



Oligonucleotides designed to inhibit TLR9 block Herpes simplex virus type 1 infection at multiple steps



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ABSTRACT

Herpes simplex virus type 1 (HSV-1) is an important human pathogen which requires activation of nuclear factor-kappa B (NFκB) during its replication cycle. The persistent nature of HSV-1 infection, and the emergence of drug-resistant strains, highlights the importance of research to develop new antiviral agents. Toll-like receptors (TLRs) play a prominent role during the early antiviral response by recognizing viral nucleic acid and gene products, activating NFκB, and stimulating the production of inflammatory cytokines. We demonstrate a significant effect on HSV-1 replication in ARPE-19 and Vero cells when oligonucleotides designed to inhibit TLR9 are added 2 h prior to infection. A greater than 90% reduction in the yield of infectious virus was achieved at oligonucleotide concentrations of 10–20 μM. TLR9 inhibitory oligonucleotides prevented expression of essential immediate early herpes gene products as determined by immunofluorescence microscopy and Western blotting. TLR9 oligonucleotides also interfered with viral attachment and entry. A TLR9 inhibitory oligonucleotide containing five adjacent guanosine residues (G-ODN) exhibited virucidal activity and inhibited HSV-1 replication when added post-infection. The antiviral effect of the TLR9 inhibitory oligonucleotides did not depend on the presence of TLR9 protein, suggesting a mechanism of inhibition that is not TLR9 specific. TLR9 inhibitory oligonucleotides also reduced NFκB activity in nuclear extracts. Studies using these TLR inhibitors in the context of viral infection should be interpreted with caution.

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1. Introduction

Herpes simplex virus type 1 (HSV-1) is a significant human pathogen infecting approximately 70% of the world's population (Fatahadeh and Schwartz, 2007). Infection with HSV-1 commonly causes ulcerative lesions on the skin or mucosal membranes (Whitley, 1996) and HSV-1 infection of the eye can result in conjunctivitis and keratitis, which may lead to blindness due to an immunopathological response (Streilein et al., 1997). A high percentage of primary infections with HSV-1 are asymptomatic and the virus can establish latent infection in the innervating sensory ganglia (Whitley, 1996).

Currently, there is no cure for persistent HSV-1 infection and prolonged therapy with anti-herpetic drugs can lead to the

emergence of drug resistant strains especially in immunocompromised individuals. Acyclovir resistant strains of HSV-1 have also been isolated from patients with herpetic keratitis and uveitis (Duan et al., 2008; van Velzen et al., 2013). Research into anti-herpetic agents, with a mechanism of action different than that of the currently available nucleoside analogs, is therefore crucial. Oligomeric nucleic acids are intriguing candidates for antiviral therapy as they have high affinity for a given target. They also have high inhibitory potential, can be selected against almost any target, and exhibit limited toxicity or immunogenicity (Hagedorn et al., 2013; Mescalchin and Restle, 2011). Studies have indicated antisense oligonucleotides may function as antiviral agents for viruses including HIV and HBV (Galderisi et al., 1999; Mescalchin and Restle, 2011), HCV (Janssen et al., 2013) and Influenza (Wong et al., 2010, 2007). Vitravene, an antisense drug used for the treatment of CMV retinitis in AIDS patients, consists of a phosphorothioate oligonucleotide designed to inhibit human CMV replication (Galderisi et al., 1999).

The antiviral properties of amphipathic phosphorothioated oligonucleotides (PS-ONs), regardless of sequence specificity, were

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demonstrated some time ago against EBV and HSV (Fennewald et al., 1995; Gao et al., 1990; Yao et al., 1993). The antiviral activity of PS-ONs is dependent on the length and hydrophobicity of the oligonucleotide as demonstrated by studies with HIV-1 (Vaillant et al., 2006), LCMV (Cardin et al., 2009; Lee et al., 2008), HCMV (Luganini et al., 2008), HCV (Counihan and Lindenbach, 2009), DHBV (Noordeen et al., 2013a,b) and HSV (Bernstein et al., 2008; Guzman et al., 2007; Shogan et al., 2006). A GT rich 20 base PS-ON (ISIS 5652) was shown to have potent antiviral activity against HSV which is thought to be mediated by a conformational change in the viral glycoprotein gB (Shogan et al., 2006).

Innate recognition of viruses by the mammalian immune system is crucial in inducing interferon and other immune responses. The most highly studied family of pattern recognition receptors (PRRs) is the Toll-like receptors (TLRs) (Huang and Yang, 2009). Multiple human TLRs have been identified to date, each recognizing a unique set of pathogen-associated molecular patterns (PAMPs) (Akira and Takeda, 2004; Imler and Zheng, 2004). TLR signaling pathways culminate in activation of the transcription factor NF κ B which controls the expression of many inflammatory cytokines and other genes (Kawai and Akira, 2006). Herpes viruses contain several PAMPs that are recognized by TLRs and this interaction is thought to play a crucial role in viral pathogenesis (Martinez-Martin and Viejo-Borbolla, 2010). The HSV-1 glycoproteins gB and gH/gL are recognized by cell surface TLR2 (Cai et al., 2013; Kurt-Jones et al., 2004; Leoni et al., 2012), TLR3 recognizes HSV-1 dsRNA (Zhang et al., 2007), and TLR9 recognizes unmethylated CpG dinucleotides in the genome of Herpes simplex virus (Lund et al., 2003). This recognition of HSV DNA by TLR9 activates NF κ B and results in the production of type 1 interferons and cytokines, including IL-6 (Huang and Yang, 2009; Krug et al., 2004; Rasmussen et al., 2007). Purified HSV-1 DNA up-regulates IL-6 in human corneal epithelial cells (HCEs) and primary human corneal fibroblasts in part via their interaction with TLR9 and TLR3 (Hayashi et al., 2006). Retinal pigment epithelial (RPE) cells express TLRs 1–7, 9 and 10 (Kumar et al., 2004), suggesting a role for these molecules in retinal immune responses.

Because some TLRs recognize nucleic acids, oligonucleotides have been developed that inhibit TLR signaling and function as competitive agonists (Barrat and Coffman, 2008; Latz et al., 2007; Peter et al., 2007). Administration of a TLR9 inhibitory oligonucleotide after HSV-1 infection of mice decreased mortality by controlling the inflammatory response in the brain (Boivin et al., 2012). A TLR9 inhibitory oligonucleotide significantly reduced HSV-1 replication in human corneal endothelial cells without affecting viral entry (Takeda et al., 2011), but this study did not determine whether the antiviral effect of the TLR9 oligonucleotide was dependent on the presence of the TLR9 protein.

In this study we investigated the effects of two commercially available TLR9 inhibitory oligonucleotides on HSV-1 infection of ARPE-19 cells. The inhibitory oligonucleotides reduced viral replication and prevented synthesis of essential herpes proteins. TLR9 oligonucleotides interfered with early stages of the viral life cycle, including attachment and entry, as well as post-entry events. The TLR9 inhibitory oligonucleotide decreased NF κ B activity in infected cells. Surprisingly, this antiviral effect was not dependent on the presence of TLR9 protein, suggesting a mechanism of inhibition that is not TLR9 specific.

2. Materials and methods

2.1. Cell culture and virus

Human adherent retinal pigmented epithelial cells (ARPE-19) (ATCC, CRL-2302) were maintained in DMEM/F-12 (Mediatech,

Herndon, VA, 10-092-CV) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μ g/ml streptomycin, and 2 mM L-glutamine at 37 °C in 5% CO₂. Vero cells (ATCC, CCL-81) were maintained in DMEM (Mediatech, 10-017-CV) supplemented with 5% fetal bovine serum, 100 units/ml penicillin, 100 μ g/ml streptomycin, and 2 mM L-glutamine at 37 °C in 5% CO₂. 293XL/null (#293xl-null) and 293XL-hTLR9-HA cells (#293xl-hTLR9ha) were obtained from Invivogen (San Diego, CA). 293XL cells were maintained in DMEM supplemented with 10% FBS, 50 U/ml penicillin, 50 μ g/ml streptomycin, 100 μ g/ml Normocin (Invivogen, #anti-nr-1) and 10 μ g/ml Blasticidin (Invivogen, #ant-bl-1). High titer, sucrose gradient purified stocks of HSV-1 KOS were prepared as described previously (Visalli and Brandt, 1993). Viral titers were determined by plaque assay in Vero cells as described previously (Grau et al., 1989). The plaque efficiency of HSV-1 KOS was equivalent on Vero and ARPE-19 cells.

2.2. TLR oligonucleotides

The TLR9 inhibitory oligonucleotide (#tlrl-hinhodn, 5'-TTT AGG GTT AGG GTT AGG G-3'), G-ODN oligonucleotide (#tlrl-godn, 5'-CTC CTA TTG GGG GTT TCC TAT-3') and TLR9 control oligonucleotide (#tlrl-hinhodnc, 5-GCT AGA TGT TAG CGT-3') were purchased from Invivogen. The oligos were resuspended in endotoxin-free water prior to use.

2.3. Antibodies and immunofluorescence

The following primary antibodies were utilized in this study: mouse anti-ICP4 (Virusys Corp. Taneytown, MD; H1A021), mouse anti-ICP8 (Santa Cruz, Dallas, TX, sc-53329), mouse anti-ICP27 (Virusys, H1142), mouse anti-gD (Virusys, HA025), and goat anti-actin (Santa Cruz, sc1616). The following secondary antibodies were utilized: donkey anti-mouse IgG Alexafluor 488 (Invitrogen, Grand Island, NY, A21202), donkey anti-mouse IgG-HRP (Santa Cruz, sc-2314) donkey anti-goat IgG-HRP (Santa Cruz, sc-2020), and goat anti-mouse IgG-HRP (Santa Cruz sc-2031).

For immunofluorescence assays, ARPE-19 cells were seeded in 8-well chamber slides (Lab-Tek, Rochester, NY). At various times post infection (PI) the supernatants were removed, the cells were rinsed with PBS, and fixed in 4% paraformaldehyde/PBS. Cells were permeabilized with 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO) in PBS, washed in PBS, and blocked in 5% normal donkey serum/PBS (Jackson ImmunoResearch Laboratories, West Grove, PA). Anti-ICP4 and anti-gD antibodies were diluted 1:1000 and applied for 1 h at RT in PBS containing 2.5% normal donkey serum. Slides were then washed with PBS containing 0.1% Tween-20 (Fisher, Fairlawn, NJ). Secondary antibody was applied at a dilution of 1:400 for 1 h at RT in PBS containing 2.5% normal donkey serum. Slides were washed in PBS/0.1% Tween-20 before incubation with 1.0 μ g/ml Hoechst (33342, Invitrogen) in PBS for 5 min. Slides were washed in PBS prior to mounting with Immu-mount (Richard Allen Scientific, Kalamazoo, MI). Fluorescence was observed with a Zeiss Axioplan 2 microscope equipped with an Axiocam HR camera with Axiovision 4.8 software (Carl Zeiss Microimaging Inc., Thornwood, NY). Photographs were taken at 200 \times magnification.

2.4. Oligonucleotide and acyclovir inhibition assays

ARPE-19, Vero, 293XLnull, or 293XLhTLR9HA cells grown in 8-well chamber slides were pretreated with culture media, various concentrations of inhibitory or control oligonucleotides, or increasing concentrations of acyclovir (SIGMA, A4669) for 2 h at 37 °C. Cells were then infected with HSV-1 KOS at a multiplicity of infection (MOI) of 1–2 and incubated ON at 37 °C. Supernatants were collected and stored at –80 °C for titrating in Vero cells

(Grau et al., 1989). Titers were calculated as plaque forming units/ml (pfu/ml). The half maximal effective concentration (EC_{50}) was defined as the concentration of oligonucleotide or acyclovir needed to reduce viral titer to 50% of media control in ARPE-19 cells.

2.5. Western blotting

ARPE-19 cells grown to confluence in 10 cm culture dishes were pretreated with media only (DMEM/F-12/10% FBS), 20 μ M TLR9 control oligonucleotide in media, or 20 μ M TLR9 inhibitory oligonucleotide in media for 2 h at 37 °C. Cells were then infected with HSV-1 KOS at a MOI of 2 for 6 or 15 h. At the designated time points, the cells were washed, collected, and pelleted for 10 min at 1000 rpm in a Beckman Model TJ-6 table top centrifuge. Cell pellets were resuspended in Laemmli sample buffer with 5% 2-mercaptoethanol, sonicated, and boiled prior to loading equal amounts onto a 10% (ICP4, ICP8) or 12% (ICP27, gD) denaturing polyacrylamide gel. Protein was electrophoretically transferred to nitrocellulose and the membrane blocked overnight at 4 °C with 5% non-fat dry milk in Genius Buffer I (100 mM maleic acid, 150 mM NaCl, pH 7.5) containing 0.3% v/v Tween-20. The primary antibodies were applied for 1 h at RT, followed by washing in Genius Buffer I containing 0.3% v/v Tween-20. Secondary antibodies were applied for 1 h at RT. After washing, the blots were developed using an enhanced chemiluminescence system (GE Healthcare, Piscataway, NJ, PRN2232). For the actin blot, the membrane was incubated for 1 h at 50 °C in stripping buffer (80 mM Tris-HCl, pH 6.7, 100 mM β -mercaptoethanol, 2% SDS), washed in PBS, blocked overnight as above, and blotted with the actin antibody.

Mouse anti-ICP4, which recognizes a 120 kDa protein, was used at a 1:1000 dilution. Mouse anti-ICP8, which recognizes a 130 kDa protein, was used at a 1:200 dilution. Mouse anti-gD, which recognizes a 55 kDa protein, was used at a 1:1000 dilution. Mouse anti-ICP27, which recognizes a 63 kDa protein, was used at a 1:100 dilution. The secondary antibody, donkey anti-mouse IgG HRP, was diluted 1:5000. Goat anti-actin, which recognizes a 42 kDa protein, was diluted 1:200. Donkey anti-goat IgG HRP was diluted 1:4000.

2.6. Cellular toxicity assay

ARPE-19 cells were seeded in 96 well plates until confluent. Triplicate wells were incubated with media or increasing concentrations of TLR9 control, TLR9 inhibitory, or G-ODN oligonucleotides overnight at 37 °C. As a positive control for cell lysis, triplicate wells were incubated with 0.5% IGEPAL in media. A CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, TB245) was performed to determine the number of viable cells per well. The mean viability of the triplicate media wells was set at 100%, and results were graphed as percent media control.

2.7. Virucidal assay

5×10^6 plaque forming units of HSV-1 KOS were incubated with media or increasing concentrations of TLR9 control, TLR9 inhibitory, or G-ODN oligonucleotide for 1 h at 37 °C. Samples were diluted, to reduce ODN concentrations well below EC_{50} values, and titered on Vero cells (Grau et al., 1989). Mean titers of triplicate samples were graphed with error bars representing standard error of the mean.

2.8. Viral attachment assay

ARPE-19, 293XLnull, and 293XLhTLR9-HA cells were plated in 6 well plates until confluent. Duplicate wells were treated with media, or 20 μ M TLR9 control, TLR9 inhibitory, or G-ODN oligonucleotides for 2 h at 37 °C. Cells were chilled on ice and infected

with HSV-1 KOS at an MOI of 5 for 1 h at 4 °C. Cells were washed with cold PBS, and collected via scraping and centrifugation prior to lysis in 20 mM Tris, 100 mM NaCl, 1 mM EDTA, 0.5% Triton-X-100, and a 1:100 dilution of protease inhibitor cocktail (SIGMA, P8340). Protein concentration of lysates was assayed by the Pierce BCA protein assay kit (Pierce, 23225). Twenty micrograms of each ARPE-19 lysate or 40 μ g of each 293XLnull and 293XLhTLR9HA lysate were run on 4–15% Mini-PROTEAN TGX Precast gels (BIO-RAD). Protein was electrophoretically transferred to nitrocellulose, and the membranes blocked overnight with 5% non-fat dry milk in Genius Buffer I (100 mM maleic acid, 150 mM NaCl, pH 7.5) containing 0.3% v/v Tween-20. The membranes were incubated with a 1:1000 dilution of mouse anti-gD primary antibody (Virusys, HA025) for 1 h at RT in blocking buffer containing 1% FBS. After incubation, the membranes were washed in Genius Buffer I with 0.3% Tween-20. A 1:5000 dilution of goat anti-mouse IgG HRP (Santa Cruz, sc-2031) in blocking buffer was applied to the membranes for one hour at RT. After washing, the blots were developed using an enhanced chemiluminescence system (GE, PRN2232).

2.9. Viral entry assay

ARPE-19, 293XLnull, or 293XLhTLR9HA cells were plated in 6 well plates until confluent. Cells were chilled on ice prior to addition of 150 pfu of HSV-1 KOS for 1 h at 4 °C to allow for viral attachment. 150 pfu of virus was chosen to allow for adequate spacing of plaques to facilitate counting of wells. Media or oligonucleotides (20 μ M) were added to triplicate wells for 15 min at 4 °C prior to increasing the temperature to 37 °C for 1 h to allow for viral entry. Cells were rinsed with PBS, treated for 1 min with pH 3 citrate buffer (40 mM Citric acid, 10 mM KCl, 135 mM NaCl, pH 3.0), rinsed with PBS, then overlaid with methylcellulose (2% in DMEM supplemented with 2% FBS) and media (DMEM/2% FBS). Cells were incubated at 37 °C for 3 days. ARPE-19 wells were stained for plaques as described previously (Grau et al., 1989), and the number of plaques per well were counted. To visualize 293XL plaques, cells were fixed in 3.7% formaldehyde/PBS, rinsed with PBS, and blocked for 1 h in 5% milk/0.1% Tween-20/PBS. Mouse anti-gD (1:1000 dilution in block solution) was then applied to wells for 1 h at RT. Wells were washed with PBS/0.1% Tween before incubating with a 1:2500 dilution of goat anti-mouse alkaline phosphatase (SIGMA, A3562) in block solution for 1 h. After washing with PBS/0.1% Tween, the wells were exposed to SIGMA-FAST BCIP/NBT reagent (SIGMA, B5655) until plaques were detectable. The data are represented as mean plaque number per well with error bars representing standard error of the mean.

2.10. NF κ B transcription factor binding ELISA

ARPE-19 cells grown to confluency in 10 cm plates were pretreated with media (DMEM/F-12/10% FBS), 20 μ M TLR9 control oligonucleotide in media, or 20 μ M TLR9 inhibitory oligonucleotide in media for 2 h at 37 °C. Plates, except uninfected control plate, were infected with HSV-1 KOS at a MOI of 5 for 6 h at 37 °C. Cells were washed twice with cold PBS, collected by scraping, and pelleted at 1000 rpm for 10 min at 4 °C in a Beckman Model TJ-6 table top centrifuge. Nuclear and cytoplasmic extracts were prepared with a SIGMA CellLytic NuCLEAR Extraction Kit, utilizing hypotonic lysis buffer and IGEPAL (NXTRACT, SIGMA). The protein concentration of each extract was determined by BCA assay (Pierce, Rockford, IL, 23225). The equivalent of 14 μ g of nuclear extract was diluted in complete transcription factor binding assay buffer and loaded, in duplicate, into wells of a NF κ B (p65) Transcription Factor Assay Kit plate (Cayman Chemical Company, Ann Arbor, MI, 100007889). To assay p65 binding in the cytoplasmic fractions, the equivalent of

91 µg of cytoplasmic extract was loaded into wells of the transcription factor assay plate. The positive control cell lysate (PC) was diluted in hypotonic lysis buffer during the cytoplasmic fraction ELISA. Plates were read at OD 450 nm on a Safire² TECAN plate reader. The OD value of blank well(s) was subtracted from the OD value of each sample. Error bars represent standard error of the mean.

3. Results

3.1. Effect of TLR oligonucleotides on HSV-1 replication

As shown in Fig. 1A, pretreatment of ARPE-19 cells with 10 or 20 µM TLR9 inhibitory oligonucleotide reduced viral titers by

approximately 50% and 99%, respectively, compared to the media control. The G-ODN oligonucleotide, which contains a stretch of five guanines, had an even greater effect on viral titer, reducing titers to less than 1% of control at concentrations of 10 µM and 20 µM (Fig. 1A). To determine if this effect was cell type specific, Vero cells were pre-incubated with TLR9 inhibitory oligonucleotide prior to HSV-1 infection. The results in Vero cells correlated with those of the ARPE-19 cells, as 10 µM TLR9 inhibitory oligonucleotide reduced viral titers by approximately 89% and 20 µM inhibitory oligonucleotide reduced viral titers by 96% of the media control (Fig. 1A). The G-ODN oligonucleotide reduced viral titers in Vero cells to less than 1% of control at 10 and 20 µM (Fig. 1A). Pre-incubation with the TLR9 control oligonucleotide resulted in an enhancement of viral replication, in that viral titers were increased approximately 6-fold over the media control in ARPE-19 cells and 3- to 4-fold in Vero cells (Fig. 1B). The positive effect on viral replication by the TLR9 control oligonucleotide was not concentration dependent, suggesting this was a nonspecific effect.

The EC₅₀ for the TLR9 inhibitory oligonucleotide in ARPE-19 cells was 10 µM, while the EC₅₀ for the G-ODN oligonucleotide was 5.5 µM. These values correspond to the data in Fig. 1A, which indicates that the G-ODN oligonucleotide is approximately twofold more effective at reducing viral titers than the TLR9 inhibitory oligonucleotide. We determined that the EC₅₀ value of acyclovir for KOS infection of ARPE-19 cells was 2.25 µM (data not shown), indicating that the TLR9 inhibitory oligonucleotides require a 2.4- to 4.4-fold higher concentration compared to acyclovir.

3.2. Synthesis of HSV-1 proteins in the presence of TLR9 oligonucleotides

To better understand the effect of the TLR9 oligonucleotides on HSV-1 replication, ARPE-19 cells were pretreated with media or 10 µM TLR9 oligonucleotide prior to infection, and the expression of viral α (ICP4 and ICP27), β (ICP8), and γ (gD, and VP5) proteins was assayed by immunofluorescence (IF). IF on uninfected cells confirmed that antibodies to these herpes proteins did not cross react with cellular proteins (data not shown). Note that similar results were obtained when using the G-ODN oligonucleotide or the TLR9 inhibitory oligonucleotide.

In media treated ARPE-19 cells, ICP4 localized to 30–40% of cell nuclei at 6 h (2A) and 90–100% of cell nuclei at 15 h (2B) post infection (PI). In ARPE-19 cells treated with the TLR9 control oligonucleotide, the distribution of ICP4 at 6 h (2C) or 15 h (2D) PI was similar to the media control at 6 and 15 h, respectively. Treatment with the TLR9 inhibitory oligonucleotide, however, resulted in very few (3%) ICP4 positive nuclei at 6 h PI (2E). At 15 h PI, small foci of cells expressed ICP4 in both the nucleus and cytoplasm (8%), but the majority of cells did not express ICP4 (2F). Similar results were obtained when staining for the HSV-1 immediate early protein ICP27 or the major DNA binding protein ICP8 (data not shown).

To determine the effect of the TLR9 inhibitory oligonucleotide on viral γ-proteins, cells were stained for the HSV-1 glycoprotein gD and the major capsid protein VP5. Fig. 3 shows that at 6 h PI, about 20% ARPE-19 cells stained positive for gD (3A), and by 15 h every cell was gD positive (3B). Treatment with the TLR9 control oligonucleotide had no effect on gD expression or distribution at 6 h (3C) or 15 h (3D) PI compared to the media control. In contrast, pretreatment with the TLR9 inhibitory oligonucleotide eliminated all gD positive cells at 6 h PI (3E), and reduced the number of gD positive cells at 15 h PI to roughly 17% (3F). In media or control oligonucleotide treated cells, the VP5 antibody stained the cytoplasm of some ARPE-19 cells at 6 h PI, and by 15 h PI, VP5 was localized in punctate nuclear clusters in all ARPE-19 cells (data not shown). Pretreatment with the TLR9 inhibitory oligonucleotide reduced

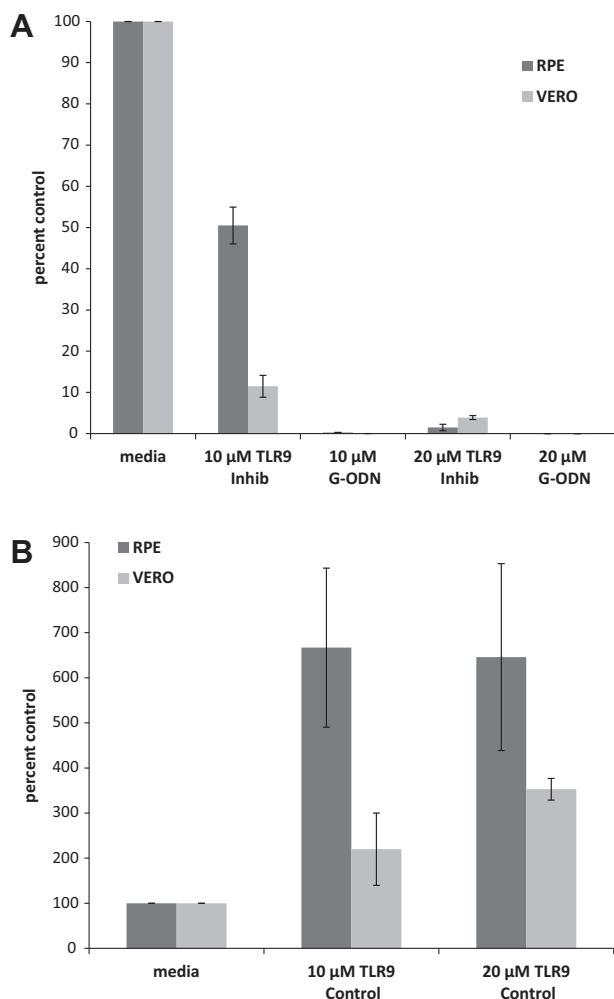


Fig. 1. TLR9 Inhibitory oligonucleotides decrease KOS titer in ARPE-19 and Vero cells. ARPE-19 (RPE) or Vero cells were incubated with 10 or 20 µM of the TLR9 inhibitory oligos TLR9i or G-ODN for 2 h at 37 °C (A). ARPE-19 or VERO cells were incubated with 10 or 20 µM of the TLR9 control oligo for 2 h at 37 °C (B). HSV-1 KOS virus was added to the wells at an MOI of 1 to 2 and cells were cultured overnight in the continued presence of the oligo and viral titers were determined. Mean titers of triplicate media controls were set at 100% and titers from triplicate experiments were averaged and graphed as percent of control. Error bars represent standard error of the mean. RPE titers (pfu/ml): Media 7.5×10^7 ; 10 µM TLR9 Control 3.8×10^8 ; 20 µM TLR9 Control 2.2×10^8 ; 10 µM TLR9 Inhibitory 3.8×10^7 ; 20 µM TLR9 Inhibitory 1.1×10^6 ; 10 µM G-ODN 1.9×10^5 ; 20 µM G-ODN 2.5×10^1 . Vero titers (pfu/ml): Media 2.0×10^7 ; 10 µM TLR9 Control 4.4×10^7 ; 20 µM TLR9 Control 7.1×10^7 ; 10 µM TLR9 Inhibitory 2.3×10^6 ; 20 µM TLR9 Inhibitory 7.8×10^5 ; 10 µM G-ODN 2.2×10^4 ; 20 µM G-ODN 2.6×10^2 . Student's *t*-test confirmed all treatments were significantly different from the media control ($p < 0.05$) except for RPE 20 µM TLR9C ($p = 0.058$) and Vero 10 µM TLR9C ($p = 0.209$).

the number of VP5 positive cells at 6 h PI, and reduced the number of cell nuclei staining positive for VP5 at 15 h PI (data not shown).

To confirm the effect of TLR9 oligonucleotides on the synthesis of HSV-1 proteins, Western blotting was performed on infected ARPE-19 lysates. A concentration of 20 μ M was chosen because at this concentration the TLR9 inhibitory oligonucleotide decreased viral titers by greater than 90% in ARPE-19 cells (Fig. 1A). Cell lysates were prepared at 6 and 15 h PI and samples were analyzed by gel electrophoresis and Western blotting. Media treated, infected ARPE-19 cells expressed ICP27, ICP4, ICP8 and gD at 6 h PI, with increased amounts of proteins evident at 15 h PI (Fig. 4). ARPE-19 cells pretreated with the TLR9 control oligonucleotide expressed viral proteins in approximately the same amounts as the media control at both 6 and 15 h PI (Fig. 4). In contrast, in cells pretreated with the TLR9 inhibitory oligonucleotide, there was no detectable ICP27, ICP4, ICP8 or gD at 6 or 15 h PI. To confirm equal loading of cell lysates, the ICP4-blotted membrane was stripped and blotted with an anti-actin antibody (Fig. 4). These data confirm that the TLR9 inhibitory oligonucleotide blocks HSV-1 protein synthesis in ARPE-19 cells.

3.3. Cellular toxicity of TLR9 oligonucleotides

To determine whether TLR9 oligonucleotides exhibit cellular toxicity, ARPE-19 cells were incubated overnight with increasing concentrations of TLR9 control, TLR9 inhibitory, or G-ODN oligonucleotides before determining cell viability. Results in Fig. 5 indicate

that the TLR9 control (TLR9C), TLR9 inhibitory (TLR9I), and G-ODN oligonucleotides are not toxic to ARPE-19 cells, as cells treated with 5, 10, or 20 μ M oligonucleotide concentrations had greater viability than cells treated with media alone. Note that cells treated with 0.5% IGEPAL had a mean viability of 13.7% compared to the media control. Overall, the negative effect of the TLR9 inhibitory and G-ODN oligonucleotides on HSV-1 replication cannot be explained by cellular toxicity.

3.4. Antiviral mechanism of TLR9 oligonucleotides

To determine whether TLR9 oligonucleotides were virucidal, we incubated increasing concentrations of oligonucleotides with HSV-1 KOS for 1 h at 37 °C. After incubation, the samples were diluted to reduce oligonucleotide concentrations to below active levels, and titered on Vero cells. Fig. 6A demonstrates no effect on viral titer for the TLR9 control or TLR9 inhibitory oligonucleotides at 5, 10, or 20 μ M. The G-ODN oligonucleotide, however, decreased viral titers by 4 logs at 5, 10, or 20 μ M. These data indicate that the G-ODN oligonucleotide exhibits virucidal activity against HSV-1 KOS, which may explain why it is more effective at inhibiting HSV-1 replication, compared to the TLR9 inhibitory oligonucleotide.

To assess whether TLR9 oligonucleotides affected HSV-1 attachment, ARPE-19 cells were incubated with media, TLR9 control, TLR9 inhibitory, or G-ODN oligonucleotide for 2 h at 37 °C, then transferred to 4 °C prior to virus infection. Immunoblotting with an antibody to the viral glycoprotein gD indicated that viral attach-

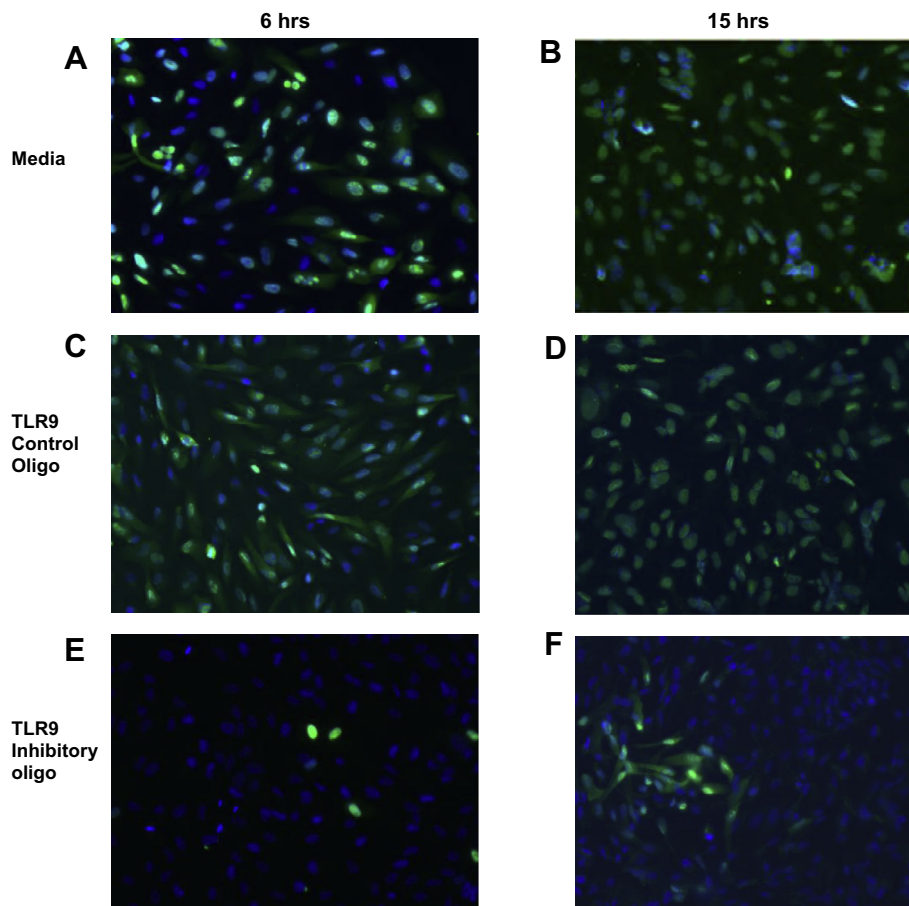


Fig. 2. Expression of HSV-1 ICP4 protein after treatment with TLR9 oligonucleotides. ARPE-19 cells were pretreated with media, 10 μ M TLR9 control oligo, or 10 μ M TLR9 inhibitory oligo for 2 h at 37 °C. Cells were then infected with HSV-1 KOS at an MOI of 2. At 6 or 15 h post infection, cells were fixed and stained with a mouse anti-ICP4 primary antibody followed by a donkey anti-mouse IgG Alexafluor 488 secondary antibody. Nuclei were visualized by Hoechst staining. Cells were photographed at 200 \times magnification.

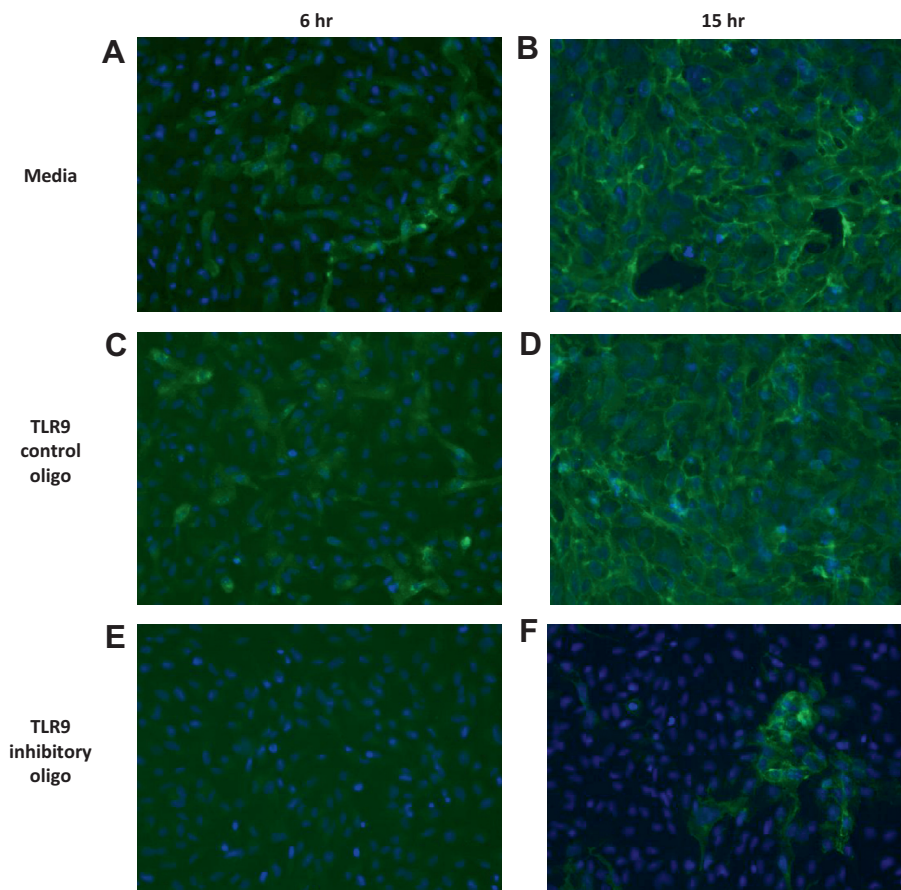


Fig. 3. Expression of HSV-1 gD protein after treatment with TLR9 oligonucleotides. ARPE-19 cells were pretreated with media, 10 μ M TLR9 control oligo, or 10 μ M TLR9 inhibitory oligo for 2 h at 37 $^{\circ}$ C. Cells were then infected with HSV-1 KOS at an MOI of 2. At 6 or 15 h post-infection, cells were fixed and stained with a mouse anti-gD primary antibody followed by a donkey anti-mouse IgG Alexafluor 488 secondary antibody. Nuclei were visualized by staining with Hoechst. Cells photographed at 200 \times magnification.

ment had occurred (Fig. 6B, Media lanes). The TLR9 control, the TLR9 inhibitory, and the G-ODN oligonucleotides significantly reduced the level of detectable gD, indicating that all TLR9 oligonucleotides reduced the ability of HSV-1 to bind to cells. Since the TLR9 control oligonucleotide had a positive effect on viral replication in ARPE-19 cells (Fig. 1B) and did not alter the expression or distribution of HSV-1 proteins during the viral life cycle (Figs. 2–4), this data suggests that the inhibition seen with our pre-addition studies was not due to inhibition of viral attachment.

Viral entry assays were also performed to determine whether TLR9 oligonucleotides were affecting entry of HSV-1 into ARPE-19 cells. Fig. 6C shows that all oligonucleotides decreased viral entry in ARPE-19 cells compared to the media control. The TLR9 control oligonucleotide decreased plaque number to about one third of the media control for ARPE-19 cells. The TLR9 inhibitory and G-ODN oligonucleotides had a greater effect, with a 10-fold (TLR9 inhibitory) or 80-fold (G-ODN) decrease in plaque number per well for ARPE-19 cells.

3.5. Post-infection treatment with TLR9 oligonucleotides

To determine whether TLR9 oligonucleotides inhibited HSV-1 replication when added post-infection, ARPE-19 cells were infected with HSV-1 KOS for 1 h prior to addition of increasing concentrations of TLR9 control, TLR9 inhibitory, or G-ODN oligonucleotides. Fig. 7 indicates that no reduction of viral titer was achieved when up to 20 μ M of the TLR9 control or TLR9 inhibitory oligonucleotides were added to ARPE-19 cells 1 h after infection. In contrast, 5 μ M of the G-ODN oligonucleotide reduced viral titers by about

1 log compared to the media control, and higher concentrations (10 and 20 μ M) reduced titers by up to 3 logs. These titers correspond to a 93% reduction at 5 μ M G-ODN compared to the media control and a greater than 99% reduction in viral titers at concentrations of 10 and 20 μ M G-ODN. These results indicate a difference in the mechanism of action of the TLR9 inhibitory versus G-ODN oligonucleotides that may be the result of sequence or charge differences.

3.6. TLR9 is not essential for antiviral activity of the TLR9 inhibitory oligonucleotides

To determine whether the antiviral effect of the TLR9 inhibitory oligonucleotides was dependent on the expression of the TLR9 protein, the TLR9 negative cell line, 293XLnull, and its TLR9 expressing counterpart, 293XLhTLR9HA, were employed. 293XLnull and 293XLhTLR9HA cells were treated with TLR9 oligonucleotides for 2 h at 37 $^{\circ}$ C, prior to overnight infection with HSV-1 strain KOS. The TLR9 inhibitory oligonucleotide decreased viral titers in 293XLnull cells to roughly 10% of media control at 10 μ M and 3.5% of media control at 20 μ M (Fig. 8A). Similar reductions were seen in the 293XLhTLR9HA cells where the TLR9 inhibitory oligonucleotide reduced titers to 15% of media control at 10 μ M and about 10% of media control at 20 μ M. The G-ODN inhibitory oligonucleotide had a profound effect on viral titer in 293XLnull cells, with decreases in viral titer to 5% of media control at 10 μ M and less than 1% of media control at 20 μ M (Fig. 8A). Viral titers in 293XLhTLR9HA cells were reduced to less than 1% of media control at the 10 and 20 μ M concentrations of G-ODN (Fig. 8A). The

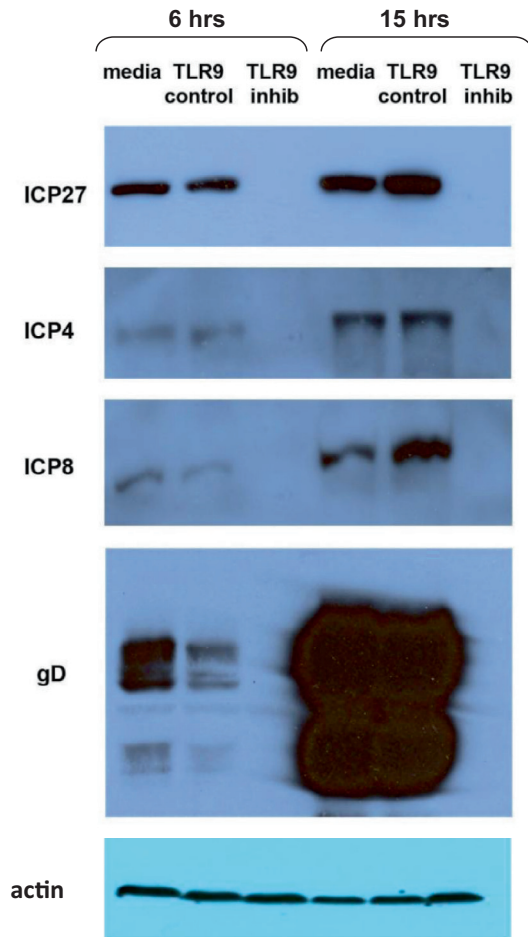


Fig. 4. TLR9 inhibitory oligonucleotides block synthesis of HSV-1 proteins. ARPE-19 cells were pretreated with media, 20 μ M TLR9 control oligo, or 20 μ M TLR9 inhibitory oligo for 2 h at 37 °C. Cells were infected with HSV-1 KOS at a MOI of 2 for 6 or 15 h. Cell lysates were prepared and loaded onto 10% (ICP4, ICP8) or 12% (ICP27, gD) denaturing polyacrylamide gels. Proteins were transferred to nitrocellulose and Western blotting was performed with HSV-1 antibodies. The ICP4 blotted membrane was stripped and re-blotted with goat-anti actin to demonstrate equivalent loading of cell lysates. Mouse anti-ICP4 recognized a 120 kDa protein. Mouse anti-ICP8 recognized a 130 kDa protein. Mouse anti-gD recognized a 55 kDa protein. Mouse anti-ICP27 recognized a 63 kDa protein. Goat anti-actin recognized a 42 kDa protein.

presence or absence of TLR9 protein did not influence the antiviral activity of these oligonucleotides, indicating that the antiviral effect was not due to inhibition of TLR9 signaling and suggesting that another target is involved. The TLR9 control oligonucleotide decreased viral titers to approximately 50% of the media control in 293XLnull cells at both 10 and 20 μ M (Fig. 8B). Similar to what was seen with ARPE-19 and Vero cells, the TLR9 control oligonucleotide increased viral titers in 293XLhTLR9HA cells to about 190% of media control at 10 and 20 μ M and the effect was not dose dependent (Fig. 8B).

We then assessed whether the TLR9 oligonucleotides affected HSV-1 attachment to 293XLnull and 293XLhTLR9HA cells. Cells were incubated with media, TLR9 control, TLR9 inhibitory, or G-ODN oligonucleotide for 2 h at 37 °C, then transferred to 4 °C prior to virus infection. Immunoblotting with an antibody to the viral glycoprotein gD showed that the gD protein was detected in the media lanes of both 293XLnull and 293hTLR9HA cells, indicating viral attachment had occurred (Fig. 9B). The TLR9 control, the TLR9 inhibitory, and the G-ODN oligonucleotides, significantly reduced the level of detectable gD in both cell types, indicating that

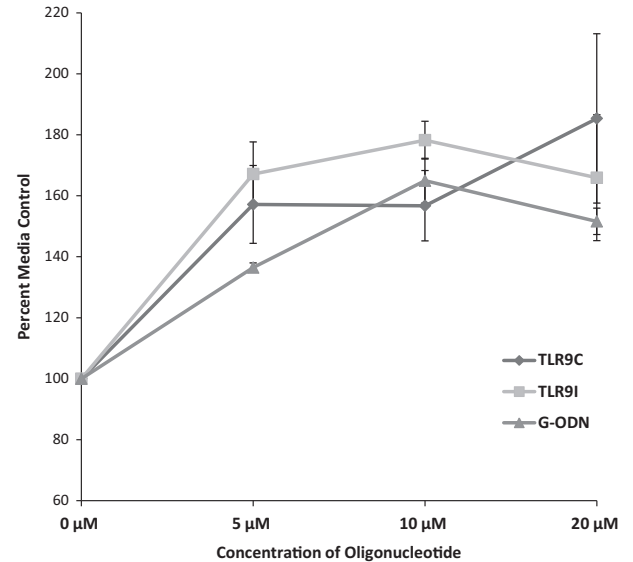


Fig. 5. Do TLR9 oligonucleotides exhibit cellular toxicity? ARPE-19 cells, plated in triplicate wells, were incubated with media or increasing concentrations of TLR9 control (TLR9C), TLR9 inhibitory (TLR9I) or G-ODN oligos overnight. Cell viability was determined via the CellTiter 96 Aqueous One Solution Cell Proliferation Assay. The mean cell viability for each oligonucleotide concentration is graphed as percent of the media control. Mean viability of cells treated with 0.5% IGEPAL was 13.7%. Error bars represent standard error of the mean. Student's *t*-test confirmed that all treatments were significantly different from the media control ($p < 0.05$).

all TLR9 oligonucleotides reduced the ability of HSV-1 to bind to both TLR9 negative and TLR9 positive cells.

Viral entry assays were performed to determine whether the effect of TLR9 oligonucleotides on HSV-1 entry in ARPE-19 cells (Fig. 6C) also occurred with the 293XLnull and 293XLhTLR9HA cells compared to the media control. The TLR9 control oligonucleotide decreased plaque number to about one half of the media control for 293XLnull, and two thirds of the media control for 293XLhTLR9HA cells. The TLR9 inhibitory and G-ODN oligonucleotides decreased plaque number in 293XLnull cells to roughly one third of the media control. In 293XLhTLR9HA cells, the TLR9 inhibitory and G-ODN oligonucleotides decreased plaque number to about one half of the media control. Thus the oligonucleotides are acting in a similar manner in all of the three cell types.

To determine whether the post-infection inhibitory effect of the G-ODN oligonucleotide observed in ARPE-19 cells (Fig. 7) was dependent on the TLR9 protein, 293XLnull and 293XLhTLR9HA cells were infected with HSV-1 KOS for 1 h prior to addition of increasing concentrations of TLR9 control, TLR9 inhibitory, or G-ODN oligonucleotides. Fig. 9C indicates that no reduction of viral titer was achieved when up to 20 μ M of the TLR9 control or TLR9 inhibitory oligonucleotides were added to 293XLnull and 293XLhTLR9HA cells 1 h after infection. In contrast, 5 μ M of the G-ODN oligonucleotide reduced viral titers by about 2.5 logs compared to the media control, and higher concentrations (10 and 20 μ M) reduced titers by up to 4 logs. These titers correspond to a greater than 99% reduction in viral titer compared to the media control at concentrations of 5, 10, and 20 μ M G-ODN. 293XL cells grow in clusters and cannot be trypsinized, making efforts to accurately count cells difficult. A large standard of deviation in the media treated 293XLhTLR9HA wells resulted in no significant difference between G-ODN and media treatments ($p > 0.05$). Despite these challenges, this experiment confirmed that the G-ODN oligonucleotide functioned post-infection to inhibit HSV-1 replication, and that this inhibition was not dependent on TLR9.

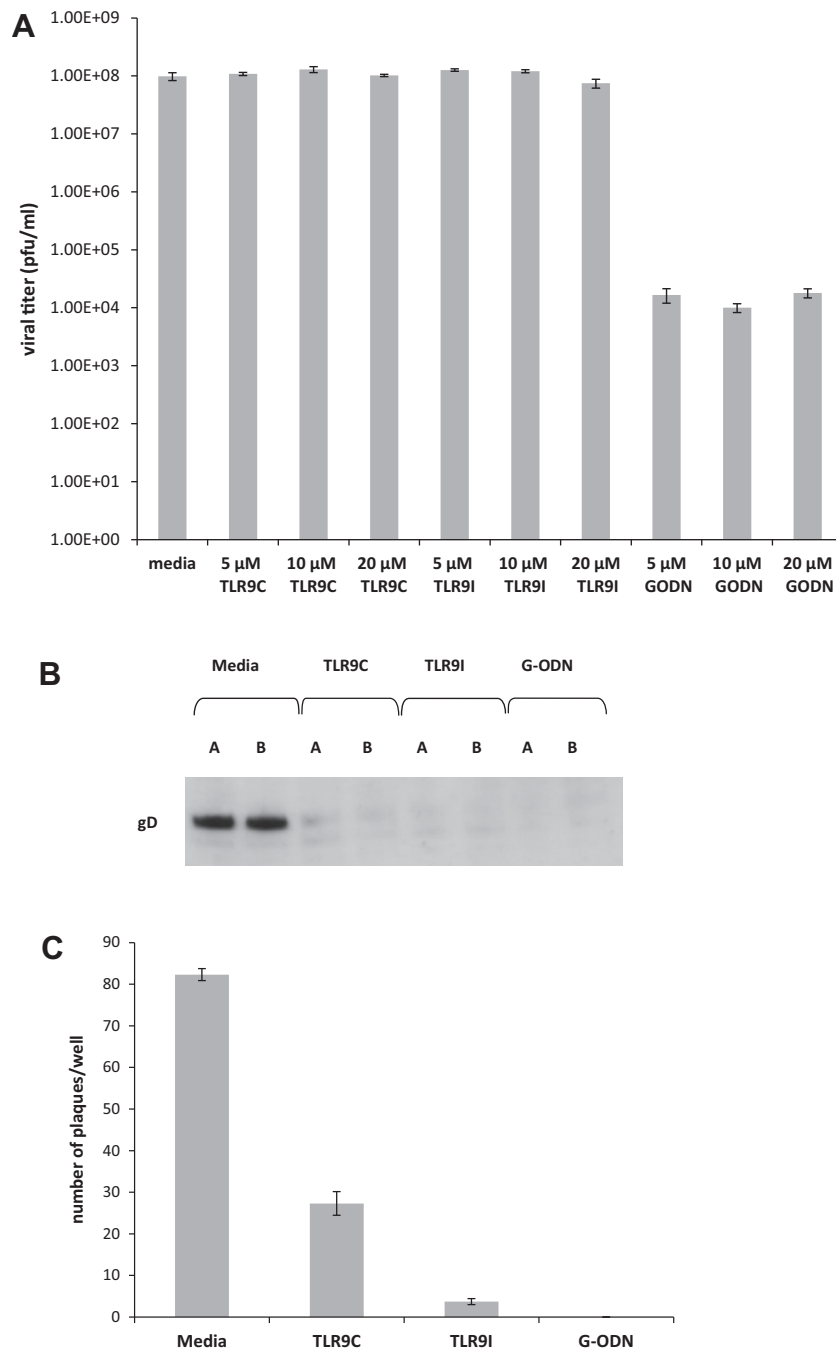


Fig. 6. Antiviral mechanism of TLR9 oligonucleotides. Virucidal activity (A): 5×10^6 pfu of HSV-1 KOS was incubated for 1 h at 37 °C with media or increasing concentrations of TLR9 control (TLR9C), TLR9 inhibitory (TLR9I), or G-ODN oligonucleotides. Triplicate samples were diluted and titered on Vero cells to determine pfu/ml. Error bars represent standard error of the mean. Student's *t*-test confirmed a statistically significant difference between all G-ODN treatments and media control ($p < 0.05$). Results for TLR9C and TLR9I treatment were not significant compared to the media control ($p > 0.05$). Viral attachment (B): Duplicate wells of ARPE-19 were treated with media, or 20 μM TLR9 control, TLR9 inhibitory, or G-ODN oligonucleotides for 2 h at 37 °C. Cells were chilled on ice, infected with HSV-1 KOS, washed with cold PBS, and lysates were prepared. Twenty micrograms of each lysate was run on a 4–15% SDS–PAGE gel. Immunoblotting was performed with mouse anti-gD primary antibody followed by goat anti-mouse IgG HRP secondary antibody. Viral entry (C): ARPE-19 cells were chilled on ice prior to addition of HSV-1 KOS at 4 °C to allow for viral attachment. Media or oligonucleotides (20 μM) were added to triplicate wells at 4 °C prior to increasing the temperature to 37 °C to allow for entry. Graph represents mean plaque number per well with error bars representing standard error of the mean. Student's *t*-test indicated a statistically significant difference between TLR9C, TLR9I, and G-ODN treatment compared to the media control ($p < 0.001$).

3.7. The effect of TLR9 oligonucleotides on NFκB binding in cellular extracts

To investigate the effect of TLR9 oligonucleotides on the ability of NFκB to bind to its response element, ARPE-19 cells were treated with media, TLR9 control oligonucleotide, or TLR9 inhibitory oligo-

nucleotide prior to infection with HSV-1 KOS. At 6 h PI, cells were collected and nuclear and cytoplasmic extracts were prepared. The uninfected nuclear extract contained low levels of NFκB binding activity (Fig. 10A, uninf). KOS infection increased the level of NFκB binding activity in the extract approximately 5-fold, consistent with NFκB activation and nuclear translocation during HSV-1

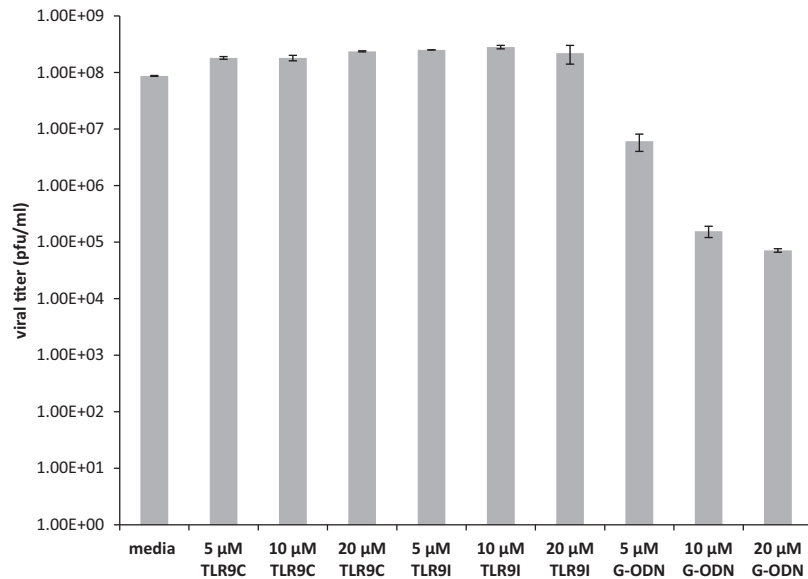


Fig. 7. Post-infection treatment with TLR9 oligonucleotides. ARPE-19 cells were infected with HSV-1 KOS at a MOI of 2 for 1 h at 37 °C. TLR9 control, TLR9 inhibitory, or G-ODN oligos were then added to a final concentration of 5, 10 or 20 μM and cells were incubated overnight at 37 °C. Supernatants were collected for titrating on Vero cells. Graph shows mean viral titer of duplicate wells with error bars representing the standard error of the mean. Student's *t*-test indicated a statistically significant difference between all treatments and media control ($p < 0.05$) except for 20 μM TLR9I ($p = 0.237$).

infection (10A, med/KOS). Pretreatment with the TLR9 control oligonucleotide had little effect on the amount of NFκB binding activity in the nuclear extract (10A, TLR9C/KOS). Pretreatment with the TLR9 inhibitory oligonucleotide, however, reduced NFκB binding activity to levels similar to the uninfected extract (10A, TLR9I/KOS). These results indicate that pretreatment with the TLR9 inhibitory oligonucleotide significantly reduced the level of NFκB activation during HSV-1 infection.

Since the TLR9 inhibitory oligonucleotide blocked activation of NFκB during HSV-1 infection, we wanted to examine whether the cytoplasmic NFκB in TLR9 inhibitory oligonucleotide treated cells was in an inactive state, or was active but not able to translocate to the nucleus. No significant NFκB binding was detected in any of the ARPE-19 cytoplasmic extracts, including KOS infected extracts, compared to the nonspecific binding control (Fig. 10B). This data indicates that cytoplasmic NFκB remains inactive, even when nuclear translocation is inhibited by the TLR9 inhibitory oligonucleotide.

4. Discussion

In summary, we have found that commercially available TLR9 inhibitory oligonucleotides are potent antivirals that result in a significant reduction in HSV-1 replication in several cell types. In the presence of TLR9 inhibitory oligonucleotides, expression of the essential viral α protein ICP4 was significantly inhibited, as was subsequent expression of viral β and γ proteins. All the TLR9 oligonucleotides, including the control, decreased viral attachment and entry, while the G-ODN oligonucleotide also exhibited virucidal activity and decreased viral titers when added post infection. The antiviral effect of the TLR9 inhibitory oligonucleotides was not dependent on TLR9, as similar reductions in viral titer were observed in a TLR9 null cell, and its TLR9 expressing counterpart. The TLR9 inhibitory oligonucleotides also decreased the ability of HSV-1 to activate NFκB during infection of ARPE-19 cells. These results indicate a potential use for TLR9 inhibitory oligonucleotides as antiviral agents during HSV-1 infection and emphasize that care must be taken when using these agents to study the role of TLRs in viral infection.

In the present study we infected ARPE-19, Vero, 293XLnull, and 293XLhTLR9HA cells with different multiplicity of infections (MOIs) in separate experiments. Titers of HSV-1 stocks were determined in Vero cells, and the plaquing efficiency of HSV-1 KOS was equivalent in ARPE-19 cells. Higher MOIs could potentially decrease the amount of oligonucleotide available at a given concentration to interact with each virion. For studies involving titrations, we infected overnight with an MOI of 1–2 to allow for adequate infection of all cells. Immunofluorescence and Western blot assays, which were performed at 6 or 15 h post-infection, also utilized an MOI of 1–2. During the attachment assay, we infected at an MOI of 5 for 1 h at 4 °C to allow for detection of viral glycoprotein bound to cells. In the entry assay, 150 pfu/well was chosen to generate a reasonable number of plaques/well to count. A high MOI of 5 was chosen for the p65 ELISA to get enough virus to activate p65 within the 6 h time frame. Regardless of the MOI chosen, the oligonucleotides (TLR9C, TLR9I, G-ODN) performed consistently across different experiments indicating that the MOIs chosen did not influence the results. In addition, the antiviral properties of the TLR9 oligonucleotides were similar in 3 different cell types (ARPE-19, Vero, 293XL) from two different species.

The cellular toxicity assay indicated that all oligonucleotides had a mitogenic effect on ARPE-19 cells. Previous studies have indicated mitogenic properties of bacterial and protozoan DNA, which is largely un-methylated and contains CpG sequences, for B cells (Brown et al., 1998; Krieg et al., 1995; Sun et al., 1997). It is thus possible that the TLR9 oligonucleotides have mitogenic potential, however it is unlikely that this effect is relevant, as all oligonucleotides are mitogenic in ARPE-19 cells, yet only the TLR9 control oligonucleotide increased viral titers.

The TLR9 control oligonucleotide increased HSV-1 titers in ARPE-19 and Vero cells, but this increase was not concentration dependent and was not accompanied by a change in the expression or localization of HSV-1 alpha (IC04, ICP27), beta (ICP8), or gamma (gD, VP5) proteins. Interestingly, the TLR9 control oligonucleotide also increased viral titers in 293XLhTLR9HA cells, but not in the TLR9 negative cell line 293XLnull, implying that the stimulatory effect may depend on the presence of the TLR9 protein. The stimulatory effect of the TLR9 control oligonucleotide on viral replica-

tion in these 3 cell lines was most likely an off-target effect, since it was not dose dependent, but further studies would be needed to determine its mechanism.

When added 2 h prior to infection, the G-ODN oligonucleotide, which contains a stretch of 5 guanines, was approximately two-fold more effective at reducing viral titers in several cell types, compared to the TLR9 inhibitory oligonucleotide. This data is consistent with studies which have indicated that G-ODN oligonucleotides are more effective at inhibiting TLR9 activation compared to TLR9 inhibitory oligonucleotides lacking the guanosine rich repeat (Peter et al., 2007). In addition, the G-ODN oligonucleotide was the only oligonucleotide to exhibit virucidal activity against HSV-1. The virucidal activity of the G-ODN oligonucleotide may be due to interaction with HSV-1 glycoproteins yielding conformational changes that affect their functionality. ISI 5652, a 20 base PS-ON, which contains 3 stretches of 4 guanines, has been shown to exhibit virucidal activity against HSV-1 KOS (Shogan et al., 2006). The G-ODN oligonucleotide decreased viral titers significantly when added to ARPE-19 post infection, while the control and TLR9 inhibitory oligonucleotides did not. Similar results were obtained with the guanosine repeat containing ISIS 5652 oligonucleotide versus the ISIS 2922, which lacks the guanosine repeat, in plaque reduction assays on Vero cells (Shogan et al., 2006). These data suggest a distinct mechanism of antiviral action for oligonucleotides containing guanosine repeats.

PS-ONs can prevent viral attachment via interaction with the α helices of viral glycoproteins such as the HIV-1 fusion protein gp41 (Vaillant et al., 2006) or the HSV-1 viral glycoprotein gB (Shogan et al., 2006). Our studies into the mechanism of TLR9 oligonucleotide antiviral activity revealed that all oligonucleotides interfered with HSV-1 attachment and entry into ARPE-19, 293XLnull, and 293XLhTLR9HA cells. These results conflict with the Takeda study (Takeda et al., 2011) in which TLR9 control and inhibitory oligonucleotides were not shown to interfere with attachment to human corneal endothelial cells (HCEn). It is possible that HCEn cells express different cell surface receptors for viral attachment, as HSV-1 is known to bind to several cellular proteins (Eisenberg et al., 2012), and these interactions may be affected differently by the TLR9 oligonucleotides. TLR9 oligonucleotides could also be triggering internalization of cell surface receptor proteins and thereby decreasing HSV-1 attachment and entry.

The inhibitory effect of the TLR9 control oligonucleotide on viral attachment and entry was surprising, as this oligonucleotide enhanced viral replication by roughly 6-fold when added to APRE-19 cells prior to infection. A PS-ON lacking guanosine stretches (ISIS 2922) was a potent inhibitor of HSV-1 attachment, but did not inhibit viral titers in plaque reduction assays (Shogan et al., 2006). These results mirror our data with the TLR9 control oligonucleotide, which does not inhibit HSV-1 replication in plaque assays, but does decrease viral attachment probably by acting as a polyanion. The observation that the control oligonucleotide inhibited attachment and entry, but enhanced replication when added to cells prior to infection, suggests that inhibition of attachment and entry was not involved in pre-infection treatment.

Our data lead us to believe that the TLR9 oligonucleotides are working on at least two and perhaps three levels (i) intracellularly to block TLR9 signaling and NF κ B activation as they were designed to do, (ii) intracellularly to inhibit some process critical for infection, and (iii) externally to interfere with early events in the viral life cycle such as attachment and entry. Thus, the order in which the oligonucleotide and virus is added during experiments is crucial for determining which mechanism of inhibition will predominate. We postulate that when oligonucleotides are added 2 h prior to infection, the majority are internalized and not available on the cell surface to interfere with viral attachment or entry, thus allowing for robust infection during these assays. Phosphorothioation of

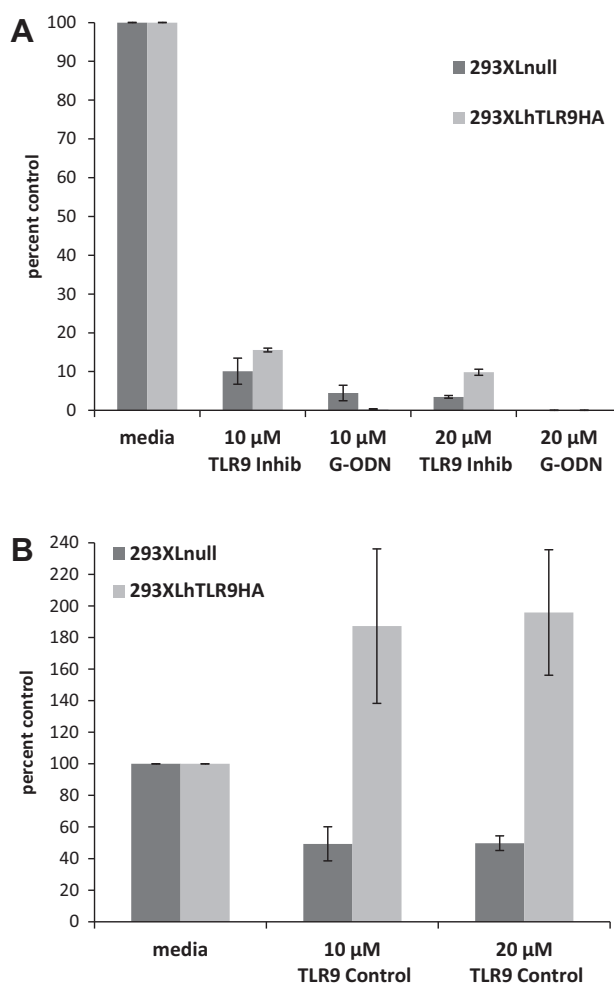


Fig. 8. TLR9 inhibitory oligonucleotides decrease HSV-1 replication independent of TLR9. Triplicate wells of 293XLnull and 293XLhTLR9HA cells were incubated with media or two concentrations of the TLR9 control, the TLR9 inhibitory, or the G-ODN oligonucleotide for 2 h at 37 °C. Cells were infected with HSV-1 KOS and cultured overnight in the continued presence of the oligonucleotide. Supernatants were collected and viral titer determined by plaque assay. Mean titers of media controls from two experiments were set at 100%, and titers from two experiments were averaged and graphed as percent of control. Error bars represent standard error of the mean. 293XLnull titers (pfu/ml): Media 6.8×10^7 ; 10 μ M TLR9 Control 1.4×10^7 ; 20 μ M TLR9 Control 2.5×10^7 ; 10 μ M TLR9 Inhibitory 4.3×10^6 ; 20 μ M TLR9 Inhibitory 9.6×10^5 ; 10 μ M G-ODN 1.1×10^6 ; 20 μ M G-ODN 4.9×10^5 . 293XLhTLR9HA titers (pfu/ml): Media 1.0×10^7 ; 10 μ M TLR9 Control 7.4×10^7 ; 20 μ M TLR9 Control 2.0×10^7 ; 10 μ M TLR9 Inhibitory 1.6×10^6 ; 20 μ M TLR9 Inhibitory 1.0×10^6 ; 10 μ M G-ODN 2.7×10^4 ; 20 μ M G-ODN 6.5×10^1 . Student's *t*-test indicated that all oligonucleotide treatments were statistically significant compared to the corresponding media control ($p < 0.04$).

nucleic acids can lead to off-target effects, which in the case of oblimersen, a phosphorothioate antisense oligonucleotide directed against *bcl-2*, results in useful therapeutic properties (Anderson et al., 2006; Gjertsen et al., 2007; Winkler et al., 2010). This may be the case for TLR9 oligonucleotides, which are potent antivirals even in TLR9 negative cells. The antiviral activity of oligonucleotides may also be dependent on temperature, as demonstrated by ISIS 5652, which loses its antiviral activity at 4 °C, probably because low temperatures inhibit oligonucleotide-induced conformational changes in the HSV-1 gB protein (Shogan et al., 2006).

Recently, the importance of several classes of DNA sensors in antiviral immunity has been elucidated (Rathinam and Fitzgerald, 2011). During viral infection, the cytosol accumulates viral RNAs or DNAs that originate from incoming viral genomes, viral transcripts, or transcription and replication intermediates (Rathinam

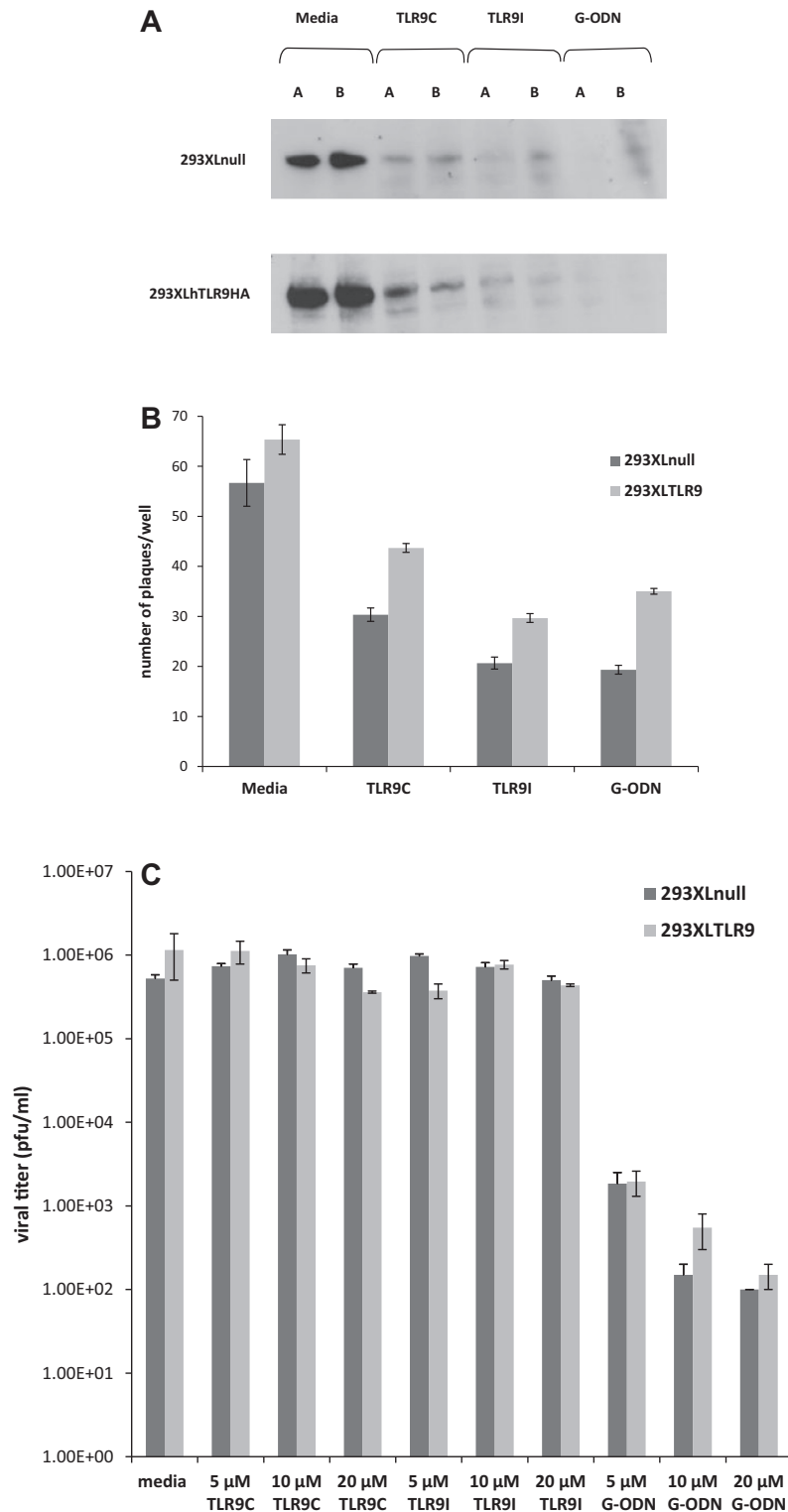


Fig. 9. Antiviral mechanism of TLR9 oligonucleotides on 293XLnull and 293XLTLR9 cells. Viral attachment (A): Duplicate wells of 293XLnull, or 293XLhTLR9HA cells were treated with media, or 20 μ M TLR9 control, TLR9 inhibitory, or G-ODN oligonucleotides for 2 h at 37 °C. Cells were chilled on ice, infected with HSV-1 KOS for 1 h, washed with cold PBS, and lysed. Forty micrograms of each lysate was run on a 4–15% SDS–PAGE gel. Immunoblotting was performed with mouse anti-gD primary antibody followed by a goat anti-mouse IgG HRP secondary antibody. Viral entry (B): 293XLnull and 293XLhTLR9HA cells were chilled on ice prior to addition of HSV-1 KOS to allow for viral attachment. Media or oligonucleotides (20 μ M) were added to triplicate wells at 4 °C prior to increasing the temperature to 37 °C to allow for entry. Graph represents mean plaque number per well with error bars representing standard error of the mean. Student's *t*-test indicated all oligonucleotide treatments were statistically significant compared to media controls ($p < 0.007$). Post-infection treatment with TLR9 oligonucleotides (C): 293XLnull and 293XLhTLR9HA cells were infected with HSV-1 KOS for 1 h at 37 °C. TLR9 control, TLR9 inhibitory, or G-ODN oligonucleotides were then added to a final concentration of 5, 10 or 20 μ M and cells were incubated overnight at 37 °C. Supernatants were collected for titrating on Vero cells. Graph shows mean viral titer of duplicate wells with error bars representing the standard error of the mean. For 293XLnull cells, Student's *t*-test indicated no statistically significant difference vs. media control for TLR9C treatments and 10 and 20 μ M TLR9I treatments ($p > 0.05$). 5 μ M TLR9I treatment and G-ODN treatments were significantly different compared to the media ($p < 0.05$). For 293XLTLR9 cells, Student's *t*-test indicated no statistically significant difference between media and any of the oligonucleotide treatments ($p > 0.05$).

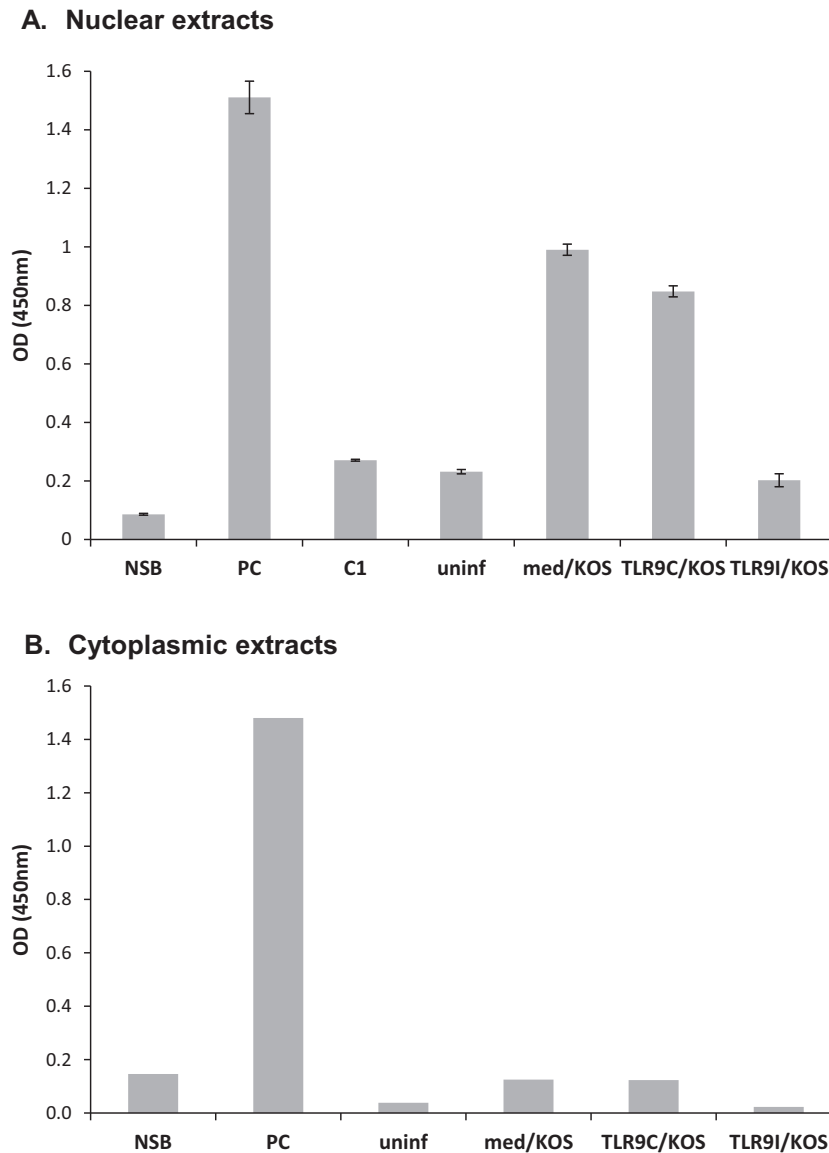


Fig. 10. TLR9 Inhibitory oligonucleotides decrease NFκB binding activity in nuclear extracts: ARPE-19 cells were pretreated with media, 20 μM TLR9 control oligo, or 20 μM TLR9 inhibitory oligo for 2 h at 37 °C prior to infection with HSV-1 KOS for 6 h. (A) The equivalent of 14 μg of nuclear extract was loaded, in duplicate, into wells of a Cayman NFκB (p65) Transcription Factor Assay Kit plate. The average OD value of duplicate blank wells was subtracted from the average OD of each duplicate sample. Error bars represent standard error of the mean. Student's *t*-test indicated all OD values for nuclear extracts were statistically significant compared to NSB ($p < 0.03$). (B) The equivalent of 91 μg of ARPE-19 cytoplasmic extract was loaded into wells of a Cayman NFκB (p65) Transcription Factor Assay Kit plate. The OD value of the blank well was subtracted from the OD value of each sample. NSB, non-specific binding; PC, positive control extract; C1, competitor dsDNA added to well prior to positive control; uninf, uninfected nuclear extract; med/KOS, media treated and KOS infected extract; TLR9C/KOS, TLR9C treated and KOS infected extract; TLR9I/KOS, TLR9I treated and KOS infected extract.

and Fitzgerald, 2011). Several classes of Interferon-inducing DNA receptors including DNA-dependent activator of interferon regulatory factors (IRFs) (Takaoka et al., 2007), the mostly nuclear interferon inducible protein IFI16 (Horan et al., 2013; Unterholzner et al., 2010), and several DExD/H-box helicases (Kim et al., 2010; Zhang et al., 2011) have been linked to an antiviral response during HSV-1 infection. Notably, the DExD/H proteins 9 and 36 are known to bind to CpG rich DNA in the cytosol and have been shown to be important in dendritic cell cytokine response, including NFκB activation, to DNA viruses (Kim et al., 2010). It is possible that the TLR9 inhibitory oligonucleotides, which do not require TLR9 for their antiviral effect, are targeting DExD/H box proteins or other DNA sensing cytosolic receptors. Further studies are needed to identify the target of the TLR9 inhibitory oligonucleotides in order to determine their mechanism of action.

The interaction of HSV with NFκB is complicated in that the virus activates NFκB to facilitate its replication but concomitantly blocks NFκB activation to limit production of antiviral and inflammatory cytokines (Liu et al., 2008). HSV-1 activation of NFκB occurs in two waves (Amici et al., 2006). The first wave of NFκB activation requires virus binding and entry and the action of several viral proteins including glycoprotein gD, which binds to HVEM to activate NFκB and suppress apoptosis (Aubert and Blaho, 1999; Cheung et al., 2009; Goodkin et al., 2003; Medici et al., 2003; Sciortino et al., 2008a,b), and the UL31 or UL37 tegument proteins (Liu et al., 2008; Roberts and Baines, 2011). Recently, it has been demonstrated that HSV-1 utilizes the recognition of its glycoproteins gB and gH/gL by the pattern recognition receptor TLR2 to activate NFκB early in infection (Leoni et al., 2012). We detected decreased activation of NFκB by HSV-1 when ARPE-19 cells were

incubated with the TLR9 inhibitory oligonucleotide prior to infection. This decrease in NF κ B activation is not likely caused by decreased viral attachment or entry as the TLR9 control oligonucleotide did not decrease NF κ B activation in nuclear extracts, yet was able to decrease both viral attachment and entry.

The second wave of NF κ B activation requires viral replication (Amici et al., 2006; Cai and Brandt, 2008) and is required for sustained HSV-1 gene expression and efficient replication. It is likely that multiple HSV-1 proteins including ICP27 and ICP0 (Gregory et al., 2004; Hargett et al., 2006), and possibly multiple pathways, are involved in NF κ B activation, providing redundancy to optimize infection. Studies in human corneal endothelial cells suggest that HSV-1 is using TLR9-mediated NF κ B activation for its own replication, yet these authors did not determine whether the oligonucleotide was acting in a TLR9-dependent manner (Takeda et al., 2011). Further studies will be needed to address any link between decreased NF κ B activation and the antiviral mechanism of TLR9 oligonucleotides.

In summary, we have shown that TLR9 inhibitory oligonucleotides exhibit a profound antiviral effect on HSV-1 replication in both TLR9 positive and TLR9 negative cell lines. The phosphorothioated oligonucleotides appear to act both externally and intracellularly to inhibit several stages of the viral life cycle and the most potent oligonucleotides contain stretches of guanosine residues. Caution must be used when utilizing these oligonucleotides to study interactions between the TLR system and viruses.

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