Extracellular vesicles derived from Hepatitis-D Virus infected cells induce a proinflammatory cytokine response in human peripheral blood mononuclear cells and macrophages

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Abstract

Hepatitis D Virus (HDV) is as a satellite virus requiring Hepatitis B Virus (HBV) envelope proteins for productive infection. Hepatitis D is the most severe form of viral hepatitis and is a global health threat affecting 15 to 20 million humans. In contrast to Hepatitis B Virus monoinfection, against which only a minor innate immune response is mounted at most, HBV-HDV coinfection is characterized by a strong activation of innate immune responses. To shed light on poorly understood mechanisms of HDV-triggered disease progression, we focussed on the question how immune cells may be activated by HDV. We hypothesized that extracellular vesicles (EVs) released from infected cells mediate this activation. We therefore purified EVs from the supernatant of HDV-infected and non-infected cells and incubated them with human peripheral blood mononuclear cells (PBMC) and macrophages. Here we show for the first time that HDV infection leads to production of EVs which subsequently mediate a proinflammatory cytokine response in primary human immune cells. These data might help to understand how HDV can be sensed by non-infected immune cells.

Introduction

Extracellular vesicles (EVs) are small membranous particles selectively transferring cargo such as nucleic acids and cytokines between cells of origin and recipient cells as an essential mechanism of intercellular communication. They are classified by their mode of biogenesis as e.g. exosomes (40 – 100 nm), which are released from multivesicular bodies, or microvesicles (50 nm – 1 µm) which directly bud from the plasma membrane. EV cargo delivery has been shown to mediate immunoregulatory effects via miRNAs, mRNAs, proteins and signalling molecules (2). Regarding viral infections, EVs have been reported to exert both pro- and antiviral properties and to be responsible for transcellular spread, apoptosis, cytokine modulation and transfer of viral nucleic acids (3-5).

HDV is a satellite virus coexisting with HBV because it requires HBV envelope proteins for productive virion release and propagation of the infection (6). In contrast to HBV monoinfection, which does not mount an interferon (IFN) response, HBV-HDV coinfection leads to a pronounced activation of the innate immune system (7, 8) and induces a robust proinflammatory cytokine release (9, 10). In the clinics, this results in a severe inflammatory liver disease with rapid progression to liver cirrhosis and hepatocellular carcinoma with high mortality. So far there is no directed therapy available, and there is an urgent need to better understand the interaction between HDV and host organism (11). The cytoplasmatic RNA sensor MDA5 has recently been identified as the major pattern recognition receptor detecting HDV and inducing an interferon (IFN) response (12, 13). But due to the lack of appropriate animal models, it remains elusive which immune cells contribute to HDV-dependent immune recognition, how these immune cells recognize HDV and how they contribute to disease pathogenesis.

Objective

This study aimed at elucidating the mode of HDV-induced innate immune activation of primary human immune cells. In particular, we asked whether EVs derived from HDV-infected cells induce a proinflammatory cytokine response in primary human immune cells, and whether this depends on HDV replication. Understanding the mode of immune cell activation by HDV may help to select therapeutic interventions to prevent or at least slow down disease progression and to combat this deadly disease.

Results and Discussion

To determine whether EVs released by HDV-infected cells (HDV-EVs) regulate innate immunity, we collected conditioned media of HDV-infected, non-infected or IFN-β treated hepatoma cell lines (14). Supernatants before purification and purified EVs (supplementary data 2A-C) were subjected to ELISA. No or only minimal amounts of proinflammatory cytokines were released from hepatoma cells in response to HDV infection, and EV preparation also contained only minute amounts of TNF-α (supplementary data 1).

Investigating the immune stimulatory role of EVs on primary human immune cells, HDV-induced EVs were incubated with human PBMC. This triggered TNF-α and IFN-γ production by the PBMC in a dose-dependent manner (Figure 1A and 1B). Cytokine release in response to EVs from non-infected cells or EVs produced in the presence of IFN-β was significantly lower. Importantly, EVs derived from cells infected with UV-inactivated HDV did not induce TNF-α or IFN-γ production indicating that

intermediates from HDV replication were a responsible cargo. Indeed, HDV mRNA could be detected in EVs released by HDV infected cells but not in EVs released by cells infected with UV-inactivated HDV (supplementary data 2D).

To find out which cell type is activated by HDV infection, we studied the effect on primary human macrophages because macrophages are the most frequently represented immune cells in the liver with up to 40 macrophages accompanying 100 hepatocytes (15). Thus, macrophages were differentiated from monocytes using macrophage colony stimulating factor mCSF and incubated with EVs. These macrophages released TNF-α and IL-6 in a dose-dependent manner after incubation with EVs obtained from HDV-infected cells, but not when HDV was treated with UV before infection, or when cells were only treated with IFN-β (Figure 1 C, D and supplementary data 3). Consequently, viral transcription or HDV replication were essential to induce the release of immune stimulatory EVs. Neither incoming HDV RNA nor the proteins contained in virions or IFN-β that is released upon HDV infection were sufficient to trigger the release of EVs from infected cells that were immune stimulatory.

To confirm results generated with EVs obtained from hepatoma-derived cells, primary human hepatocytes (PHH) were infected with HDV, UV-inactivated HDV or treated with IFN-β. EVs were purified and used to stimulate mCSF Mϕ. (Figure 1 E,F). Although the signal was weak due to low numbers of EV secreting cells, mCSF Mϕ showed a trend to release interferon gamma-induced protein 10 (IP-10) and IL-6 in response to EVs from HDV-infected cells only.

Verifying availability of immunostimulatory entities in the blood of HDV-infected patients, EVs were purified from sera of HDV-positive (pos) or cured patients (neg 6y and neg 6m) and used to stimulate mCSF Mϕ (supplementary data 4). Both IL-6 and TNF-α were induced by EVs from HDV-positive samples only. However, EV samples purified from patient sera most likely still contain HBV and HDV virions not removed by SEC, which could also be immune activating. Taken together, these results demonstrate, that after productive HDV infection, i.e. HDV gene expression or replication, EVs are produced that activate a proinflammatory cytokine release from primary human immune cells. Most likely these EVs contain HDV RNA or replication intermediates as cargo and activate a pattern recognition response in the immune cells.

So far, it has not been clarified whether and to which extend HDV-induced innate immune activation occurs in infected hepatocyte or non-infected immune cells and which cells secrete the proinflammatory cytokine that contribute to disease progression (11). PBMC comprise a mixture of various cell types. One main component of PBMC are monocytes, which were reported to be recruited to the inflamed liver where they differentiate to macrophages (16). The liver itself, as a part of the mononuclear phagocyte system, is the organ harbouring the highest percentage of macrophages in the body and has been shown to accumulate the largest proportion of EVs injected into the bloodstream (15, 17). Consequently, we stimulated primary human macrophages with EVs purified from differentially conditioned media. In line with our results obtained in PBMC stimulation experiments, HDV-EVs specifically lead to proinflammatory cytokine release from mCSF-differentiated macrophages. This proves that macrophages respond to EV cargo affected by HDV infection but does not rule out that other cell types may be involved in a proinflammatory response in the liver upon HDV infection.

In our experiments, the conditioned media of HDV-infected hepatoma cell lines did only contain a minute amount of TNF-α and no other proinflammatory cytokines. Release was not evoked by HDV infection, as cytokine levels were higher in the media of non-infected than in the media of infected

cells.It has been reported previously that transfection of large Hepatitis Delta antigen can enhance hepatocellular NFκB signalling in response to co-stimuli like TNF-α or plasmid DNA (9, 10). By inactivating HDV with UV-light treatment prior to infection, however, we observed a complete loss of the immune stimulatory potential of EVs. Also mimicking pattern recognition of HDV infection by IFN-β treatment and subsequent immune activation did not activate a proinflammatory response. Consequently, incoming viral genomes and proteins are not sufficient to trigger the release of immune stimulatory EVs. Although we cannot completely rule out an effect of the higher amount of Hepatitis Delta antigen produced in infected cells, most likely viral replication intermediates, HDV mRNA or genomes are required.

It is very likely that response to HDV infection is provoked by EV mediated transfer of HDV-derived cargo. We could show that cytokine production from PBMC and macrophages in response to HDV-EVs was dose dependent and most likely not due to EV associated cytokines. In line with our results, other studies report that EVs derived from various cell types can mediate proinflammatory effects in target cells (18) and that EVs induce cytokine secretion in response to HIV or HCV infection (19, 20). EV-mediated transfer was even shown to play a special role in infection with non-enveloped viruses. While naked Hepatitis A Virus (HAV) failed to trigger plasmacytoid dendritic cell (pDC) activation, uptake of pseudo-enveloped HAV particles induced IFN-α production from human pDCs (21). Hereby, RNA cargo was suspected to be responsible for immune activation. For HCV, type I IFN secretion of pDCs in response to stimulation with EV preparations containing HCV RNA has been reported (19). In HIV infection, a proinflammatory cytokine response was shown to be mediated by exosomal Trans-activating Response (TAR) RNA (20).

Interestingly, during the course of infection with HDV´s “host virus”, HBV, EVs released by HBV monoinfected cells seemed to have an immune-inhibitory function affecting monocytes, differentiated monocytic THP-1 and natural killer (NK) cells as well as IFN-γ production and RIG I expression (22-24). Consequently, innate immune activation by EVs is not a general mechanism linked to viral infections but specifically linked to HDV-induced EVs, and may even be hampered by HBV-coinfection *in vivo*. Upon others, this may be one reason why evolution selected HBV as a donor for the HDV envelop.

Conclusions

We could show that EVs released from HDV-infected hepatoma cell lines and primary human hepatocytes induce a dose-dependent proinflammatory cytokine response in primary human immune cells like mCSF-differentiated macrophages and PBMC. As this effect was blocked by previous inactivation of HDV, functional viral genomes seem to be crucial for innate immune activation. Further studies shall clarify which component is responsible for this effect. Understanding the mode of virus mediated danger signal transmission will allow treating severe immunopathology and tissue damage in HBV-HDV coinfection.

Limitations

The exact immune stimulatory content of HDV-EVs remains to be identified, as this was limited by the low amount of EVs released from our cell lines. An exclusion of potential cytokine mediated effects is also hindered by the fact that an inactivation of cytokines by protease digestion or denaturation would also affect EV-associated proteins and thus the uptake of EVs that depends on their surface proteins (25). Although our experiments demonstrate that macrophages respond to

HDV-EVs, we cannot currently not rule out that other cells also contribute to the response observed in PBMC. This study does not address impact of HDV-EVs under natural conditions like HBV-HDV coinfection or *in vivo* models, as only HDV-monoinfection of hepatoma cell lines and primary human hepatocytes was used. EVs purified from patient sera are most likely contaminated with HBV and HDV virions, so observed proinflammatory cytokine induction only supports *in vitro* results. Development of reliable methods to remove virions from EV samples is urgently needed. Additionally, usage of plasma instead of sera for EV purification would be desirable but was not possible due to the lack of untreated HDV patients and conditions in blood banks.

Alternative Explanations

Most recently, cytokine and growth factors have been reported as EV cargo (26). TGF-β, for example, seems to be more stable in a membrane-bound form than in a soluble form, indicating an important role of EVs as TGF-β transporting vehicles (27). Thus, an effect of cytokines not included in our assays cannot be ruled out. In addition, transfer of second messengers via EVs is also conceivable, as transfer of cGAMP by HIV virions has already been reported (28).

Conjectures

Based on our data we hypothesize that RNA intermediates generated during HDV replication in infected cells become cargo of EVs released and can stimulate immune cells inside and outside of the liver. Further investigations will have to follow proving this hypothesis and defining the immune stimulatory EV cargo in HDV infection.

Methods

Antibodies and reagents. Western blot samples were lysed in Pierce RIPA Buffer (Thermo Scientific, Waltham, USA). Primary antibodies targeting Syntenin were purchased from Abcam (Cambridge, UK) and anti-CD63 antibodies were from SBI (Palo Alto, California). Cellular proteins were stained using anti-Calnexin (BD Biosciences, San Jose, California) and anti-GAPDH antibodies (Acris Antibodies, Herford, Germany). Secondary antibodies (anti-mouse IgG Peroxidase and anti-rabbit IgG Peroxidase) were purchased from Sigma-Aldrich (St. Louis, Missouri). EVs were removed from 1:2 diluted FCS (Gibco, Dreieich, Germany) by consecutive centrifugation (5 min, 2000g, RT), 0.45µm and 100kDa filtration.

HDV production. Huh7 cells were transfected with HDV-encoding plasmid pSVL(D3) (29) and HBV-surface protein encoding plasmid pT7HB2.7 (30). Transfection was performed with FuGENE® HD Transfection Reagent (Promega, Madison, USA). Supernatant was collected for two weeks, purified with HiTrap Heparin HP affinity columns (GE Healthcare, Chalfont St Giles, UK) and subsequently concentrated via centrifugation in Vivaspin® Turbo 15 columns (MWCO 50kDa) (Sartorius, Göttingen, Germany). HDV genome equivalents were determined via RT-qPCR.

HDV infection. NTCP-expressing hepatoma cell lines (HepG2-NTCP or Huh7-NTCP cells) were infected with HDV at a moi of 25 vp/cell under the conditions described for HBV infection (14) and maintained in advanced DMEM (Gibco, Dreieich, Germany) supplemented with EV-free FCS, 2mM L-Glutamine,

100 Units/ml Penicillin and 10 µg/ml Streptomycin. EV-containing supernatants were collected for two weeks from day 3 post infection (p.i.) and fresh medium was provided every other day.

Purification of EVs. Apoptotic bodies were removed by centrifugation of the supernatants collected (1000xg, 5 min) and a 0.45µm filtration step before EVs were purified. EVs were purified by size exclusion chromatography using qEVoriginal columns (Izon Science, Oxford, UK) according to the manufactures protocol and stored at -80°C. Quality of EV preparations was confirmed in Dynamic Light scattering (DLS) by dynamic light scattering, Western Blot and electron microscopy (supplementary figure 2-4) according to the recommendations of International Society for Extracellular Vesicles (MISEV2018) (1)

HDV RNA detection in EVs. EV RNA cargo was isolated using Total Exosome RNA and Protein Isolation Kit (Invitrogen, Carlsbad, California) according to the manusfactures protocol and stored at -80°C. Presence of HDV RNA was shown by nested PCR and subsequent agarose gel elextrophoresis as described (31).

Dynamic light scattering. Particle measurements were performed by dynamic light scattering (DLS) using a Zetasizer Nano ZS machine (ZEN3600, Malvern, Kassel, Germany) with detection at an angle of 173° (back-scattering). Samples were diluted 1:10 in 10 mM dust-free NaCl and measured in disposable, low-volume cuvettes (Malvern). For each sample, three measurements were run consecutively, each one for 50 x 10 seconds at 20 °C (10 minutes between individual measurements) and measurements were averaged for analysis. Instrument settings were set to automatic attenuation and automatic positioning. For all EV measurements, a Refractive Index of 1.39 and sample absorption of 0.01 were assumed. High measurement quality was assured by analyzing raw correlation data, resulting correlation fits and polydispersity indices.

Isolation and activation of human immune cells. Peripheral blood mononuclear cells (PBMC) were isolated by Biocoll (Merck, Darmstadt, Germany) based density centrifugation of heparinized blood from healthy human donors after informed consent and approval by the local ethics committee. Monocytes were selected by adherence for 1 h at 37°C in serum-free medium and differentiated into macrophages in RPMI supplied with 10 % FCS, 2 mM L-Glutamine, 100 Units/ml Penicillin, 10 µg/ml Streptomycin, amino acids, sodium pyruvate and 50 ng/ml human macrophage-colony stimulating factor (mCSF) (PeproTech, Hamburg, Germany). Medium was exchanged after five days and macrophages were detached with 5mM EDTA in PBS on day 7. Macrophages were re-seeded at 10^5 cells/well on a 96-well plate in medium supplied with EV-free human serum and rested overnight prior to immune stimulation, PBMC were seeded at $3*10^5$ cells/well and stimulated the same day. For immune stimulation, primary human immune cells were incubated with EVs normalized to the number of secreting cells and supernatants were harvested 24h post stimulation. Release of human cytokines was determined by ELISA according to the manufacturer's instructions (BD Biosciences, Heidelberg, Germany for TNF and Invitrogen, Schwerte, Germany for IL-6 and IFN-γ).

Electron Microscopy. Negative stain transmission electron microscopy (TEM) of EVs was performed on a JEOL JEM-1400 Plus microscope (camera: JEOL CCD Ruby, 8 Mpix), operating at 120 kV with a 60.000x magnification (0.275nm/pix). For staining, the side blotting method was followed. A continuous carbon film coated 400 mesh copper grid was treated by glow discharge for 30s. 5µL of a 0.1mg/mL EV-containing suspension was loaded on the grid and allowed to adsorb for 5 min. The liquid was pulled off by pressing filter paper (Whatman, grade 1) against the grid edge. The specimen was immediately stained with 5µL of a filtered 2% uranyl acetate (UA) solution for 30s. After completely removing the excess UA solution with filter paper, the grid was allowed to dry at room temperature before TEM measurements.

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Conflict of interest

The authors declare no conflicts of interest.

Ethics Statement

The use of human blood was approved by the local ethical board of the Klinikum rechts der Isar and written informed consent was obtained from all participants.

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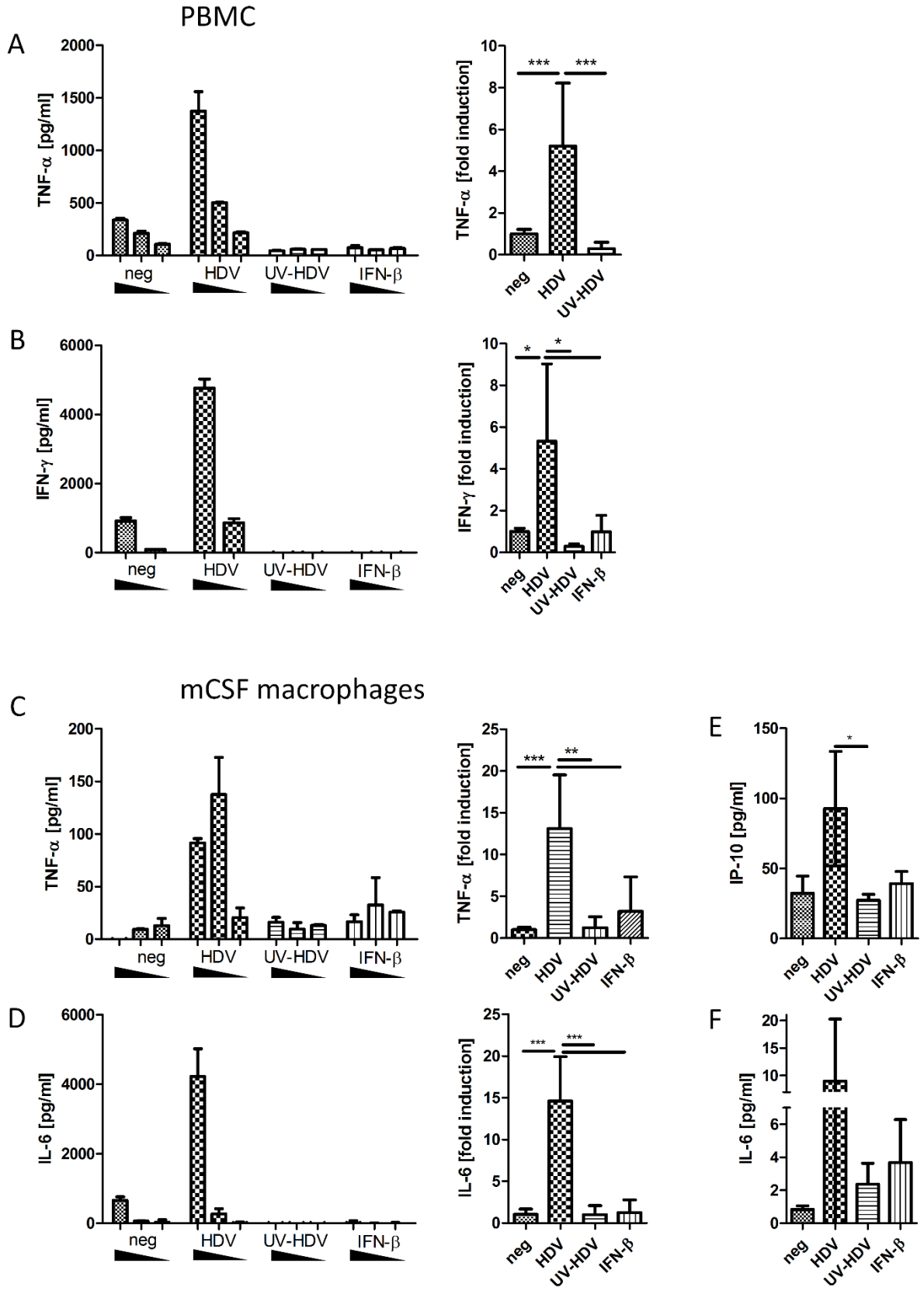
Figures and figure legends



Impact of Hepatitis D-Virus(HDV)-primed extracellular vesicles (EV) on primary human immune cells

(A-D) Human peripheral blood mononuclear cells (PBMC) (A,B) or macrophage-colony stimulating factor differentiated macrophages (mCSF Mϕ) (C,D) were stimulated with EVs purified from cell culture medium by Size-exclusion chromatography (SEC). Cell culture medium was collected either from untreated hepatoma cells (neg), HDV-infected cells (HDV), cells infected with UV-inactivated HDV (UV-HDV) or interferon β (IFN-β). The inoculum was normalized to the number of secreting cells and added in 5-fold dilutions. Supernatants collected from PBMC and mCSF Mϕ were analysed by ELISA. The left panel shows dose-dependent results from individual experiments with NTCP-expressing HepG2 (A, B, D) or Huh7 (C) derived EVs. The right panel depicts relative induction of cytokines by EVs released from treated versus non-stimulated hepatoma cells (neg). EVs were released by NTCP-expressing HepG2-cells only (A,B) or NTCP expressing HepG2 and NTCP-expressing Huh7 cells (C,D). Mean ±SD from three (B,C, D) or six (A) independent experiments is given. Data were analyzed for normality using D'Agostino-Pearson test, statistical analysis of the normally distributed data was done using paired t-tests. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. A) Tumor-necrosis factor-α (TNF-α) released by PBMC. B) Interferon-γ (IFN-γ) released by PBMC. C) TNF-α released by mCSF Mϕ. D) Interleukin-6 (IL-6) released by mCSF Mϕ
(E,F) mCSF Mϕ were stimulated with EVs purified from cell culture medium of primary human hepatocytes (PHH) which were untreated (neg), HDV-infected (HDV), infected with UV-inactivated HDV (UV-HDV) or treated with interferon β (IFN-β). The inoculum was normalized to the number of secreting cells. Supernatants were analysed by ELISA and results are depicted as mean ±SD from two independent experiments. Statistical analysis was performed using Mann Whitney test. * $p < 0.05$.
E) Interferon gamma-induced protein 10 (IP-10) released by mCSF Mϕ. F) IL-6 released by mCSF Mϕ

Supplementary information

A) EVs subset a	IFN-γ 1	IFN-γ 2	TNF-α 1	TNF-α 2	IP-10 1	IP-10 2
neg-EVs	< LLOD	< LLOD	< LLOD	< LLOD	12,1	< LLOD
HDV-EVs	< LLOD	< LLOD	< LLOD	< LLOD	< LLOD	< LLOD

B) EVs subset b	IFN-γ 1	IFN-γ 2	TNF-α 1	TNF-α 2	IL-6 1	IL-6 2
neg-EVs	< LLOD	< LLOD	173,6	13,0	< LLOD	< LLOD
HDV-EVs	< LLOD	< LLOD	54,4	18,1	< LLOD	< LLOD
UV-HDV-EVs	< LLOD	< LLOD	59,6	49,2	< LLOD	< LLOD

C) EVs subset c	IFN-γ 1	IFN-γ 2	TNF-α 1	TNF-α 2	IL-6 1	IL-6 2
neg-EVs	< LLOD	< LLOD	< LLOD	< LLOD	< LLOD	< LLOD
HDV-EVs	< LLOD	< LLOD	< LLOD	< LLOD	< LLOD	< LLOD
UV-HDV-EVs	< LLOD	< LLOD	< LLOD	< LLOD	< LLOD	< LLOD
IFN-β-EVs	< LLOD	< LLOD	< LLOD	< LLOD	< LLOD	< LLOD

D) supernatants	IFN-γ 1	IFN-γ 2	TNF-α 1	TNF-α 2	IL-6 1	IL-6 2
neg day 3	< LLOD	< LLOD	< LLOD	< LLOD	< LLOD	< LLOD
HDV day 3	< LLOD	< LLOD	< LLOD	< LLOD	< LLOD	< LLOD
UV-HDV day 3	< LLOD	< LLOD	< LLOD	< LLOD	< LLOD	< LLOD
IFN-β day 3	< LLOD	< LLOD	< LLOD	< LLOD	< LLOD	< LLOD
neg day 5	< LLOD	< LLOD	< LLOD	< LLOD	< LLOD	< LLOD
HDV day 5	< LLOD	< LLOD	< LLOD	< LLOD	< LLOD	< LLOD
UV-HDV day 5	< LLOD	< LLOD	< LLOD	< LLOD	< LLOD	< LLOD
IFN-β day 5	< LLOD	< LLOD	< LLOD	< LLOD	< LLOD	< LLOD
neg day 8	< LLOD	< LLOD	< LLOD	< LLOD	< LLOD	< LLOD
HDV day 8	< LLOD	< LLOD	< LLOD	< LLOD	< LLOD	< LLOD
UV-HDV day 8	< LLOD	< LLOD	< LLOD	< LLOD	< LLOD	< LLOD
IFN-β day 8	< LLOD	< LLOD	< LLOD	< LLOD	< LLOD	< LLOD
neg day 10	< LLOD	< LLOD	< LLOD	< LLOD	< LLOD	< LLOD
HDV day 10	< LLOD	< LLOD	9,8	< LLOD	< LLOD	< LLOD
UV-HDV day 10	< LLOD	< LLOD	< LLOD	< LLOD	< LLOD	< LLOD
IFN-β day 10	< LLOD	< LLOD	< LLOD	< LLOD	< LLOD	< LLOD
neg day 12	< LLOD	< LLOD	< LLOD	< LLOD	< LLOD	< LLOD
HDV day 12	< LLOD	< LLOD	< LLOD	< LLOD	< LLOD	< LLOD
UV-HDV day 12	< LLOD	< LLOD	< LLOD	< LLOD	< LLOD	< LLOD
IFN-β day 12	< LLOD	< LLOD	< LLOD	< LLOD	< LLOD	< LLOD
neg day 15	< LLOD	< LLOD	< LLOD	< LLOD	< LLOD	< LLOD
HDV day 15	< LLOD	< LLOD	< LLOD	< LLOD	< LLOD	< LLOD
UV-HDV day 15	< LLOD	< LLOD	< LLOD	< LLOD	< LLOD	< LLOD
IFN-β day 15	< LLOD	< LLOD	< LLOD	< LLOD	< LLOD	< LLOD

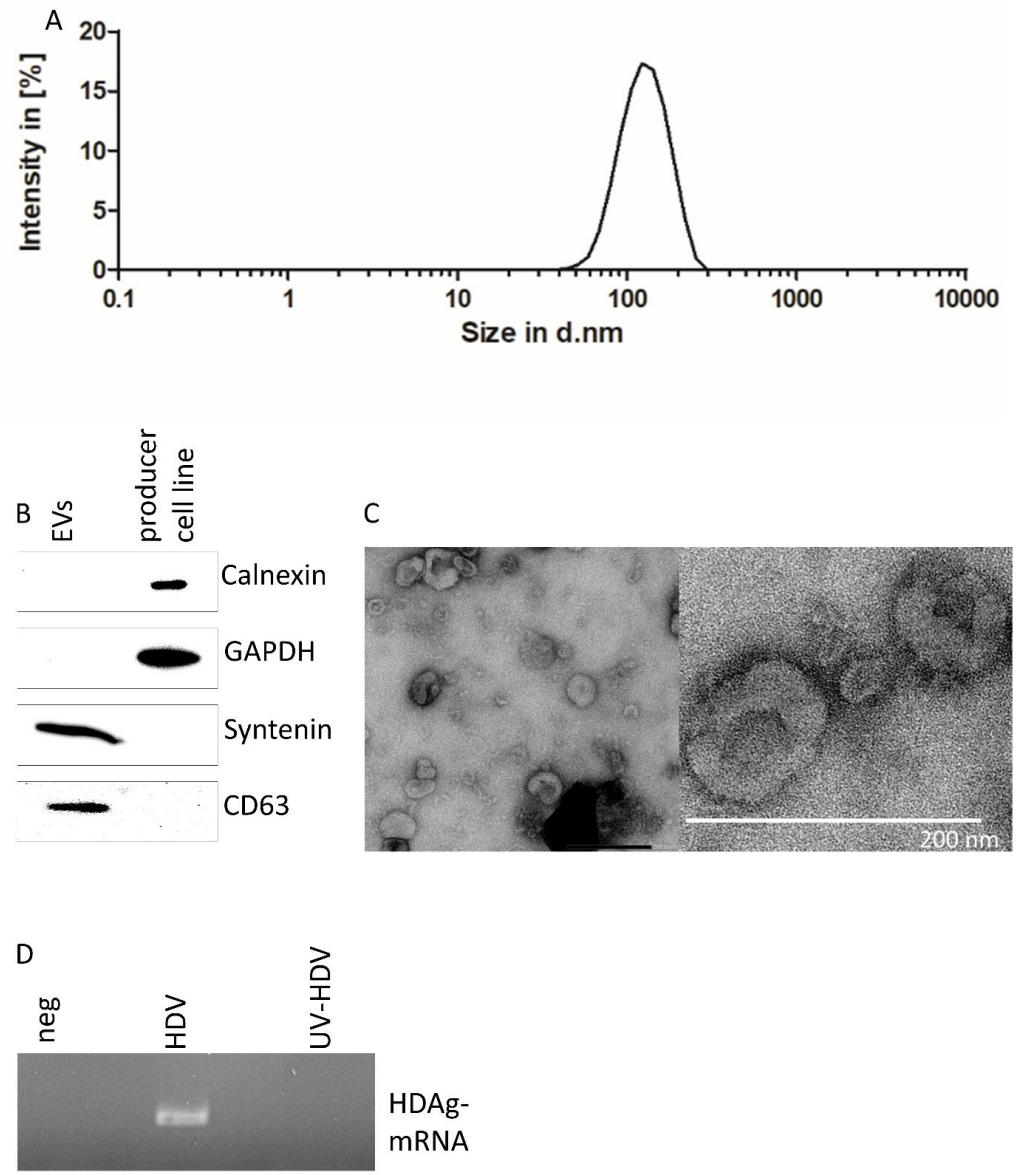
E) supernatants	IFN-γ 1	IFN-γ 2	IL-6 1	IL-6 2
neg day 4	< LLOD	< LLOD	< LLOD	< LLOD
HDV day 4	< LLOD	< LLOD	< LLOD	< LLOD
UV-HDV day 4	< LLOD	< LLOD	< LLOD	< LLOD
IFN-β day 4	< LLOD	< LLOD	9,40	13,52
neg day 7	< LLOD	< LLOD	< LLOD	< LLOD
HDV day 7	< LLOD	< LLOD	< LLOD	< LLOD
UV-HDV day 7	< LLOD	< LLOD	< LLOD	< LLOD
IFN-β day 7	< LLOD	< LLOD	< LLOD	< LLOD
neg day 9	< LLOD	< LLOD	< LLOD	< LLOD
HDV day 9	< LLOD	< LLOD	< LLOD	< LLOD
UV-HDV day 9	< LLOD	< LLOD	< LLOD	< LLOD
IFN-β day 9	< LLOD	< LLOD	13,70	< LLOD
neg day 11	< LLOD	< LLOD	< LLOD	< LLOD
HDV day 11	< LLOD	< LLOD	< LLOD	< LLOD
UV-HDV day 11	< LLOD	< LLOD	< LLOD	< LLOD
IFN-β day 11	< LLOD	< LLOD	< LLOD	< LLOD

F) supernatants	TNF-α 1	TNF-α 2	IL-6 1	IL-6 2
neg day 5	< LLOD	< LLOD	< LLOD	< LLOD
HDV day 5	28,9	29,8	< LLOD	< LLOD
UV-HDV day 5	< LLOD	< LLOD	< LLOD	< LLOD
neg day 7	< LLOD	< LLOD	< LLOD	16,3
HDV day 7	< LLOD	< LLOD	< LLOD	< LLOD
UV-HDV day 7	< LLOD	< LLOD	< LLOD	< LLOD
neg day 11	< LLOD	< LLOD	< LLOD	< LLOD
HDV day 11	< LLOD	< LLOD	< LLOD	< LLOD
UV-HDV day 11	< LLOD	0,6	< LLOD	< LLOD
neg day 13	31,1	14,9	< LLOD	< LLOD
HDV day 13	9,5	12,5	< LLOD	< LLOD
UV-HDV day 13	< LLOD	< LLOD	< LLOD	< LLOD

Supplementary data 1: Extracellular vesicle subsets and conditioned media do not contain proinflammatory cytokines in response to HDV infection.

EV preparations and conditioned media used to purify EVs were subjected to ELISA measurement. NTCP-expressing HepG2-cells were used as producer cell line.
Detectable values are highlighted in grey, values below lower limit of detection (7,8 pg cytokine/ml) are marked with < LLOD.

A) IFN-γ, TNF-α and 10 kDa interferon gamma-induced protein 10 (IP-10) content in size-exclusion chromatography (SEC)-purified EV preparation. Each column represents the duplicate measurement of a single sample.
B) IFN-γ, TNF-α and IL-6 content in SEC-purified EV preparation. Each column represents the duplicate measurement of a single sample.
C) IFN-γ, TNF-α and IL-6 content in SEC-purified EV preparation. Each column represents the duplicate measurement of a single sample. NTCP-expressing HepG2-cells were used as producer cell line.
D) IFN-γ, TNF-α and IL-6 content in conditioned media collected on different days p.i.. Each column represents the duplicate measurement of a single sample. NTCP-expressing HepG2-cells were used as producer cell line.
E) IFN-γ and IL-6 content in conditioned media collected on different days p.i.. Each column represents the duplicate measurement of a single sample. NTCP-expressing Huh7-cells were used as producer cell line.
F) TNF-α and IL-6 content in conditioned media collected on different days p.i.. Each column represents the duplicate measurement of a single sample. NTCP-expressing HepG2-cells were used as producer cell line.



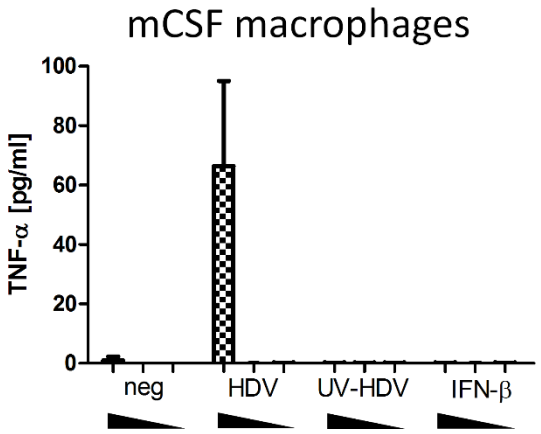
Supplementary data 2: Quality control of size-exclusion chromatography purified extracellular vesicles

A) EVs purified from media of Na^+ taurocholate cotransporting polypeptide *(*NTCP)-expressing hepatoma cells (shown: HepG2) were subjected to dynamic light scattering (DLS) measurement as size control.

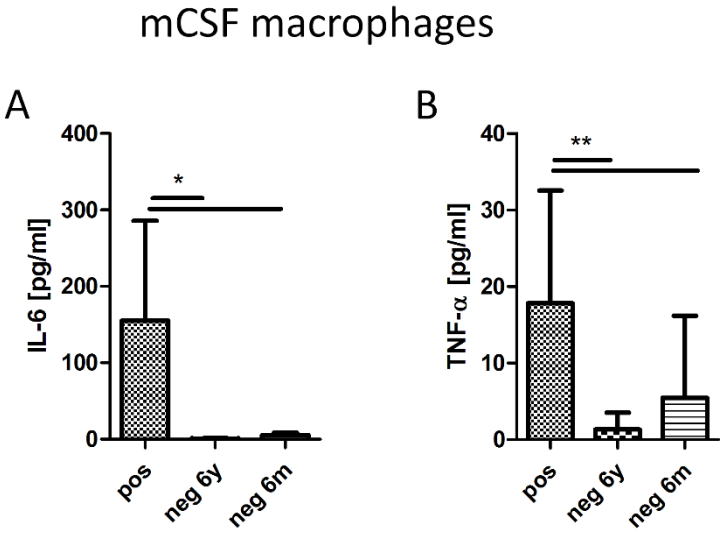
B) Lysates obtained from purified EVs and the NTCP-expressing producer cell line Huh7 were subjected to SDS-polyacrylamide gel electrophoresis and visualized by Western blotting and subsequent antibody staining to control for EV purity (1). As characteristic proteins deprived from EV, Calnexin and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were stained. As proteins typically enriched in EVs, Syntenin and Cluster of Differentiation (CD)63 were stained.

C) EVs were collected from media of NTCP-expressing HepG2-cells, fixed in 2% PFA and analysed by negative-stain electron microscopy. Scale bars 200 nm.

D) EVs were collected from media of untreated NTCP-expressing HepG2-cells (neg), HDV-infected cells (HDV) or cells infected with UV-inactivated HDV (UV-HDV). RNA was isolated and presence of HDV-Antigen mRNA (HDAg mRNA) was shown by nested PCR and agarose gelelectrophesis.



Supplementary data 3: Impact of HepG2-derived HDV-primed EVs on primary human macrophages
Macrophage-colony stimulating factor differentiated macrophages were stimulated with EVs purified from cell culture medium by Size-exclusion chromatography (SEC). Cell culture medium was collected either from untreated NTCP-expressing HepG2-cells (neg), HDV-infected cells (HDV), cells infected with UV-inactivated HDV (UV-HDV) or interferon β (IFN-β). The inoculum was normalized to the number of secreting cells and added in 5-fold dilutions. Supernatants collected from macrophages were analysed by ELISA for TNF-α content. Graph shows dose-dependent results from one individual experiment.



Supplementary data 4: Size-exclusion chromatography purifies immunostimulatory entities from patient sera
Human macrophage-colony stimulating factor differentiated macrophages (mCSF Mϕ) were stimulated with EVs purified from patient sera by Size-exclusion chromatography (SEC). Sera contained at least $1{,}61*10^5$ Genom equivalents HDV RNA / ml (pos), were HDV-negative for 6 years (neg 6y) or HDV-negative for 6 months (neg 6m). The inoculum was normalized to the plasma volume and supernatants collected from mCSF Mϕ were analysed by ELISA. Mean ±SD from three independent experiments is given. Statistical analysis was done using Mann Whitney test. * $p < 0.05$, ** $p < 0.01$. A) Interleukin-6 (IL-6) released by mCSF Mϕ B) TNF-α released by mCSF Mϕ.