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**Inhibitory effect of the combination of CpG-induced cytokines with  
lamivudine against HBV replication *in vitro***

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Short title: Combination of CpG and lamivudine inhibit HBV replication

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**Abstract**

**BACKGROUND:** Currently approved antiviral monotherapies against chronic hepatitis B fail to eradicate hepatitis B virus (HBV), to overcome the defects in HBV-specific immune responses, and cannot prevent HBV relapse after cessation of therapy. CpG oligodesoxynucleotides (CpG ODN) are synthetic agonists of Toll-like receptor 9 (TLR9) and potent inducers of innate and acquired immunity. Our aim was to establish the proof of concept of the antiviral benefit of combining a nucleoside analog with CpG-induced cytokines on HBV replication *in vitro*. **METHODS:** Peripheral blood mononuclear cells from naïve individuals were stimulated with CpG ODN to generate CpG-induced cytokine supernatants. Proliferating HepaRG and HepG2 cells were transduced with recombinant HBV baculovirus and differentiated HepaRG cells were inoculated with HBV virions. Antiviral effects of CpG-induced cytokine +/- lamivudine were evaluated by analysing HBV DNA, RNA and antigen secretion (HBsAg, HBeAg). **RESULTS:** Following transduction or HBV-inoculation, CpG-induced cytokines strongly inhibited HBV viral intermediates of replication, as well as HBsAg and HBeAg secretion from infected cells. Strikingly, in transduced-HepaRG cells, the combination of CpG-induced cytokines with lamivudine reduced by one hundred fold the EC<sub>50</sub> of lamivudine. Importantly, CpG-induced cytokines treatment prior to HBV inoculation conferred to hepatocytes a partial protection against infection. **CONCLUSION:** CpG-induced cytokines associated with polymerase inhibitor do represent a promising combination to suppress HBV replication. Such an immunotherapeutic strategy should be evaluated *in vivo* to assess restoration and duration of anti-HBV specific immune responses.

**Keywords:** CpG, lamivudine, Hepatitis B Virus, inhibition

Hepatitis B virus (HBV) is a major public health problem and chronic hepatitis B is the leading cause of cirrhosis and hepatocellular carcinoma worldwide [1]. Current available monotherapies include the use of interferon-alpha (IFN- $\alpha$ ) or nucleos(t)id analogs (NA; Lamivudine, Adefovir, Entecavir, Telbivudine). IFN- $\alpha$  combines immunomodulatory and antiviral properties in about 30% of chronic carriers but adverse side effects restrict its use for short term therapy [2]. By contrast, NA are well tolerated, strongly suppress viral replication but require long term therapy that invariably leads to the emergence of drug resistant mutants [2]. The currently recommended antiviral therapies are confronted to the slow clearance of infected-hepatocytes and HBV genetic variability [3]. Importantly, NA can only transiently and partially overcome the defects of HBV-specific T cell immunity [4], TH1-like responses being associated with the resolution of acute HBV infection [3]. Thus, these limitations highlight an urgent need for the development of a combined therapy, aiming at targeting both HBV replication and HBV-specific immune responses, essential to maintain HBV suppression in chronic carriers after cessation of therapy.

An emerging class of immunomodulatory drugs targeting toll-like receptor 9 (TLR9) has become very attractive in the field of infectious diseases as vaccine adjuvants and anti-infective agents [5]. Indeed, oligonucleotides containing unmethylated CpG motifs (CpG ODN), mimicking pathogen-associated molecular patterns, are synthetic agonists of TLR9 and potent inducers of innate and adaptive immunity [6]. CpG ODN (A/C-class) trigger plasmacytoid dendritic cells to secrete tremendous amounts of IFN- $\alpha$ . Indirect antiviral mechanisms of CpG ODN also include the induction of pro-inflammatory cytokines (TNF- $\alpha$  and IL-6), chemokines (CCL2/3/4/5, CXCL10) and activation of natural killer cells (IFN- $\gamma$ ) [6]. Importantly, preclinical and ongoing clinical studies are providing evidences that TLR9 activation can induce strong TH1-like and antigen-specific immune responses [5, 6].

Taken together, this provides the rationale for the development of immunotherapeutic approaches combining NA and TLR9 agonists to accelerate viral clearance and restore anti-HBV specific immune responses. The aim of this study was therefore to establish the proof of concept of the antiviral efficacy of a combination between a nucleoside analog and CpG-induced cytokines *in vitro*.

For this purpose, peripheral blood mononuclear cells (PBMC) were isolated from healthy blood donors and stimulated with CpG ODN (CpG 2216; type A) or control ODN (2216C) for 24h. The presence of IFN- $\alpha$ , IFN- $\gamma$  and TNF- $\alpha$  was determined in supernatants (SN PBMC CpG 2216 or 2216C) using ELISA. CpG-stimulated PBMC produced high levels of IFN- $\alpha$  (11755 $\pm$ 650 pg/ml), moderate levels of IFN- $\gamma$  (1580 $\pm$ 460 pg/ml), TNF- $\alpha$  (195 $\pm$ 123pg/ml) and 2216C-stimulated PBMC did not induce detectable levels of such cytokines. Hepatocyte cell lines (HepG2, HepaRG, Huh7) showed no response following CpG ODN stimulation, suggesting an absence of TLR9 expression in human hepatocyte cell lines (data not shown). The antiviral potential of CpG-induced cytokines was evaluated using an HBV baculovirus vector, that was designed to trigger high levels of HBV replication in HepG2 cells [7]. The antiviral effect of CpG-induced cytokines, using PBMC SN, was demonstrated by 73% to 82% inhibition of intracellular encapsidated HBV DNA, respectively in HepG2 and HepaRG transduced-cells (Figure 1A). Such a powerful and significant ( $p < 0,001$ ) inhibition was obtained using several concentrations of IFN- $\alpha$ -induced by CpG stimulation (200 to 1000 pg) and is similar to a previous study using HepG2.2.15 cells [8]. IFN- $\alpha$  has been shown to inhibit HBV replication by eliminating viral RNA-containing capsids in the cytoplasm of hepatocytes [9]. Other studies showed that IFN- $\gamma$ , as well as IFN- $\beta$ , prevent the formation of replication-competent HBV capsids [10]. Moreover, antiviral properties of TNF- $\alpha$  have also been demonstrated through the destabilisation of HBV nucleocapsids [11, 12]. *In vivo*, TLR9 stimulation impairs HBV-replication in HBV-transgenic mice, however TLR9 expression

strongly differs between human and mouse cells, which makes the extrapolation of results difficult [13].

Experiments were next performed to evaluate the antiviral potential of CpG-induced cytokines combined with Lamivudine on HBV replication *in vitro* (Figure 1B). Proliferating HepG2 and HepaRG cells were transduced with HBV baculovirus and treated simultaneously with various concentrations of Lamivudine and with fixed concentrations of supernatant from CpG-stimulated PBMC. Effective concentrations of Lamivudine to inhibit HBV by 50% ( $EC_{50}$ ) were calculated after Southern blot analysis of encapsidated DNA (Figure 1B, Table 1). Dose-response curves demonstrate that combination of CpG-induced cytokines with Lamivudine reduced by almost one hundred fold the Lamivudine  $EC_{50}$  in HepaRG cells, whereas no difference was observed in HepG2 cells (Figure 1B, Table 1). Previous antiviral studies suggested that transformed hepatoma cells (HepG2, Huh7) are partially deficient in the IFN signaling with a poor ability to produce and respond to type I IFNs [14-16]. By contrast, HepaRG, that are liver progenitor and non-transformed cells, have been shown to be more competent for the IFN pathway as compared to the transformed HepG2 cell line [17, 18]. However, the discrepancy in the antiviral effect observed in HepG2 and HepaRG cells, which was only detectable when the combination treatment was applied, remains to be elucidated.

We next evaluated the ability of CpG-induced cytokines to inhibit HBV replication in differentiated HepaRG cells (Figure 2A), which represent an unique cellular model for HBV infection [19]. HepaRG cells were inoculated with HBV virions produced in HepG2.2.15 cells [19], followed by treatment at day 7 post-inoculation with CpG-induced cytokines for 48 hours. HBV RNA, DNA, HBs and HBeAg were analyzed using northern and Southern blots and ELISA. The inhibitory effect was more pronounced against HBV DNA and HBeAg ( $p < 0,001$ ), similar to previous study in transfected hepatoma cell lines [20].

Finally, we addressed the ability of CpG-induced cytokines to protect hepatocytes from HBV infection (Figure 2B). Differentiated HepaRG cells were thus treated with supernatants from CpG-stimulated PBMC, or recombinant IFN- $\alpha$ 2B, 24 hours prior to HBV inoculation. At day 9 post-inoculation, the reduction in viral genome intermediates and secreted antigens was more pronounced in HBV-infected HepaRG cells treated with CpG-induced cytokines, as compared to IFN- $\alpha$ 2B-treated cells ( $p < 0,001$  for HBV DNA, HBs and HBeAg). The inhibition of viral intermediates was similar to that observed in HepaRG cells treated post-inoculation (Figure 2A), and more pronounced for HBsAg secretion ( $p < 0,001$ ). These results provide evidence that CpG-induced cytokines can induce an antiviral state in HepaRG cells and confer a partial protection against HBV infection.

Overall, our study demonstrates that CpG-induced cytokines associated with Lamivudine do represent a powerful combination to inhibit HBV replication in HepaRG cells. Despite the absence of additive/synergic effect of the combination in HepG2 cells, the main interest in targeting TLR9 through CpG ODN resides in their ability to stimulate immune-specific responses. Indeed, formulation of HBV vaccine with CpG ODN was able to achieve higher rates of seroconversion and conferred long term protection in HIV patients, usually hyporesponsive to HBV vaccination [21]. Moreover, combination of CpG ODN with chronic hepatitis C therapy (PEG-IFN + ribavirine) was associated with immune marker activities which correlated with antiviral efficacy in relapser patients [22]. Therefore, it is tempting to speculate that manipulation of TLR9 in chronic HBV individuals might increase the non-cytolytic control of HBV, through induction of type I and II IFNs, TNF- $\alpha$  and TH1-like responses. Thus, the use of TLR9 agonists combined with nucleoside analogs should be evaluated *in vivo*, in order to assess the restoration and duration of anti-HBV specific immune responses.

Taken together, ongoing clinical trials using CpG ODN [6] are paving the way for the evaluation of promising novel combination strategies with NA to achieve an immunomediated and sustained control of HBV replication in chronic hepatitis B patients.

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**Table 1**

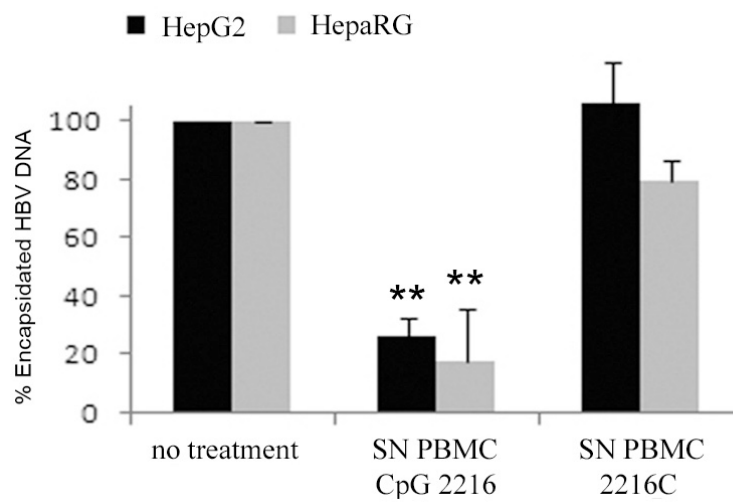
	EC50 Lamivudine ( $\mu\text{M}$ )	
	HepG2	HepaRG
no treatment	0,269 $\pm$ 0,152	0,507 $\pm$ 0,203
SN PBMC CpG 2216	0,367 $\pm$ 0,058	0,007 $\pm$ 0,002
SN PBMC 2216C	0,313 $\pm$ 0,103	0,340 $\pm$ 0,174

**Figure legends**

**Figure 1. CpG-induced cytokines alone or in combination with Lamivudine inhibit HBV replication in transduced-HepG2 and -HepaRG cells.** Proliferating HepG2 and HepaRG cells were transduced at a multiplicity of infection of 100 PFU/cell with Bac-HBV-1.1-WT [7]. Cells were immediately treated with medium (no treatment), supernatants from CpG-stimulated PBMC (SN PBMC CpG 2216) or control ODN-stimulated PBMC (SN PBMC 2216C), alone (A) or in combination with various concentrations of Lamivudine (B, dose-response curves of Lamivudine). Encapsidated DNA was extracted 48 hours post-treatment and quantification of HBV DNA following Southern blot analysis was performed by PhosphorImager (ImageQuant software). In (A), values obtained for “SN PBMC CpG 2216 and SN PBMC 2216C” were divided by “no treatment” value, which was assumed to represent 100 % of HBV DNA. Note that in (B), encapsidated HBV DNA was standardized in reference to values obtained in the absence of Lamivudine (set at 100% for each condition “no treatment, SN PBMC CpG 2216 and SN PBMC 2216C”). Values are the mean of at least three independent experiments. Statistical analysis was performed by ANOVA test followed by posthoc Tukey test ( $p$  value of  $<0,001^{**}$  was considered significant).

**Table 1. Lamivudine effective concentration in combination with CpG-induced cytokines.** EC50 (Lamivudine effective concentration which inhibits 50% of HBV replication) were calculated from experiments performed in figure 1B. Values are the mean of at least three independent experiments.

**Figure 2. CpG-induced cytokines inhibit HBV replication in infected-HepaRG cells and protect hepatocytes from HBV infection.** Differentiated HepaRG cells were treated with medium (no treatment), supernatants from CpG-stimulated PBMC (SN PBMC CpG2216) or control ODN-stimulated PBMC (SN PBMC 2216C) 7 days after (A) or 24 hours prior to (B) HBV inoculation with virions from HepG2.2.15 [19]. Nine days post-inoculation, quantification of viral RNA and encapsidated DNA was performed following northern and Southern blot analysis. Supernatants of infected-HepaRG cells were tested for HBsAg and HBeAg secretion using ELISA. Values obtained for each treatment were divided by “no treatment” value, which was assumed to represent 100 % of viral replication. Values are the mean of at least three independent experiments. Statistical analysis was performed by ANOVA test followed by posthoc Tukey test. CpG-induced cytokines and IFN- $\alpha$ 2B-treated values were compared to “no treatment and 2216C-stimulated supernatants” values ( $p < 0,05$  \* or  $<0,001$ \*\*). CpG-induced cytokines values were compared to IFN- $\alpha$ 2B-treated values ( $p < 0,05$  # or  $<0,001$ ##).

**A****B**