Research article

JHEP|Reports

A novel therapeutic HBV vaccine candidate induces strong polyfunctional cytotoxic T cell responses in mice

Robbert Boudewijns,[1] Ji Ma,[1] Johan Neyts,[1] Kai Dallmeier[1,*]

[1]KU Leuven Department of Microbiology, Immunology and Transplantation, Rega Institute, Laboratory of Virology and Chemotherapy, Leuven, Belgium

JHEP Reports **2021.** https://doi.org/10.1016/j.jhepr.2021.100295

Background & Aims: Current standard-of-care suppresses HBV replication, but does not lead to a functional cure. Treatment aiming to cure chronic hepatitis B (CHB) is believed to require the induction of strong cellular immune responses, such as by therapeutic vaccination.
Methods: We designed a therapeutic HBV vaccine candidate (YF17D/HBc-C) using yellow fever vaccine YF17D as a live-attenuated vector to express HBV core antigen (HBc). Its ability to induce potent cellular immune responses was assessed in a mouse model that supports flavivirus replication.
Results: Following a HBc protein prime, a booster of YF17D/HBc-C was found to induce vigorous cytotoxic T cell responses. In a direct head-to-head comparison, these HBc-specific responses exceeded those elicited by adenovirus-vectored HBc. Target-specific T cells were not only more abundant, but also showed a higher degree of polyfunctionality, with HBc-specific $CD8^+$ T cells producing interferon γ and tumour necrosis factor α in addition to granzyme B. This immune phenotype translated into a superior cytotoxic effector activity toward HBc-positive cells in YF17D/HBc-C vaccinated animals *in vivo*.
Conclusions: The results presented here show the potential of YF17D/HBc-C as a vaccine candidate to treat CHB, and warrant follow-up studies in preclinical animal models of HBV persistence in which other candidate vaccines have been unable to achieve a sustained virologic response.
Lay summary: Resolution of CHB requires the induction of strong cellular immune responses. We used the yellow fever vaccine as a vector for HBV antigens and show that it is capable of inducing high levels of HBV-specific T cells that produce multiple cytokines simultaneously and are cytotoxic *in vivo*.

Introduction

HBV affects billions of people worldwide, with about 257 million cases of chronic HBV (CHB) infection at high risk of developing decompensating liver disease, such as cirrhosis and hepatocellular carcinoma, resulting in an estimated 887,000 deaths per year.[1] HBV is a small enveloped DNA virus with a pronounced tropism for human hepatocytes, in which it can establish its genome as a covalently closed circular DNA (cccDNA) episome, which in turn serves as continuous source for the synthesis of viral antigens and virus progeny. Current standard of care, direct-acting nucleos(t)ide analogues, typically require life-long treatment to prevent viral rebound from the cccDNA reservoir.[2] However, the combined evidence that some patients can achieve cure (i) during interferon (IFN) treatment; (ii) after bone marrow transplantation from an immune donor;[3] or (iii) even spontaneously[4] demonstrates that HBV infection is amenable to immunological control.

Clearance of HBV infection, although mechanistically not fully understood, is considered to be achieved by a concerted action of cytolytic and noncytolytic mechanisms,[5] involving virus-specific $CD8^+$ T lymphocytes as key players. Such $CD8^+$ T cells are abundant and show a vigorous and polyfunctional phenotype in those patients who clear the virus, but are hardly detectable and functionally impaired in patients with chronic disease.[6] Such defective T cell function is likely the result of a combination of the tolerogenic liver environment,[7] and the expression of high quantities of viral antigens by infected hepatocytes,[8] excessively triggering cognate T cell receptors. This can lead to functional inactivation of T cells, known as T cell exhaustion, or even their deletion.[9] Therefore, currently therapies that (i) reduce antigen expression; or (ii) reverse T cell exhaustion; as well as (iii) therapeutic vaccination to increase or boost the pool of HBV-specific T cells[10,11]; or (iv) combinations thereof[12] are considered to achieve CHB cure. Attempts to repurpose the prophylactic HBV vaccine as a therapeutic vaccine have failed in clinical trials. However, more immunogenic virally vectored vaccines have shown promising results in preclinical models.[10,11]

The live-attenuated yellow fever vaccine (YF17D) has been proposed as a highly immunogenic viral vector[13,14] containing multiple genomic sites for insertion of sequences encoding foreign epitopes or larger transgenes. Importantly, in contrast to many other vaccines and vector platforms,[14] YF17D is known to

Keywords: Chronic hepatitis B; HBV; Therapeutic vaccination; Yellow fever vaccine.
Received 6 January 2021; received in revised form 2 April 2021; accepted 13 April 2021; available online 22 April 2021
* Corresponding author. Address: KU Leuven Department of Microbiology and Immunology, Rega Institute, Laboratory of Virology and Chemotherapy, Herestraat 49, Box 1030, 3000 Leuven, Belgium. Tel.: +32-16-32-18-64
E-mail address: kai.dallmeier@kuleuven.be (K. Dallmeier).

elicit exceptionally broad and multifaceted innate, humoral, and cellular responses that translate into a vigorous polyfunctional and long-lasting immunity.[14–16]

Here, we report the engineering of a recombinant YF17D construct that expresses the HBV core antigen (HBc) and induces vigorous cytotoxic T lymphocyte (CTL) responses in mice. This response was even more pronounced when combined with a HBc protein prime and superior to adenovirus-vectored HBc in a direct head-to-head comparison.

Materials and methods

Vaccine virus and antigen

The construction of YF17D/HBc-C and YF17D/E-HBc-NS1 was accomplished by inserting a copy DNA (cDNA) encoding the HBc (genotype D, subtype ayw) as translational fusion into the N terminus and at the E/NS1 junction of the YF17D polyprotein, respectively (Fig. 1). Replication-deficient E1/E3-deleted adenovirus serotype 5 expressing HBc amino acids (aa) 1–155 (AdV5-HBc) was purchased from SIRION Biotech GmbH (Martinsried, Germany). Stamaril® (lot G5400; Sanofi-Pasteur, Paris, France) was passaged twice in VeroE6 cells before storage at −80°C. Antigen for vaccine priming was prepared by diluting 5 μg recombinant HBc (rHBc) protein (HBc_{1-183}, subtype ayw, American Research Products, Waltham, MA, USA) and 10 μg saponin-based adjuvant Quil-A® (InvivoGen, San Diego, CA, USA) in 100 μl PBS (Life Technologies, Carlsbad, CA, USA). For a detailed description of the generation and *in vitro* characterisation of these constructs, please see the Supplementary data.

For the data presented in the main figures, mice were first primed with 5 μg rHBc and 10 μg Quil-A® as adjuvant. Two weeks (Fig. 2) or 4 weeks (Figs 3–5) post-prime, mice were boosted with either 5 μg rHBc and 10 μg Quil-A®; 10^5 plaque-forming units (pfu) YF17D/HBc-C; 10^5 pfu YF17D/E-HBc-NS1;

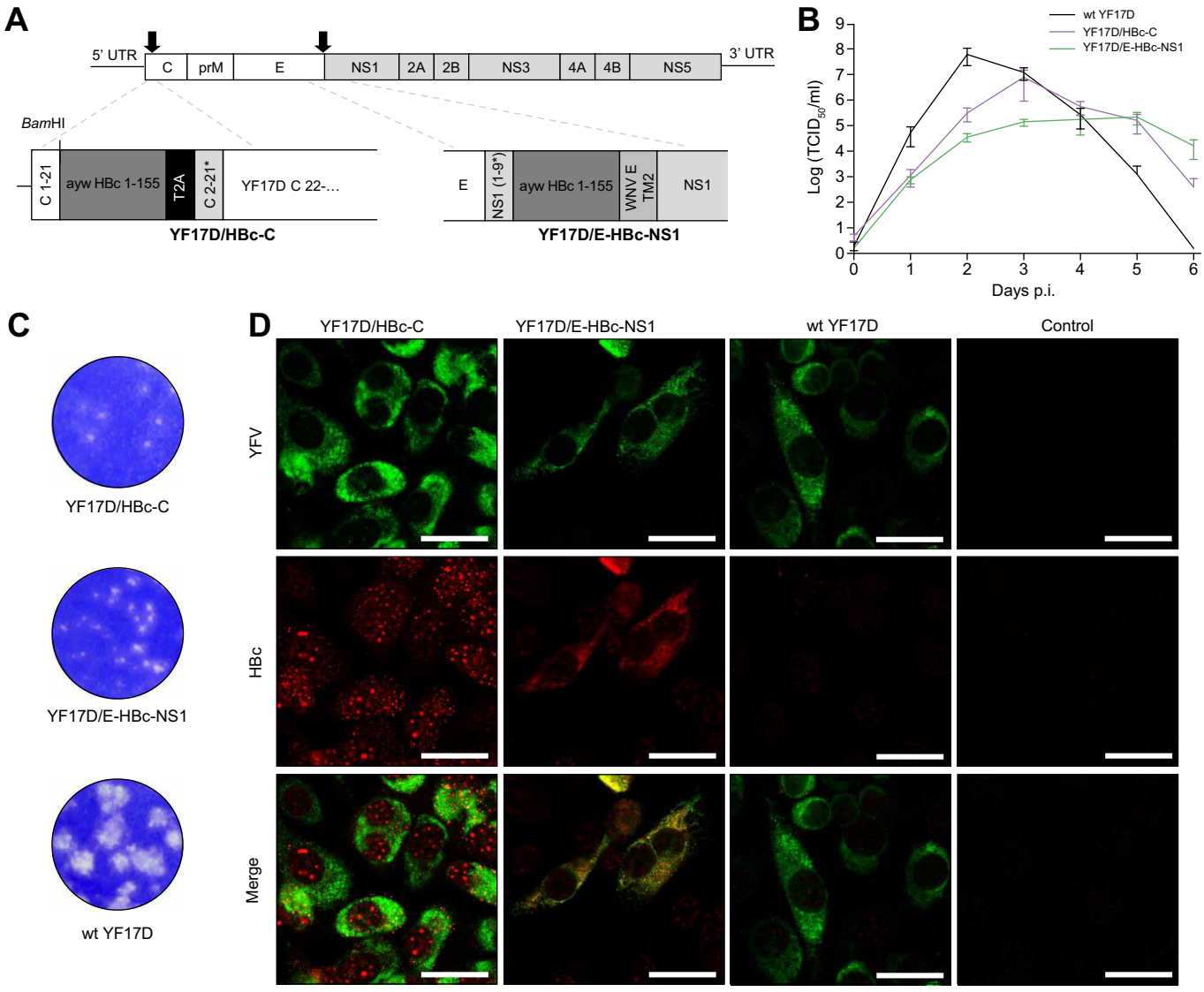

Fig. 1. *In vitro* characterisation of vaccine candidates. (A) Schematic representation of yellow fever (YF)17D/HBc-C and YF17D/E-HBc-NS1. YF17D sequence repeats (indicated by asterisks) were included for efficient expression from each site. (B) *In vitro* growth kinetics of wt versus transgenic YF17D variants. Error bars indicate SEM (n = 3). (C) Plaque phenotype of wt and transgenic YF17D variants (VeroE6 cells, 6 dpi). (D) YFV and HBc antigen-specific immunofluorescence staining of YF17D variants (VeroE6 cells, 3 dpi; scale bars = 25 μm). dpi, days post-infection; HBc, HBV core antigen; wt, wild-type; YF, yellow fever.

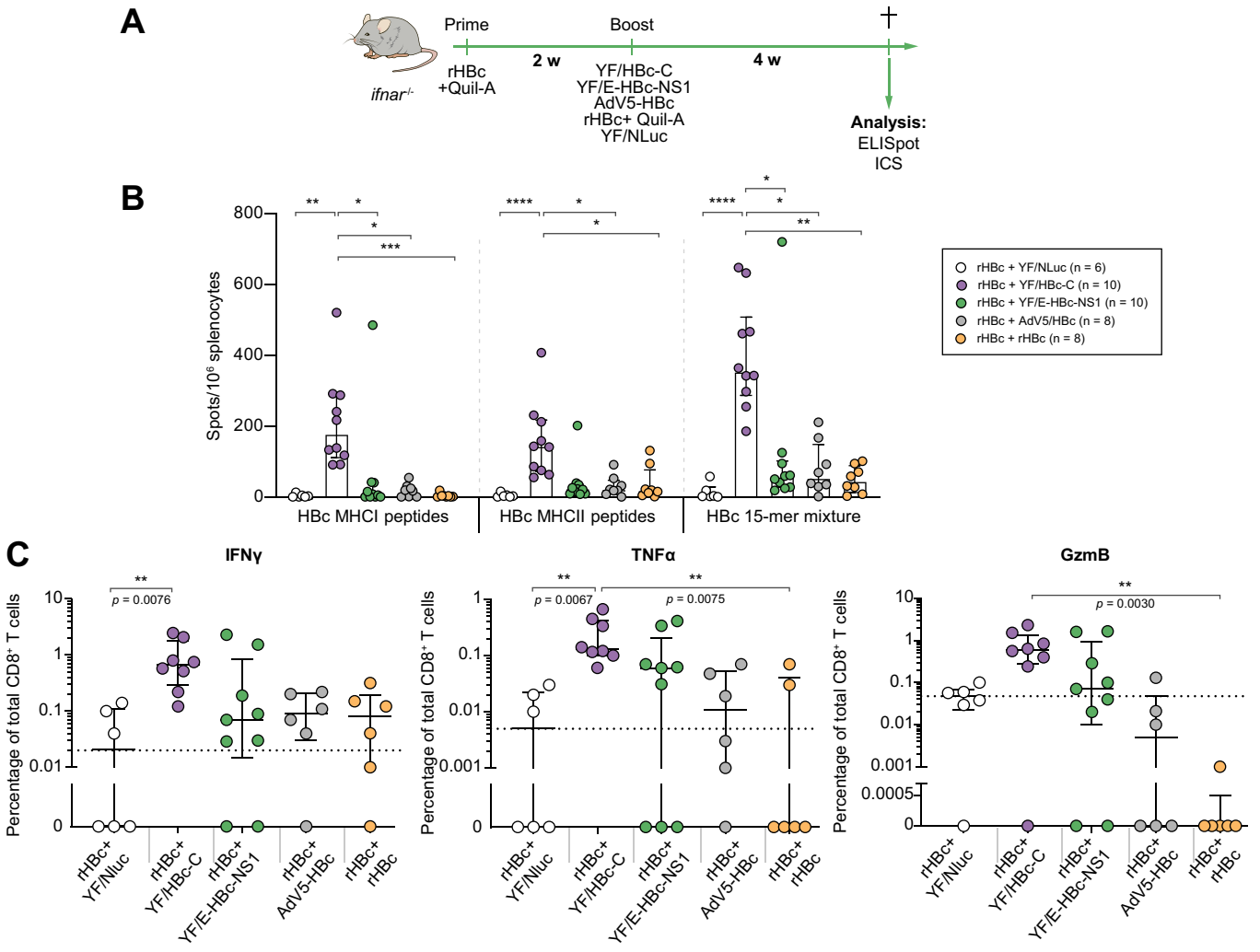

Fig. 2. Heterologous prime-boost vaccination (analysis 4 weeks post-boost). (A) Experiment time-course. (B) IFNγ ELISPOT for splenocytes of vaccinated *ifnar*$^{-/-}$ mice (n = 6–10, from 2 independent experiments) after stimulation with HBc-derived peptides (MHCI or MHCII) or a pool of HBc 15-mers. Bars represent medians ± IQR. **p* ≤0.05, ***p* ≤0.01, ****p* ≤0.001, and *****p* ≤0.0001, respectively (Kruskal–Wallis with Dunn's test). (C) Cytokine/granzyme-positive CD8$^+$ T cells, following overnight stimulation with HBc-derived peptides (n ≥6 for each group, from 2 independent experiments). Bars represent median ± IQR. Dotted lines represent median expression of control mice (rHBc primed, YF17D/NanoLuc boosted). ***p* ≤0.01 (Kruskal–Wallis with Dunn's test). ELISPOT, enzyme-linked ImmunoSpot; HBc, HBV core antigen; IFN, interferon; ifnar, IFN-α/β receptor; MHC, major histocompatibility complex; NanoLuc, nanoluciferase; rHBc, recombinant HBc; YF, yellow fever.

10^8 pfu AdV5-HBc; or 10^5 pfu YF17D/NanoLuc. Splenocytes were analysed 4 weeks (Fig. 2) or 10 days (Figs 3–5) post-boost, respectively.

Cells and medium

BHK-21J and VeroE6 cells were maintained in minimum essential medium (MEM; Life Technologies) with 10% FBS (Hyclone, GE Healthcare, Chicago, IL, USA), 2 mM L-glutamine (Life Technologies), and 0.075% sodium bicarbonate (Life Technologies) (seeding medium). For cell culture assays, medium containing only 2% FBS was used (assay medium). Cells were maintained at 37°C, 5% CO_2, and 95–99% humidity.

Animals

Six–eight-week-old female IFN-α/β receptor knockout (*ifnar*$^{-/-}$) mice [C57BL/6 mice homozygous for an *Ifnar1*-knockout mutation, B6.129S2–*Ifnar1tm1Agt*, breeding couples provided by Claude Libert (VIB–UGent IRC, BE) n = 158] or wild-type (wt) C57BL/6 mice (Janvier Labs, Le Genest-Saint-Isle, France, n = 16) were used throughout this study. For a detailed description of the housing conditions and guidelines, see the Supplementary data.

ELISA

Anti-HBc antibodies were detected using an anti-HBc competitive ELISA (Anti-HBc ELISA 4.0, MP Biomedicals, Irvine, CA, USA), according to the manufacturer's instructions. The cut-off was calculated as 0.5 × the average absorbance in negative control wells. The limit of detection was defined as the average absorbance in positive control wells.

Enzyme-linked ImmunoSpot assay

Preparation of recall antigen is described in the Supplementary data. Per well, 6×10^5 splenocytes were plated with antigen: either VeroE6 cell lysate (YF17D infected or non-infected, 50 μg/ml), YF17D NS3 peptide (ATLTYRML, $NS3_{268–275}$, 5 μM,

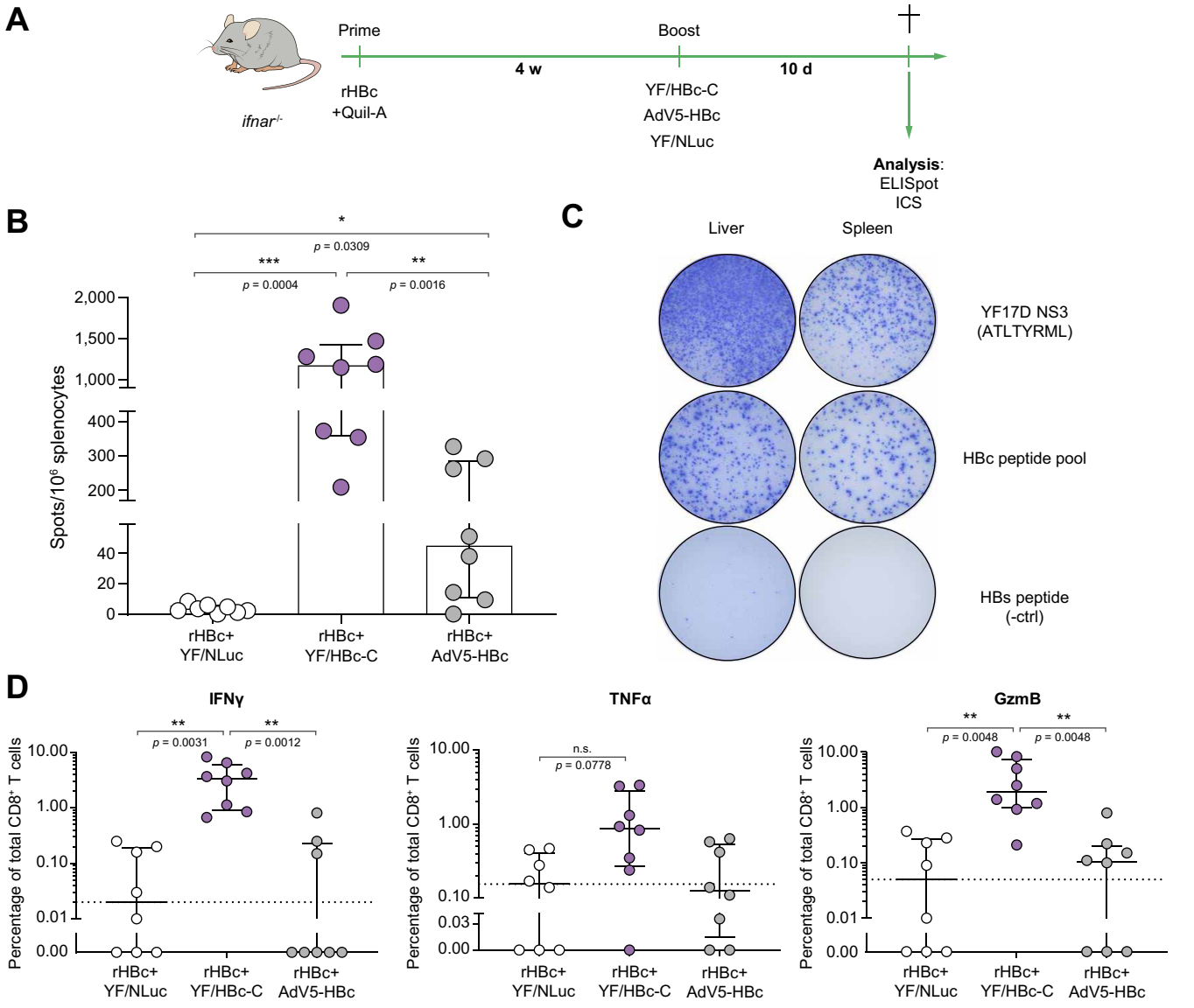

Fig. 3. Heterologous prime-boost vaccination (analysis 10 days post-boost). (A) Experiment time-course. (B) IFNγ ELISPOT for splenocytes of mice vaccinated with different prime-boost regimens (n = 8) after stimulation with a HBc 15-mer peptide pool. Bars represent medians ± IQR. *$p \leq 0.05$, **$p \leq 0.01$, and ***$p \leq 0.001$ (Kruskal–Wallis with Dunn's test). (C) Representative ELISPOT wells from liver-associated lymphocytes and splenocytes of YF17D/HBc-C boosted mice. (D) Cytokine/granzyme-positive $CD8^+$ T cells, following overnight stimulation with HBc-derived peptides (n = 8). Bars represent median ± IQR. Dotted lines represent median expression of control mice (rHBc primed, YF17D/NanoLuc boosted). **$p \leq 0.01$, n.s. not significant (Kruskal–Wallis with Dunn's test). ELISPOT, enzyme-linked ImmunoSpot; HBc, HBV core antigen; IFN, interferon; NanoLuc, nanoluciferase; rHBc, recombinant HBc; YF, yellow fever.

Eurogentec, Seraing, Belgium), HBc peptide pool (PepMix™ HBV Ultra, 0.25 μM/peptide, JPT Peptide Technologies GmbH, Berlin, Germany), or HBc- or HBs-derived peptides (5 μM/peptide, Eurogentec) in RPMI 1640 medium (Life Technologies) supplemented with 10% FCS, 2 mM L-glutamine, and 0.075% sodium bicarbonate. After 48 h at 37°C and 5% CO_2, spots were visualised by subsequent incubation with detection antibody, enzyme, and substrate. Plates were analysed using an ImmunoSpot S6 Universal Reader (CTL Europe, Bonn, Germany). Finally, spot counts were normalised by subtraction of the number of spots in corresponding control wells ($HBs_{179–186}$ as the control for peptide conditions, and non-infected VeroE6 lysate as control for YF17D cellular lysate).

Intracellular cytokine staining and t-SNE

Intracellular cytokine staining (ICS) was performed as described elsewhere.[17–19] The gating strategy is detailed in Fig. S1. The percentages of responding cells were normalised by subtracting the percentage of responders in samples stimulated with negative control peptide ($HBs_{179–186}$, Eurogentec) from those in

corresponding stimulated samples. Dimensionality reduction was performed by *t*-stochastic neighbour embedding (*t*-SNE) as plug-in in FlowJo V10 on concatenated subpopulations (Barnes-Hut, 1000 iterations, perplexity 30).

In vivo HBc-specific cytotoxic T cell-killing assay

An *in vivo* killing assay was performed as described previously.[20] Fresh mouse splenocytes were isolated from naïve mice and pulsed for 30 min with a HBc peptide pool (PepMix™ HBV Ultra, JPT, 0.25 μM/peptide) at 37°C, or were left untreated. Cells were washed twice with PBS, and labelled with 5 μM (HBc-pulsed cells) or 0.5 μM (unpulsed cells) of carboxyfluorescein succinimidyl ester (CFSE, Merck, Darmstadt, Germany). The mixture of pulsed and unpulsed splenocytes (ratio 1:1) was further labelled with an anti-CD45 APC antibody (0.5 μg/spleen, clone 30-F11, Biolegend, San Diego, CA, USA), and $5x10^6$ cells were then injected i.v. into vaccinated or naïve mice. Six hours later, fresh splenocytes were isolated from these mice and pulsed and unpulsed cells were identified by flow cytometry as $CFSE^{high}$ and $CFSE^{low}$ populations of APC-positive cells, respectively (for the full gating strategy, see Fig. S2), and the percentage of specific lysis was calculated using Equation 1:% of HBc-specific lysis = 100 – [100×(% pulsed/% unpulsed)vaccinated/(% pulsed/% unpulsed)naïve]

Data analysis

All statistical analyses were performed using GraphPad Prism 7 software (GraphPad, San Diego, CA, USA). Results are presented as medians ± IQR. Statistical differences between more than 2 groups were analysed using Kruskal–Wallis with Dunn's multiple comparisons test, and between 2 groups with the 2-tailed Mann–Whitney *U* test or Wilcoxon signed rank test for unpaired and paired data, respectively, and considered statistically significant at p values ≤0.05 ($^{**}p$ ≤0.01, $^{***}p$ ≤0.001, and $^{****}p$ ≤0.0001).

Results

Construction and characterisation of YF17D/HBc-C and YF17D/E-HBc-NS1

Two constructs were generated using a molecular cDNA clone of YF17D as a viral vector, expressing the HBc aa 1–155 (genotype ayw) as translational fusion either to the N terminus (YF17D/HBc-C) or at the E/NS1 junction of the YF17D polyprotein (YF17D/E-HBc-NS1) (Fig. 1A). The HBc polypeptide was accommodated by additional elements to guarantee proper processing of the transgene while maintaining the replication competence of the YF17D vector.[13,21,22]

Transfection of both constructs into BHK-21J cells yielded replication competent viruses, with somewhat reduced growth kinetics, as seen in a 1-step growth curve (Fig. 1B), and by a reduction in plaque size on VeroE6 (Fig. 1C) and BHK-21J (data not shown) cells as compared to the parental YF17D vector. Virus was collected as cell culture supernatant and passaged on VeroE6 cells to generate virus stocks (first passage, P1). Virus from P1, used throughout the study, was shown to be genetically stable and to retain the complete transgene (Fig. S3). To confirm the correct expression of HBc, VeroE6 cells infected with YF17D/HBc-C or YF17D/E-HBc-NS1 (Fig. 1D) were analysed by immunofluorescence. Of note, the expression pattern of HBc after YF17D/E-HBc-NS1 appeared to overlap with that of YFV antigens, suggesting that HBc remained associated with the endoplasmic reticulum (ER),[17,23] whereas HBc from YF17D/HBc-C appeared to be mainly localised in the nuclei of infected cells, suggesting the release of HBc from the YF17D polyprotein and its assembly into capsid-like particles. Such differential subcellular expression of HBc is in line with the respective vaccine design, where soluble HBc is released by the activity of the T2A peptide (YF17D/HBc-C) or remains membrane anchored by fusion to an extra membrane-targeting domain (WNV E TM2, YF17D/E-HBc-NS1) (Fig. 1A).

YF17D/HBc-C induces immunity more efficiently than does YF17D/E-HBc-NS1

We next tested whether these 2 viable HBc-expressing YF17D variants induce a humoral and cellular immune response. Given that IFN type I signalling severely restricts flavivirus replication in mice and, hence, also hampers vaccination with YF17D (or constructs derived thereof) (Fig. S4),[17,23] *ifnar*$^{-/-}$C57BL/6 mice, in which YF17D replicates and, thus, induces an immune response, were used throughout the study.

To induce strong cellular immune responses directed against HBc, mice were vaccinated in a heterologous prime-boost regimen (Fig. 2A). First, 6–8-week-old *ifnar*$^{-/-}$ mice received a s.c. prime immunisation with 5 μg particulate rHBc adjuvanted with 10 μg saponin-based adjuvant (Quil-A®). Two weeks post-prime, mice received a booster dose of either 10^5 pfu YF17D/HBc-C, 10^5 pfu YF17D/E-HBc-NS1, or, as matched control, 10^5 pfu YF17D/NanoLuc, a YF17D construct that carries the nanoluciferase (NanoLuc) gene as a nonrelevant insert of comparable size to HBc. The effect of YF17D/HBc-C and YF17D/E-HBc-NS1 was directly compared with that of a booster dose of 10^8 pfu AdV5-HBc serving as benchmark (Fig. S5). As another control condition, a homologous booster with a second dose of 5 μg rHBc and 10 μg Quil-A® was included. Four weeks after boosting, mouse splenocytes were assessed for antigen-specific recall responses by both enzyme-linked ImmunoSpot (ELISPOT) and ICS. Among the different tested prime-boost combinations, the HBcAg prime together with the YF17D/HBc-C boost regimen was most efficient at eliciting HBc-specific Th1 cell responses in a quantitative IFNγ ELISPOT assay (Fig. 2B and Figs S6–S7). For all tested HBc-derived recall antigens, only a booster regimen using YF17D/HBc-C significantly increased the number of HBc-specific IFNγ-secreting T cells over priming alone. In fact, YF17D/HBc-C boosting outcompeted a booster dose of AdV5-HBc or rHBc for every stimulation condition. Notably, booster immunisation using YF17D as a vector also enhanced HBc-specific Th2 responses, with either YF17D/HBc-C or YF17D/E-HBc-NS1 yielding similar levels of IL-4-secreting T cells (Fig. S8). However, the marked increase in IFNγ-secreting T cells observed particularly in YF17D/HBc-C vaccinated mice suggests a more pronounced Th1 polarisation of HBc-specific cellular immunity induced by latter variant.

When $CD8^+$ T cells were next analysed by ICS 4 weeks after boosting, the median number of cells expressing markers such as IFNγ, tumour necrosis factor alpha (TNFα), and granzyme B (GzmB) was highest after the YF17D/HBc-C boost (Fig. 2C). Moreover, immunisation with YF17D/HBc-C also resulted in a high degree of polyfunctionality within the HBc-specific $CD8^+$ T cell population (Fig. S9). Of note, although lower in absolute numbers, use of YF17D/E-HBc-NS1 in an identical heterologous prime-boost regimen resulted in an expression profile of $CD8^+$ T

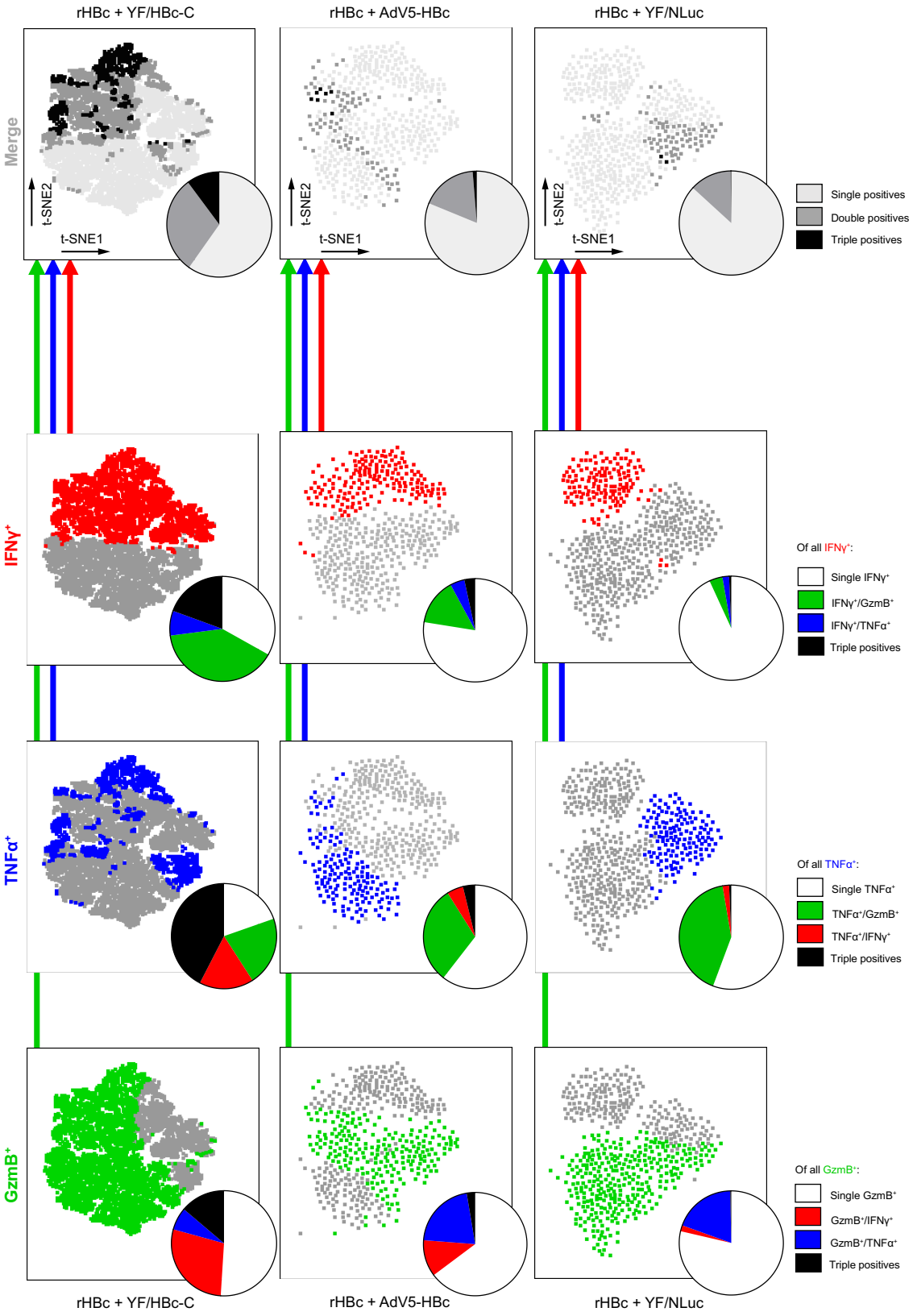

Fig. 4. CD8⁺ T cell t-SNE analysis. CD8⁺ T cells positive for at least 1 intracellular marker (IFNγ, TNFα, or GzmB), from splenocytes of *ifnar*$^{-/-}$ mice vaccinated with different prime-boost regimens (n = 8 per group) after overnight stimulation with HBc-derived peptides (10 days post-boost), were subjected to *t*-SNE analysis. Circles in grey shades (top) indicate fractions of these cells that were positive for 1, 2, or all 3 markers. Coloured circles (bottom rows) indicate the poly-functionality within each marker-positive population. GzmB, granzyme B; HBc, HBV core antigen; IFN, interferon; TNF, tumour necrosis factor; *t*-SNE, *t*-stochastic neighbour embedding

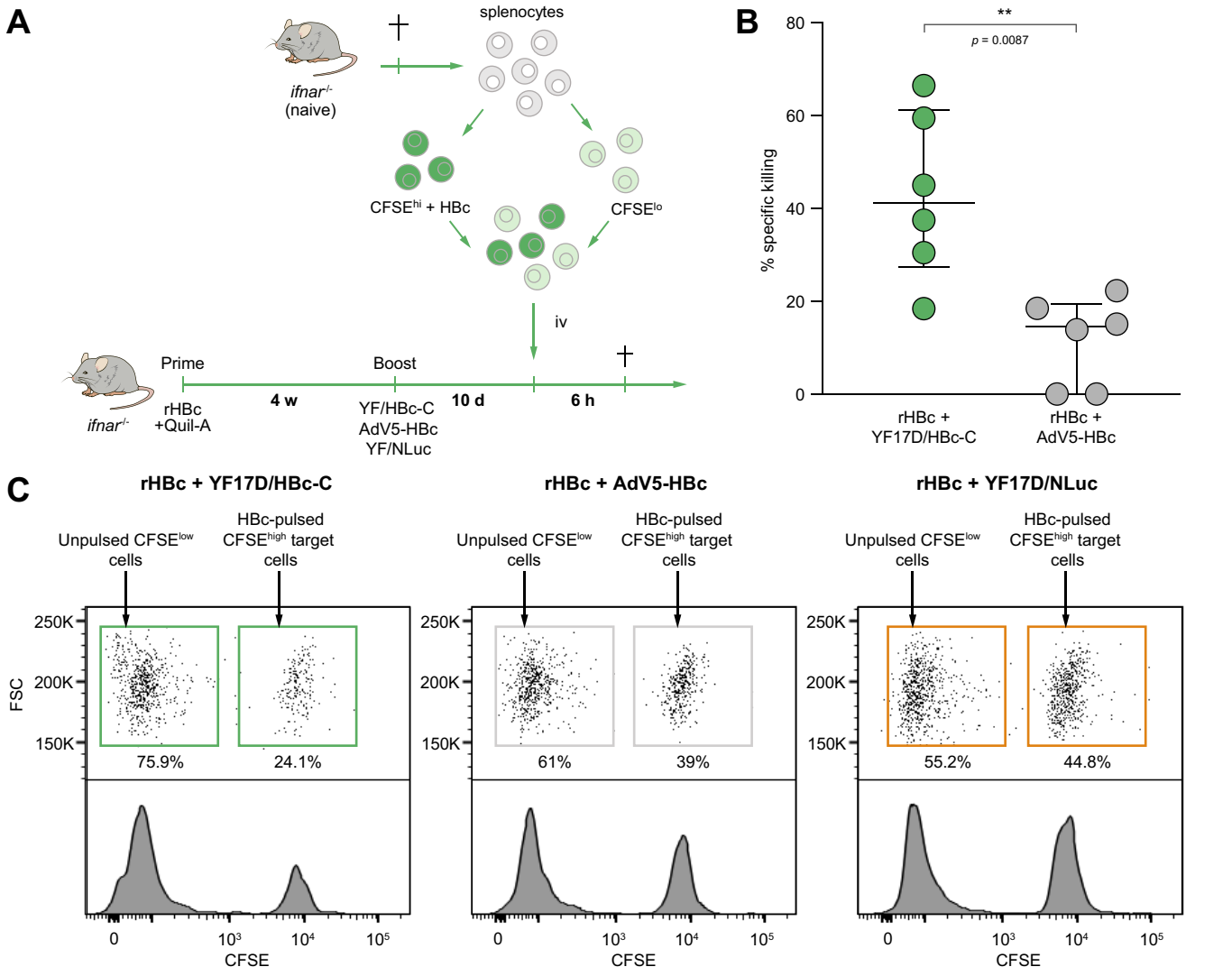

Fig. 5. *In vivo* cytotoxicity assay. (A) Six–eight-week-old *ifnar*$^{-/-}$ mice (n = 6, from 2 independent experiments) were rHBc primed and boosted with YF17D/HBc-C, AdV5-HBc, or YF17D/NanoLuc. Ten days post-boost, killing of i.v.-transferred HBc-pulsed splenocytes was monitored by flow cytometry. (B) Percentage specific killing of HBc-pulsed target cells in boosted mice compared with negative controls (boosted with YF17D/NanoLuc). Bars represent median values ± IQR. $^{**}p \leq 0.01$ (Mann–Whitney *U* test). (C) Representative dot plots and histograms of CFSE^{low} (unpulsed) and CFSE^{high} (HBc-pulsed) populations, 6 h after transfer (1:1) into vaccinated mice. CFSE, carboxy-fluorescein succinimidyl ester; HBc, HBV core antigen; ifnar, IFN-α/β receptor; NanoLuc, nanoluciferase; rHBc, recombinant HBc; YF, yellow fever.

cells that was comparable (in terms of their polyfunctionality) to that observed following YF17D/HBc-C boost. However, although heterologous prime-boost immunisation yielded the strongest HBc-specific responses among all tested regimens, YF17D/HBc-C in a homologous boost setting (repeated dosing) induced quantitatively more vigorous HBc-specific cellular immune responses (Fig. S6) compared with YF17D/E-HBc-NS1. In addition, CD4^{+} T cell responses toward HBc were elicited most efficiently by rHBc priming and subsequent boosting with YF17D/HBc-C (Fig. S10).

The presence of anti-HBc antibodies in patients with CHB treated with antiviral therapy has been linked to higher HBeAg conversion rates, a surrogate marker of treatment response.[24] Analysis of humoral responses by ELISA revealed that vaccination with YF17D/HBc-C alone elicited clearly detectable levels of anti-HBc antibodies (Fig. S11). Nevertheless, anti-HBc levels were further increased in regimens where mice were first primed with rHBc, highlighting the impact of protein priming on the magnitude of not only the cellular, but also the humoral immune responses induced.

In summary, in mice that have been primed with HBc (rHBc plus Quil-A® adjuvant), YF17D-vectored HBc expression can induce cellular immune responses that exceed in terms of magnitude and polyfunctionality those responses triggered by a repeated rHBc protein dose[25] or by a heterologous booster immunisation using an adenovirus-vectored HBc antigen. In particular, this appears to be the case when HBc is expressed as soluble protein from the N terminus of the YF17D polyprotein.

YF17D/HBc-C elicits strong, polyfunctional, and cytotoxic cellular immune responses in *ifnar*$^{-/-}$ mice

After having established an rHBc prime followed by YF17D/HBc-C boost as a potent regimen for inducing HBc-specific T cells that

last for several weeks (Fig. 2), we next tested whether immunisation by this combination also translates into vigorous effector functions by analysing cellular immunity more shortly after boosting. Six–eight-week-old *ifnar*$^{-/-}$ mice were immunised by s.c. prime of 5 μg rHBc (+10 μg Quil-A®), and then boosted 4 weeks later with 10^5 pfu YF17D/HBc-C, 10^8 pfu AdV5-HBc, or 10^5 pfu of the negative control YF17D/NanoLuc (Fig. 3A). At 10 days post-boosting, T cell responses were analysed after collection of splenocytes and overnight stimulation with an HBc-derived peptide pool. Again, boosting with YF17D/HBc-C and, to a lesser extent, AdV5-HBc, significantly increased (as measured by IFNγ ELISPOT) the HBc-specific T cell responses over priming alone (Fig. 3B). In fact, for YF17D/HBc-C, this was to more than 0.1% of all splenocytes that express IFNγ [when assessed *ex vivo* without any further enrichment by prolonged culture before analysis; median spots per million splenocytes ± IQR = 1163 (358–1421) for YF17D/HBc-C *vs.* median ± IQR = 45 (11–285) for AdV5-HBc]. Compared with analysis 4 weeks after boost (Fig. 2), there appeared to be a trend of higher absolute amounts of HBc-specific T cells when the analysis was performed only 10 days after boost (p = 0.0545). Of note, the HBc-specific cellular immunity was not restricted to the spleen, but could also be detected in liver-associated lymphocytes of *ifnar*$^{-/-}$ mice (Fig. 3C and Fig. S4B).

Next, *ex vivo* CD4$^+$ (Fig. S12) and CD8$^+$ (Fig. 3D) T cell responses were analysed by ICS and flow cytometry. The number of cells expressing IFNγ, TNFα, and GzmB in CD8$^+$ T cells after YF17D/HBc-C boosting were all markedly increased over priming (Fig. 3D), whereas boosting with AdV5-HBc did not result in an obvious increase in cells expressing either marker (p >0.05). To analyse differences in polyfunctionality levels between conditions, all HBc-specific CD8$^+$ T cells were subjected to a dimensionality reduction analysis, *t*-SNE, based on 3 parameters: expression levels of IFNγ, TNFα, and GzmB (Fig. 4). Cell populations that were positive for any of the 3 markers showed a substantial overlap in the YF17D/HBc-C-boosted condition, indicating a high level of polyfunctionality (Figs S13 and S14). By contrast, in control conditions, this overlap between IFNγ-, TNFα- and GzmB-producing cells remained limited, including in AdV5-HBc-boosted animals (p = 0.0002 comparing YF17D/HBc-C *vs.* AdV5-HBc regarding the percentage of triple-positive CD8$^+$ T cells). Thus, the quantitative increase in virus-specific T cells following YF17D/HBc-C booster immunisation was also accompanied by a considerable improvement in quality.

Finally, we aimed to determine whether, in addition to pronounced *ex vivo* activity, the HBc-specific cellular immunity raised by YF17D/HBc-C also exhibited a clear cytolytic function *in vivo*. To this end, we performed an *in vivo* cytotoxicity assay (Fig. 5), in which equivalent numbers of HBc-pulsed or unpulsed target splenocytes were injected intravenously into vaccinated mice (n = 6). Flow cytometric analysis after 6 h revealed that YF17D/HBc-C boosting resulted in specific killing of the HBc-pulsed population compared with sham-boosted controls [% specific killing: median ± IQR 41.25 (27.48–61.25)], which was almost 3-fold higher than that achieved following AdV5-HBc boosting [% specific killing: median ± IQR 14.50 (0–19.45); n = 6, p = 0.037].

Discussion

We constructed a YF17D-vectored therapeutic HBV vaccine candidate comprising HBc, which was replication competent and stably expressed its transgene. The aim of any therapeutic HBV vaccine is to induce potent T cell responses toward HBV antigens that can ultimately lead to cure of chronic infection, either by boosting of existing T cell immunity and/or by *de novo* generation of T cells. The latter is of particular interest in CHB because HBV-specific T cells can be exhausted or deleted.

In this regard, the use of YF17D offers several advantages. As a live-attenuated viral vector, it can amplify and spread recombinant antigens throughout, leading to long-lasting and polyvalent immune responses. In particular, YF17D infects and activates several subsets of dendritic cells (DCs),[15] which ultimately results in a balanced Th1/Th2 type immune response, hallmarked by broad CD8$^+$ T cell responses in addition to neutralising antibodies. In this respect, YF17D has been proposed to be superior to other vector platforms.[14] The anti-YFV immunity might in turn restrict the use of YF17D as vaccine vector in a therapeutic context in previously vaccinated patients. However, even in vaccinated individuals (who likely constitute only a minority of patients with CHB), revaccination with YF17D results in increased anti-YFV antibody and T cell levels,[26] warranting its use as vector also in cases of (some degree) of pre-existing immunity. Of note, the YF17D vaccine has an excellent safety record,[27] including in patients with HIV,[28] who form a relevant target group for CHB therapy given the high incidence of HIV–HBV co-infections. Likewise, CHB is not contraindicated for YF17D vaccination. Expression of heterologous genes is expected to increase safety further. In our study, IFN type I and II receptor-knockout mice (AG129 mice, an otherwise lethal model of YF17D infection)[17,19] showed no signs of morbidity after YF17D/HBc-C administration (data not shown). The favourable safety profile of YF17D-vectored vaccine candidates was recently confirmed in several preclinical models, including immunodeficient (IFN-I and -II receptor-deficient) mice and [signal transducer and activator of transcription 2 (STAT2)-deficient] hamsters, and suckling mice.[22] Moreover, chimeric vaccines based on YF17D have been licensed for use in children.[29] In fact, YF17D is the only vaccine that forms the basis of any licensed recombinant live-attenuated virus-vectored vaccine, in contrast to other vectors still in (pre-) clinical development.

Throughout the study, C57BL/6 *ifnar*$^{-/-}$ mice were used to cope with the severe restriction of type I IFNs on the replication of YF17D in mice.[17,23] Comparison of vaccination in *ifnar*$^{-/-}$ *vs.* wt C57BL/6 (with or without temporal *ifnar* knockdown) showed clearly that T cell responses (toward YF17D and HBc) were most prominent in *ifnar*$^{-/-}$ mice.

In this study, we focused on a single HBV antigen, HBc. T cell responses toward HBc are associated and coincide with the recovery of CHB in patients,[30] an observation also made in the woodchuck HBV model.[31] In HBV transgenic mice, the administration of HBc alone, as particulate antigen,[25] or as HBc-pulsed DCs[32] resulted in seroconversion to HBsAg, and the induction of HBs- and HBc-specific T cells. Immunisation with HBc results in Th1 polarisation,[33,34] hallmarked by the secretion of cytokines, such as TNFα and IFNγ, which are considered particularly important in HBV clearance.[35] Compared with HBs and HBp antigens, HBc-specific T cells appear to be less exhausted and present at higher levels in patients with HBV.[36,37] Therefore, core-derived therapeutic vaccines might rely on some existing anticore immunity in patients with chronic HBV. Additionally, as in our study, such immunity can be installed by priming with the antigen before boosting. However, the observation that *de novo* HBs-specific T cells could also be generated by therapeutic

vaccination in a mouse model of HBV persistence[38] suggests that other HBV antigens, for which T cells are comparatively more exhausted, can be included as target antigens in the context of a therapeutic HBV vaccine; however, there is neither experimental nor clinical evidence that cure of CHB requires T cell responses to extend to more than 1 viral antigen. By contrast, several advanced immune therapies, such as chimeric antigen receptor T cells (CAR-T) used for cancer treatment, show a promising effect by targeting even single-peptide epitopes.[39]

In this study, HBc_{1-155} was used as opposed to single epitopes. Although the inclusion of a larger antigen likely increases the attenuation of the vector, it provides a complete epitope repertoire, which allows multiple HLA haplotype specificities and/or variation between HBV genotypes to be covered. Notably, a comparison of HBc_{1-155} from 3 major genotypes (A, C, and D) revealed a high degree of sequence conservation with only 8 unique amino acid changes identified. Likewise, vaccination with HBc from 1 genotype raised cellular immune responses to nonidentical epitopes from other genotypes.[25] Nevertheless, in analogy to the observation that naturally occurring polymorphisms in HBc can confer resistance to HBc inhibitors,[40] a consensus HBc sequence might improve immunogenicity across the entire HBV genetic spectrum. Finally, inclusion of HBc_{1-155} favours the induction of anti-HBc antibodies in addition to T cells. We show here that antibody production was achieved by vaccination with YF17D/HBc-C alone, but was most pronounced after administration of adjuvanted rHBc (although HBc is known to elicit strong antibody responses adjuvant independently).[33]

Depending on the HBc insertion site in the YF17D genome, we observed that HBc was expressed in different cellular compartments. As postulated for other transgenic YF17D, this can result in activation of distinct immune responses through different antigen-presenting pathways.[41,42] In the particular case of HBc, insertion in the capsid gene of YF17D resulted in stronger Th1 T cell responses and higher HBc-specific antibody levels compared with insertion in between the E and NS1 genes. Qualitative differences between the 2 variants were not observed.

Comparing different therapeutic HBV vaccine candidates comprising HBcAg is challenging, given the variety of readouts and *in vivo* models (HBV naïve or persistent), and the possible influence of other included HBV antigens. Hence, for reference, we benchmarked the YF17D platform to an AdV5-vectored HBc. AdV5 is a commonly used nonreplicative viral vaccine vector and forms the basis of a therapeutic HBV vaccine currently in clinical trials.[43] AdV5-HBc was confirmed to express HBc *in vitro* and, when used as a boost after HBcAg priming, induced significant increases in T cell responses over priming alone. However, YF17D/HBc-C induced T cell immunity that was superior in both magnitude and polyfunctionality to that induced by AdV5-HBc (appropriate route and dosing as established by others),[43,44] with the enhanced immunogenicity of YF17D-vectored HBc possibly resulting from the replicative nature of YF17D and/or more favourable immune presentation of the transgene. We showed that vaccination with YF17D/HBc-C elicited high levels of CTLs that simultaneously expressed mediators of cytolytic (GzmB) and noncytolytic clearance (TNFα and IFNγ).[35]

In summary, in this study we show that a YF17D-based vaccine dose, following a protein prime, elicits strong, polyfunctional, and antigen-specific T cells that are able to selectively kill HBV antigen-positive target cells *in vivo*. These results warrant further study of this vaccine regimen as a therapeutic agent in HBV-carrier mice, as well as the development of additional transgenic YF17D carrying other HBV antigens. The YF17D-based live-attenuated recombinant vector expressing HBc described here could be a promising addition to the arsenal of therapeutic vaccine candidates aiming at (super-)charging cellular immunity for the functional cure of CHB.

Abbreviations

aa, amino acids; CAR-T, chimeric antigen receptor T cells; cccDNA, covalently closed circular DNA; CFSE, carboxy-fluorescein succinimidyl ester; CHB, chronic hepatitis B; CTL, cytotoxic T lymphocyte; DCs, dendritic cells; ELISPOT, enzyme-linked ImmunoSpot; GzmB, granzyme B; HBc, HBV core antigen; HBs, HBV surface antigen; HBp, HBV polymerase antigen; *ifnar*, IFN-α/β receptor; IFNγ, interferon γ; ICS, intracellular cytokine staining; MHC, major histocompatibility complex; NanoLuc, nanoluciferase; pfu, plaque-forming units; rHBc, recombinant HBc; STAT2, signal transducer and activator of transcription 2; *t*-SNE, *t*-stochastic neighbour embedding; TNFα, tumour necrosis factor α; wt, wild-type; YF, yellow fever.

Financial support

JM has received funding from the Chinese Scholarship Council (CSC) under grant number 201706760059. This project has received funding from the Belgian Science Policy Office (BELSPO) programme 'Interuniversitaire Attractiepolen' (IUAP), and the European Union Infect-ERA hepBccc project.

Conflicts of interest

RB, JN, and KD have filed a patent application comprising the discovery and use of live-attenuated flaviviruses with heterologous antigens. JM declares no competing interests.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

Conceived and designed the experiments: RB, KD. Performed cloning and *in vitro* work: RB. Conducted animal experiments and downstream analysis: RB, JM. Wrote the manuscript: RB, JN, KD.

Data availability statement

All data are available from the authors upon request.

Acknowledgements

We are grateful to Peter Bredenbeek for providing cell lines, Claude Libert for providing *ifnar*$^{-/-}$ breeding couples, and EUROSCARF for plasmid pAG61. We thank Carolien De Keyzer, Katrien Geerts, Madina Rasulova, and Catherina Coun for their excellent technical assistance; Jolien De Munck for helping to establish the ELISPOT assay; and Geert Schoofs and Tania Mitera for their assistance with the flow cytometry set-up and instrumentation.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhepr.2021.100295.

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Author names in bold designate shared co-first authorship

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