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Induction of immune activation by a novel immunomodulatory oligonucleotide without thymocyte apoptosis

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Abstract

Bacterial DNA and synthetic oligodeoxynucleotides (ODN) containing unmethylated CpG motifs (CpG DNA) can potently stimulate innate immunity. While the actions of CpG DNA resemble those of LPS, these molecules stimulate distinct Toll-like receptors as well as cell types. In a previous study, we showed that a CpG ODN could induce cytokine production but, unlike LPS, did not induce thymocyte apoptosis. In this study, we have further investigated these differences using as a model a second-generation immunostimulatory oligonucleotide called HYB2048. Following administration to normal BALB/c mice, HYB2048-induced IL-12 but not IL-6 production. Under conditions in which LPS induced thymocyte apoptosis, HYB2048 did not cause significant cell death and, furthermore, did not block apoptosis induced by LPS. The levels of corticosterone induced by HYB2048 were also significantly lower than those induced by LPS. This pattern of activation could distinguish CpG DNA from LPS in its effects on the immune system.

Keywords: CpG DNA; Oligonucleotide; Thymocyte; Apoptosis; LPS

Bacterial and synthetic DNA, depending on base sequence and backbone chemistry, can exert powerful immune actions to trigger innate immunity and promote host defense [1,2]. Among sequences conferring activity, CpG motifs occur much more commonly in bacterial than mammalian DNA and can serve as pathogen associated molecular patterns (PAMPs) to activate B cells, macrophages, and dendritic cells (DCs) [3–5]. While the activity of CpG DNA resembles that of LPS, these molecules trigger different Toll-like receptors (TLRs), with LPS stimulating TLR4 and CpG DNA stimulating TLR9 [6,7]. Both LPS and CpG DNA, however, involve similar downstream signaling systems, including myeloid differentiation factor 88 (MyD88), NF-κB and the mitogen-activated protein kinases (MAPKs) [8–10]. The consequences of stimulating different TLRs are not known, although they could be relevant in the encounter of bacteria and the mammalian host.

Because of its unique molecular properties and pattern of immune stimulation, CpG DNA in the form of oligonucleotides (ODNs) has been explored as a novel immunomodulator to promote immune responses in various contexts, including vaccines, malignancy, and infection [11–13]. To develop such agents, the influence of sequence and backbone modifications has been extensively investigated, with the most experience gained with the phosphorothioate (Ps) backbone. This modification, which involves the substitution of a sulfur for a non-bridging oxygen, leads to increased stability and resistance to nuclease digestion. Ps CpG ODN resembles bacterial DNA in activity, although differences in the stimulation of various cell types can occur depending on nucleotide sequence and backbone chemistry. Thus, CpG ODNs with a Ps backbone preferentially stimulate B cells [1,3], while CpG ODNs with phosphodiester [2] or mixed phosphodiester and Ps backbone and poly(dG) sequences either directly or indirectly stimulate NK cell or DC activities [3,14,15].

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Recent studies on structure–activity relationships of ODN have shown that a number of synthetic pyrimidine (Y) and purine (R) nucleotides are accepted by TLR9 as substitutes for natural deoxycytidine and deoxyguanosine in a CpG dinucleotide [16,17]. These studies have led to the development of synthetic immunostimulatory motifs, YpG, CpR, and YpR, and have established a nucleotide motif recognition pattern (NMRP) for TLR9 [16,18]. A number of site-specific chemical modifications in the flanking sequences of the CpG dinucleotide have also allowed modulation of immunostimulatory activities [18–21]. Consistent with findings that TLR9 reads the CpG DNA sequence from the 5′-end [16,17,22], CpG ODNs with two 5′-ends, immunomers, show potent immunostimulatory activity with distinct cytokine profiles in vitro and in vivo [16,23–26].

In a previous study, we compared patterns of immune cell activation by LPS and a conventional CpG ODN that is active on B cells, macrophages, and DCs [27]. Specifically, we were interested in the capacity of these molecules to induce apoptosis since LPS administration leads to significant thymocyte death which may contribute ultimately to immunosuppression. In normal mice, we found that, while both LPS and CpG ODN induced similar levels of IL-12, LPS stimulation led to significant thymocyte death under conditions in which the CpG ODN had much less effect; this difference was observed using both FACS analysis and assay of blood nucleosomes which can represent the remnants of dead and dying cells. These findings pointed to fundamental differences in the stimulation by these classes of PAMP.

In the current study, we have used as a model for CpG DNA, HYB2048, an immunomer containing a synthetic CpR (R = 2′-deoxy-7-deazaguanosine) motif, and tested its effects on cytokine production and thymocyte apoptosis in mice. HYB2048 has been shown to induce higher IL-12 and lower IL-6 secretion in vitro and in vivo as well as rapid and enhanced activation of the transcription factor NF-κB [16]. In results presented herein, we show that HYB2048, while inducing cytokine production and lymphocyte activation in the spleen, nevertheless did not cause significant thymocyte apoptosis. Furthermore, levels of corticosterone, which can lead to thymocyte apoptosis, were much lower in mice treated with HYB2048 than LPS. Together, these findings indicate that a synthetic ODN can induce a distinct pattern of immune activation that may be relevant in the context of drug development as well as the diverse roles played with LPS and DNA in the setting of infection.

Materials and methods

Animals. Female BALB/c mice were purchased at the age of 4–6 weeks from the Jackson Laboratory (Bar Harbor, ME) and were kept in isolated cages. Food and water were provided ad lib. All animal procedures were conducted in conformity with protocols approved by the Durham Veterans Affairs Medical Center.

DNA and LPS. LPS (*Escherichia coli* 0111:B4) was purchased from Sigma Chemical (St. Louis, MO). A conventional CpG ODN (5′-TCCATGACGTTCCTGACGT-3′, denoted as SAK2) was purchased from Midland Certified Reagent (Midland, TX) HYB2048 (5′-TCTGACRTTCT-X-TCTTRCAGTCT-5′, wherein X and R in HYB2048 stand for a glycerol linker and 2′-deoxy-7-deazaguanosine) was synthesized and characterized as described previously [16]. Specific stimulatory motifs are shown as underlined.

Treatment and sample collection. Mice received either 50 μg LPS or 50 μg HYB2048, i.p. in 0.2 ml sterile PBS (pH 7.4). A vehicle control group was also included. Mice were anesthetized by methoxyflurane and plasma was collected at 4 and 24 h after treatment. For the experiments on corticosterone induction, a conventional CpG ODN (SAK2) group was also tested using the same injection route and dose as HYB2048. All mice were anesthetized by isoflurane inhalation and serum samples were collected 4 h after treatment.

Each experiment was performed at least twice. Results are presented from a representative experiment.

Cytokine assays. Cytokine concentrations (IL-6 and IL-12) were determined by enzyme linked immunoabsorbent assays (ELISA). Capture antibodies, anti-IL-6 and anti-IL-12 p40/p70 (BD Pharmingen, San Diego, CA), were coated overnight at 4 °C on Immunlon 96-well plates at a concentration of 3 μg/ml in 0.1 M phosphate buffer (pH 9.0). Plates were washed three times, plasma samples were added in duplicate and incubated at room temperature for 2 h. Biotinylated anti-IL-6 or anti-IL-12 p40/p70 detection antibodies (BD Pharmingen, San Diego, CA) were added and incubated for an additional 2 h at room temperature. Avidin conjugated horseradish peroxidase was added for a 30 min incubation at room temperature followed by TMB substrate (0.015% 3,3′,5,5′-tetramethylbenzidine, 0.01% H_2O_2 in 0.1 M citrate buffer, pH 4.0) until color development. Three PBS washes were performed between steps and plates were read at OD380 using an automated ELISA plate reader (UV MAX, Molecular Devices, Sunnyvale, CA).

Apoptosis analysis. Mice were euthanized by cervical dislocation under anesthesia and the thymuses were aseptically removed. Single cell preparations were prepared by mincing thymuses with sterile glass slides and rinsed with PBS. Thymocytes were then stained with Annexin V-FITC (BD Pharmingen, San Diego, CA) and propidium iodide (PI) (Sigma Chemical, St. Louis, MO) and incubated at room temperature for 15 min. Spleens were also collected at the time of euthanasia. Splenocytes were also stained with Annexin V-FITC and PI. Stained cells were then subjected to FACS analysis (FACScan, BD Pharmingen, San Diego, CA) and FACS results were analyzed by CellQuest version 3.3.

Thymocyte and splenocyte phenotype distribution. Thymuses were collected 24 h after either HYB2048 or LPS treatment. A vehicle control group was also included. A single cell preparation was prepared and stained with anti-CD4-FITC and/or anti-CD8-PE (BD Pharmingen, San Diego, CA). Splenocytes were also collected and stained with anti-B220-Cy-Chrome or anti-Thy1.2-FITC (BD Pharmingen, San Diego, CA). To determine the phenotype distribution of activated splenocytes, cells were stained with anti-CD69-PE, anti-B220-CY-Chrome or anti-Thy1.2-FITC. Stained cells were subjected to FACS analysis and results were analyzed by CellQuest version 3.3.

Serum corticosterone assay. Serum corticosterone levels were assayed using a commercial corticosterone enzyme immunoassay (EIA) kit (Assay Designs, Ann Arbor, MI). Serum samples were diluted 1:100 and assayed according to manufacturer's suggestion. Assays were read at OD405 and corticosterone concentration was determined by plotting against standards.

Statistical analysis. All data collected were analyzed by two-tailed, unequal variance Student's t test. P value less than 0.05 was considered significant.

Results

Cytokine levels after DNA or LPS administration

To determine cytokine production by HYB2048 or LPS, plasma samples were collected at different time points after administration and analyzed by ELISA. In these experiments, we tested a single concentration of both compounds on the basis of prior dose–response experiments. With compounds at 50 μg per mouse (2.5 mg/kg), both LPS and HYB2048 stimulated IL-12 production as early as 4 h after in vivo administration (Fig. 1A). HYB2048 at this dose induced higher levels of IL-12 than LPS. The plasma IL-12 levels declined over time and returned to baseline levels at around 24 h after stimulation as previously observed using a conventional CpG ODN [27].

IL-6 is another cytokine induced during stimulation by CpG ODN both in vitro and in vivo [28,29]. In the current study, LPS increased plasma IL-6 levels as early as 4 h after stimulation (Fig. 1B). HYB2048, however, did not induce appreciable IL-6 production at the time points assayed. Similar to the pattern of IL-12 induction, IL-6 production induced by LPS decreased to background levels 24 h after stimulation.

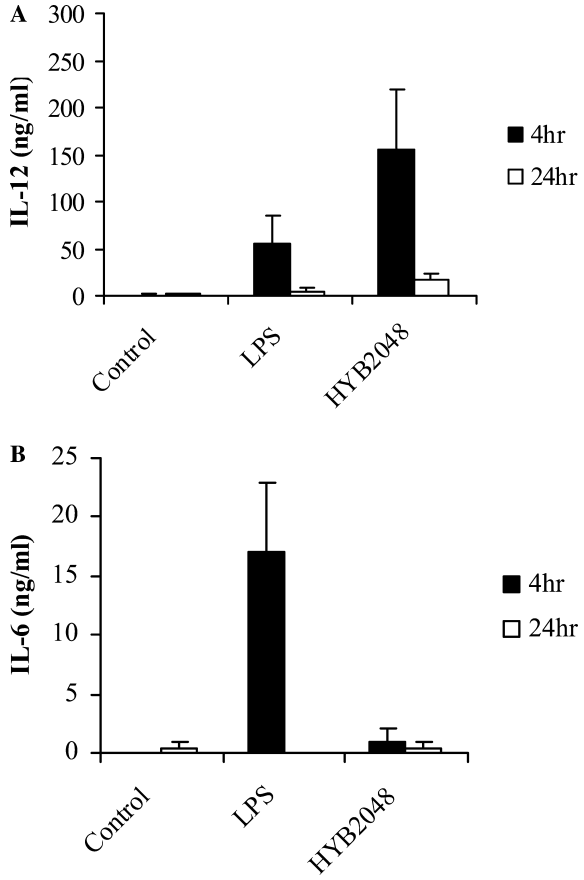

Fig. 1. Plasma cytokine levels after HYB2048 and LPS stimulation. Plasma IL-12 (A) and IL-6 (B) levels were determined by ELISA at various time points indicated. Each bar represents the mean of five mice. Error bars depict the standard deviation.

Effect of treatments on thymocyte survival

To analyze the influence of LPS and HYB2048 on thymocyte survival, FACS analysis was performed on thymocytes stained with Annexin V-FITC and PI. Twenty-four hours after stimulation, surviving thymocyte numbers in LPS-treated group were significantly lower than those of the control group ($P < 0.05$) (Fig. 2). In contrast, the HYB2048 treated group did not show a significant decrease in the number of cells surviving. In a similar experiment, a conventional CpG ODN also did not induce thymocyte apoptosis (Fig. 3). Neither conventional CpG ODN nor HYB2048 prevented LPS-induced thymocyte death (Figs. 2 and 3). At a lower dose of 5 μg/animal, neither ODN caused thymocyte deaths, although levels of cytokines were lower (data not shown).

Thymocyte and splenocyte phenotype distribution

To determine the effect of HYB2048 on the phenotype distribution of immune cells, FACS analysis was performed on thymocytes and splenocytes. The administration of either LPS or HYB2048 did not affect the $CD4^+$ and $CD8^+$ cell distribution based on the percentage of total thymocytes as shown in Fig. 4. The effect of HYB2048 and LPS on spleen cell populations was also tested. Although the administration of either

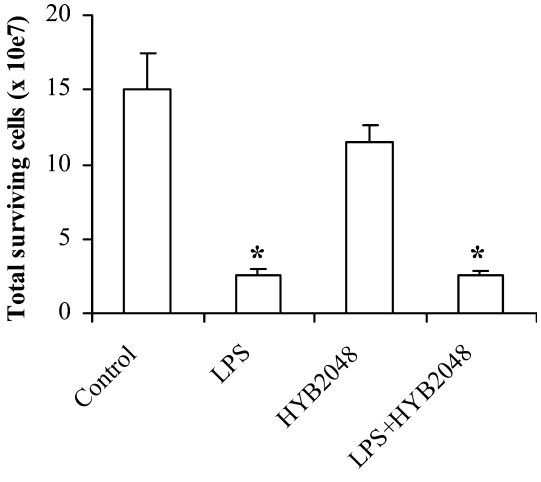

Fig. 2. Total surviving thymocytes after LPS and HYB2048 stimulation. Thymocytes were collected 24 h after administration of LPS, HYB2048, LPS plus HYB2048 or vehicle, and stained for Annexin V and PI. All cells were analyzed by two-color FACS and each bar represents the mean of the surviving cells which were identified as negative for both Annexin V and PI. There were five mice in each treatment group. Error bars depict the standard deviations. The asterisk indicates a result statistically significant when compared to either vehicle control or HYB2048 group ($P < 0.05$).

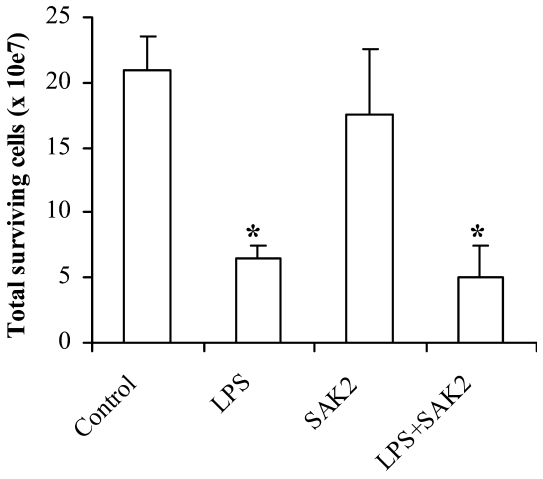

Fig. 3. Total surviving thymocytes after LPS and conventional CpG ODN stimulation. Thymocytes were collected 24 h after LPS, a conventional CpG ODN (SAK2), LPS plus SAK2 or vehicle administration, and stained for Annexin V and PI. All cells were analyzed by two-color FACS and each bar represents the mean of total number of cells negative for both Annexin V and PI staining from five mice in each treatment group. Error bars depict the standard deviations. The asterisk indicates a value statistically significant when compared to either vehicle control or SAK2 group ($P < 0.05$).

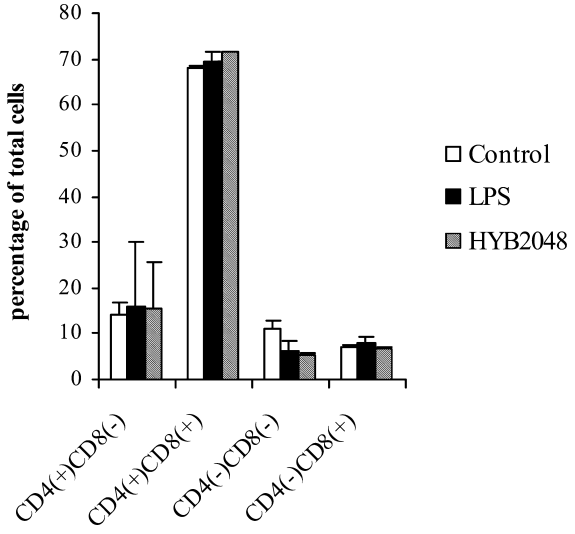

Fig. 4. The effects of HYB2048 and LPS on thymocyte phenotype. Thymocytes were collected 24 h after either HYB2048 or LPS treatment. Cells were stained with anti-CD4 and/or -CD8 conjugates and subjected to FACS analysis. Each bar represents percentage of total thymocytes.

HYB2048 or LPS did not reduce the number of spleen cells or modify the splenocyte phenotype distribution (Fig. 5), administration of HYB2048 did modify the activated splenocyte phenotype distribution. Both $B220^+$ and $Thy1.2^+$ cell populations showed an increase in the number of activated cells ($CD69^+$) in mice treated with HYB2048 compared to either the control or LPS group (Fig. 6). In contrast, the conventional CpG ODN

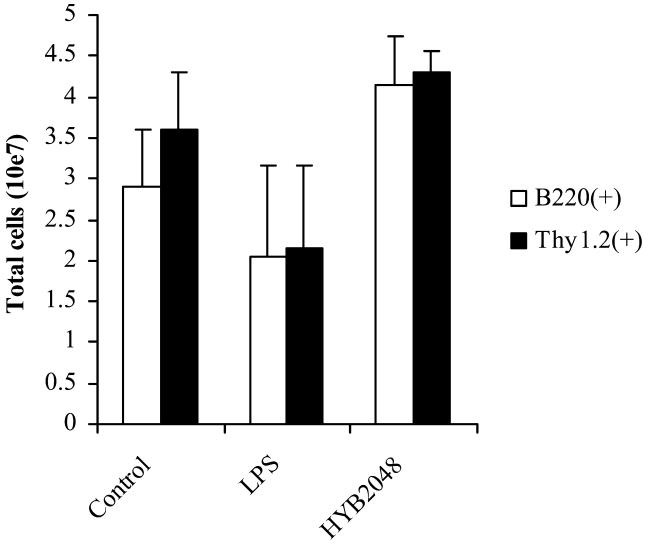

Fig. 5. The effects of HYB2048 on splenocyte phenotype. Splenocytes collected 24 h after stimulation were stained with anti-B220 and anti-Thy1.2 conjugates. Open and closed bars represent $B220^+$ cells and $Thy1.2^+$ cells, respectively. Error bars depicts the standard deviation.

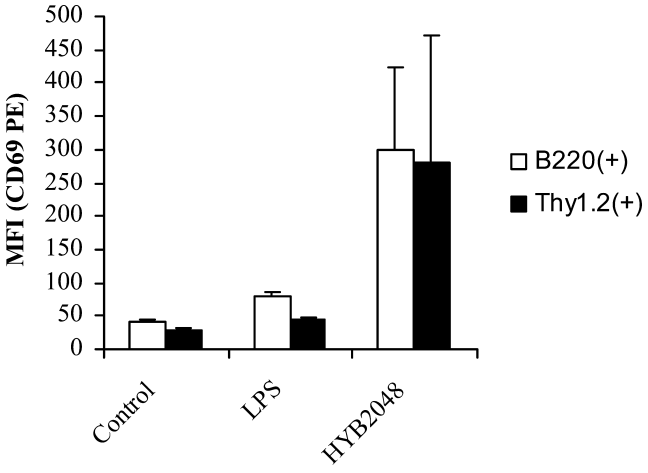

Fig. 6. The effects of HYB2048 on splenocyte CD69 expression. Splenocytes collected 24 h after stimulation were stained with anti-CD69, anti-B220, and anti-Thy1.2 conjugates. Open bars and closed bars represent the mean fluorescence intensity of $B220^+$ and $Thy1.2^+$ cells, respectively.

administration only increased the $B220^+$ population (data not shown).

Corticosterone analysis

Systemic administration of bacterial endotoxin can activate the hypothalamus–pituitary–adrenal (HPA) axis and increase glucocorticoid levels [30]. Since glucocorticoids play an important role in immune regulation and induce thymocyte apoptosis, we investigated whether the differences in the effects of HYB2048 and LPS were associated with glucocorticoid levels.

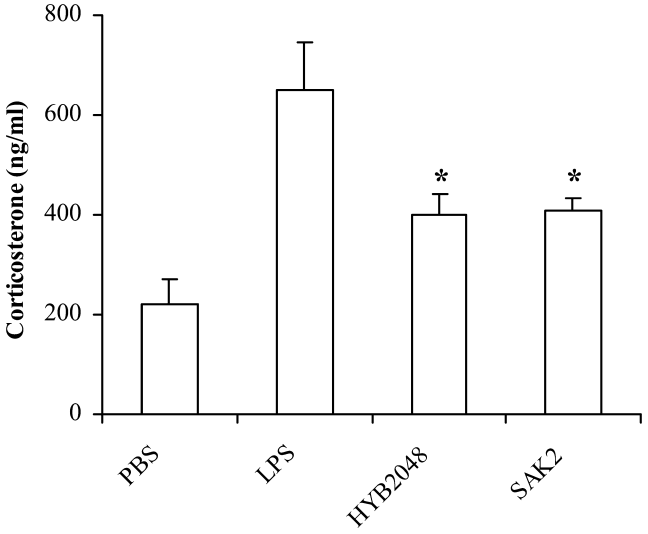

Fig. 7. Corticosterone induction by DNA and LPS. Serum samples collected 48 h after vehicle, LPS, or CpG ODN (HYB2048 and conventional CpG ODN) administration were analyzed for corticosterone levels by EIA. Each bar represents mean of corticosterone levels in five mice. Error bars depict the standard deviations. The asterisk indicates a value statistically significant when compared to vehicle or LPS administered mice ($P < 0.05$).

We therefore analyzed serum levels of corticosterone, a major murine glucocorticoid, at 4 h after administration. Serum corticosterone levels increased in both LPS and HYB2048 groups when compared to PBS controls (Fig. 7); the LPS group, however, had a higher corticosterone level than the HYB2048 group ($P < 0.05$). A similar increase in corticosterone level was observed with the conventional CpG ODN (Fig. 7). These results indicate that HYB2048 and LPS, in addition to differences in their effects on lymphocyte populations, also differ in the magnitude of corticosterone stimulation which may relate to effects on thymocytes.

Discussion

Results presented herein provide new insights into the immune effects of DNA and point to potentially important differences between stimulation by CpG DNA and LPS. Thus, we found that, while both LPS and a second-generation immunomer induced a significant cytokine response, they differed in the occurrence of thymocyte apoptosis as well as glucocorticoid induction. Coupled with previous studies on the in vivo effects of CpG ODN [27], these findings suggest that stimulation by these compounds may lead to a pattern of immune activation that favors responsiveness since the consequences of thymocyte loss may be averted.

Although CpG DNA and LPS utilize some common signaling pathways, they involve different toll receptors with distinct patterns of cellular expression. In humans, TLR4 is expressed on myeloid DCs while plasmacytoid DCs preferentially express TLR9 after stimulation [31]. In addition, LPS signaling also has MyD88-independent pathways [32], while CpG DNA signaling is MyD88-dependent [31]. As a result, the in vivo consequences of stimulation by CpG ODN and LPS may differ because of the distribution of these molecules and regulation of responses by other cytokines such as IL-10 [33].

In the current study, we evaluated stimulation of IL-6 and IL-12. Whereas LPS and HYB2048 both induced IL-12 production, at the dose tested, HYB2048 induced higher IL-12 levels than LPS. In contrast, despite more robust stimulation of IL-12, HYB2048 did not stimulate production of IL-6, a cytokine induced by other Ps ODNs. Since HYB2048 has been developed as a second-generation ODN, these findings suggest that structural modification allows a more selective pattern of immune activation [16].

While LPS causes potent immune stimulation, it has deleterious effects as exemplified by septic shock. As shown in in vivo experiments, stimulation by LPS can lead to apoptosis, with the thymus a major target tissue [34,35]. The loss of thymocytes can lead to immune suppression and thus impair further host responses following an initial wave of immune activation. In contrast to LPS, CpG ODNs are generally much less toxic and, in in vitro experiments, can even prevent the induction of apoptosis in certain cell populations [36,37]. The current study shows that the systemic administration of an IMO fails to induce thymocyte apoptosis following stimulation with a compound that leads to levels of IL-12 even higher than LPS.

Previous experiments on the induction of shock by CpG ODN were based on mice treated with galactosamine to increase their sensitivity to TNF that can be induced by CpG ODN or LPS [38]. In our experiments, we have used normal mice and showed that doses of the CpG DNA as high as 200 μg/mouse are well tolerated whereas mice treated with LPS at doses of 50 μg/animal showed evident signs of sickness as well as mortality.

Apoptosis is a complex, regulated cellular process that can be induced by a wide variety of stimuli. In the thymus, apoptosis can occur in both physiologic and pathologic settings, with glucocorticoids implicated as a major factor. Glucocorticoids are usually produced by the adrenal gland through the operation of the HPA axis. There is evidence, however, that immune cells such as B and T cells can produce adrenocorticotropic hormone (ACTH) which can induce glucocorticoids [39,40]. During septic shock or treatment with LPS, cortisol increases may result from the effects of LPS-induced cytokines, such as IL-1, IL-6, and TNF-α [41], on the HPA axis as well LPS induction of leukocyte-derived bioactive corticotropin [30]. Proinflammatory cytokines such as TNF-α and IFN-γ may also promote thymocyte apoptosis [42–44].

While glucocorticoids have anti-inflammatory action that could counter events during sepsis, excessive production of these hormones could lead to immunosuppression resulting from cell loss. Since cytokines induced by LPS have been linked to glucocorticoid induction [44–47], it is surprising that CpG DNA, which also stimulates the production of these cytokines, does not induce thymocyte death. The lack of thymocyte death has been observed with both a conventional CpG ODN as well as HYB2408.

While the basis for the differences between LPS and CpG DNA on thymocyte apoptosis is not known, results from the current study suggest a relationship to the glucocorticoid induction. Thus, HYB2048 induced significantly lower corticosterone levels compared to LPS. A conventional CpG ODN showed a similar result as HYB2048, suggesting that low glucocorticoid induction may be a property of CpG DNA. A previous study showed that stimulation of mice by bacterial DNA also did not cause corticosterone induction [48]. Similarly, the previous study showed that, although bacterial DNA itself did not induce corticosterone, it did not prevent LPS-induced corticosterone induction [48].

Taken together, these findings suggest that stimulation by CpG DNA, depending of structure, leads to a unique pattern of activity that differs from that of LPS. These differences can be categorized in terms of the array of cytokines induced, the pattern of lymphocyte activation in the spleen, the induction of thymocyte apoptosis, and the induction of glucocorticoid production. These differences may reflect the cell types stimulated and the signaling pathways induced [31,49].

In the setting of infection, CpG DNA may activate innate immunity with fewer adverse effects than LPS, causing limited "danger" that leaves the immune system more intact. In the pharmacologic use of DNA, this spectrum of activity may be particularly favorable, with the adjuvant effects resulting from immune cell activation not counterbalanced by immune cell loss via apoptosis. Furthermore, the much less profound effects on glucocorticoids induction may also allow greater T cell responsiveness. Future studies will clarify the signaling pathways involved in these various responses and promote the development of ODN optimized for therapeutic use.

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