Nucleic acid-based polymers effective against hepatitis B virus infection in patients do not harbour immune stimulatory properties in primary isolated blood or liver cells

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BACKGROUND

Nucleic acid polymers (NAPs) block the release of HBsAg from infected hepatocytes *in vivo*. Although this mechanism is assumed to be responsible for the activity of NAPs against hepatitis B virus (HBV) in patients, the role of putative immunostimulatory effects have not been explored.

OBJECTIVES

Examine the immune stimulatory properties of NAPs in primary isolated human blood, parenchymal, and non-parenchymal liver cells.

MATERIALS & METHODS

- Peripheral blood mononuclear cells (PBMC, n=3) were isolated from healthy volunteers. Primary human hepatocytes (PHH, n=4) and Kupffer cells (KC, n=4) were isolated after liver perfusion and digestion.
- Cells were treated with NAPs (see table) for 6 h (gene expression) or 24h (cytokine secretion) prior to analysis.
  - REP 2006 is the prototypic degenerate NAP and contains residual CpG (TLR-9 stimulatory) sequences.
  - REP 2055 and REP 2139 are clinically active NAPs.
  - REP 2139 is a derivative of REP 2055 containing additional modifications (2'O methylation of ribose and 5-methylcytosine) known to supress immunoreactivity.
  - REP 2165 is a derivative of REP 2139 designed to degrade more rapidly.
- Analysis of IFN (IFNA4, IFNB1, IFNG, IFNL2) and inflammatory (TNF, IL6, and IL10) gene expression upon NAPs treatment was performed by RT-qPCR on total cellular RNA after a 6 h NAP treatment.
- Analysis of cytokine secretion was conducted after a 24 h NAP treatment by specific ELISA on supernatants.
- Immune responsiveness was confirmed by cell treatment with known oligonucleotide-based ligands for TLR-3 (poly I:C, 25µg/ml), TLR-7 (ssRNA40, 10µg/ml) and TLR-9 (CpG ODN2216, [2µM]) in both studies.
- The intracellular uptake of CY3-labelled NAPs was confirmed using fluorescence microscopy.

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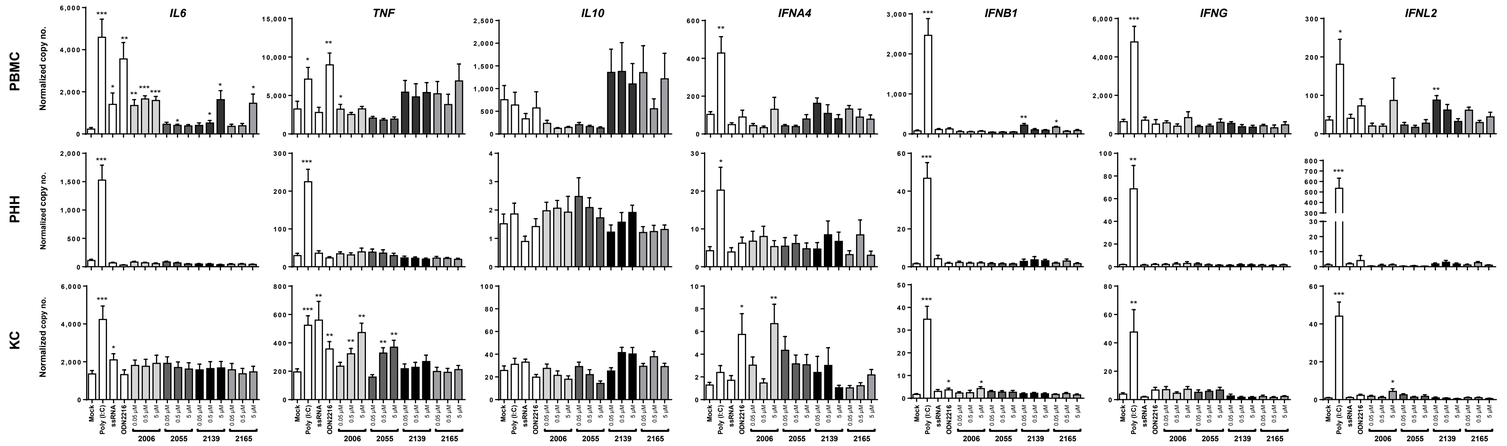
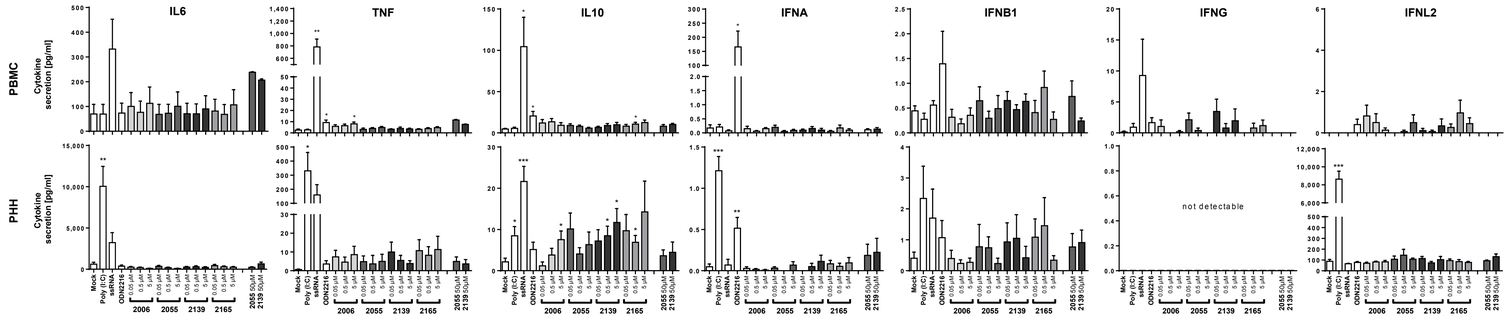
RESULTS

Cell type-specific expression of innate immune genes in response to NAP treatment *in vitro*



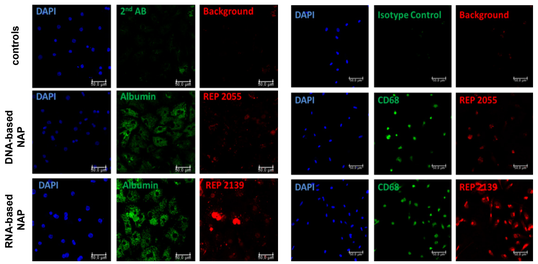
Peripheral blood mononuclear cells (PBMC, n=3), primary human hepatocytes (PHH, n=4) and Kupffer cells (KC, n=4) were stimulated with DNA-based (REP 2006 [2006] and REP 2055 [2055]) and RNA-based (REP 2139 [2139] and REP 2165 [2165]) NAPs or immune stimulatory controls (ODN 2216, TLR9 agonist; poly(I:C), TLR3 agonist; and ssRNA40 a TLR7 ligand) for 6h. Gene expression was assessed by RT-qPCR. REP 2006, REP 2139 and REP 2165 induced pro-inflammatory responses in PBMCs but displayed no significant antiviral activity. In PHH, no significant inflammatory or antiviral responses were detected for any NAP. In KC, pro-inflammatory activity (restricted to *TNF*) was observed with REP 2006 and REP 2055, whereas a weak but significant induction of interferon genes (*INFA4* and *IFNL2*) was only observed with REP 2006 at the highest concentration. These signals were comparable to those induced by ODN2216 stimulation Values represent mean ± SEM (normalized to 100,000 copies of beta actin mRNA). Statistically significant changes compared to untreated controls are reported for p<0.001 (***), p< 0.01 (**) and p< 0.05 (*). Mock, no treatment

Cell type-specific secretion of cytokines in response to NAP treatment *in vitro*



Peripheral blood mononuclear cells (PBMC, n=3), and primary human hepatocytes (PHH, n=3) were stimulated with DNA-based (REP2006 [2006] and REP2055 [2055]) and RNA-based (REP2139 [2139] and REP2165 [2165]) NAPs or immune stimulatory controls (ODN 2216, TLR9 agonist; poly(I:C), TLR3 agonist; and ssRNA40 a TLR7 ligand) for 24h.Supernatants were collected and secretion of IL6, TNF, IL10, IFNA, IFNB1, IFNG and IFNL2 was quantified by ELISA.. No significant increase of cytokine secretion was observed upon NAPs treatment in both PBMC and PHH. Importantly, ODN2216, a phosphorothioate oligonucleotide (similar to NAPs) containing CpG sequences stimulated the secretion of IFNA and IFNB1 in both PBMC and PHH. High dose treatment in PBMC (n=1) and PHH (n=3) did not significantly increased cytokine secretion. Values represent mean ± SEM. Statistically significant changes compared to untreated controls are reported for p<0.001 (***), p< 0.01 (**) and p< 0.05 (*). Mock, no treatment.

PHH and KC efficiently take up NAPs *in vitro*



The identity of PHH and KC was assessed by immuno-fluorescence staining of cell type-specific markers albumin and CD68 (green), respectively. Nuclei were counterstained with DAPI (blue). Uptake of NAPs was visualized using Cy3-labeld (red) REP 2055 [0.01µM] and REP 2139 [0.05µM]. Fluorescence images were acquired using Axiovert 100M microscope (Zeiss).

NAPS USED IN THE STUDY

NAP	Sequence 5' - 3'	Length	Modifications		
			PS	2'OMe	5'MeC
REP 2006	dN_{40} (degenerate, contains CpG)	40	✔		
REP 2055	$(dAdC)_{20}$	40	✔		
REP 2139	$(AC)_{20}$	40	✔	✔	✔
REP 2165	$(AC)_{20}$	40	✔	✔ *	✔

d= DNA
PS = phosphorothioation
2'OMe = O-linked methylation at 2' position in ribose
5'MeC = methylation of 5' position in cytidine base
* Positions 11, 21 and 31 have 2'OH ribose to facilitate degradation.
• Green boxes indicate clinically active NAPs

CONCLUSIONS

- ✓ Oligonucleotide uptake into primary cultured PMBC, KC and PHH is functional for immune stimulation.
- ✓ Results suggest that clinically active NAPs do not induce significant antiviral responses in primary isolated blood or parenchymal and non-parenchymal liver cells.
- ➢ We therefore hypothesize that the antiviral activity of NAPs against HBV infection cannot be explained by direct induction of innate antiviral responses