Abschlussbericht Teilprojekt 16.1

Projekttitel: In vivo Inhibition der Hepatitis B Virus (HBV)-Infektion durch acylierte pre-S Peptide unter Verwendung des Urokinase-Typ Plasminogen Aktivator (uPA/ RAG-2) Mausmodells

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I. Kurze Darstellung

1. Aufgabenstellung

Ziel des Projektes war die Überprüfung, ob eine Klasse zuvor in der AG von SU *in vitro* charakterisierter Eintrittsinhibitoren der HBV-Infektion auch *in vivo* in einem von JP entwickelten Mausmodell wirksam sind. Dazu wurde eine Kooperation der beiden Gruppen etabliert.

2. Voraussetzungen

Das Projekt wurde größtenteils in der Arbeitsgruppe von Jörg Petersen. Die Infrastruktur zur Durchführung und Auswertung der *in vivo* Infektionsversuche an transplantierten uPA/RAF-2 Mäuse sind dort seit vielen Jahren etabliert. In Heidelberg wurden die für die Versuche benötigten Peptide synthetisiert, das HBV-Inocculum präpariert, sowie Biodistributions-analysen durchgeführt. Hep-Net hat das Projekt mit einer BAT IIa/2 Stelle und Sachmitteln unterstützt. Als sehr vorteilhaft für die Kooperation war der bereits über lange Jahre bestehende wissenschaftliche Kontakt der beiden Arbeitsgruppen.

3. Planung und Ablauf des Vorhabens

Planung und Ablauf des Vorhabens sind detailliert im Antrag sowie im Zwischenbericht beschrieben. Aufgrund der Klarheit der Fragestellung („proof of principle“ im Tiermodell) war die experimentelle Vorgehensweise klar:

1 Synthese der für die Inhibition notwendigen Peptide
2 Test der Peptide in vitro in HepaRG Zellen
3 Bestimmung der inhibitorischen Potenz (IC50 und IC90)
4 Bestimmung kritischer Parameter, die für die in vivo Applikation wichtig waren. Dazu gehörten Halbwertszeit im Serum und Organverteilung.
5 Durchführung von 3 Runden von in vivo Infektionsexperimenten mit intraperitonealer und subkutaner Gabe von Peptiden
6 Analyse viraler Marker aus Blut und Lebergewebe infizierter Tiere.
7 Anfertigung von Schnitten und IF und Autoradiographie von Leberschnitten.

Die Ergebnisse wurden auf zahlreichen wissenschaftlichen Tagungen vorgestellt und sind zurzeit im re-revisions Prozess bei der Fachzeitschrift „Nature Biotechnology“. Von allen 4 Gutachtern lagen positive Beurteilungen vor, sodass wir von einer Annahme durch den Editor ausgehen.

4. wissenschaftlicher und technischer Stand

Beide Laboratorien verfügen über die „state of the art“ Methodologie die für die Durchführung der beiden experimentellen Teilprojekte notwendig waren. Alle mit der Quantifizierung der Infektionsmarker (HBsAg, HBeAg, real-time PCR, Immunoblot etc.) im Zusammenhang stehenden Methoden entsprachen dem neuesten Stand. Die notwendigen Substanzen (Antikörper, Peptide, Tetramere, molekularbiologische Reagenzien) wurden kommerziell erworben oder sind selbst erzeugt und seit längerem in Gebrauch. Literatursuche wurde mittels Medline/PubMed durchgeführt. Bei der verwendeten Fachliteratur handelte es sich um Zeitschriften der Universitätsbibliotheken der Universitäten Hamburg und Heidelberg.

5. Zusammenarbeit mit anderen Stellen

Im Zuge der Arbeiten wurde außerdem eine enge Kooperation mir der Arbeitsgruppe von Walter Mier, Nuklearmedizin, Universität Heidelberg etabliert. Die Expertise seiner Arbeitsgruppe hinsichtlich Peptidsynthese, Peptidanalyse und in vivo Biodistribution sind in die Arbeiten eingeflossen. Dr. Mier ist auf der Basis dieser Kooperation als

Teilantragsteller in das aus dem „start-up Fond“ erfolgreich hervorgegangenen Projektes „Innovative Therapieverfahren -Myrcludex B“ integriert worden.

II. Eingehende Darstellung

1. des erzielten Ergebnisses

Die Darstellung der Ergebnisse ist bereits aus dem Zwischenbericht ersichtlich. Sie ist weiterhin in Form des zurzeit in re-revision befindlichen Manuskriptes dem Anhang beigefügt. Hier die deutschsprachige Zusammenfassung:

360 Millionen Menschen sind chronisch mit dem Hepatitis B Virus infiziert und tragen damit ein stark erhöhtes Risiko an Leberzirrhose oder Leberkrebs zu erkranken. Zugelassene Arzneimittel zielen darauf ab, die Immunantwort zu stimulieren oder inhibieren die virale Polymeraseaktivität. Da diese Therapieansätze meistenteils nicht kurativ sind ist es notwendig, Therapeutika zu entwickeln, die andere Replikationsschritte des Virus adressieren. Kürzlich gelang es uns acylierte Peptidfragmente des großen HBV Hüllproteins zu charakterisieren die sehr effizient den Viruseintritt in Hepatozyten verhindern können. In der vorliegenden Arbeit zeigen wir die Anwendbarkeit dieser Peptide für die Prävention der Hepatitis B Virus Infektion in vivo. Dazu verwendeten wir immundefiziente uPA-Mäuse die zuvor mit primären humanen Hepatozyten bzw. mit Hepatozyten von Tupaia belangeri transplantiert worden waren als Tiermodell. Die Anreicherung der Peptide in der Leber in Kombination mit ihrer außergewöhnlichen inhibitorischen Potenz und deren spezifischer Mechanismus der Infektionsinhibition erlaubte eine subkutane Applikation in sehr geringer Dosis. Die Inhibition des hepadnaviralen Eintritts stellt damit einen neuen therapeutischen Ansatz zur Behandlung akuter (z.B. nach Lebertransplantation) und möglicherweise auch chronischer HBV Infektionen dar.

Prevention of hepatitis B virus infection in vivo by envelope-
protein-derived entry inhibitors

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360 million people are chronically infected with the human hepatitis B virus (HBV) and are consequently prone to develop liver cirrhosis and hepatocellular carcinoma[1]. Approved therapeutic regimens modulate patients´ antiviral defences or inhibit the viral reverse transcriptase. Since these therapies are generally non-curative, strategies interfering with other HBV replication steps are required. We recently identified acylated peptides derived from the large HBV envelope protein that block virus entry[2-5]. Here we demonstrate their applicability for the prevention of hepatitis B virus infection *in vivo*, using immunodeficient uPA-mice repopulated with primary human or tupaia hepatocytes as animal models[6, 7]. Accumulation of the peptides in the liver combined with an extraordinary inhibitory potency and a specific mode of action permits subcutaneous application at very low doses. Inhibition of hepadnavirus entry thus constitutes a novel therapeutic approach to prevent primary HBV infection, e.g. after liver transplantation but also for the treatment of chronic hepatitis B.
Specific inhibition of virus entry is an attractive therapeutic concept to control and eventually eliminate acute and chronic infections. For HIV, interference with virus entry has been successfully accomplished by a gp41 protein-derived peptide consisting of 36 a.a. (Fuzeon®) which prevents fusion of the viral and the cellular membrane[8].

In spite of the availability of a prophylactic vaccine, reverse transcriptase (RT) inhibitors and immune globulins used for post-exposure prophylaxis, the number of HBV-infected people and the number of HBV-related deaths worldwide (presently about 500.000 per year) is increasing. About 2/3 of primary liver cancers are attributable to persistent HBV infection[9]. Current treatment pursues two strategies: (i) interferon (IFN alpha) treatment modulates immune responses against HBV and displays a direct antiviral effect, which leads to long-term clinical benefit in about 30% of treated patients without eradication of the virus; (ii) administration of viral reverse transcriptase-inhibitors suppresses viral replication and is accompanied by significant biochemical and histological improvements after one year of treatment. However, long-term treatment is associated with the emergence of resistant virus strains[10].

HBV is the prototype of a family of small, enveloped DNA viruses of mammals and birds[11]. The HBV envelope encloses three proteins termed L-(large), M-(middle) and S-(small) (**Fig 1a**). They share the C-terminal S-domain with four transmembrane regions. The M- and L-protein carry additional N-terminal extensions of 55 and, genotype-dependent, 107 or 118 a.a. (preS2- and preS1) **(Fig. 1b)**. In virions the stoichiometric ratio of L, M and S is about 1:1:4, while the more abundantly secreted non-infectious subviral particles (SVPs) contain almost exclusively S- and only traces of L-protein[12]. During synthesis, the preS1-domain of L is myristoylated and translocated through the ER. This modification and the integrity of the first 77 amino acids of preS1 are essential for infectivity[13, 14].

Studies of the early events of HBV infection have been limited, since neither cell culture systems nor small animal models were available until recently[15]. The development of the HBV susceptible cell line HepaRG facilitated systematic investigations of HBV entry and resulted in the discovery of envelope protein-derived entry inhibitors[4]. The prototype of these peptides (HBVpreS/2-48^{myr}) comprises the N-terminal 47 preS1 amino acids linked to a myristoyl-moiety at Gly-2. It inhibits HBV infection with an $IC_{50} \approx 8$ nM (**Fig. 1b**). A peptide with an increased specific activity, HBVpreS/2-48^{stearoyl} ($IC_{50} \approx 250$ pM), comprises the same sequence but is fused to stearic acid. HBVpreS/2-39^{myr}, the third peptide used in this study, is C-terminally truncated by 9 a.a. which results in an IC_{50} of ≈ 300 nM[3, 5].

The restriction of HBV infections to chimpanzees demands for small hepadnavirus animal models[16]. Urokinase-type plasminogen activator (uPA) transgenic mice represent a useful system, in which hepatocyte-targeted expression of albumin-uPA induces death of the transgene-carrying hepatocytes and thus provides a growth advantage for transplanted cells[17]. To test the antiviral activity of HBVpreS-derived lipopeptides we utilized uPA$^{+/-}$ transgenic mice crossed with RAG-2$^{-/-}$/pfp$^{-/-}$ mice lacking mature T-, B- and NK-cells to allow xenotransplantation with primary hepatocytes isolated from the asian tree shrew *Tupaia belangeri* (PTH)[7] or with primary human hepatocytes (PHH)[6].

Tupaia belangeri is phylogenetically related to primates[18] and PTH have been shown to be susceptible for HBV and woolly monkey hepatitis B virus (WMHBV) infection[19]. Because of the higher repopulation rates (up to 95 %) and the possibility to maintain PTH without signs of metabolic disorders over the full life span of the animals, we tested the antiviral activity of the peptides using mice repopulated with PTH[6, 7]. WMHBV belongs to the genus *Orthohepadnavirus* sharing 78% sequence identity with HBV[20]. WMHBV and HBV L-proteins exhibit high homology within a receptor recognition site of the preS1 domain **(Fig.1c)** and previous studies indicated that entry of both viruses follows indistinguishable pathways including the sensitivity against acylated HBVpreS-peptides[5]. Based on these findings and taking advantage of the higher replicative activity of WMHBV in PTH we performed most of our study using WMHBV and PTH-transplanted mice. Nevertheless, we validated the applicability of this system in mice repopulated with PHH which were subsequently infected with HBV.

In total 25 hemizygous uPA$^{+/-}$/RAG-2$^{-/-}$/Pfp$^{-/-}$ mice highly repopulated with PTH were used in two sets of experiments. The percentage of repopulation was estimated by quantification of serum alpha1 anti-trypsin (tu-□). Ten mice (**Fig. 2a**) were used in the first experimental setup (**Fig 2b)**. The two peptides HBVpreS/2-48^{stearoyl} and HBVpreS/2-39^{myr}, which differ by a factor of ≈1000-fold in their specific activities, were chosen to assess their efficacy *in vivo*. As a control, a myristoylated preS-peptide from the heron hepatitis B virus (HHBV) (HHBVpreS/2-44^{myr}, **Fig 1c**)[21], which is inactive against HBV infection, was administered. Six mice received the two HBV preS-peptides and two mice the control. An initial injection (10 mg/kg body weight) was administered into the spleen 30 min prior to intraperitoneal (i.p.) administration of 10^7 genome equivalents (g.e.) of WMHBV. Two subsequent i.p. administrations of peptides were carried out 24 h and 5 days p.i. As an additional control, two animals were infected in the absence of peptide. Infection was monitored through quantification of HBsAg and WMHBV-DNA levels in the serum at week 2, 5, 8, 11, 14, 17 and 20, by ELISA and quantitative real time PCR, respectively. As shown in **Fig. 2c** viremia was detected in the controls (≥ 2 x 10^4 WMHBV DNA g.e./ml serum) with titres of 10^7 and 10^9 g.e./ml for mouse #25 and #26 respectively. In control mouse #28 viremia was lower (10^5 g.e./ml), possibly due to the lower repopulation rate (**Fig 2a**). Mouse #23 died for unknown reasons. In contrast, none of the 3 mice that received HBVpreS/2-48^{stearoyl} and also none of those treated with HBVpreS/2-39^{myr}, developed detectable levels of HBV-DNA and HBsAg (data not shown) at any time point during 24 weeks. To examine whether these animals established a low level infection with only few transplanted hepatocytes expressing HBV-specific markers, we performed a histological analysis of mouse #15 (receiving HBVpreS/2-39^{myr}) and #19 (receiving HBVpreS/2-48^{stearoyl}) in comparison to a control (mouse #25). To locate infection of transplanted PTH, serial livers sections were examined by cytokeratin 18 (Cyt-18) and HBcAg staining. HBcAg was detected in virtually all repopulated cells in control mouse #25, but not in the murine parenchyma (**Fig. 2d and e)**. In contrast, none of the transplanted hepatocytes stained positive for HBcAg in mouse #19 (**Fig 2 f and g**) and #15 (data not shown). To ascertain that the previously treated mice were principally susceptible to infection, we re-infected the 4 remaining mice (#16, 17, 20 und 22) at week 22. As shown in the **supplementary Fig. 1**, 3 of the 4 mice established viral titres of 10^5-10^6 WMHBV DNA g.e./ml at week 16.

To inquire whether acylated HBVpreS-peptides are also detectable in the liver after subcutaneous (s.c.) administration, we performed pharmacokinetic studies in both repopulated and naïve mice. For this purpose we synthesized myristoylated and stearoylated HBVpreS/2-48-peptides tagged with a C-terminal tyrosine allowing specific labelling by iodine-131 (^{131}I). As a control we used the approved HIV gp41-derived T-20 peptide Fuzeon®. 1.9-2.5 x 10^7 MBq (≈ 2µg) of each peptide was s.c. administered into male NMRI mice. 10 min, 1 h, 4 h, and 24 h post injection 3 mice per time point were sacrificed and ^{131}I in the organs (liver, blood, heart, lung, spleen, kidney and muscle) was quantified. Unlike Fuzeon® the HBV-derived lipopeptides are enriched in the liver with about 15-25 % of the injected dose/g tissue detected 4 h post injection and at least 4 % of HBVpreS/2-48^{stearoyl} still being present after 1 day (**Fig 3a**). For HBVpreS/2-48^{stearoyl} (**supplementary Fig. 2)** the maximal levels detectable

in heart, spleen and muscle were below 0.5% of the ID/g, for blood and the lung below 1% and for kidney below 2%**.** This biodistribution did not change substantially in hemizygous uPA$^{+/-}$/RAG-2$^{-/-}$/Pfp$^{-/-}$ mice (**supplementary Fig. 3**). Interestingly, when using transplanted mice, the peptide is not enriched in areas of transplanted cells (**Fig. 3b and c**) indicating an organ- but not species specific accumulation of HBVpreS-derived lipopetides. To investigate the stability of HBVpreS/2-48^{myr} in the liver of uPA$^{+/-}$/RAG-2$^{-/-}$/Pfp$^{-/-}$ mice, we extracted the ^{131}I-labelled lipopeptide 24 h after s.c. injection from the liver and analysed its retention behaviour on a reversed phase HPLC column. As shown in **Fig. 3d** about 50% of the eluted activity is still associated with the full-length peptide after 24 h while the other 50% elute at lower hydrophobicity indicating partial endoproteolytic degradation.

Since s.c. injection resulted in efficient transport of the peptides to the liver, 15 repopulated uPA$^{+/-}$/RAG-2$^{-/-}$/Pfp$^{-/-}$ mice (**Fig 4a**) were selected for an inhibition experiment using lower peptide doses and s.c. application. Two groups of 5 mice received either 2 mg/kg or 0.2 mg/kg body weight of HBVpreS/2-48^{myr}, while a third group of 5 animals received 2 mg/kg of the control peptide HHBVpreS/2-44^{myr}. 1h p.i. the mice were infected with 2 x 10^7 g.e. of WMHBV. S.c. peptide administrations were repeated 24 h later and at day 2, 3 and 5 **(Fig 4b)**. 4 out of 5 control mice developed viral titres ranging from 10^5 to 5 x 10^7 WMHBV DNA g.e./ml within 9 weeks, while the treated mice (N=10) remained negative (detection limit: 5 x 10^4 g.e./ml) **(Fig 4c)**. Among the 5 animals receiving 0.2 mg HBVpreS/2-48^{myr}/kg body weight, one mouse developed viral titres of 2 x 10^5 by week 15, and one of the 5 mice treated with a 10-fold higher dose of HBVpreS/2-48^{myr} (2 mg/kg) reached borderline levels (10^5 g.e./ml at week 15). However, in contrast to the control mice, all treated mice remained negative in serum HBsAg (**Fig 4d**) and HBcAg expression, as determined by immunohistochemistry of liver sections at week 15 (**supplementary Fig 4 a-d**).

To validate whether acylated preS-peptides are able to also impede HBV infection in PHH-repopulated uPA$^{+/-}$/RAG-2$^{-/-}$/Pfp$^{-/-}$ mice, 8 animals with repopulation indices > 10% were analysed (**Fig 4e, f**). They received either 2 mg/kg body weight HBVpreS/2-48^{stearoyl} s.c. (N=4) or the same amount of a scrambled stearoylated HBVpreS control peptide (N=4). 1 h later, 2 x 10^8 HBV-DNA g.e. were i.p. injected. S.c. peptide administrations were repeated 24 h later and at day 2, 3 and 5. As depicted in **Fig 4e**, control mice (left) developed HBV serum titres ranging from 7 x 10^4 to 2 x 10^7 g.e./ml within 10 weeks, while establishment of HBV infection was constrained in mice treated with HBVpreS/2-48^{stearoyl} (right). Though one animal (#1833) died shortly after blood withdraws at week 4, HBV titres stayed below 10^5 HBV-DNA g.e./ml in the remaining mice with only mouse #1829 showing barely detectable viremia (6 x 10^4 HBV g.e./ml). Ten weeks after infection, mouse #1834 treated with HBVpreS/2-48^{stearoyl} and one control mouse (#1821) were sacrificed to determine human liver cell composition by human Cyt-18 staining and intrahepatic viral loads by real-time PCR[22]. Intrahepatic HBV-DNA remained below the detection limit (100 HBV-DNA copies/10.000 cells) in the treated animal, while establishment of productive HBV infection with 6 rcDNA and 0.5 cccDNA copies per human hepatocyte was estimated in a control. Positive HBcAg-staining was also detected in human hepatocytes (**Fig 4g**) of mouse #1821 (**Fig 4 h**) but not in the HBVpreS/2-48^{stearoyl} treated mouse (not depicted).

We here prove the principle that hepadnavirus infection (WMHBV and HBV) can be efficiently restrained through s.c. application of HBV envelope protein-derived lipopeptides *in vivo.* This opens new perspectives for the future therapy of acute and probably chronic hepatitis B. Since the uPA/RAG-2/Pfp mice used here lack B-, T-, and NK cells, a direct inhibitory effect of the peptides on susceptible hepatocytes must be assumed. This is supported by accumulation of acylated preS-peptides in the liver, followed by a slow clearance possibly by proteolytic degradation in hepatocytes. Both properties permit s.c. application at very low doses and low frequencies. Given that 5 injection of 0.2 mg/kg HBV/preS2-48^{myr} within 5 days strongly inhibited the establishment of an infection, administration of the about 30-fold more active peptide HBV/preS2-48^{stearoyl} might be effective at doses < 7 µg/kg ≈ 13 nmol/kg when given daily or every two days. Taking into account that the efficient pharmacological dose per body weight obtained in mice has to be corrected for

humans[23] by a factor of about 10, the efficient dose per person is expected to be < 100µg/day.

The nucleos(t)ide analogue-based regimen for treatment of chronic HBV infection frequently results in the selection of resistant mutants[10, 24]. This demands for alternative strategies and new drugs that address different steps of the HBV replication cycle. Entry inhibition with HBV lipopeptides represents such an approach. Due to the mode of action we assume efficacy against any kind of nucleos(t)ide resistant mutant. Moreover, since the activity of the peptides requires a conserved sequence in the preS1-domain[2], we expect activity against any HBV-genotype. In contrast to Fuzeon® which targets the HIV gp41 fusion protein thus allowing the emergence of intramolecular compensatory mutations, previous studies indicate that acylated HBV-lipopeptides address a cellular component preventing interaction of HBV with its receptor[5, 25]. Therefore, emergence of resistant mutants might be improbable.

Apparent indications for future clinical applications of HBVpreS-derived lipopeptides are the prevention of not yet established HBV infections (e.g. post exposure prophylaxis, vertical transmission or prevention of reinfection of the liver transplant). However, entry inhibitors may be effective also in chronically infected patients, possibly in combination with IFNα or inhibitors of the viral RT. Since maintenance of HBV-chronic infection may depend on a dynamic turnover of infected hepatocytes cleared by the immune system on one hand and (re)infection of cured/naive cells on the other hand[26], it will be interesting to evaluate the efficacy of such therapeutic approaches in the uPA chimeric system to repress spreading of infection and emerging of resistant strains under antiviral treatment[7].

HBVpreS-derived lipopeptides also inhibit *in vitro* infection of HDV, a satellite virusoid utilizing HBV envelope proteins for the entry into hepatocytes[2, 25, 27]. Since to date no effective therapy for HDV infection exists, preS-derived lipopeptides may represent the first selective therapy for this often complicated liver disease.

Application of HBVpreS-derived lipopeptides in immune competent patients might elicit cellular and humoral immune reactions. This might be beneficial for the therapeutic outcome, since it is known that antibodies recognizing epitopes within HBVpreS/2-48 neutralize HBV infection *in vitro*[28]. Moreover, it has been speculated that virus elimination in a natural infection requires the successful establishment of HBVpreS-specific immunity. Thus, in addition to the direct interference with virus entry stimulation of preS-specific immune responses by the peptide could contribute to virus elimination through cytolytic or non-cytolytic immune reactions, especially in combination with IFNα. However to assess the effects of this complicated interplay between direct and immune system-mediated antiviral effects of HBVpreS-derived lipopeptides, clinical studies in immune competent chronically infected patients are necessary.

2. Methods

Animals and transplantation procedures. Alb-uPA transgenic mice (Jackson Laboratories, ME, USA) crossed with RAG-2-/-/Pfp-/- double knockout mice (Taconic Farms, Denmark, EU) were housed and maintained under specific pathogen-free conditions in accordance with institutional guidelines under approved protocols. Asian tree shrews (Tupaia belangeri) were obtained from the German Primate Centre in Göttingen, Germany, and maintained in the animal facility of the University of Freiburg in accordance with institutionally approved protocols. For the preparation of PTH animals were anesthetized by intramuscular injection of ketamine (50 mg/kg body weight) and xylazine (10 mg/kg body weight) and the liver was perfused in situ by collagenase perfusion19. 1 x 106 PTH were injected into the spleen of 15 to 25 day-old uPA+/-/RAG-2-/-Pfp-/- anesthetized mice. PHH were isolated from surgical liver tissues remaining after partial hepatectomy. Informed consent was obtained from patients according to the principles of the Declaration of Helsinki and approved by the Ethical Committee of the city and state of Hamburg. 1 x 106 viable (90%) freshly isolated hepatocytes were transplanted into uPA+/-/RAG-2-/-Pfp-/- mice using the same procedure as reported for PTH6.

Determination of liver repopulation and viral titres in mice. Serum samples were diluted 1:50 in SDS-Laemmli buffer subjected to SDS-PAGE and transferred to PVDF membranes. To estimate mouse liver repopulation with PTH, blots were probed with a human alpha-1-antitrypsin (AAT) mouse antibody (1 : 2,000 dilution; Bio-trend, Köln, Germany) which cross-reacts with tupaia but not mouse AAT. Semi-quantitative determination of AAT levels in mice were determined with a streptavidin-HRP conjugate and chemo-luminescent substrate (Pierce, Rockford, Illinois) as described7. To estimate the repopulation with human hepatocytes serum samples diluted in SDS buffer were heat-denatured and directly blotted on nitrocellulose membranes. After incubation with a mouse anti human albumin antibody (1 : 30.000 dilution; Sigma, St.Louis), HSA was detected by using Trueblot Ultra HRP-coupled anti-mouse IgG antibody (1 : 100.000 dilution, Bioscience, San Diego) and the same chemoluminescence system. Semi quantitative determination of the HSA-amounts in mice was performed using a series of mixtures composed of known ratios of human and mouse sera. Viral DNA was extracted from serum samples (20 µl) using the MasterPure DNA purification kit (Epicentre, Germany). Real-time PCR was performed in a LightCycler (Roche, Basel, Switzerland)29 using either HBV-specific primers and hybridization probes as reported22, 29 or the WMHBV-specific primers F: 5´-CTCGTGGTGGACTTCTCTC-3´ and Rev: 5´-CAGCAGGATGAAGAGGAA-3´ and specific fluorescence hybridization probes (FL-CACTCACCAACCTGCTGTCCACCGA and LCRed640-TGTCCTGGGTATCGCTGGATGTGTTT). Known references of HBV-DNA or WMHBV DNA were amplified in parallel to establish a standard curve for quantification. Intrahepatic HBV-DNA values were normalized for cellular DNA content using the beta-globin gene kit (Roche DNA control Kit; Roche Diagnostics). To estimate intrahepatic amounts of replicating virus (rcDNA), total intracellular HBV DNA values were modified to exclude cccDNA22. Hepatitis B surface antigen (HBsAg) was measured by ELISA.

Treatment with acylated HBV-preS peptides and infection experiments. Mice repopulated with tupaia hepatocytes were injected intra-splenically with 50 µl of a saline phosphate buffered solution (PBS) containing 10 mg/kg body weight of either the HBV-specific (HBVpreS/3-39^{myr} or HBVpreS/2-48^{stearoyl}) peptides or control heron peptide (HHBVpreS/2-44^{myr}). In the following days, peptides were re-administered by i.p. injection as indicated in **Fig. 2a**. For the infection studies, mice repopulated with PTH received a single i.p. injection of 20 µl of WMHBV-positive mouse serum (1 x 10^9 WMHBV DNA g.e./ml of serum). Mouse serum samples were obtained from previous infection experiments and stored at -70°C.

For s.c. injection, 100 µl of PBS containing 0.2 mg/kg body weight (N=5) or 2 mg/kg body weight (N=5) of HBVpreS/2-48myr was applied. As a control, 5 repopulated mice received 2 mg/kg body weight of HHBVpreS/2-44myr. Experimental WMHBV infection (2 x 107 g.e.) was performed 1 h later (Fig. 4b). The same amount of peptide solution was further injected s.c. one day later and on days 2, 3 and 5. To infect mice harbouring PHH, 2 x 108 HBV g.e. purified by heparin affinity chromatography from the supernatant of HepG2.2.15 cells30 were inoculated i.p 1 h after subcutaneous administration of 100 µl of PBS containing 2 mg/kg body weight (N=4) of HBVpreS/2-48myr. The same amount of peptide solution was given subcutaneously 24 h later and on days 2, 3 and 5. As control, similar amounts of scramble preS peptides were given to 4 mice repopulated with hepatocytes obtained from the same human liver.

Biodistribution of acylated HBV preS-peptides in mice. The biodistribution of HBVpreS/2-48^{stearoyl} and HBVpreS/2-48^{myr} was studied in male NMRI and uPA$^{+/-}$/RAG-2$^{-/-}$/Pfp$^{-/-}$ mice. All experiments were performed in compliance with German laws. The peptides, containing an additional Tyr-residue at position 49 were labelled with ^{131}I (Amersham Biosciences, Freiburg, Germany) by the chloramine-T method and purified by HPLC. The labelled peptides were s.c. administered by injection of a solution in 10 % DMSO containing 1 % BSA. At selected times mice were sacrificed and the radioactivity contained in the blood, heart, lung, spleen, liver, kidney, muscle, intestine and brain was measured in a γ-counter (Canberra Packard, Rüsselsheim, Germany) and expressed as a percentage of injected dose per gram of tissue (%ID/g). To determine the peptide stability in the liver ^{131}I labelled

HBVpreS/2-48^{myr} was extracted from one liver lobe 24 h post s.c. injection. To that aim, 1 ml water per gramm frozen liver tissue was added to the sample. After homogenization an equal volume of acetonitrile was added and the homogenization was repeated. After centrifugation (2 x 10 min at 4000g) this solution was separated on a reverse phase HPLC column and the radioactivity of each fraction was quantified in a gamma counter.

Immunohistochemistry. Serial cryostat sections of transplanted and non transplanted uPA+/-/RAG-2-/-/Pfp-/- mouse livers were immunostained with a human -specific cytokeratin 18 (Cyt-18) monoclonal antibody (1:200), which specifically recognizes tupaia and human Cyt-18 but not mouse Cyt-18, or with a rabbit HBcAg antiserum (1:200), (both from DAKO Diagnostika, Hamburg, Germany). Specific signals were then detected using Envision anti mouse or anti rabbit HRP, (Dako, Hamburg, Germany) immunoflurescence staining (Invitrogen).

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Figure 1. Schematic representation of the HBV particle, the HBV L-protein and the HBVpreS-derived peptides used in this study. **(a)** The partially double stranded DNA is covalently associated with the viral polymerase complex, consisting of the terminal protein, (TP), the reverse transcriptase (RT) and the RNaseH. The genome is encapsulated by an icosahedral shell, built of 120 core-protein dimers. The 3 HBV surface proteins L-, M- and S- are embedded into an ER-derived lipid bilayer. The L- and M-proteins contain the complete S-domain (red) serving as a membrane anchor. **(b)** Domain structure of the HBV L-protein with its N-terminally myristoylated preS1-domain (pink), the 55 a.a. preS2-domain (orange) and the S-domain (red) containing the 4 transmembrane segments (yellow). The 3 acylated HBVpreS-derived lipopeptides HBVpreS/2-48^{myr}, HBVpreS/2-48^{stearoyl} and HBVpreS/2-39^{myr} used in this study and their *in vitro* inhibitory activities are depicted below. **(c)** Sequence alignment of the first 48 preS-amino acids of the HBV and WMHBV L-protein (above) compared to the corresponding preS-part of HHBV. Note that the sequence identity between HBVpreS 1-48 and WMHBV preS 1-48 is 67% while HHBV preS1-48 shows no significant homology.

Figure 2. Repopulation rates, experimental design and analysis of in vivo inhibition experiments with mice repopulated with tupaia hepatocytes.. **(a)** alpha-1-antitrypsin specific Western Blot analysis of serum samples of the 10 PTH transplanted uPA+/-/RAG-2-/-Pfp-/- mice (left) and 3 control sera containing 0%, 4% and 40% *Tupaia belangeri* serum diluted in mouse serum (right). *Tupaia belangeri* alpha-1-antitrypsin (tu-α) migrates with a slightly lower electrophoretic mobility when compared with its mouse homologue (mu-α) and reacts much stronger with the antibody recognizing human alpha-1-antitrypsin. The peptides HBVpreS/2-39^{myr}, HBVpreS/2-48^{stearoyl} and HHBVpreS/2-44^{myr} (control) or PBS (-) used in different mice are specified below. **(b)** Application scheme and time points for sample preparation. **(c)** Serological analysis of WMHBV infection. WMHBV serum titres 2, 5, 8, 11, 14, 17, 20 and 24 weeks after infection of the control mice (23, 25, 26 and 28) and those treated with HBVpreS/2-39^{myr} (15, 16, 17) and HBVpreS/2-48^{stearoyl} (19, 20, 22). Note that the kinetics of HBV infection is slow but reaches high serum levels at about week 11 p.i. **(d)** HBV core antigen (HBcAg) specific staining (brown) of a liver section of mouse 25 at week 22 p.i. Note that only transplanted hepatocytes express HBcAg and that virtually all of them are infected. The indicated areas of murine and *Tupaia belangeri* hepatocytes were identified by Cyt-18 staining **(e)**. **(f)** *Tupaia belangeri*-specific Cyt-18 staining of a cryostatic liver section of

mouse 19 (treated with HBVpreS/2-48^{stearoyl}) 22 weeks p.i. The deep brown staining indicates the area of the transplanted hepatocytes; V indicates the crossing of central veins. **(g)** Subsequent cryostat liver section of mouse 19 after HBcAg-specific staining. The position of the veins and dotted lines mark the two areas of different hepatocyte populations (PTH, tupaia and MH, murine hepatocytes). Note that no cell stained positive for HBcAg.

Figure 3. Biodistribution and liver stability of HBVpreS-derived lipopetides after s.c. application. **(a)** Liver accumulation of myristoylated (C_{14}) versus stearoylated (C_{18}) HBV preS/2-48 peptides after s.c. injection in male NMRI mice in comparison to the control Fuzeon® (T-20). Per animal approximately 2.25 µg of the ^{131}I-labelled peptides were subcutaneously injected. At the indicated time points animals were sacrificed and the liver specific peptide accumulation (%ID/g) was determined. **(b)** Cyt-18 staining of a liver section of a low populated, *Tupaia belangeri* hepatocyte transplanted uPA/RAG-2 mouse 4 h after injection of ^{131}I-labelled HBVpreS/2-48^{myr}. Islands of transplanted hepatocytes are visible by darker staining. **(c)** Autoradiography of an adjacent slice of the same mouse liver as shown in c. Note that areas with enriched susceptible hepatocytes do not preferentially accumulate the labelled peptide. (d) Reversed phase HPLC of pure ^{131}I-labelled HBVpreS/2-48^{myr} (brown curve) in comparison to liver extracts (orange curve) obtained 24 h post s.c. injection into uPA+/-/RAG-2-/-Pfp-/- mice of the same peptide. Note that 50% of detectable activity is eluting at fraction 13. Since the hydrophobic myristoyl residue is located at the N-terminus and the iodinated Tyr-residue at the C-terminus fraction 13 represents the unaltered full-length peptide.

Figure 4. ***Experimental design and serological analysis of hepadnaviral markers in PTH and PHH-transplanted mice after s.c. application of HBVpreS/2-48-lipopeptides.*** **(a)** alpha-1-antitrypsin specific Western Blot analysis of serum samples of the 15 PTH transplanted uPA+/-/RAG-2-/-Pfp-/- mice used for s.c. applications of 2.0 mg/kg HBVpreS/2-48^{myr} (mouse # 774, 775, 776, 777 and 779), 0.2 mg/kg HBVpreS/2-48^{myr} (mouse # 819, 752, 755, 757 and 759) and the control peptide derived from the heron hepatitis B virus envelope, HHBVpreS/2-44^{myr} (820, 824, 826, 827 and 828). **(b)** Application scheme and time points for sample preparation. **(c)** Time course of WMHBV serum titres after infection of 15 mice, subcutaneously treated with 2mg/kg per dose for 5 days of the control peptide HHBVpreS/2-44^{myr} (violet), 2mg/kg HBVpreS/2-48^{myr} (orange) and 0.2 mg/kg HBVpreS/2-48^{myr} (yellow). Note that 4 out of 5 control mice develop increasing viremia 9 weeks p.i., while treatment even with low doses of HBVpreS/2-48^{myr} lead to abrogation or pronounced repression of infection. **(d)** Serum HBsAg of the 15 subcutaneously treated mice at week 15 post infection. **(e)** HBV g.e. in the sera of PHH-transplanted mice 4 (orange) and 10 (red) weeks after infection. The mice with the numbers 1819, 1821, 1830, and 1831 were treated with a scrambled stearoylated HBVpreS control peptide. Mice with the numbers 1828, 1829, 1834 and 1833 received s.c. HBVpreS/2-48^{stearoyl} according to the scheme in Fig 4b. The repopulation rate of the respective mouse is indicated in an HSA-specific Dot Blot below the bars. **(f)** Standard curve of the immune dot blot with concentrations of 0, 1, 5, 10, 25 and 50 % of human serum. **(g)** Cyt-18 staining of cryostat liver section of mouse #1821 treated with scramble stearoylated HBVpreS control peptide. Areas repopulated with human hepatocytes (HH) are indicated; MH: murine hepatocytes. **(h)** HBcAg (red) and Cyt-18 (green) double staining of serial section from the same mouse liver used in panel g.

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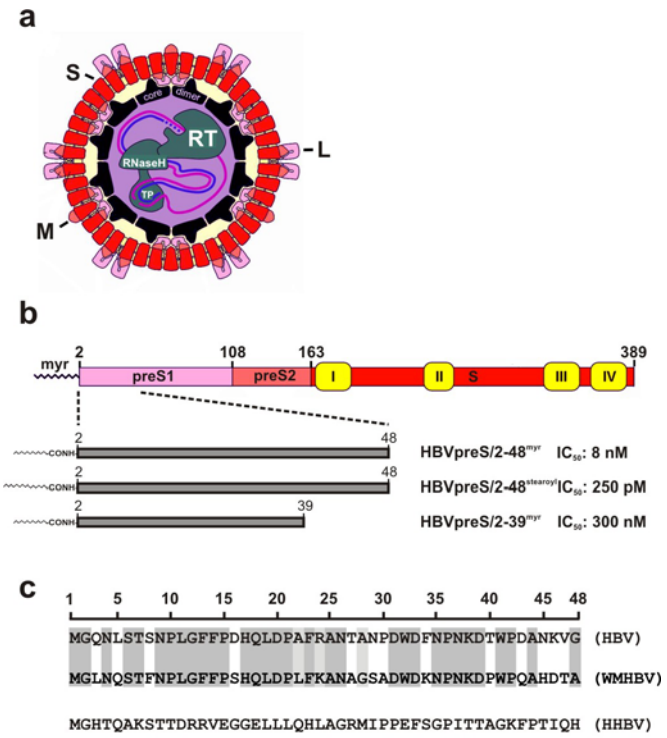

Fig. 1

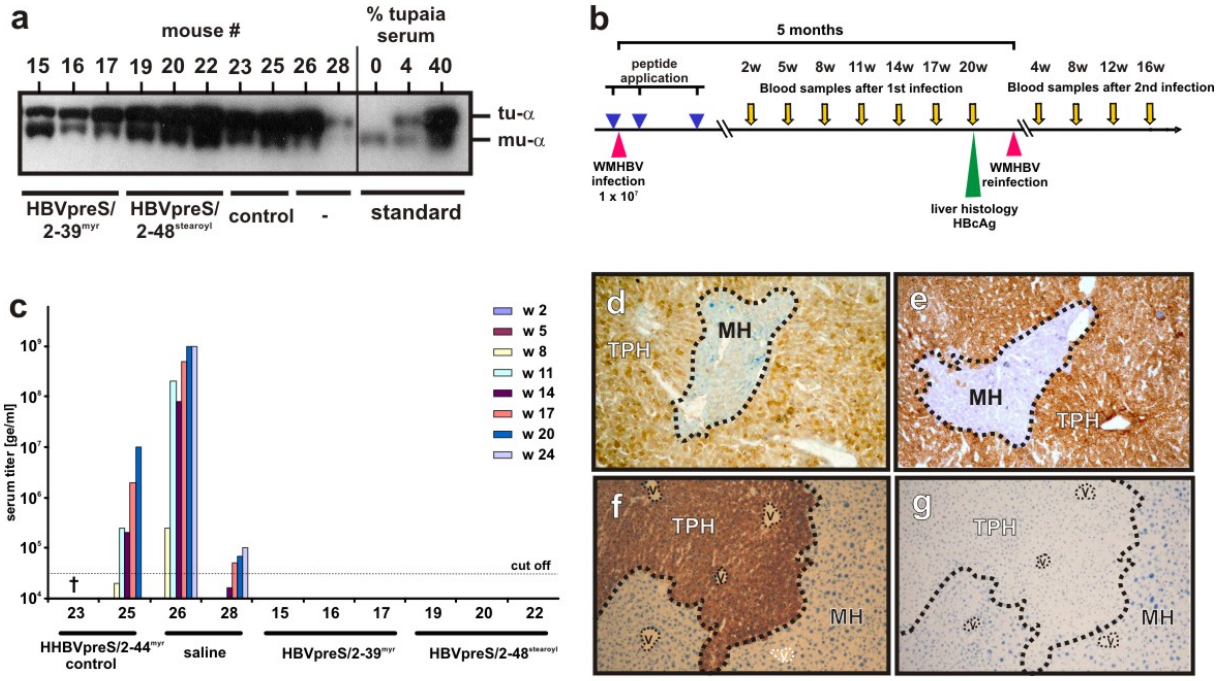
a
mouse #
% tupaia serum
15 16 17 19 20 22 23 25 26 28 0 4 40
tu-α
mu-α
HBVpreS/ 2-39myr
HBVpreS/ 2-48stearoyl
control
-
standard
b
5 months
peptide application
2w 5w 8w 11w 14w 17w 20w
Blood samples after 1st infection
4w 8w 12w 16w
Blood samples after 2nd infection
WMHBV infection 1 x 10⁷
WMHBV reinfection
liver histology HBcAg
c
serum titer [ge/ml]
w 2
w 5
w 8
w 11
w 14
w 17
w 20
w 24
cut off
†
23 25 26 28 15 16 17 19 20 22
HHBVpreS/2-44myr control
saline
HBVpreS/2-39myr
HBVpreS/2-48stearoyl
d
e
f
g
TPH
MH

Fig. 2

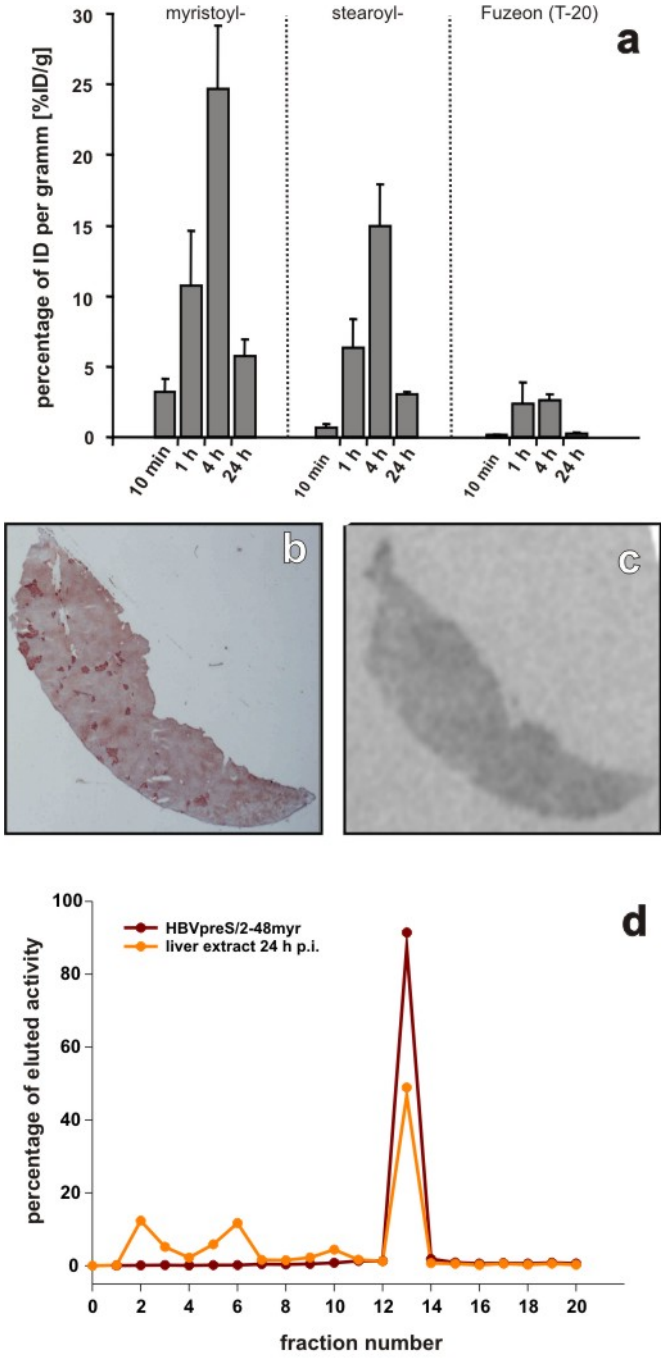
a
myristoyl-
stearoyl-
Fuzeon (T-20)
percentage of ID per gramm [%ID/g]
30
25
20
15
10
5
0
10 min
1 h
4 h
24 h
b
c
d
HBVpreS/2-48myr
liver extract 24 h p.i.
percentage of eluted activity
100
80
60
40
20
0
0
2
4
6
8
10
12
14
16
18
20
fraction number

Fig. 3

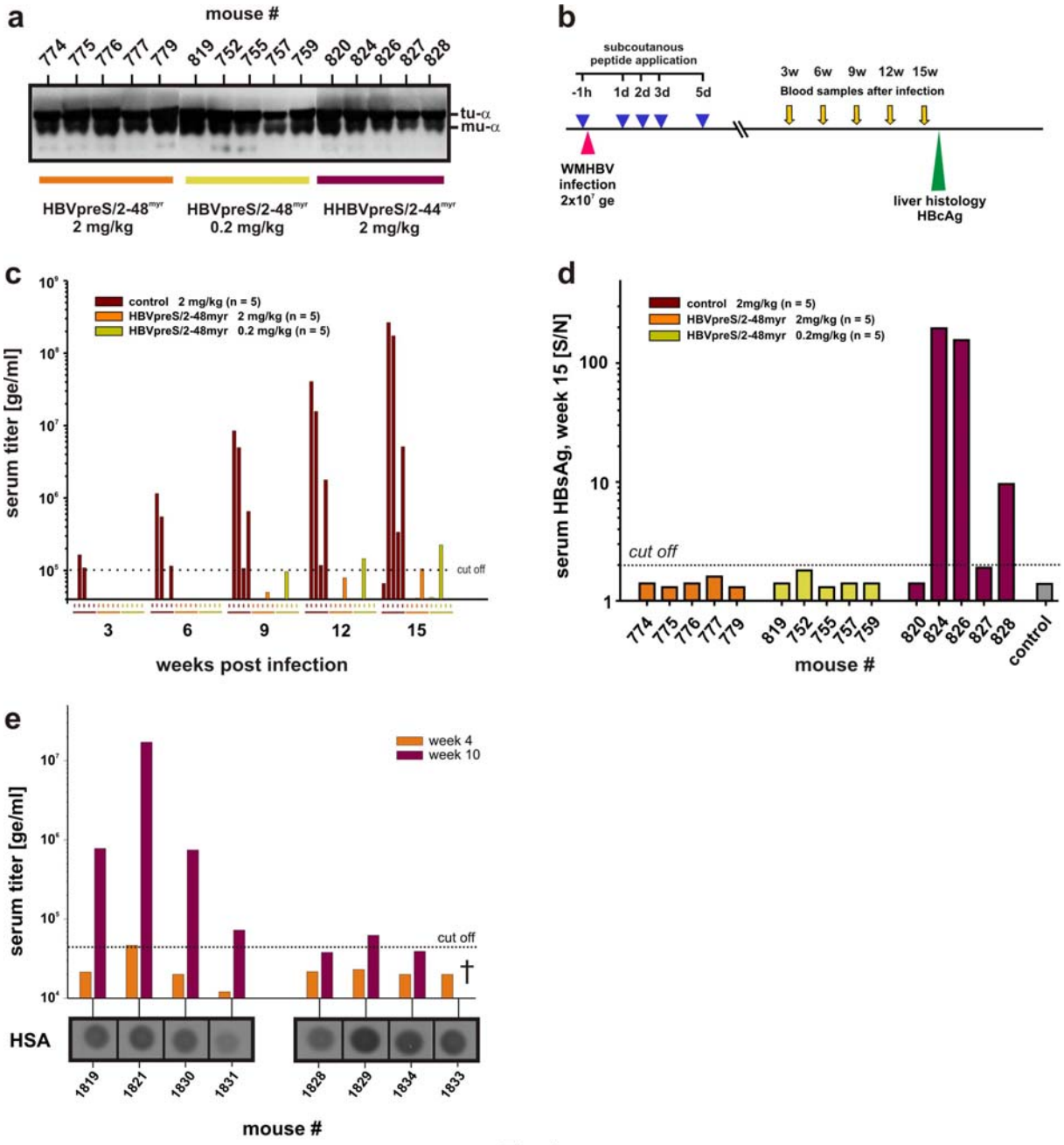
a
mouse #
774 775 776 777 779 819 752 755 757 759 820 824 826 827 828
tu-α
mu-α
HBVpreS/2-48myr 2 mg/kg
HBVpreS/2-48myr 0.2 mg/kg
HHBVpreS/2-44myr 2 mg/kg
b
subcoutanous peptide application
-1h 1d 2d 3d 5d
3w 6w 9w 12w 15w
Blood samples after infection
WMHBV infection 2x10⁷ ge
liver histology HBcAg
c
control 2 mg/kg (n = 5)
HBVpreS/2-48myr 2 mg/kg (n = 5)
HBVpreS/2-48myr 0.2 mg/kg (n = 5)
serum titer [ge/ml]
cut off
3 6 9 12 15
weeks post infection
d
control 2mg/kg (n = 5)
HBVpreS/2-48myr 2mg/kg (n = 5)
HBVpreS/2-48myr 0.2mg/kg (n = 5)
serum HBsAg, week 15 [S/N]
100
10
1
cut off
774 775 776 777 779 819 752 755 757 759 820 824 826 827 828 control
mouse #
e
week 4
week 10
serum titer [ge/ml]
cut off
†
HSA
1819 1821 1830 1831 1828 1829 1834 1833
mouse #

Fig. 4