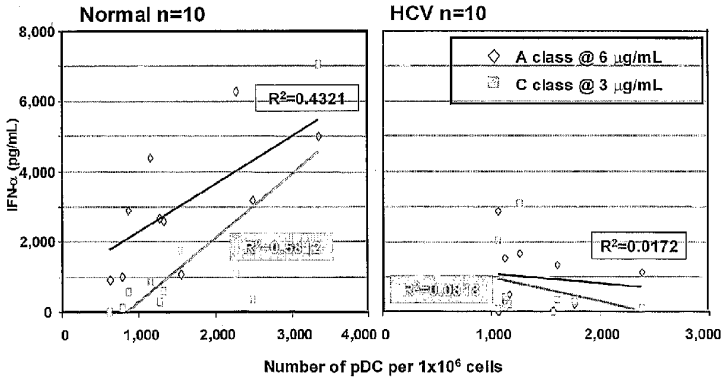

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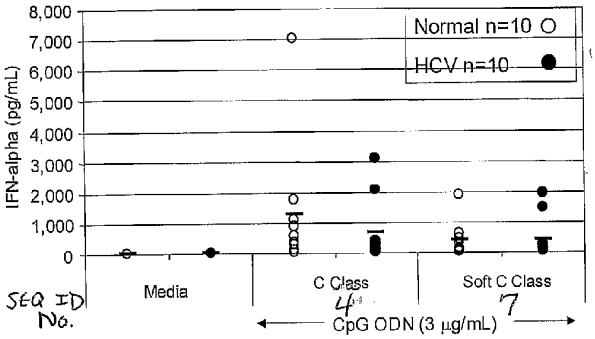

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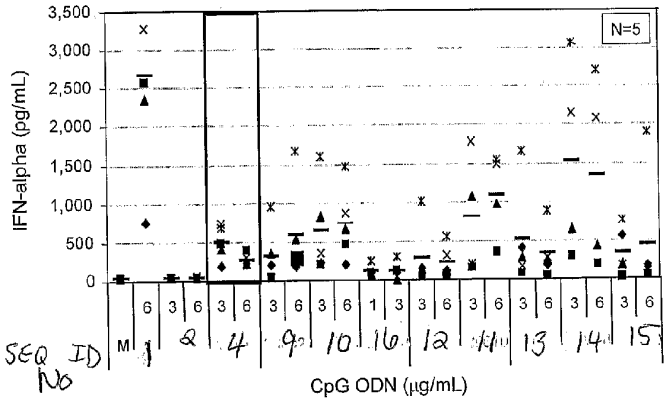

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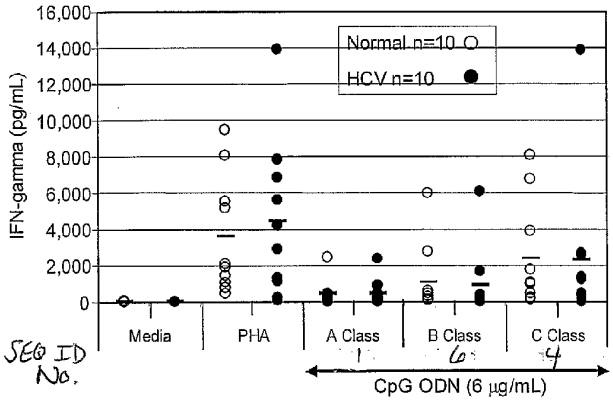

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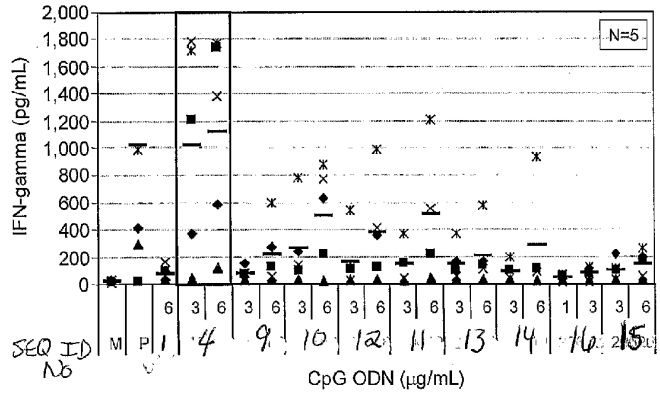

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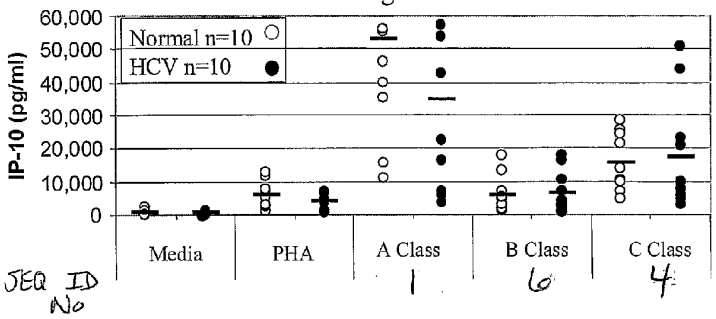

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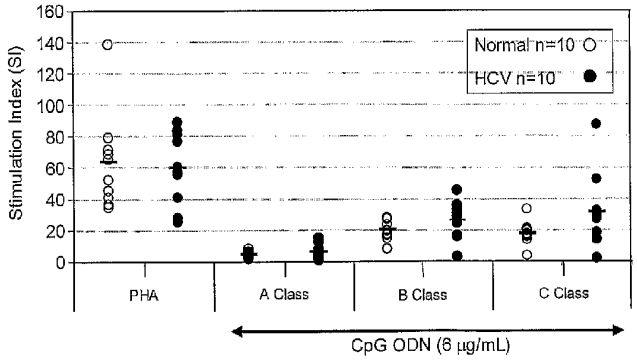

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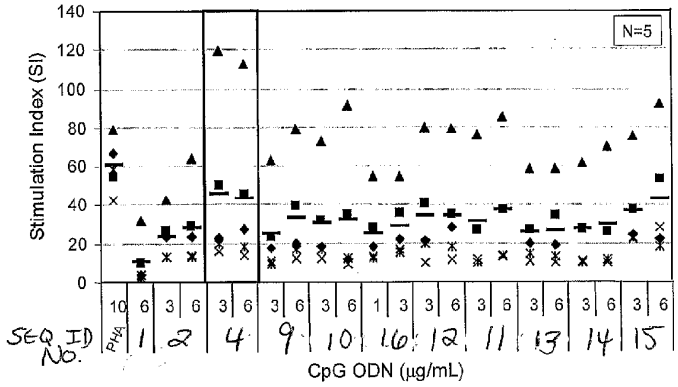

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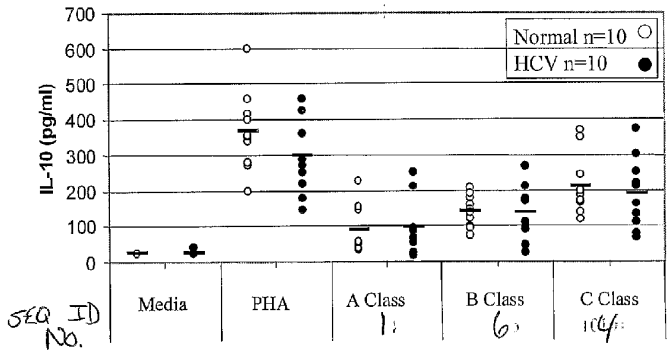

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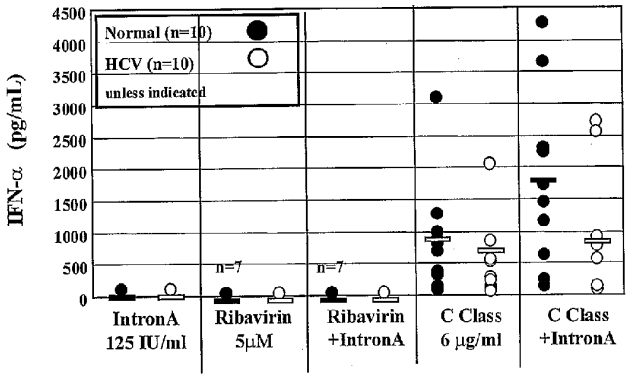

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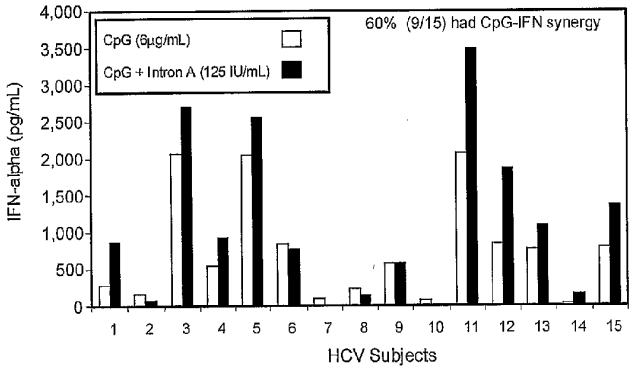

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