

## SUPPLEMENTARY MATERIALS

### **Toll-like receptor dual-acting agonists are potent inducers of PBMC-produced cytokines that inhibit hepatitis B virus production in primary human hepatocytes**

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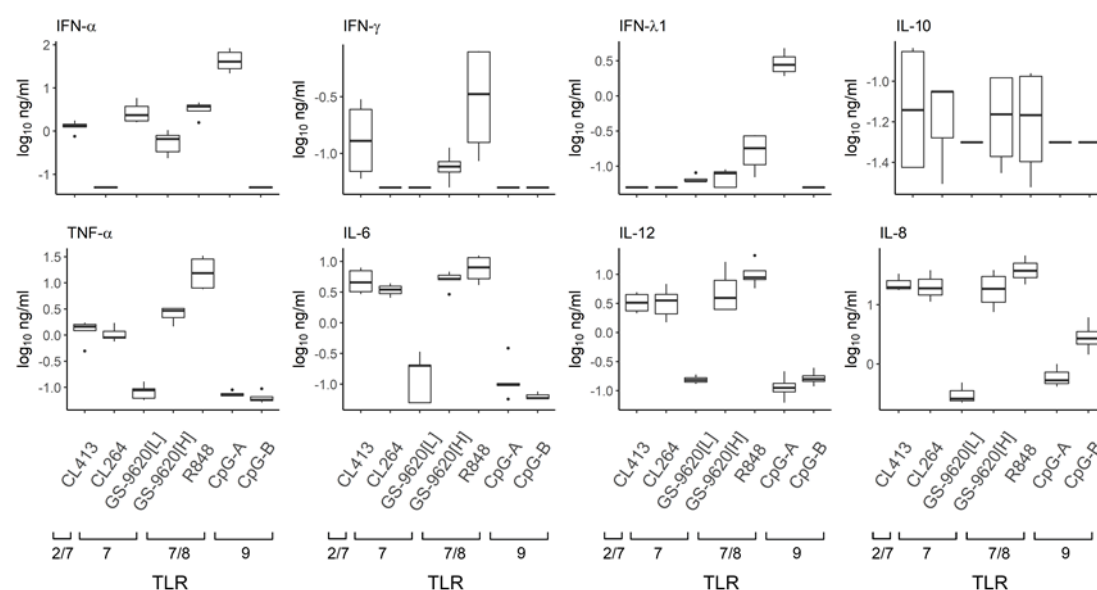
## Supplementary Methods

**Determination of NF- $\kappa$ B p65 phosphorylation by dynamic phospho-flow cytometry.** PBMCs stimulated for 1 h with TLR agonists were fixed in 4% formaldehyde for 15 min, permeabilized by 90% methanol for 30 min, and incubated with Phospho-NF- $\kappa$ B p65 (Ser536) (93H1) rabbit mAb (Cell Signaling, Danvers, MA). APC-conjugated Goat anti-Rabbit IgG polyclonal antibody (Thermo Fisher Scientific) was used as a secondary antibody. Viable PBMCs were gated according to Live/Dead Zombie Green kit. Samples were analyzed using a BD LSR FORTRESSA cytometer (BD Biosciences, San Jose, USA) and data were processed using FLOWJO software (Treestar, San Carlos, USA).

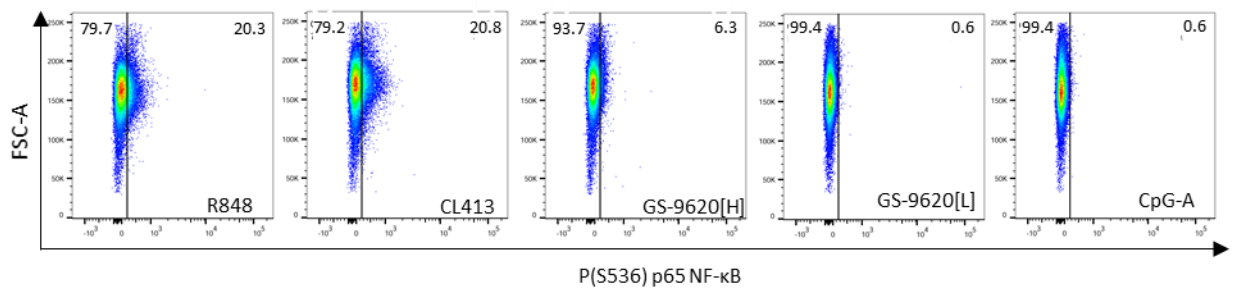
**XTT test.** Cytotoxic effect was analyzed by XTT colorimetric assay. Briefly, 50  $\mu$ l of 50:1 mixture of XTT labeling reagent (1 mg/ml; Sigma-Aldrich, Life Science) and PMS electron-coupling reagent (0.383 mg/ml; Sigma-Aldrich, Life Science) was added to the wells with HBV-infected PHH and incubated for 4 h in 37  $^{\circ}$ C in 5 % CO<sub>2</sub>. Formation of orange formazan dye was measured in Victor X3 plate reader.

## Supplementary Results

**Differential potencies of TLR2/7, TLR7, TLR7/8 and TLR9 agonists to induce repertoire of cytokines secreted by stimulated PBMCs.**

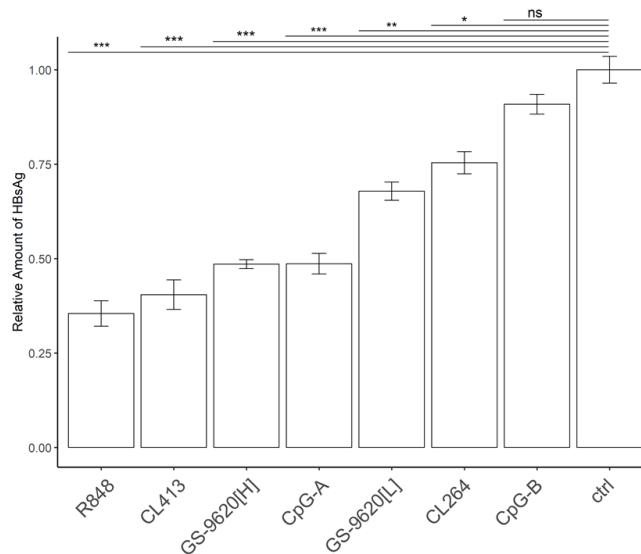


**Figure S1.** Amounts of cytokines secreted by PBMCs stimulated by different TLR2/7, TLR7, TLR7/8 and TLR9 agonists. PBMCs (N>3) were stimulated with either no agonist, the dual agonist CL413 (5  $\mu$ g/ml), the TLR7 agonists CL264 (5  $\mu$ g/ml) or GS9620[L], the TLR7/8 agonists GS9620[H] or R848 (4  $\mu$ g/ml), or the TLR9 agonists CpG-A (4  $\mu$ g/ml) or CpG-B (4  $\mu$ g/ml) for 16 h, and the cytokine levels were determined by ELISA.



**Figure S2.** PBMCs were stimulated with the dual-acting agonists R848 (4  $\mu\text{g/ml}$ ), CL413 (5  $\mu\text{g/ml}$ ) and GS9620[H], or the single-acting agonists of TLR7, GS9620[L], or of TLR9, CpG-A (4  $\mu\text{g/ml}$ ), for 1 h, and the expression levels of P(S536) p65 NF- $\kappa$ B cytokine were determined by phospho-flow cytometry. Distribution of phosphorylated p65 NF- $\kappa$ B is shown as a percentage in the upper half of dot-plot diagrams.

### Inhibition of HBsAg production from infected PHH exposed to CM from PBMCs stimulated by TLR2/7, TLR7, TLR7/8 and TLR9 agonists



**Figure S3.** Inhibition of HBsAg production from HBV-infected PHH exposed to PBMC CM. A total of 30,000 PHH were infected with 2000 VGE of HBV and cultured for 3 days before CM (diluted 1:100) from  $3 \times 10^6$  PBMCs per ml stimulated by agonists of TLR2/7 (CL413), TLR7 (CL264, GS-9620[L]), TLR7/8 (R848, GS-9620[H]), TLR9 (CpG-A, CpG-B) was added. CM was added again 6 DPI. Production of HBeAg was determined by ELISA 9 DPI and normalized to production by HBV-infected PHH in the absence of CM. CM from unstimulated PBMCs was used as a control. Cells were grown for 3 more days. The HBsAg data are shown as mean  $\pm$  SEM of 5 independent experiments with PHH from three donors. \*,  $p < 0.05$ , \*\*,  $p < 0.01$ , \*\*\*,  $p < 0.001$  pairwise Post hoc Mann-Whitney-Wilcoxon test. Kruskal-Wallis  $p < 3.6 \times 10^{-16}$ .

**Table S1.** Viability of PHH treated with PBMC CM<sup>1)</sup>

| TLR agonist   | R848      | CL413     | GS-9620[H] | CpG-A    | GS-9620[L] | CL264    | CpG-B    | Ctrl    |
|---------------|-----------|-----------|------------|----------|------------|----------|----------|---------|
| Viability (%) | 106.4±7.6 | 104.9±4.1 | 114.0±4.1  | 95.5±2,5 | 97.0±2.4   | 87.3±1.4 | 97.2±1.5 | 100±4.5 |

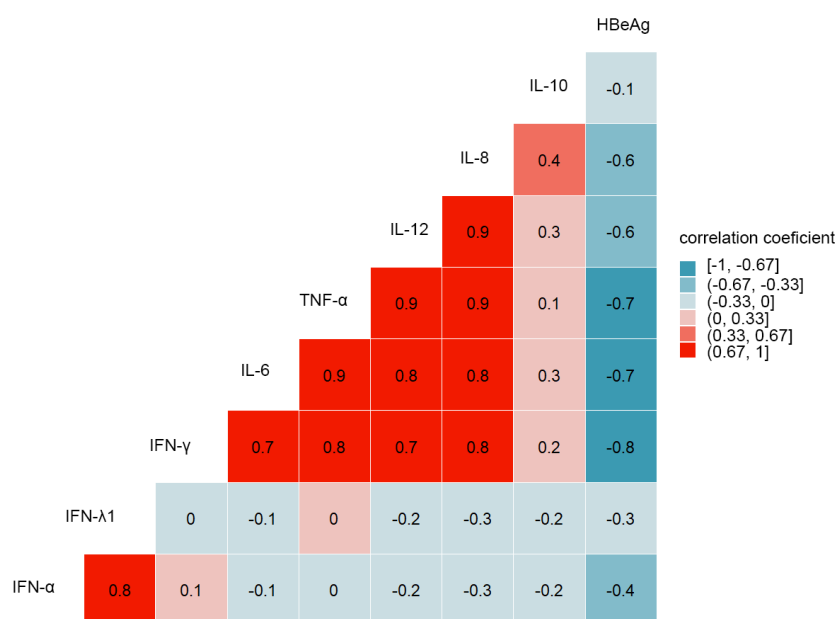
<sup>1)</sup>Determined by XTT test. Conditioned medium (CM) diluted 1:10.

**Table S2.** Multivariate statistics for HBeAg measurements<sup>1)</sup>

|            | CL413    | CL264    | GS-9620[L] | GS-9620[H] | R848     | CpG-A    | CpG-B    |
|------------|----------|----------|------------|------------|----------|----------|----------|
| CL413      | -        | -        | -          | -          | -        | -        | -        |
| CL264      | 5.70e-09 | -        | -          | -          | -        | -        | -        |
| GS-9620[L] | 5.70e-09 | 1.40e-07 | -          | -          | -        | -        | -        |
| GS-9620[H] | 2.26e-02 | 5.70e-09 | 5.70e-09   | -          | -        | -        | -        |
| R848       | 4.00e-07 | 5.70e-09 | 5.70e-09   | 6.70e-09   | -        | -        | -        |
| CpG-A      | 1.20e-07 | 5.70e-09 | 5.70e-09   | 2.40e-04   | 5.70e-09 | -        | -        |
| CpG-B      | 9.10e-09 | 1.50e-08 | 7.10e-09   | 9.10e-09   | 7.10e-09 | 7.10e-09 | -        |
| Ctrl       | 5.70e-09 | 5.70e-09 | 5.70e-09   | 5.70e-09   | 5.70e-09 | 5.70e-09 | 2.24e-03 |

<sup>1)</sup>The Kruskal-Walis (p-value <2.2e-16) test indicates differences between groups of agonists. Post hoc Mann-Whitney-Wilcoxon test indicate that all groups of agonists differ.

**Table S3.** Correlation across PBMC-secreted cytokines<sup>1)</sup>



<sup>1)</sup>The data show correlation coefficients across PBMC-secreted cytokines and HBeAg produced from HBV-infected PHH.

**Table S4.** Determination of HBV cccDNA by droplet digital PCR

| Sample                 | no T5 treatment             |                         | T5 treatment                |                         |
|------------------------|-----------------------------|-------------------------|-----------------------------|-------------------------|
|                        | cccDNA primer               |                         | cccDNA primer               |                         |
|                        | non specific<br>(copies/μl) | Specific<br>(copies/μl) | non specific<br>(copies/μl) | Specific<br>(copies/μl) |
| GS-9620[H]/CM sample 1 | 7200                        | 86.9                    | 80.8                        | 28.6                    |
| GS-9620[H]-CM sample 2 | 6130                        | 75.5                    | 46.5                        | 26.4                    |
| R848-CM sample 1       | 7290                        | 89.5                    | 105                         | 33.5                    |
| R848-CM sample 2       | 8400                        | 101.5                   | 110                         | 37.4                    |
| CL413-CM               | 6560                        | 78                      | 80.7                        | 32                      |
| CTRL-CM                | < 10000                     | 121                     | 129                         | 37.3                    |
| NO HBV                 | 44.1                        | No Call                 | No Call                     | No Call                 |