Published in final edited form as:

Cell Immunol. 2008 February ; 251(2): 78–85. doi:10.1016/j.cellimm.2008.04.001.

IN VIVO EFFICACY OF A PHOSPHODIESTER TLR-9 APTAMER AND ITS BENEFICIAL EFFECT IN A PULMANARY ANTHRAX INFECTION MODEL

Christina C.N. Wu^{a,*}, Mojgan Sabet^{b,1}, Tomoko Hayashi^a, Rommel Tawatao^a, Joshua Fierer^{b,c}, Dennis A. Carson^a, Donald G. Guiney^b, and Maripat Corr^d

^aRebecca and John Moores UCSD Cancer Center, University of California San Diego, La Jolla CA

^bDivision of Infectious Diseases, University of California San Diego, La Jolla CA

^cVA San Diego Healthcare System, University of California San Diego, La Jolla CA

^dDivision of Rheumatology, Allergy and Immunology, Department of Medicine, University of California San Diego, La Jolla CA

Abstract

Immunostimulatory oligonucleotide (ISS-ODN) used as adjuvants are commonly modified with phosphorothioate (PS). The PS backbone prevents nuclease degradation, but confers undesired side effects, including systemic cytokine release. Previously, R10-60, a phosphodiester (PO) ISS-ODN, was structurally optimized as an intracellular Toll-like receptor-9 agonist. Here intravenous, intradermal and intranasal administration of PO R10-60 elicit local or adaptive immune responses with minimal systemic effects compared to a prototypic PS ISS-ODN in mice. Furthermore, prophylactic intranasal administration of PO R10-60 significantly delayed death in mice exposed to respiratory anthrax comparable to the PS ISS-ODN. The pattern of cytokine release suggested that early IL-1 β production might contribute to this protective effect, which was replicated with recombinant IL-1 β injections during infection. Hence, the transient effects from a PO TLR-9 agonist may be beneficial for protection in a bacterial bioterrorism attack, by delaying the onset of systemic infection without the induction of a cytokine syndrome.

Keywords

phosphodiester oligonucleotide; TLR-9; cytokines; lung; anthrax

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*Address correspondence and reprint requests to Dr. Christina C.N. Wu, Moores UCSD Cancer Center, 3855 Health Sciences Drive, La Jolla, CA 92093-0820, TEL: 858 534-8798, FAX: 858 534-5399, c5wu@ucsd.edu (C.C.N. Wu).

¹C.C.N.W. and M.S. contributed equally to this work.

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1. Introduction

The prophylactic use of immunostimulatory oligonucleotides (ISS-ODN) in vaccines has been validated in clinical trials [1, 2]. These ODN contain unmethylated CpG dinucleotides, which bind to TLR-9 and signal through MyD88. The most commonly used ODN are modified with phosphorothioate (PS) to render them less susceptible to nuclease digestion. While PS ISS-ODN are generally safe, a number of studies have reported side effects associated with the use of these slowly degraded ODN. Long-lasting immune-stimulating effects include non-sequence specific B cell activation [3–6], lymph node swelling, and sustained cytokine release in the systemic circulation [7, 8], leading to a flu-like “cytokine syndrome” [9].

Phosphodiester oligonucleotides (PO ISS-ODN) also stimulate TLR-9, but have less commonly been used due to rapid degradation by nucleases [10] limiting their *in vivo* activity due to a short half-life [11, 12]. Their shorter half-life may limit their potency, but also their potential toxicity. However, several studies have shown that specific modifications of PO ISS-ODN improve their efficacy as *in vivo* adjuvants. Examples of bioeffective alterations include conjugation of the PO ISS-ODN to a hexameric deoxyriboguanosine (3′dG₆) at the 3′-end [13], and chemically linking two PO ISS-ODN via their 3′ ends. We recently reported the structural requirements for PO ISS-ODN to penetrate cells and to elicit a functional TLR-9 response [14, 15]. Serial selection from a random library and optimal structural modifications resulted in the generation of a PO ISS-ODN, R10-60, which contains three CpG dinucleotides, a hairpin secondary structure near the core CpG motif, and a guanine rich 3′ tail. The 3′ guanine rich tail allows multimerization necessary for cellular uptake, while the rigid secondary structure allows for presentation of the core CpG motif to TLR-9. The PO ISS-ODN R10-60 stimulated IL-6 and IL-12 production in TLR-9 and MyD88 expressing macrophages and dendritic cells, but not the corresponding null cells, confirming its selection specificity as a TLR-9 aptamer [15]. However, a larger dose of this PO ISS-ODN was required to stimulate cytokine release from these antigen presenting cells than the well-characterized PS ISS-ODN, 1018. These *in vitro* experiments suggested that the *in vivo* efficacy of R10-60 may be limited by dose and the rapidity of degradation. Hence the PO ISS-ODN should be tested for a variety of potential applications in varying dose ranges.

An optimized immunostimulatory agent may prove critical in promoting an immediate and rapid response against a highly virulent pathogen. In general, an antibody or cellular immune response may protect against these agents; however, generating these protective responses requires prior immunization against each organism. In addition, emerging pathogens may become mutated to evade vaccines, either naturally or by design. In contrast, the innate immune system has evolved to rapidly respond to products of microbial organisms that are relatively invariant. Hence, prophylactic activation of innate immune receptors at the portal of entry could increase the critical window before antibiotics can be instituted. Such a strategy would not be limited to a particular microbe and would be useful in mixed as well as single agent attacks.

In recent times inhaled *Bacillus anthracis* has been an organism of choice for bioterrorists. *Bacillus anthracis* is a gram-positive, spore-forming organism that is the etiologic agent of anthrax infection. The infective spores, following inhalation, are phagocytized by host alveolar macrophages and are transported to the regional lymph nodes. These spores germinate inside these macrophages and become vegetative bacilli that upon release from the cells produce virulence factors, including lethal toxin and edema toxin. Mouse models of this infection have been established, however the mortality is strain dependent. C57BL/6 and BALB/c mice are resistant to challenge with nonencapsulated strains of *B. anthracis* [16–18], however, complement-deficient mice, such as A/J mice are sensitive to aerosol challenge with nonencapsulated Sterne spores [19–21]. Given the ability of PO ISS-ODN R10-60 to stimulate bronchial cytokine production, this ODN was tested in comparison with a PS ISS-ODN in a murine pulmonary anthrax model. In this model equivalent doses of the different forms of ODN enhanced survival.

2. Materials and Methods

2.1. Mice

Female C57BL/6 mice (8 – 12 wk of age) were obtained from Harlan West Coast (Germantown, CA). Female A/J mice (6 – 8 wk old) and IL-1R1^{-/-} (on the C57BL/6 background) mice were purchased from The Jackson Laboratories (Bar Harbor, ME), and housed in the animal facility of Veterans Affairs Medical Center, San Diego, California. MyD88^{-/-} mice were a generous gift of Dr. Akira (Osaka University, Japan) [22] and were backcrossed for ten generations onto the C57BL/6 background. The mice were bred and maintained under standard conditions in the University of California, San Diego Animal Facility that is accredited by the American Association for Accreditation of Laboratory Animal Care. Anthrax experiments were performed at the Veterans Affairs Medical Center, San Diego, California. All animal protocols received prior approval by the respective institutional review boards.

2.2. Oligonucleotides

Single-stranded PO R10-60 (TLR-9 aptamer, 5'-CCA GTC GTA CAG GAA ACA TGC GTT CTA GAT GTT CGG GGC-3') and PO R10-5 (aptamer without CpG, 5'-CCA GCC ACC TAC TCC ACC AGT GCC AGG ACT GCT TGA GGG G-3') were synthesized by Integrated DNA Technologies (IDT, Corvallis, OR). PS-modified 1018 (5'-TGA CTG TGA ACG TTC GAG ATG A-3') and PS-1019 (ODN without CpG, 5'-TGA CTG TGA AGG TTG GAG ATG A-3') were kindly provided by Dr. Eyal Raz (UCSD, San Diego, CA). Endotoxin contamination in all ODN was <0.02 EU/mg as measured by a *Limulus* amebocyte lysate assay (BioWhittaker, Walkersville, MD).

2.3. Oligonucleotide administration to mice

For systemic administration female age-matched C57BL/6 mice were injected with 100 µl saline solution containing indicated doses of either PO R10-60 or PS-1018 via the tail vein, and sera were collected at various time points from individual mice. To assess mucosal effects, A/J mice were treated with 20 µl saline, 25 µg of PO R10-60 or PS-1018 intranasally (i.n.) after anesthesia with intramuscular Ketamine/xylazine. After varying time points, mice

were sacrificed, sera and bronchial alveolar lavage fluids (BALF) were collected and analyzed for the indicated cytokines using the Beadlyte Mouse MultiCytokine kit (Upstate, Charlottesville, VA), according to the manufacturer's instructions and a Luminex technology fluorescent reader (Austin, TX). The lower limit of detection was 2.3 pg/ml for IL-6, and 6.9 pg/ml for IL-1 β , IL-10, IL-12p40, IFN- γ and TNF- α based on the kit standard curve.

In adjuvant studies C57BL/6 mice were immunized intradermally in the tail with 50 μ g of chicken ovalbumin (OVA, Sigma, St. Louis, MO) either alone or together with 50 μ g PS-1018 as positive control or 500 μ g PO R10-60 on days zero and 14. At six weeks the mice were given a boost of 20 μ g OVA intravenously and then sacrificed three days later. Sera were collected at weeks 2, 4 and 6.5 (at sacrifice). Spleens were harvested, weighed, and total splenocytes isolated.

2.4. OVA-specific IgG2a ELISA

Plates were coated with OVA at 5 μ g/ml in 1 x BBS (boric acid-buffered saline, pH 7.9 – 8.2) overnight at 4°C, washed and incubated with 1% BSA (Sigma) in BBS at 37° C for 2 h. Serum samples were serially diluted and incubated at 4° C overnight. Bound IgG2a was detected with alkaline phosphatase-conjugated goat anti-mouse IgG2a (Southern Biotechnology Associates, Birmingham, AL), and 4-nitrophenyl phosphate substrate (Roche Diagnostics, Basel, Switzerland). Absorbance values were read at 405 nm with a multi-well reader (Molecular Devices, Sunnyvale, CA).

2.5. Cellular response to DNA immunization

Cells were cultured in RPMI1640 medium supplemented with 10% FBS, glutamine, and 100 U/ml penicillin and 100 μ g/ml streptomycin (RP10; all from Invitrogen, Carlsbad, CA) at 37°C, and 5% CO₂. For the cytokine production studies, 2 x 10⁵ splenocytes per well in 96 well plates were restimulated with 100 μ g OVA for 3 days. Culture supernatants were tested for IFN- γ , IL-6, and TNF- α by ELISA (BD PharMingen).

Cytotoxic T lymphocyte (CTL) responses were assayed as previously described [23]. Briefly, 2 x 10⁶ effector splenocytes were restimulated in culture for five days with 1.8 x 10⁷ OVA SIINFEKL peptide-pulsed irradiated splenocytes and 50 U/ml recombinant human IL-2 (Sigma). After restimulation, viable lymphocytes were recovered by centrifugation over Ficoll lympholyte M (Cedarlane Laboratories, Ltd., Hornby, Ontario, Canada) at room temperature for 20 min. Cells were dispersed into 96 well plates at several effector to target cell ratios. Lysis of EL-4 target cells pulsed with OVA peptide (SIINFEKL) [24], or negative control influenza virus nucleoprotein (NP) peptide (ASNENMETM) [25] (Molecular Research Laboratories, Durham, NC) was assayed using CytoTox 96 kit (Promega, Madison, WI) according to the manufacturer's instructions.

2.6. Challenge with *Bacillus anthracis* spores

Bacteria challenge experiments were performed using non-encapsulated *Bacillus anthracis* 32F2 (Sterne strain; pXO1⁺pXO2⁻) [19]. Spores were prepared as previously described [26] and purified spores were stored in PBS at 1 – 4 x 10⁸ CFU/ml at 4° C. Prior to infection, the

spores were heated to 65°C for 30 min to kill vegetative forms and activate spores. Mice were anesthetized with intramuscular Ketamine/xylazine and infected with $2 - 8 \times 10^5$ of *B. anthracis* spores i.n. in 20 μ l. Survival was determined after 9 days. In some studies, A/J mice were pretreated i.n. one day prior to anthrax infection with saline, 25 μ g of PO R10-60, or PS-1018 after anesthesia with intramuscular Ketamine/xylazine. In other studies mice were injected intraperitoneally with 0.125 μ g recombinant mouse IL-1 β (R & D Systems, Minneapolis, MN) 1 h prior to infection and daily after infection for 3 days. For the cytokine profile study, infected and uninfected control mice were treated with ODN as above. Mice were sacrificed and sera and BALF were collected at 24, 48 and 96 h after ODN exposure or at days 1 and 3 post-infection. Cytokine (IL-1 β , IL-6, IL-10, IL-12p40, IFN- γ and TNF- α) levels in serum and BALF were determined by multiplex bead based assay using the Luminex platform (Upstate).

2.7. Determination of spores and bacilli load in lungs of anthrax-infected mice

Lungs from mice pretreated with either saline, PO R10-60 or PS-1018 were harvested aseptically at days 1 and 3 after infection with *B. anthracis* spores. Tissues were homogenized in 1 ml of sterile saline that was serially diluted in saline and plated on BHI agar to determine total viable organisms. The same samples were also heated for 30 min at 65°C prior to plating to determine the number of surviving ungerminated spores.

2.8. Statistics

Data are expressed as mean $\pm$ standard error of the mean. Statistical analysis was performed according to the Mann Whitney U-test when comparing two groups or ANOVA for multiple group comparisons. Bonferonni post hoc testing was used for pairwise between group comparisons. Kaplan-Meier survival curves and log rank tests were performed using GraphPad Prism software version 4.0c (San Diego, CA) to compare differences in survival after anthrax infection at day 9 post-infection. $P < 0.05$ was considered significant.

3. Results

3.1. Intravenous PO R10-60 elicited a short-lived systemic cytokine response

R10-60, a CpG containing PO ISS-ODN, was previously selected as a TLR-9 aptamer and shown to efficiently permeate cells and to elicit cytokine secretion in a TLR-9-dependent manner [14, 15]. To examine the *in vivo* kinetics of systemic cytokine induction by PO R10-60, mice were injected intravenously with varying doses of PO R10-60 and PS-1018. In pilot studies 25 μ g PS-1018 i.v., but not higher doses, gave the maximal cytokine release. The lowest dose of PO R10-60 that gave a detectable cytokine response is 125 μ g. Shown are the results for 125 μ g of PO R10-60 and 25 μ g of PS-1018 (Figure 1). The sera from injected mice were collected at serial time points and analyzed for cytokine production. In mice treated with 125 μ g PO R10-60, levels of IL-6, IL-12 and TNF- α were detectable between two and six hours and then quickly declined. IFN- γ had a delayed peak and was observed in the group with PS-1018 starting at 24 h whereas only low levels were found in mice treated with PO R10-60. By 24 h little or no detectable cytokine levels were measurable in the PO R10-60 injected mice. In contrast, cytokine levels were still detectable

at 48 h post-injection in mice that received 25 µg PS-1018, indicating a longer stimulatory effect presumably due to protection from nuclease degradation by the PS modification.

3.2. Intradermal PO R10-60 as an effective Th1 adjuvant with minimal splenomegaly

Previously we and others demonstrated the need for a CpG motif in ISS oligonucleotides used as a generic vaccine [27]. As PO R10-60 could stimulate bone marrow derived dendritic cells *in vitro* [15], we evaluated its use as an adjuvant *in vivo* using ovalbumin (OVA) as a model antigen. C57BL/6 mice were immunized intradermally with OVA alone or OVA mixed with either PO R10-60 (OVA+R10-60) or PS-1018 (OVA+PS-1018) on days 1 and 14. The use of PO R10-60 as an adjuvant required a higher dose than the PS-ODN to achieve a similar biologic effect. Although 125 µg of i.v. PO R10-60 was able to raise systemic serum cytokine levels, this dose did not enhance immune responses when given intradermally as an adjuvant (data not shown). However, a four fold higher dose (500 µg) of PO R10-60 given with OVA as an intradermal adjuvant was able to elicit an immune response similar to 50 µg of PS-1018 (Figure 2). Mice immunized with 500 µg PO R10-60+OVA generated antigen specific T cells that secreted IFN- γ , IL-6 and TNF- α (Figure 2A–C), consistent with a Th1 response. Furthermore, comparable cytotoxic T lymphocyte activity was observed in both ISS-ODN injected groups of mice (Figure 2D).

The Th1 profile was also reflected in the robust IgG2a antigen specific humoral response. Sera were collected at weeks 2, 4 and 6.5 and assayed for OVA-specific IgG2a by ELISA (Figure 2E). Mice that received 50 µg PS-1018 or 500 µg PO R10-60 with OVA exhibited a significant increase in anti-OVA IgG2a, while only low levels of anti-OVA IgG2a were induced in mice that received OVA alone.

At concentrations where comparable anti-OVA IgG2a responses were induced, there were fewer secondary effects in the mice treated with PO R10-60 than with PS-1018. As a surrogate measure of cytokine induced extramedullary hematopoiesis, the spleens were weighed [28]. Only a slight increase in splenic mass was observed in mice treated with PO R10-60 compared to those treated with OVA alone (Figure 2F). In contrast, significant splenomegaly was observed in mice that received PS-1018 even at a 10-fold lower dose compared to PO R10-60. Collectively, the data suggested that *in vivo* PO ISS-ODN required higher doses to induce cellular and humoral immune responses than PS ISS-ODN, and effective adjuvant activity with PO ISS-ODN resulted in less splenomegaly, an undesired effect.

3.3. Mucosal delivery of PO R10-60 stimulates local cytokines release and enhances survival against anthrax infection

Although systemic cytokine release induced by i.v. PO R10-60 waned by 24 h to basal levels, local administration of this PO ISS-ODN to the skin still provided a sufficient immunostimulatory effect to prime an antigen specific immune response. Thus, different routes of administration and different tissues might elicit varied responses to PO and PS ISS-ODN. For aerosolized pathogens the respiratory mucosa would provide the first line of defense. Therefore, we investigated the clinical efficacy of PO R10-60 in a pulmonary murine model of anthrax, using highly susceptible A/J mice.

The C57BL/6 and A/J mouse strains differ in their resistance to anthrax [19, 20]. Since our *in vivo* cytokine data were established based on C57BL/6 mice, it was necessary to evaluate the mucosal cytokine response of A/J mice to ISS-ODN treatment. Groups of A/J mice treated i.n. with saline or 25 µg of either forms of ISS-ODN were sacrificed at 24, 48 and 96 h, and sera and BALF were harvested for cytokine assays. As shown in Table 1, mice that received PO R10-60 generally released maximal amounts of cytokines into the BALF and serum at 24h. However, with the exception of IL-1 β the level of cytokine production was less than that seen with PS-1018. These results demonstrate that A/J mice are capable of responding to mucosal ISS-ODN treatment by local and systemic cytokine production.

Based on the temporal cytokine induction profile following i.n. administration of ISS-ODN (Table 1), we selected an *in vivo* pretreatment regimen with ISS-ODN one day before challenging A/J mice with *B. anthracis*. A dose response study using PS-1018 from 25 – 100 µg or PO R10-60 from 5 – 125 µg per mouse suggested that the optimal dose for prolonged survival in this anthrax model was 25 µg for both ISS-ODN (data not shown). Hence, A/J mice were treated with saline only or with 25 µg of either PO R10-60 or PS-1018 i.n. one day prior to infection with anthrax spores and we followed the mice for survival (Figure 3). The saline treated mice succumbed as early as day three post-infection and most control mice died by day six. By day nine post-infection all control mice were dead, and the survival experiments were terminated. A significant, albeit modest reduction in mortality in mice pretreated with either form of ISS-ODN was seen (Figure 3; $p = 0.01$). Pretreatment with a control PS ODN lacking the CpG motif (PS-1019) did not afford any protection (Figure 3A; $p = 0.11$). No clear difference was found between the PO R10-60 and PS-1018 pre-treated groups (Figure 3B; $p = 0.54$). The median survival rates of PO R10-60 and PS-1018 pre-treated mice were 6 and 7 days, respectively, compared to 4 days in the saline control group (Figure 3B; $p < 0.01$). At the end of each experiment, lungs of the remaining mice were harvested for visual inspection and the bacterial load measured to ensure that all the survivors became infected from spore challenge.

To investigate whether early bacterial clearance may play a role in ISS-ODN protection in treated mice infected with *B. anthracis* spores, in a separate experiment one and three days after infection we cultured the lungs of mice that had or had not been pretreated with ISS-ODN. Analysis at longer time intervals beyond the first three days after infection was not possible due to the death of untreated mice. Bacterial cultures from the lungs of infected animals at one and three days after inoculation showed no effect of ISS-ODN on the early clearance of spores (data not shown).

3.4. Anthrax challenge sustains cytokine production initiated by mucosal ISS-ODN delivery

To further investigate the role of cytokine induction in the protection afforded by i.n. ISS-ODN pre-treatment, BALF and sera were collected from separate groups of mice that were pretreated with saline or ISS-ODN on days 1 and 3 following infection (Figure 4). The serum levels of cytokines from PS-1018 treated mice remained elevated at day 1 and in some cases day 3 post-infection, respectively. Although the saline treated mice did not have elevated cytokine levels after infection, the presence of the infection appeared to sustain elevated cytokine levels that were activated by the administered ISS-ODN. The levels of

cytokine in the BALF gradually peaked at day 3 post-infection in contrast to the peak in serum cytokine at day 1 post-infection. There were minimal differences in most of the serum and BALF cytokines in the PO R10-60 treated groups. Interestingly, IL-1 β was the only cytokine significantly increased in the BALF and sera by day 3 in both ISS-ODN pre-treated and infected groups. These mice were similarly treated as the mice in Figure 3, suggesting that the prolonged survival in the ODN treated mice involves early IL-1 β induction.

3.5. IL-1 β treatment prolongs survival in *B. anthracis* infection in mice

There is conflicting evidence in the literature regarding the role of IL-1 β in mortality from anthrax infection [17, 29–34]. We confirmed that both MyD88 and IL-1 receptor deficient mice are relatively susceptible to respiratory anthrax infection compared to the resistance demonstrated by the C57BL/6 background strain (Figure 5A) [16, 17]. Neither the MyD88 nor the IL-1R deficient mice succumbed as quickly as the A/J mice, suggesting that these mice have other susceptibility factors. High levels of systemic IL-1 β and IL-6 were previously reported to be associated with increased mortality from anthrax in mice [33], and passive immunization against IL-1 β was protective [32]. We hypothesized that the role of IL-1 β may paradoxically shift from protection early in infection to exacerbating pathogenicity late in infection. To test this, susceptible A/J mice were treated with recombinant IL-1 β prior to and during the early phase of infection. Indeed, the survival of recombinant IL-1 β treated mice was significantly enhanced compared to saline control group (Figure 5B). Although the initial observation was made in mice that were mucosally stimulated with ISS-ODN, the IL-1 β treated mice were also protected with systemic treatment.

4. Discussion

Although PO R10-60 was demonstrated as a potent stimulus for *in vitro* cytokine release the *in vivo* efficacy had yet to be tested. The present study extended our *in vitro* findings, demonstrating that not only could structurally optimized PO ISS-ODN be used as an adjuvant in an active immunization (Figure 2), they can also be used as prophylaxis against lethal infection (Figure 3). Higher doses of PO ISS-ODN compared to PS ISS-ODN were required to elicit systemic immune responses. There were significantly less side effects compared to the splenomegaly and high rise in serum TNF- α seen in the PS ISS-ODN treated mice (Figures 1–2). Although there may have been transient splenomegaly in the PO ISS-ODN treated mice that was not detected, as the “natural” PO ISS-ODN differed from the PS ISS-ODN in the kinetics of cytokine production. First intravenous administration resulted a rapid rise in serum inflammatory cytokines that was not sustained even at higher doses. Similarly, a higher dose of intradermal PO R10-60 was required than a prototypic PS ISS-ODN to achieve an equivalent adaptive immune response when used as an adjuvant with a test antigen. However, the mucosal administration of PO R10-60 stimulated local innate cytokine production with the same potency as the comparator PS ISS-ODN with minimal systemic cytokine release, suggesting that this PO ISS-ODN might be biologically advantageous during an acute upper respiratory or pulmonary infection.

Cytokine release after PO ISS-ODN treatment was brief. In contrast, cytokine levels, including TNF- α , persisted longer after PS ISS-ODN treatment, especially in serum. The

short half-life of the PO ISS-ODN limit their potential for systemic cytokine release, however, may be beneficial when quick and transient activation of the innate immune system is desirable thus concentrating their efficacy at the site of administration. A transient cytokine response, and a lack of non-specific lymphoproliferation following PO ISS-ODN administration, has also been seen after footpad injection [7]. The mainly local effects of PO ISS-ODN could potentially enhance their efficacy, by creating cytokine and chemokine gradients at the site of administration, which more closely mimic a local infection. Most PS ISS-ODN, but not PO ISS-ODN, bind avidly to plasma proteins [15, 35], resulting in a larger volume of distribution [36, 37]. Thus, PS would be preferable to PO ISS-ODN when systemic or prolonged activation of innate immunity is needed, whereas PO ISS-ODN may be preferable where localized and transient effects are preferred. Innate immunotherapy using optimized PO ISS-ODN may generate a local concentrated response in the microenvironment of the delivery site, limiting untoward systemic effects.

In the past DNA has been used as an adjuvant in active immunization strategies to generate specific adaptive immune responses against anthrax [24, 38–41]. DNA vaccination with a plasmid encoding full-length anthrax protective antigen (PA) protein was effective in a mouse anthrax model [24, 40, 42]. In addition, PS ISS-ODN have been used to enhance the potency of developmental anthrax vaccines in rodents and non-human primates [43–45]. However the activation of TLR-9 by PS ISS-ODN without specific antigen input has also been reported to partially protect experimental animals against a variety of infections [46–49]. Broad protection from the lethal effects of microbial organisms that have been genetically manipulated for resistance to antibiotics or increased virulence is a critical goal in biodefense. Interventions that are not specifically targeted to individual organisms may be more widely used at times of possible infection. However, safety is a critical requirement for prophylactic treatments. Here, administration to the respiratory tract of a “natural” PO ISS-ODN was shown to be effective in delaying the lethal consequences of anthrax infection in a murine model without causing the accumulation of potentially toxic levels of circulating TNF- α (Figures 3–4). In a clinical setting, such a delay could allow for antibiotic treatment, before the release of lethal bacterial toxins.

Exactly how prophylactic TLR-9 activation in the respiratory tract retards the progression of lethal *Bacillus anthracis* infection is not clear. We have shown previously that pre-treatment with TLR9 agonists decreases the cytotoxicity of *Bacillus anthracis* for macrophages in cell culture [50]. Here we demonstrated that natural resistance is diminished in MyD88 deficient mice (Figure 5A). This signaling intermediary is shared by the TLRs and the IL-1 receptor. The protection seen with mucosal TLR-9 agonists was associated with a rise in IL-1 β in the BALF and serum after both forms of ISS-ODN treatment (Figure 4). Similarly systemic IL-1 β administration early in disease prolonged survival in susceptible A/J mice (Figure 5B). Hence the effects from the ISS-ODN may not have been exclusively local. Furthermore, the present study suggests that IL-1 β has an early beneficial effect during inhalation anthrax, likely as a result of its immunomodulatory activity rather than a direct enhancement of antibacterial defense.

Although the survival benefit shown by ISS-ODN pre-treatment was modest, it was encouraging and the effect may be improved upon optimization of the interval between ISS-

ODN administration and anthrax spore challenge. It might be possible to extend the protection by repeated administration of ISS-ODN, although PO R10-60 might be better tolerated with lower systemic cytokine levels. Moreover, it is possible that different TLR activators, administered via different routes, will influence the progression of infection in contrasting ways, depending upon the kinetics and sites of innate immune system activation [51, 52]. Only direct comparisons can discern the most effective and least toxic strategy for immune protection against *Bacillus anthracis*, and other pathogens important for biodefense.

Acknowledgments

We are grateful to Drs. Eyal Raz and Jongdae Lee for their helpful advice, and to Andrea Aguirre, Agnes Fermin, Cyprian Rossetto, Carol Shin, Sharon Okamoto, Christine Gray and Chuong Dang for technical assistance. This work was supported in part by National Institutes of Health Grants AI56453, AI57436, and AI40682.

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Figure 1. Kinetics of systemic cytokine induction by mice injected with different forms of ISS-ODN

C57BL/6 mice were injected intravenously with either 125 μ g of PO R10-60 or 25 μ g of PS-1018 per mouse. Sera were harvested from individual mice at 2, 4, 6, 24 and 48 h, and cytokine levels determined by Luminex assay. Data are averaged from 6 – 17 mice per group $\pm$ SEM pooled from five separate experiments.

Figure 2. Intradermal administration of PO R10-60 as an adjuvant promotes a Th1-biased immune response and an effective CTL response without splenomegaly

Eight female age-matched C57BL/6 mice per group were immunized intradermally in the tail base on days zero and 14 with either OVA alone (50 μ g), OVA + PS-1018 (50 μ g each), or OVA + PO R10-60 (50 μ g + 500 μ g) diluted in sterile saline. (A–C)

Splenocytes were isolated after six weeks and restimulated in culture for three days. Culture supernatants were collected to determine IFN- γ , IL-6, and TNF- α production by cytokine ELISA. Significant differences by ANOVA and Bonferroni post hoc comparisons are indicated. (D) OVA-specific CTL activity was determined by LDH release from SIINFEKL or control peptide pulsed EL-4 target cells. Means $\pm$ SEM are shown. * $p < 0.001$ compared to OVA alone by ANOVA with Bonferroni post hoc multiple group comparisons. (E) Serum samples were collected at weeks 2, 4, and 6.5, and relative anti-OVA IgG2a titers were

determined by isotype-specific ELISA. Averages $\pm$ SEM are shown. * $p < 0.01$ compared to OVA alone by ANOVA of the areas under the curves and Bonferroni post hoc multiple comparisons testing. The +1018 and +R10-60 curves were not significantly different from each other. (F) Spleens were harvested and weighed at time of sacrifice. Significant differences by ANOVA and Bonferonni post hoc testing are indicated.

Figure 3. Intranasal pretreatment with ISS-ODN significantly reduces mortality in *B. anthracis* infected mice

(A) Eight female A/J mice per group were treated one day prior to infection with i.n. saline or 25 μ g of ISS-ODN PS-1018 or a non-stimulatory ODN PS-1019. Kaplan-Meier survival curves are shown. (B) To compare the efficacy of between PS and PO ISS-ODN, female A/J mice were treated one day prior to infection with i.n. saline or 25 μ g of either PO R10-60 or PS-1018 per mouse. Data are combined from 3 separate experiments for a total of 28 mice per group. Significant differences are assessed by Kaplan-Meier survival curves and log rank tests.

Figure 4. Cytokine profiles of sera and BALF from mice pretreated with ISS-ODN one day prior anthrax infection
A/J mice were treated i.n with saline or 25 μ g of ISS-ODN in either form one day prior to infection with ~ 7 LD₅₀ of *B. anthracis* spores. Mice were sacrificed at 1 and 3 days post-infection and sera and BALF were collected. Cytokine levels were determined by Luminex assay. Data are mean levels from 6 – 8 mice per group $\pm$ SEM pooled from two separate experiments. * $p < 0.05$ compared to the saline treated group.

Figure 5. IL-1 β is protective in early murine anthrax infection

(A) Groups of adult A/J ($n = 6$), MyD88 $^{-/-}$ ($n = 8$), IL-1R1 $^{-/-}$ ($n = 16$), and C57BL/6 ($n = 10$) were infected by i.n. delivery of 1×10^5 *Bacillus anthracis* spores per mouse and survival observed up to 19 days. Results are combined from 2 separate experiments. (B) A/J mice were treated i.p. with 0.125 μ g recombinant IL-1 β h before anthrax infection and daily after infection for 2 – 3 days. Data are combined from two separate experiments with a total of 10 mice per group. Significant differences are assessed by Kaplan-Meier survival curves and log rank tests.

Table 1

Intranasal PO R10-60 stimulates local cytokine production efficiently with minimal systemic cytokine release

Cytokine	Time (hrs)	BALF Cytokine (pg/ml±SEM) ^a			Serum Cytokine (pg/ml±SEM) ^a		
		Saline	PO R10-60	PS-1018	Saline	PO R10-60	PS-1018
IL-1β	24	<6.9	7.6±3.1	15.2±5.9 ^b	<6.9	30.6±12.39 ^b	15.2±5.9
	48	<6.9	8.9±2.7 ^c	14.6±3.4 ^c	<6.9	<6.9	<6.9
	96	<6.9	42.7±17.8 ^b	28.5±11.8	<6.9	<6.9	<6.9
IL-6	24	<2.3	3.7±2.1	156.4±116.1 ^c	<2.3	2.3±0.9	25.0±5.4 ^c
	48	<2.3	<2.3	7.3±2.9 ^c	<2.3	3.8±1.2	9.7±3.3 ^b
	96	<2.3	<2.3	25.7±10.9 ^c	<2.3	<2.3	5.4±2.4
IL-10	24	<6.9	<6.9	<6.9	<6.9	<6.9	47.6±3.7 ^c
	48	<6.9	<6.9	<6.9	<6.9	<6.9	25.7±4.9 ^c
	96	<6.9	<6.9	9.0±1.6 ^c	<6.9	<6.9	6.9±1.9 ^b
IL-12p40	24	<6.9	221.8±93.5 ^b	1475.0±354.1 ^c	108.5±14.7	383.1±100.1	749.3±110.4 ^c
	48	<6.9	70.0±32.9	464.0±82.0 ^c	50.26±18.9	119.9±15.4	340.3±87.0
	96	<6.9	16.1±2.5	671.2±162.2 ^c	53.6±14.2	25.8±6.5	73.3±22.1
IFN-γ	24	<6.9	16.0±6.1	24.1±6.0 ^b	29.2±6.8	62.9±19.3	206.2±39.5 ^c
	48	<6.9	<6.9	10.9±1.1 ^c	<6.9	14.7±2.7	94.2±31.5 ^b
	96	<6.9	<6.9	200.0±97.8 ^b	14.3±6.7	17.1±5.8	35.2±11.1
TNF-α	24	<6.9	17.2±9.1	44.4±23.7 ^b	<6.9	6.9±1.7	25.9±3.6 ^b
	48	<6.9	11.9±4.1	27.9±7.7 ^c	<6.9	9.6±2.9	20.0±6.7 ^b
	96	<6.9	3.3±0.1	41.2±10.6 ^c	<6.9	<6.9	7.6±2.0 ^b

^a A/J mice were treated i.n. with saline alone or 25 µg of either PO R10-60 or PS-1018. Groups of mice were sacrificed at 24, 48 and 96 h. BALF and sera were collected, and cytokine levels were measured by multiplex bead assay. Data are averaged from two separate experiments with 4 – 11 mice per group.

^b $p < 0.05$ or

$p < 0.01$ ODN vs. saline treatment by ANOVA.