Evaluation of a fully human, hepatitis B virus-specific chimeric antigen receptor in an immunocompetent mouse model

Marvin M. Festag[1], Julia Festag[1], Simon P. Fräßle[2], Theresa Asen[1], Julia Sacherl[1], Sophia Schreiber[1], Martin Mück-Häusl[1], Dirk H. Busch[2,3], Karin Wisskirchen[1,3*] and Ulrike Protzer[1,3*].

*both authors contributed equally to the work

1 Institute of Virology, Technische Universität München / Helmholtz Zentrum München, 81675 Munich, Germany.

2 Institute for Medical Microbiology, Immunology and Hygiene, Technical University of Munich, 81675 Munich, Germany.

3 German Center for Infection Research (DZIF), Munich partner site, 81675 Munich, Germany.

Corresponding Author: Prof. Ulrike Protzer, MD
Institute of Virology
Trogerstrasse 30, 81675 Munich, Germany
Phone: +49-89-4140-6821, Fax: +49-89-4140-6823
E-mail: protzer@tum.de; protzer@helmholtz-muenchen.de

Karin Wisskirchen, PhD
Institute of Virology
Trogerstrasse 30, 81675 Munich, Germany
Phone: +49-89-4140-6814, Fax: +49-89-4140-6823
E-mail: karin.wisskirchen@helmholtz-muenchen.de

Short title: Evaluating a human CAR in immunocompetent mice

ABSTRACT

Chimeric-antigen-receptor (CAR) T-cell therapy is a promising novel therapeutic approach for cancer but also for chronic infection. We have developed a fully human, second generation CAR directed against the envelope protein of hepatitis B virus on the surface of infected cells (S-CAR). The S-CAR contains a human B cell-derived single-chain antibody fragment and human IgG-spacer, CD28 and CD3 signaling domains that may be immunogenic in mice. Because immunosuppression will worsen the clinical course of chronic hepatitis B, we aimed at developing a preclinical mouse model that is immunocompetent and mimics chronic hepatitis B but nevertheless allows evaluating efficacy and safety of a fully human CAR. The S-CAR grafted on T cells triggered antibody responses in immunocompetent animals, and a co-expressed human-derived safeguard EGFRt even induced B- and T-cell responses - both limiting the survival of S-CAR-grafted T cells. Total body irradiation and transfer of T cells expressing an analogous, signaling-deficient S-CAR-decoy and the safeguard induced immune tolerance towards the human-derived structures. S-CAR T cells transferred after immune recovery persisted and showed long-lasting antiviral effector function. The approach we describe herein will enable preclinical studies of efficacy and safety of fully human CARs in the context of a functional immune system.

INTRODUCTION

T-cell therapies utilizing chimeric antigen receptors (CARs) have emerged as a revolutionary approach to treat cancers and infections with a high specificity during the last two decades.[1] Anti-CD19 CAR T-cell therapy has a significant benefit for patients with B-cell malignancies not responding to first-line chemo- and immune therapies and recently two CAR T-cell products have been approved for clinical use.[2, 3] CAR T cells for other cancer types including solid tumors have also been developed and are currently evaluated in clinical trials. However, it became obvious that CAR T-cell therapy of solid tumors is a more complex scenario.[4] Targets for CAR T-cell therapies include tumor-associated antigens but also viral antigens displayed on the surface of malignant or infected cells. CARs that exploit binders recognizing viral envelope proteins have been developed for chronic infections with hepatitis B virus (HBV),[5] human cytomegalovirus,[6] hepatitis C virus,[7] and human immunodeficiency virus.[8, 9]

A CAR is composed of a single-chain variable fragment (scFv) that determines the target specificity, an extracellular spacer linking the scFv to the signaling domains, a transmembrane domain and intracellular signaling domains. In clinical application, ideally a fully human CAR should be utilized to prevent rejection of CAR T cells by the patient's immune system. Furthermore, accessory molecules co-expressed to purify CAR T cells or as a safeguard to be able to deplete T cells if needed could also be recognized as foreign if they contain non-human-derived domains. In fact, CAR T cells carrying a murine scFv were rejected and their use lead to decreased response rates in patients.[10]

Before advancing to clinical application, CAR constructs have to be studied in preclinical models. In particular, immunocompetent preclinical models are urgently needed to study the efficacy but also potential side effects of a CAR T-cell therapy, because these can be largely influenced by bystander effects of other immune cells or mediators. Immune competence of the animals, however, can limit the preclinical investigation of a CAR with human domains since allogenic immune rejection could limit CAR T-cell persistence in these models. To prevent such an immune response by the endogenous murine immune system, most preclinical studies are performed in immunodeficient mouse models.[11] In the case of anti-CD19 CAR T-cell transfer for hematological malignancies, patients are preconditioned with chemotherapeutic lymphodepleting regimens and hence, using immunodeficient mice mimics this particular clinical situation.[12] However, immunosuppressive or lymphodepleting regimens will most likely not be applied in the clinics for CAR T-cell approaches targeting certain solid tumors or fighting viral diseases. We therefore aimed at generating an experimental, preclinical system in which the recipient is immunocompetent and which at the same time allows the study of CARs with human-derived sequences.

The target of the CAR T-cell approach presented here is HBV. Chronic hepatitis B and HBV-associated hepatocellular carcinoma (HCC) are a major health concern with >250 million humans affected and 880,000 deaths per year due to HBV-associated liver diseases.[13] Current treatment regimens suppress viral replication but are curative only in rare cases. HBV still is the major cause of HCC development worldwide and mainly due to a lack of therapeutic options, HCC became the number two cause of cancer-related death.[14] CAR T-cell therapy is a promising approach to address this medical need [15].

We have generated a fully human CAR that is specific for the small envelope protein "S" of HBV and targets the S-domain (S-CAR) of all HBV envelope proteins,[5] which are found on the surface of HBV virions and subviral particles therefore called hepatitis B surface antigen (HBsAg), but are also located on the surface of HBV-infected hepatocytes and HBV-induced hepatoma cells.[16] Previous results from our laboratory indicated that the S-CAR can redirect T cells against HBV-infected hepatocytes *in vitro* and eliminate HBV from infected cell cultures[5] but that the therapeutic effect of adoptively transferred murine S-CAR T cells into HBV-transgenic immunocompetent mice was limited.[17] After an initial expansion and a very good antiviral effect, S-CAR T cells vanished and viral parameters rose again.

In the study presented here, we show that an immune response against the human domains of the S-CAR limited CAR T-cell persistence in immunocompetent preclinical mouse models but not in immunocompromised animals. We were able to overcome the problem of S-CAR T-cell rejection by specifically tolerizing immunocompetent mice against the allogenic CAR domains. In this setting, S-CAR T cells persisted at high numbers and induced a sustained antiviral effect.

RESULTS

A repeated transfer of S-CAR T cells into immunocompetent mice does not lead to quantitative or functional reconstitution of S-CAR T cells

A loss of S-CAR T-cell function that has been observed after transfer into HBV-transgenic mice[17] could be due to either T-cell exhaustion or activation-induced cell death or an immune response against the transferred cells. To address this question,

121 we investigated whether a second transfer of S-CAR T cells would maintain the
122 antiviral effect. We engineered murine CD45.1$^+$ CD8$^+$ T cells to express the S-CAR
123 (schematically depicted in Figure S1) and transferred them on day 0 and again on
124 day 20 into CD45.1-negative HBV-transgenic mice. A second group of mice received
125 the T-cell product only on day 20 (Figure 1A). The congenic marker CD45.1 allowed
126 to differentiate transferred cells from endogenous, CD45.2$^+$ cells. We detected
127 comparable numbers of total CD45.1$^+$ transferred cells on day 25, i.e. five days after
128 first or second transfer that dropped until day 33, i.e. two weeks after transfer, in both
129 groups (Figure 1B). In contrast to total transferred cells, S-CAR-expressing T cells
130 were only detected after the first but not after the second transfer (Figure 1C).
131 Concomitantly, liver damage indicated by a rise in serum alanine amino transferase
132 (ALT) levels five to seven days after transfer was exclusively detected after the first
133 but not after the second injection of S-CAR T cells (Figure 1D). On day 33,
134 lymphocytes from liver and spleen were isolated and stimulated on plate-bound
135 HBsAg, or anti-CD3/anti-CD28 antibodies as positive control. Phosphate-buffered
136 saline (PBS)-treated plates served as negative control. Intracellular cytokine staining
137 (ICS) did not reveal HBsAg-specific activation of lymphocytes (Figures 1E and S2),
138 although a comparable S-CAR signal was still detected by qPCR in both groups
139 (Figure S3). The fact that neither S-CAR T cells nor liver cytotoxicity were detected
140 after the second adoptive transfer suggested an immune response against the
141 transferred cells rather than a lack of antigenic stimulation or T-cell exhaustion – both
142 of which would not lead to reduced cell numbers in a short term - or activation-
143 induced cell death.

144

Adaptive immunity limits S-CAR T-cell persistence

To find out if the murine immune system would react to the human-derived domains on S-CAR T cells, we first transferred T cells that expressed the S-CAR or a non-functional S-decoy (Δ)-CAR, both in combination with a truncated human epidermal growth factor receptor (EGFRt), into HBV-naïve C57BL/6J mice. The SΔ-CAR construct[17] contains the same extracellular domains as the S-CAR, but intracellular T-cell signaling domains have been exchanged to the cytoplasmic domain of the nerve growth factor receptor rendering the SΔ-CAR uncapable of activating T cells (Figure S1). EGFRt can be targeted by depleting antibodies and serves as a potential safeguard when *in vivo* toxicity is observed, but also as an additional transduction and selection marker.[18, 19] Both CARs contain a mutated, human IgG1 spacer with decreased Fc-receptor binding capacity (Figure S1).[20] The T-cell products had transduction rates of 85 % (S-CAR$^+$/EGFRt$^+$) or 74 % (SΔ-CAR$^+$/EGFRt$^+$) as determined by flow cytometry (Figure 2A). Expansion and persistence of S-CAR and SΔ-CAR T cells were limited in immunocompetent animals compared to that of mock T cells without transgene expression, although all cell products were detected at comparable numbers on day 3 (Figure 2B). When we transferred S-CAR or SΔ-CAR T cells into B- and T-cell deficient Rag2$^{-/-}$ mice, cells expanded and persisted at least as well as mock T cells (Figure 2B). Hence, S-CAR and SΔ-CAR T cells vanished irrespectively of presence of antigen or ability of CAR T cells to be activated, but only in immunocompetent mice and not in immunodeficient mice. This indicated that neither a lack of antigenic stimulation nor tonic signaling is the leading cause of S-CAR T-cell depletion but that an immune response caused the fate of S-CAR T cells.

To investigate the antiviral potential of S-CAR T cells in the absence of anti-CAR immunity, we adoptively transferred the cells into AAV-HBV-infected Rag2$^{-/-}$/IL-2Rγ$^{-/-}$ mice. AAV-mediated HBV genome transfer to the mouse liver allows persistence of the HBV genome over months [21] generating a preclinical model that *a priori* allows to study not only HBV persistence but also “HBV cure”. A cure can be achieved in AAV-HBV-infected mice because only a proportion of hepatocytes is infected and the HBV genome remains episomal allowing elimination of infected hepatocytes. In AAV-HBV-infected Rag2$^{-/-}$/IL-2Rγ$^{-/-}$ mice, S-CAR T cells expanded and were detected for >30 days after transfer (Figure 2C). S-CAR T-cell therapy induced moderate liver damage indicated by a transient increase of serum transaminase activity (ALT) to 70-190 U/l (Figure 2D). To assess the antiviral activity of S-CAR T cells, we determined viral HBsAg (Figure 2E) and e antigen (HBeAg) (Figure 2F) in serum. Notably, HBsAg significantly decreased by about 2 $\log_{10}$ until day 13 and then remained detectable at a low level. HBeAg decreased more slowly by 60 % until day 38. These results indicated that S-CAR T cells expand and exhibit a continuous antiviral effect if they are not targeted by an adaptive immune response.

Immunocompetent mice mount CD8^{+} T-cell and antibody responses against S-CAR T cells

As shown in Figure 2B, S-CAR T cells persisted in Rag2$^{-/-}$ mice that harbor functional NK cells, but neither B nor T cells. Hence, both B and T cells could be responsible for reduced survival of S-CAR T cells. When we analyzed expression of either S-CAR or EGFRt by flow cytometry after T-cell transfer, both markers disappeared in immunocompetent but not in immunodeficient mice (Figures 3A and S4), confirming

the loss of S-CAR T cells. Next, we determined if T-cell responses contributed to S-CAR T-cell rejection in the immunocompetent animals. Thus, we co-cultured splenocytes from wildtype recipient mice that had received 2.7 x 10^6 T cells grafted with both the S-CAR and the EGFRt overnight with $CD8^+$ T cells as target cells expressing either the S-CAR or the EGFRt. ICS revealed that endogenous $CD45.2^+$ $CD8^+$ T cells from the treated mice became activated and expressed IFN-γ if co-cultured with EGFRt- but not with S-CAR-expressing target cells (Figure 3B). This indicated a $CD8^+$ T-cell response against the human-derived EGFRt. In contrast, we did not detect a $CD8^+$ T-cell response against the S-CAR although it also contains human-derived domains, namely the extracellular scFv C8, a human IgG1 spacer, a transmembrane domain from human CD28 and intracellular signaling domains of human CD28 and CD3ζ.

This finding, together with the time kinetics of vanishing S-CAR- or EGFRt-stainings on day 5 or 7, respectively (Figure 3A), indicated an additional immunological mechanism playing a role in S-CAR T-cell rejection. We therefore decided to also test for anti-S-CAR and anti-EGFRt antibodies, incubated our target cells expressing either the S-CAR or EGFRt with mouse sera of recipient mice and stained for bound murine IgG antibodies. Flow cytometry analysis revealed antibody production against both the S-CAR and EGFRt molecules (Figure 3C). To confirm anti-S-CAR antibodies and to investigate which domains of the S-CAR were targeted by the antibodies, an enzyme-linked immunosorbent assay (ELISA) was established. This ELISA confirmed the presence of antibodies against both extracellular domains of the S-CAR, namely the human IgG1 spacer (Figure 3D) and the scFv C8 (Figure 3E).

As an attempt to prevent an antibody response against the S-CAR, the number of immunogenic extracellular epitopes was reduced by exchanging the spacer to a murine IgG1 domain (Figure S1) and excluding the EGFRt. The intracellular signaling domains were left unaltered as no T-cell response against the S-CAR had been detected. Thus, in this murine IgG1 S-CAR construct only the human-derived scFv C8 remained as a potentially immunogenic extracellular epitope. To study the effect of an antibody response against the scFv C8, we transferred murine IgG1 S-CAR T cells into HBV-transgenic mice. In murine IgG1 S-CAR T cell-treated mice, numbers of transferred cells declined with kinetics comparable to those in mice treated with S-CAR T cells containing a human IgG1 (Figure 3F). While no anti-hIgG1 antibodies were detected anymore, we still detected antibodies directed against the human-derived scFv C8 domain (Figure 3G).

Taken together, the EGFRt elicited B- and T-cell responses in immunocompetent mice, while the S-CAR elicited only antibody responses. Antibody responses were directed against the scFv C8 binder, which is of human origin, as well as the human IgG1 spacer domain within the S-CAR. Since the human scFv binder was also targeted by antibodies, a reduction of immunogenic epitopes did not prevent rejection of S-CAR T cells by the endogenous immune system. Exchanging the scFv, however, does not allow preclinical evaluation of a CAR anymore. Thus, alternative models are required for preclinical evaluation of a human CAR.

Irradiation allows long-term persistence of S-CAR T cells

Sublethal total body irradiation is an option to prevent rejection of cells expressing alloantigens.[22] To establish tolerance against S-CAR- and EGFRt-expressing T cells

239 in AAV-HBV-infected immunocompetent mice, recipients were irradiated one day
240 before T-cell transfer (Figure 4A). Under this condition, S-CAR T cells expanded and
241 persisted until day 140 in peripheral blood (Figure 4B and C, gating strategy depicted
242 in Figure S5). Importantly, even when B cells and $CD8^+$ T cells (Figure 4D) as well as
243 $CD4^+$ T cells and NK cells (Figure S6) had reached physiological concentrations
244 again 80 days after irradiation, the concentration of S-CAR T cells remained stable.

245 At day 140 after transfer, S-CAR T cells were still detected at high numbers in liver
246 and spleen (Figure 4E) and allowed characterizing their phenotype. The majority of
247 S-CAR T cells (60-70 %) in both organs exhibited an effector phenotype ($CD62L^-$
248 $CD127^-$) (Figures 4F and S7A). Mock-transduced $CD8^+$ T cells, in contrast, showed a
249 phenotype of naïve or central-memory T cells ($CD62L^+$ $CD127^+$, 60-70 % in liver, 89-
250 94 % in spleen). When exhaustion markers were analyzed, a high percentage of S-
251 CAR T cells were positive for PD-1 but only about 25 % expressed Tim-3, and CTLA-
252 4 was barely detected at all (Figures 4G and S7B). To investigate the functionality of
253 S-CAR T cells after *in vivo* circulation for more than four months, cells from liver and
254 spleen isolated on day 140 after transfer were re-stimulated *ex vivo* on plate-bound
255 HBsAg. ICS revealed that the transduced S-CAR T cells could still be activated and
256 expressed the proinflammatory cytokines IFN-γ and to a lower extent TNF-α upon
257 antigen encounter (Figure 4H). Furthermore, irradiation prevented the development
258 of anti-human-IgG1 and anti-scFv C8 antibodies upon S-CAR T-cell transfer (Figure
259 4I). In summary, irradiation of immunocompetent mice prior to T-cell transfer allowed
260 expansion and long-term persistence of S-CAR T cells, which developed an effector
261 phenotype and to a considerable proportion remained functional.

262

S-CAR T cells have long-term antiviral function in irradiated, immunocompetent mice

Having shown that transferred S-CAR T cells could expand and survive in AAV-HBV-infected and irradiated wildtype mice, we next determined their antiviral effect in this model. Mice that received S-CAR T cells, both with and without prior irradiation, displayed 2- to 4-fold elevated serum ALT levels on day 7 (Figure 5A). Around day 40, irradiated mice treated with S-CAR T cells but not the other groups showed a moderate ALT elevation again. Serum HBsAg levels dropped by 1 $\log_{10}$ until day 30 in both S-CAR T cell-treated groups independent of prior irradiation. However, HBsAg subsequently rebounded in mice without prior irradiation reaching pretreatment levels again around day 80 (Figure 5B). One mouse (not irradiated) developed spontaneous HBV-immunity >80 days after S-CAR T-cell treatment and seroconverted with a drop in HBsAg of >3 $\log_{10}$ and anti-HBsAg antibodies detectable on day 140 (Figure 5C). In all irradiated and S-CAR T cell-treated mice, HBsAg continued to decrease to <1 % of pretreatment values until day 140 (Figure 5B) and HBeAg continuously dropped (Figure 5D). This was not observed in non-irradiated or mock T cell-treated animals. The antiviral effect was confirmed by qPCR analysis of liver DNA. AAV as well as HBV DNA copies in the liver were significantly reduced in irradiated and S-CAR T cell-treated mice compared to the other groups (Figure 5E). Thus, when initiation of immune responses against the human-derived S-CAR was prevented by irradiation, S-CAR T cells expanded, persisted long-term and elicited a significant antiviral effect in AAV-HBV-infected mice. Whether low-level persistence of HBsAg and HBeAg was due to the fact that the observation period was limited to 140 days or to the fact that the largely reduced antigen levels were not sufficient to stimulate the S-CAR anymore cannot be clarified.

S-CAR-specific tolerization of mice allows T-cell persistence and antiviral efficacy

To further improve the model and allow S-CAR T-cell transfer into fully immunocompetent animals, we aimed at inducing antigen-specific tolerance to the human-derived CAR domains and EGFRt before S-CAR T-cell transfer. To this end, non-functional SΔ-CAR T cells co-expressing EGFRt were transferred into AAV-HBV-infected mice one day after irradiation (Figure 6A). SΔ-CAR T cells should neither proliferate nor show any effector function in AAV-HBV-infected mice. We hypothesized that the presence of the human alloantigens from SΔ-CAR and EGFRt during recovery of the endogenous immune system would allow to induce specific immune tolerance.

As observed before, irradiation of mice induced a depletion of endogenous B- and T-cell populations that were restored in numbers after two months (Figure S8A-C). SΔ-CAR/EGFRt T cells injected at the time of irradiation persisted at low concentration for more than three months (Figure S8D). After three months functional S-CAR T cells that were additionally grafted with EGFRt were injected. Mice that had been irradiated and had received SΔ-CAR T cells neither mounted an antibody response against the human IgG1 or scFv C8 domains of the S-CAR (Figure 6B) nor developed a $CD8^+$ T-cell response against the EGFRt (Figure 6C). In contrast, mice only irradiated but not tolerized using SΔ-CAR T cells mounted B- and T-cell responses against the human alloantigens. Functional S-CAR T cells proliferated well and persisted in tolerized mice until the end of the study, i.e. day 110 after S-CAR T-cell transfer (Figure 6D), but rapidly vanished from peripheral blood in mice that had not been tolerized. This shows that functionality of the endogenous immune system

against the foreign antigens S-CAR and EGFRt had been re-established after irradiation at the time point when functional S-CAR T cells were transferred. On the other hand, it proves that antigen-specific immune tolerance induced by the SΔ-CAR T-cell transfer after irradiation was sufficient to allow persistence of S-CAR T cells.

In the tolerized animals, in which S-CAR T cells expanded and survived, we then determined the antiviral effect of S-CAR T cells. Serum ALT levels remained slightly elevated after S-CAR T-cell treatment, although statistically significant only on day 110 (Figure 6E). Viral HBsAg decreased by 2 $\log_{10}$ in tolerized animals, but remained unaltered in the other groups (Figure 6F). Both serum HBeAg levels (Figure 6G) as well as AAV-DNA and HBV-DNA copies in the liver (Figure 6H) decreased about 60 % in comparison to control groups. Again, despite being quite efficient, S-CAR T-cell therapy did not fully cure mice from HBV infection and a proportion of HBV-positive hepatocytes persisted.

DISCUSSION

Appropriate mouse models are needed for thorough preclinical investigations of CAR T-cell products. While a CAR ideally consists of only human-derived domains when applied to a patient, the same construct may be recognized as foreign in immunocompetent mice. Hence, therapeutic efficacy and safety profiles might be altered due to limited CAR T-cell persistence. Here we show that induction of adaptive immunity is indeed an issue when investigating CAR T cells harboring human domains in an immunocompetent mouse model. After initial expansion, HBV-specific S-CAR T-cell numbers rapidly declined and a second S-CAR T-cell transfer

was unable to induce an antiviral effect anymore. Cells that express either the CAR or EGFRt as a safeguard, could be used to detect antibodies in serum of treated mice and $CD8^+$ T-cell responses via flow cytometry-based assays. These experiments showed that EGFRt was targeted by both humoral and cellular immune responses. While we could only detect humoral immune responses against the S-CAR these were unfortunately at least partially directed against the scFv binder as confirmed by specifically developed ELISAs. The scFv C8 binder as the only essential domain of human origin was still sufficient to induce antibody responses and a loss of S-CAR T cells. The rapid decrease of CAR T cells also occurred in HBV-negative mice, or when CAR T cells lacked signaling domains or harbored a spacer with reduced Fc-receptor binding capacity (Figure S1).[20] Hence, we concluded that neither T-cell exhaustion, nor activation induced cell death due to overactivation by antigen or by binding of Fc-receptors to the CAR, could have played a role in reduced S-CAR T-cell persistence.

When mice were irradiated directly before T-cell transfer, we were able to induce long-term tolerance to the S-CAR and EGFRt alloantigens with S-CAR T cells being detected at high numbers even 140 days after transfer in peripheral blood, spleen and liver. Since S-CAR T cells were transferred only one day after irradiation, when the immune system was strongly depressed, one could argue that the mice were not fully immunocompetent. Therefore, we investigated if we could induce a specific tolerance to the alloantigens by an immediate transfer of non-functional SΔ-CAR T cells. Although these cells persisted only at low numbers due to a lack of an activation signal, their numbers were sufficient to induce specific tolerance against the human-derived domains, and functional S-CAR T cells were able to persist for

more than three months in constantly high numbers and to elicit an antiviral function even when encountering a fully reconstituted immune system.

Our results show that a specific immune tolerance has been induced by transfer of the non-functional SΔ-CAR T cells preventing rejection of S-CAR T cells after immune reconstitution. Full reconstitution of the immune system at the time point of S-CAR T cell transfer was indicated since mice that had only been irradiated but did not receive an early transfer of SΔ-CAR T cells rejected the S-CAR T cells. Immune tolerance can be achieved by two distinct means, namely central and peripheral tolerance. We propose that central tolerance is the mode-of-action of tolerance induction to transferred S-CAR T cells.

Central tolerance is induced in the thymus when during T-cell development and after T-cell receptor gene rearrangement, T cells are assessed for their specificity.[23] Only T cells with a non-self T-cell receptor specificity can leave the thymus and become part of the pool of mature peripheral T cells. Since auto-reactive T cells are excluded this way, the T-cell pool usually does not target self-tissue and auto-immune diseases remain a rare event.[23] If autoreactive T cells escape negative selection in the thymus or an antigen is only encountered later in life, peripheral tolerance comes into play.[24] Tissue damage is prevented by conversion of T cells to Tregs, induction of T-cell apoptosis, T-cell exhaustion or anergy by e.g. metabolic alteration. B cells experience similar selection mechanisms.[25] In their case, central tolerance is achieved during maturation in the bone marrow. If autoreactive B cells escape negative selection, absent $CD4^+$ T-cell help in the periphery prevents B-cell activation and antibody production.

In our setting, the alloantigens expressed on transferred T cells (namely extracellular domains of the S-CAR and the EGFRt) were present during replenishment of the immune cell pool after irradiation. It was previously reported that intrathymic antigen inoculation after total body irradiation can induce selective non-responsiveness to bovine gamma globulin as an alloantigen in rats.[22] Similarly, intrathymic transplantation of pancreatic islet allografts after lymphodepletion led to acceptance of islet grafts both in- and outside the thymus.[26] Hereby, clonal deletion induced by recognition of alloantigens was identified as mode-of-action for selective non-responsiveness.[27] For our case, we propose the following concept of non-responsiveness to the human-derived antigens: adoptively transferred SΔ-CAR T cells are distributed throughout the body and will migrate to the thymus. Here, cross-presentation of peptides by thymic dendritic cells[28] induces negative selection for both $CD4^+$ and $CD8^+$ T cells with specificities for S-CAR or EGFRt epitopes. This would directly prevent $CD8^+$ T-cell responses, and indirectly B-cell responses because of a lacking $CD4^+$ T-cell help. Our data indicate that the presence of the alloantigens during recovery from total body irradiation deluded the immune system in a way that the human-derived domains are considered self-antigens and must not be targeted.

The situation may be comparable to the clinical setting. Murine components of CAR T-cell products have been reported to be immunogenic in humans. Stronger lymphodepleting preconditioning with fludarabine instead of only cyclophosphamide-based regimens lead to improved and sustained engraftment of CAR T cells. However, the mechanism by which fludarabine increased CAR T-cell survival and whether immune responses against murine domains were delayed or prevented

remains unclear.[10] It may well be the same mechanism we describe in our mouse model.

In our setting, S-CAR T-cell therapy had a sustained antiviral effect without inducing apparent therapy-limiting side effects; however, it was not yet able to “cure” the AAV-HBV infection during the 110 days of treatment. One possible explanation would be an insufficient antigenic stimulation, i.e. that the affinity of the scFv C8 is not high enough to detect low amounts of S protein on the membrane. This has been described for an anti-CD20 CAR.[29] Alternatively, S protein may not be present on the membrane in a proportion of hepatocytes. T-cell cytokines can downregulate HBV protein expression[30, 31] and can even deplete the HBV persistence form[32, 33] without killing infected cells. This will largely reduce the antigen expression level in HBV-positive cells and prevent elimination by T cells. In a clinical setting this limitation may be overcome if patients were selected for high and homogenous expression of viral proteins on the cell surface of HBV-infected cells or HBV-induced HCC tissue in liver biopsies.

A second explanation could be inefficient endogenous bystander immunity targeting HBV in the irradiated mice. Since HBV antigens are also continuously present in high amounts when the immune system recovers from irradiation, clonal deletion resulting in selective non-responsiveness to HBV is possible.[27] A third explanation could be the liver micro-environment where the anti-HBV immunity needs to become effective. To preserve integrity of the liver as an essential organ, its microenvironment is particularly prone to allow foreign antigens to escape immunity. This is e.g. exploited by pathogens like HBV, hepatitis C virus or malaria sporozoites that persist in the liver and by this frequently escape immune clearance but also explains the

extraordinary tolerance of orthotopic liver transplants.[34, 35] This may be overcome if the S-CAR T cell-induced antiviral immune response paved the way for endogenous immune cells to fight the infection.

For reliable preclinical assessment of T-cell therapies, it seems important that the chosen preclinical model closely reflects the anticipated clinical scenario. In particular if chronic viral infections or particular solid cancers shall be treated, there will be a need to apply CAR T cells without prior immunosuppression although lymphodepletion seems to support CAR T-cell efficacy. As a potential reason for the beneficial effect of lymphodepletion, competition for cytokines with endogenous immune cells but also alteration of the tumor microenvironment or simply a lack of space for the transferred cells to expand have been discussed.[36] To fully understand the benefit of lymphodepletion prior to T-cell transfer, orthotopic and immune competent preclinical models are required and will help to improve clinical efficacy of CAR T-cell therapy in settings other than hematological malignancies.[37]

For adoptive T-cell therapy of chronic HBV infection and HBV-associated HCC it is essential to rely on preclinical models using immunocompetent animals as this reflects the clinical situation. For both diseases, lymphodepleting regimes are contraindicated. Several studies have reported that chemotherapy in chronic HBV carriers leads to virus reactivation.[38-40] Especially lymphodepletion via cyclophosphamide, which is commonly used before T-cell transfer, leads to reactivation of HBV in up to one third of patients.[41] Already the depletion of B cells using anti-CD20 antibodies results in life-threatening HBV reactivation.[42] Therefore, preclinical evaluation of S-CAR T-cell therapy in an immunocompetent rather than an

immunocompromised mouse model is needed, since it is likely to provide an efficacy and safety profile that has relevance for clinical application.

Importantly, the model described here is transferable to other CAR T-cell approaches, e.g. the treatment of solid tumors, that shall be evaluated in immunocompetent preclinical models and utilize human scFv. In comparison to models using immunodeficient mice, the tolerized animals offer the advantage that they have a fully functional immune system at the time of CAR T-cell transfer allowing to investigate interactions with and activation of the endogenous immune system and how this influences efficacy and safety of the therapy. Tumor infiltration by bystander immune cells will certainly contribute to an anti-tumor response but potentially also to a cytokine storm.[37] To evaluate all consequences of a cytokine storm, cytokine receptors matching between transferred T cells and host tissue are required as it is the case in our model. Furthermore, in this model a combination therapy with checkpoint inhibitors targeting CAR T cells but also endogenous immune cells can be evaluated.

The model described here is the only model that allows studying a CAR with human-derived domains in the context of an intact immune system besides humanized mouse models harboring human immune cells. In contrast to humanized mouse models, our model allows to transfer syngeneic murine T cells which is of special importance for long-term studies of cell-cell interactions and to avoid misinterpretation of anti-tumor efficacy by graft-versus-host reactions.[43] Compared to humanized mouse models, our model is less laborious, cheaper and more physiological.

Taken together, by irradiation and subsequent tolerization with a signaling-deficient CAR, we were able to induce long-lasting, specific tolerance to human-derived CAR domains, and could study the engraftment, proliferation, long-term persistence and antiviral effector function of S-CAR T cells in fully immunocompetent mice. We believe that this model can be transferred to other CAR T-cell approaches in case they require preclinical evaluation in the context of a fully functional immune system. It will allow for the study of interactions with the different arms of the endogenous immune system, bystander immune cell activation and combination therapies with checkpoint inhibitors. Thus, it will help to bring better characterized, more efficient and safer cell products into the clinics.

MATERIALS AND METHODS

Animal models

HBVtg HBV1.3xfs mice (HBV genotype D, serotype *ayw*), $Rag2^{-/-}$ mice, $Rag2^{-/-}$/IL-$2R\gamma^{-/-}$ mice, and $CD45.1^{+}$ C57BL/6 donor mice were bred in-house in specific pathogen-free animal facilities. Adeno-associated virus (AAV) serotype 2 containing the 1.2 overlength genome of HBV genotype D (AAV-HBV) was packed with an AAV serotype 8 capsid as previously described.[21] Viral vector was produced by Plateforme de Thérapie Génique (Nantes, France). For the AAV-HBV model, 8-week-old male wildtype C57BL/6J mice were purchased from Janvier (Le Genest-Sain-Isle, France) and infected with 2×10^{10} viral particles 3-4 weeks before T-cell transfer. The study was conducted according to the German Law for the Protection of Animals.

Retroviral transduction and adoptive T-cell transfer

Splenocytes were isolated from donor mice and enriched for $CD8^+$ T cells using CD8a MACS beads (Miltenyi Biotec, Bergisch Gladbach, Germany). A total of 1.5 x 10^6 $CD8^+$ T cells/well were stimulated for 24 hours with 5 ng/ml IL-12 (kindly provided by E. Schmitt, University of Mainz) on tissue-treated 12-well plates that were precoated with anti-CD3 and anti-CD28 antibodies (kindly provided by R. Feederle, Helmholtz Zentrum München) for 2 hours at 37 °C (10 µg/ml in PBS). The next day, cells were transferred to uncoated 12-well plates and retrovirally transduced two days in a row. Retroviral supernatants were obtained from Platinum-E packaging cells transfected with MP71 retroviral plasmids containing CAR coding sequences. Activated $CD8^+$ T cells and retroviral supernatants were supplemented with 2 µg/ml protamine sulfate (Leo Pharma, Neu-Isenburg, Germany) and spinoculated (850 x g, 32 °C, 2 hours). The day after the second transduction, cells were analyzed by flow cytometry and the transduction rate determined as described below. Cells were washed twice with PBS, resuspended in PBS and transferred intraperitoneally in 200 µl into mice that were grouped by HBsAg and HBeAg levels. If applicable, mice were irradiated with 5 Gray one day before T-cell transfer.

Isolation of splenocytes, liver-associated lymphocytes and peripheral blood mononuclear cells

Spleens were mashed through a 100 µm cell strainer and erythrocytes lysed using ACK lysis buffer (8 g NH_4Cl, 1 g $KHCO_3$, 37 mg Na_2EDTA, add to 1 l H_2O, pH 7.2-7.4) for 2 min at RT. Livers were perfused with PBS to eliminate circulating lymphocytes in blood and mashed through a 100 µm cell strainer. Mashed liver tissue was digested with 4500 U collagenase type 4 (Worthington, Lakewood, USA) (20

min, 37 °C) and leukocytes were purified in an 80 %/40 % Percoll (GE Healthcare, Solingen, Germany) gradient (1400 x g, 20 min, RT, without brake). For peripheral blood mononuclear cell isolation, peripheral blood was collected into Microvette 500 LH-Gel tubes (Sarstedt, Nümbrecht, Germany) and 15 µl of blood was incubated with 250 µl ACK lysis buffer for 2 min at RT, and then resuspended in fluorescence-activated cell sorting (FACS) buffer (0.1 % bovine serum albumin in PBS).

Flow cytometry

Staining of cells was performed for 30 min in the dark on ice in FACS buffer (0.1 % bovine serum albumin in PBS). Antibodies were purchased from different suppliers: CD4, CD8, CD19, CD45.1, IFN-γ, TNF-α (BD Biosciences, Heidelberg, Germany); CD3, CD45.2, CD62L, CD127, NK1.1, PD-1 (Thermo Fisher Scientific, Germering, Germany); CTLA-4, Tim-3 (Biolegend, Koblenz, Germany). Viable cells were determined with live/dead cell marker (Thermo Fisher Scientific). For intracellular cytokine staining cells were permeabilized using Cytofix/Cytoperm (BD Biosciences) prior to incubation with antibodies following manufacturer's instruction. Total cell numbers were determined by the addition of CountBright™ Absolute Counting Beads (Thermo Fisher Scientific). Cells were analyzed on a FACS Canto II (BD Biosciences) or CytoFLEX S (Beckman Coulter, Munich, Germany). If a CAR, EGFRt and other surface markers were analyzed, first the CAR was stained with an anti-human-IgG (Abcam, Cambridge, UK) or anti-murine-IgG (BD Biosciences) antibody, followed by the primary staining of EGFRt with biotin-labelled cetuximab (Merck, Darmstadt, Germany). In a last step, bound cetuximab was stained with fluorochrome-labelled streptavidin (Thermo Fisher Scientific) together with additional antibodies against surface markers.

549 Cultivation of murine cells

550 Primary murine cells were cultured in murine T-cell medium (RPMI Dutch modified,
551 10 % FCS, 1 % glutamine, 1 % Pen/Strep, 1 % sodium pyruvate and 50 µM β-
552 mercaptoethanol; Thermo Fisher Scientific).

553 *Ex vivo* T-cell stimulation

554 Functionality of S-CAR T cells was determined by culturing 5 x 10^5 splenocytes or
555 liver-associated lymphocytes/well on tissue-treated 96-well plates precoated with
556 HBsAg (2.5 µg/ml in PBS, overnight, 4 °C; Roche Diagnostics, Mannheim, Germany)
557 or anti-CD3 and anti-CD28 antibodies (10 µg/ml in PBS, overnight, 4 °C). To
558 determine an immune response against the S-CAR and EGFRt, 1 x 10^6 splenocytes
559 were cultured with 1 x 10^5 S-CAR$^+$ or EGFRt$^+$ CD8$^+$ T cells. After 1 hour of culture, 1
560 µg/ml Brefeldin A (Sigma-Aldrich, Munich, Germany) was added. Cytokine
561 expression was determined the following day via an intracellular cytokine staining
562 and flow cytometry analysis.

563 Cell-based anti-S-CAR and anti-EGFRt antibody detection

564 Platinum-E cells were transfected with MP71 plasmids encoding the S-CAR or
565 EGFRt. After 48 hours, cells were harvested, and a flow cytometry staining was
566 performed. Cells were stained with serum diluted 1:200 in FACS buffer and in a
567 subsequent staining step incubated with PE-labeled anti-mouse-IgG antibody (12-
568 4010-82; Thermo Fisher Scientific). Median fluorescence intensity was determined
569 on a CytoFLEX S (Beckman Coulter).

570 ELISA-based anti-C8 and anti-IgG1 antibody detection

ELISA plates were precoated overnight with recombinant scFv C8 (1 µg/ml in PBS) or IgG1 cetuximab antibody (1 µg/ml in PBS; Merck) at 4 °C. The next day, plates were blocked with assay diluent (1 % bovine serum albumin in PBS) for 1 hour at RT. Diluted serum of treated mice was incubated on wells for 2 hours and bound antibodies were detected with an HRP-labelled anti-mouse-IgG antibody (1:1000, Sigma-Aldrich). TMB substrate (Thermo Fisher Scientific) conversion (OD_{450} nm - OD_{560} nm) was measured on an infinite F200 photometer (Tecan, Männedorf, Switzerland) and the signal of serum on uncoated wells subtracted.

Intrahepatic AAV and HBV DNA copies

DNA was extracted from approximately 20 mg of liver issue using the Nucleo Spin Tissue Kit (Macherey-Nagel, Berlin, Germany) following manufacturer's instructions. Quantitative PCR was performed with SyBrGreen (Roche Diagnostics) on a LightCycler® 480 II (Roche Diagnostics) using the following primers: AAVfw: AACCCGCCATGCTACTTATCTACGT; AAVrev: CACACAGTCTTTGAAGTAGGCC; HBVfw: GCCTCATCTTCTTGTTGGTTC; HBVrev: GAAAGCCCTACGAACCACTGAAC. Results were normalized to cell numbers using the single copy gene *PRNP*: PRNPfw: TGCTGGGAAGTGCCATGAG; PRNPrev: CGGTGCATGTTTTCACGATAGTA.

Serological analyses

Peripheral blood was collected into Microvette 500 LH-Gel tubes (Sarstedt) and centrifuged to separate serum (10 min, 5000 x g, RT). ALT activity was determined 1:4 diluted with PBS using the Reflotron ALT test (Roche Diagnostics). Serum HBsAg, HBeAg and anti-HBsAg antibody were quantified in different dilutions with PBS on an Architect^{TM} platform using the quantitative HBsAg test (Ref.: 6C36-44;

595 Cutoff: 0.25 IU/ml), the HBeAg Reagent Kit (Ref.: 6C32-27) with HBeAg Quantitative
596 Calibrators (Ref.: 7P24-01; Cutoff: 0.20 PEI U/ml) and the anti-HBs test (Ref.: 7C18-
597 27; Cutoff: 12.5 mIU/ml) (Abbott Laboratories, Wiesbaden, Germany).

598 Production of recombinant scFv C8

599 *E. coli* XL1-blue were transformed with scFv C8-encoding pHOG21 plasmid and a
600 single clone colony inoculated overnight in 5 ml LB media (10 g Tryptone, 5 g yeast
601 extract, 10 g NaCl, add to 1 l H_2O). The next day, 3 l of 2x YT (17 g Tryptone, 10 g
602 yeast extract, 5 g NaCl, add to 1 l H_2O) were inoculated 1:1000 and grown for
603 approximately 10 hours until the OD_{600} reached 0.6. Induction was performed
604 overnight at 18 °C with 0.1 mM IPTG (Carl Roth, Karlsruhe, Germany). Large-scale
605 protein purification was performed by fast protein liquid chromatography under native
606 conditions on an ÄKTA avant (GE Healthcare). Bacterial cells of the overnight
607 induction culture were harvested (15 min, 5000 x g, RT) and resuspended in 10 ml
608 ÄKTA binding buffer (20 mM disodium phosphate, 500 mM NaCl, 20 mM imidazole,
609 pH 7.4) per 1 g of bacterial pellet. 3 U/ml benzonase (Merck) and 0.2 mg/ml
610 lysozyme (Thermo Fisher Scientific) were added, followed by incubation for 20 min
611 on ice. The samples were then submitted to five cycles of sonication 1 min each, and
612 centrifuged (30 min, 5000 x g, 4 °C). Samples were continuously kept on ice.

613 A HisTrap™ FF crude 5 ml column (GE Healthcare) was connected to the ÄKTA
614 avant (GE Healthcare) and loaded with lysate. Samples were eluted by gradually
615 increasing the proportion of elution buffer (20 mM disodium phosphate, 500 mM
616 NaCl, 500 mM imidazole, pH 7.4) with at flow rate of 5 ml/min collecting 1 ml
617 fractions. The protein content of the eluent was measured by UV monitoring at 280
618 nm. According to the chromatographic peaks, the respective fractions were analyzed

by SDS-PAGE and Coomassie staining in order to confirm protein presence. Positive fractions were pooled and dialyzed to storage buffer (25 mM Tris-HCl, 100 mM KCl, 1 mM EDTA, 1 mM $MgCl_2$, 10 % Glycerol, pH 7.4) overnight at 4 °C. The final sample was filtered and protein concentration was measured via a Nanodrop One (Thermo Fisher Scientific).

AUTHOR CONTRIBUTIONS

MMF, JF, TA and KW conducted the experiments; SS and JS produced critical reagents and helped to perform experiments; SPF helped with experimental set-up and performed irradiation; DHB provided essential infrastructure and technical support; MMF, KW, MMH and UP designed the experiments; MMF, KW and UP wrote the paper.

CONFLICT OF INTEREST

The work was funded by the German Research Foundation (DFG) via TRR36 and the German Center for Infection Research (DZIF). UP and KW are co-founders of and MF was part-time employed by SCG Cell Therapy, Singapore. KW is consulting for LION TCR / SCG Cell Therapy, Singapore. UP serves as ad-hoc scientific advisor for Roche, GILEAD; J&J, Abbvie, Merck, Arbutus and VIR Biotechnology.

ACKNOWLEDGMENTS

We are grateful to Michael Jensen for providing the EGFRt construct, Hinrich Abken for providing CAR constructs, and Marie-Louise Michel for providing the AAV-HBV1.2 construct. We thank Philipp Hagen, Natalie Röder and Romina Bester for excellent technical assistance and Claudia Dembek for critical reading of the manuscript.

REFERENCES

1. Gill, S., Maus, M.V., and Porter, D.L. (2016). Chimeric antigen receptor T cell therapy: 25 years in the making. Blood reviews *30*: 157-167.
2. Brower, V. (2017). First Chimeric Antigen Receptor T-Cell Therapy Approved. J Natl Cancer Inst *109*.
3. Mullard, A. (2017). Second anticancer CAR T therapy receives FDA approval. Nat Rev Drug Discov *16*: 818.
4. Hartmann, J., Schüßler- Lenz, M., Bondanza, A., and Buchholz, C.J. (2017). Clinical development of CAR T cells—challenges and opportunities in translating innovative treatment concepts. EMBO molecular medicine: e201607485.
5. Bohne, F., Chmielewski, M., Ebert, G., Wiegmann, K., Kurschner, T., Schulze, A.*, et al.* (2008). T cells redirected against hepatitis B virus surface proteins eliminate infected hepatocytes. Gastroenterology *134*: 239-247.
6. Full, F., Lehner, M., Thonn, V., Goetz, G., Scholz, B., Kaufmann, K.B.*, et al.* (2010). T cells engineered with a cytomegalovirus-specific chimeric immunoreceptor. J Virol *84*: 4083-4088.
7. Sautto, G.A., Wisskirchen, K., Clementi, N., Castelli, M., Diotti, R.A., Graf, J.*, et al.* (2016). Chimeric antigen receptor (CAR)-engineered T cells redirected against hepatitis C virus (HCV) E2 glycoprotein. Gut *65*: 512-523.
8. Hale, M., Mesojednik, T., Romano Ibarra, G.S., Sahni, J., Bernard, A., Sommer, K.*, et al.* (2017). Engineering HIV-Resistant, Anti-HIV Chimeric Antigen Receptor T Cells. Molecular therapy : the journal of the American Society of Gene Therapy *25*: 570-579.
9. Leibman, R.S., Richardson, M.W., Ellebrecht, C.T., Maldini, C.R., Glover, J.A., Secreto, A.J.*, et al.* (2017). Supraphysiologic control over HIV-1 replication mediated by CD8 T

669 cells expressing a re-engineered CD4-based chimeric antigen receptor. PLoS pathogens
670 *13*: e1006613.

671 10. Turtle, C.J., Hanafi, L.A., Berger, C., Gooley, T.A., Cherian, S., Hudecek, M.*, et al.*
672 (2016). CD19 CAR-T cells of defined CD4+:CD8+ composition in adult B cell ALL
673 patients. J Clin Invest *126*: 2123-2138.

674 11. Siegler, E.L., and Wang, P. (2018). Preclinical Models in Chimeric Antigen Receptor-
675 Engineered T-Cell Therapy. Human gene therapy *29*: 534-546.

676 12. Hay, K.A., and Turtle, C.J. (2017). Chimeric Antigen Receptor (CAR) T Cells: Lessons
677 Learned from Targeting of CD19 in B-Cell Malignancies. Drugs *77*: 237-245.

678 13. WHO (2017). Hepatitis B, Fact sheet, Updated July 2017 http://www.who.int/en/news-
679 room/fact-sheets/detail/hepatitis-b.

680 14. Sartorius, K., Sartorius, B., Aldous, C., Govender, P.S., and Madiba, T.E. (2015). Global
681 and country underestimation of hepatocellular carcinoma (HCC) in 2012 and its
682 implications. Cancer Epidemiol *39*: 284-290.

683 15. Gehring, A., and Protzer, U. (2019). Targeting Innate and Adaptive Immune Responses
684 to Cure Chronic HBV Infection. Gastroenterology, in press (ePub ahead of print).

685 16. Safaie, P., Poongkunran, M., Kuang, P.P., Javaid, A., Jacobs, C., Pohlmann, R.*, et al.*
686 (2016). Intrahepatic distribution of hepatitis B virus antigens in patients with and without
687 hepatocellular carcinoma. World journal of gastroenterology *22*: 3404-3411.

688 17. Krebs, K., Bottinger, N., Huang, L.R., Chmielewski, M., Arzberger, S., Gasteiger, G.*, et*
689 *al.* (2013). T cells expressing a chimeric antigen receptor that binds hepatitis B virus
690 envelope proteins control virus replication in mice. Gastroenterology *145*: 456-465.

691 18. Wang, X., Chang, W.C., Wong, C.W., Colcher, D., Sherman, M., Ostberg, J.R.*, et al.*
692 (2011). A transgene-encoded cell surface polypeptide for selection, in vivo tracking, and
693 ablation of engineered cells. Blood *118*: 1255-1263.

694 19. Paszkiewicz, P.J., Frassle, S.P., Srivastava, S., Sommermeyer, D., Hudecek, M.,
695 Drexler, I.*, et al.* (2016). Targeted antibody-mediated depletion of murine CD19 CAR T
696 cells permanently reverses B cell aplasia. J Clin Invest *126*: 4262-4272.

697 20. Hombach, A., Hombach, A.A., and Abken, H. (2010). Adoptive immunotherapy with
698 genetically engineered T cells: modification of the IgG1 Fc 'spacer' domain in the
699 extracellular moiety of chimeric antigen receptors avoids 'off-target' activation and
700 unintended initiation of an innate immune response. Gene therapy *17*: 1206-1213.

21. Dion, S., Bourgine, M., Godon, O., Levillayer, F., and Michel, M.L. (2013). Adeno-associated virus-mediated gene transfer leads to persistent hepatitis B virus replication in mice expressing HLA-A2 and HLA-DR1 molecules. J Virol *87*: 5554-5563.

22. Staples, P.J., Gery, I., and Waksman, B.H. (1966). Role of the thymus in tolerance: III. Tolerance to bovine gamma globulin after direct injection of antigen into the shielded thymus of irradiated rats. Journal of Experimental Medicine *124*: 127-139.

23. Starr, T.K., Jameson, S.C., and Hogquist, K.A. (2003). Positive and negative selection of T cells. Annu Rev Immunol *21*: 139-176.

24. Mueller, D.L. (2010). Mechanisms maintaining peripheral tolerance. Nat Immunol *11*: 21-27.

25. Nemazee, D. (2017). Mechanisms of central tolerance for B cells. Nat Rev Immunol *17*: 281-294.

26. Posselt, A.M., Barker, C.F., Tomaszewski, J.E., Markmann, J.F., Choti, M.A., and Naji, A. (1990). Induction of donor-specific unresponsiveness by intrathymic islet transplantation. Science *249*: 1293-1295.

27. Turvey, S.E., Hara, M., Morris, P.J., and Wood, K.J. (1999). Mechanisms of tolerance induction after intrathymic islet injection: determination of the fate of alloreactive thymocytes. Transplantation *68*: 30-39.

28. Proietto, A.I., Lahoud, M.H., and Wu, L. (2008). Distinct functional capacities of mouse thymic and splenic dendritic cell populations. Immunology and cell biology *86*: 700-708.

29. Watanabe, K., Terakura, S., Martens, A.C., van Meerten, T., Uchiyama, S., Imai, M.*, et al.* (2015). Target antigen density governs the efficacy of anti-CD20-CD28-CD3 zeta chimeric antigen receptor-modified effector CD8+ T cells. Journal of immunology (Baltimore, Md : 1950) *194*: 911-920.

30. Guidotti, L.G., Ishikawa, T., Hobbs, M.V., Matzke, B., Schreiber, R., and Chisari, F.V. (1996). Intracellular inactivation of the hepatitis B virus by cytotoxic T lymphocytes. Immunity *4*: 25-36.

31. Guidotti, L.G., Rochford, R., Chung, J., Shapiro, M., Purcell, R., and Chisari, F.V. (1999). Viral clearance without destruction of infected cells during acute HBV infection. Science *284*: 825-829.

731 32. Lucifora, J., Xia, Y., Reisinger, F., Zhang, K., Stadler, D., Cheng, X.*, et al.* (2014).
732 Specific and nonhepatotoxic degradation of nuclear hepatitis B virus cccDNA. Science
733 *343*: 1221-1228.

734 33. Xia, Y., Stadler, D., Lucifora, J., Reisinger, F., Webb, D., Hosel, M.*, et al.* (2016).
735 Interferon-gamma and Tumor Necrosis Factor-alpha Produced by T Cells Reduce the
736 HBV Persistence Form, cccDNA, Without Cytolysis. Gastroenterology *150*: 194-205.

737 34. Protzer, U., Maini, M.K., and Knolle, P.A. (2012). Living in the liver: hepatic infections.
738 Nat Rev Immunol *12*: 201-213.

739 35. Knolle, P.A., and Thimme, R. (2014). Hepatic immune regulation and its involvement in
740 viral hepatitis infection. Gastroenterology *146*: 1193-1207.

741 36. Berger, C., Turtle, C.J., Jensen, M.C., and Riddell, S.R. (2009). Adoptive transfer of
742 virus-specific and tumor-specific T cell immunity. Curr Opin Immunol *21*: 224-232.

743 37. Srivastava, S., and Riddell, S.R. (2018). Chimeric Antigen Receptor T Cell Therapy:
744 Challenges to Bench-to-Bedside Efficacy. Journal of immunology (Baltimore, Md : 1950)
745 *200*: 459-468.

746 38. Cheng, J.C., Liu, M.C., Tsai, S.Y., Fang, W.T., Jer-Min Jian, J., and Sung, J.L. (2004).
747 Unexpectedly frequent hepatitis B reactivation by chemoradiation in postgastrectomy
748 patients. Cancer *101*: 2126-2133.

749 39. Lok, A.S., Liang, R.H., Chiu, E.K., Wong, K.L., Chan, T.K., and Todd, D. (1991).
750 Reactivation of hepatitis B virus replication in patients receiving cytotoxic therapy. Report
751 of a prospective study. Gastroenterology *100*: 182-188.

752 40. Nakamura, Y., Motokura, T., Fujita, A., Yamashita, T., and Ogata, E. (1996). Severe
753 hepatitis related to chemotherapy in hepatitis B virus carriers with hematologic
754 malignancies. Survey in Japan, 1987-1991. Cancer *78*: 2210-2215.

755 41. Ma, B., Yeo, W., Hui, P., Ho, W.M., and Johnson, P.J. (2002). Acute toxicity of adjuvant
756 doxorubicin and cyclophosphamide for early breast cancer–a retrospective review of
757 Chinese patients and comparison with an historic Western series. Radiotherapy and
758 oncology *62*: 185-189.

759 42. Perceau, G., Diris, N., Estines, O., Derancourt, C., Levy, S., and Bernard, P. (2006).
760 Late lethal hepatitis B virus reactivation after rituximab treatment of low-grade cutaneous
761 B-cell lymphoma. Br J Dermatol *155*: 1053-1056.

762 43. Shultz, L.D., Brehm, M.A., Garcia-Martinez, J.V., and Greiner, D.L. (2012). Humanized
763 mice for immune system investigation: progress, promise and challenges. Nat Rev
764 Immunol *12*: 786-798.

765

766

767 **FIGURE LEGENDS**

768 **Figure 1: Sequential transfers of S-CAR T cells into HBV-transgenic mice. A)**
769 Scheme of the experimental procedure. CD45.2$^+$ HBV-transgenic mice were
770 injected once (day 20, grey symbols) or twice (day 0 and day 20, black symbols)
771 with 4 x 10^6 CD45.1$^+$ S-CAR$^+$ T cells each (n=7 per group). Transferred, CD45.1$^+$
772 cells in peripheral blood and serum parameters were monitored over time. **B)**
773 Numbers of CD45.1$^+$ T cells per µl peripheral blood, **C)** numbers of S-CAR$^+$ T cells
774 in peripheral blood and **D)** ALT activity in sera at indicated time points. **E)**
775 Lymphocytes were isolated from liver and spleen on day 33 and cultured on
776 HBsAg- or anti-CD3/anti-CD28- or PBS-coated control plates overnight. Activation
777 of CD45.1$^+$ T cells was determined by intracellular staining or IFN-γ and TNF-α
778 followed by flow cytometry analysis. B, C, D: Data points represent individual
779 animals, mean values ± SD are indicated. E: Data are given as mean values ± SD.
780 ns = not significant, * = p<0.05, ** = p<0.01, *** = p<0.001 (Mann Whitney test).

781

782 **Figure 2: S-CAR T-cell engraftment in immunocompetent and**
783 **immunodeficient mice.** 2.7 x 10^6 CD45.1$^+$ S-CAR$^+$/EGFRt$^+$, SΔ-CAR$^+$/EGFRt$^+$ or
784 mock T cells were transferred into HBV-naïve CD45.2$^+$ wildtype C57BL/6J (n=5
785 per group) or Rag2$^{-/-}$ mice (n=3 per group). **A)** CAR and EGFRt expression on
786 CD8$^+$ T cells determined by flow cytometry at day of transfer. **B)** Numbers of

transferred CD45.1^{+} cells in peripheral blood were determined over time by flow cytometry. **C)-F)** AAV-HBV-infected CD45.2^{+} Rag2$^{-/-}$/IL-2Rγ$^{-/-}$ mice received 1 x 10^{6} S-CAR^{+}/EGFRt^{+} T cells each (n=5, grey triangles) or remained untreated (n=3, open triangles). CD45.1^{+} cells in peripheral blood and serum parameters were monitored over time. **C)** Numbers of S-CAR^{+} or EGFR^{+} cells per µl blood, **D)** serum ALT activity, **E)** HBsAg and **F)** HBeAg levels were determined. All data are given as mean values ± SD. ns = not significant, * = $p<0.05$ (Mann Whitney test).

Figure 3: B- and T-cell responses against S-CAR and EGFRt after T-cell transfer. A)-E) 2.7 x 10^{6} CD45.1^{+} S-CAR^{+}/ EGFRt^{+} CD8^{+} T cells were transferred into CD45.2^{+} HBV-naïve wildtype (wt, mock = open circles, S-CAR = black boxes, n=5 per group) or Rag2$^{-/-}$ mice (S-CAR = grey boxes, n=3) (see also Fig. 2B). **A)** Surface expression levels of S-CAR and EGFRt were determined by median fluorescence intensity (MFI) on CD45.1^{+} CD8^{+} T cells in peripheral blood at indicated time points after transfer. **B)** Splenocytes were isolated from treated wt mice on day 18 post transfer and co-cultured overnight with mock, S-CAR or EGFRt-expressing CD8^{+} T cells. IFN-γ expression by endogenous CD45.2^{+} CD8^{+} T cells was determined via ICS. **C)** Detection of S-CAR- and EGFRt-specific antibodies in serum of mice on day 18 post transfer. Binding of antibodies to S-CAR- or EGFRt-expressing target cells was determined via bound fluorochrome-labeled secondary anti-mouse IgG antibody by flow cytometry. **D)** Detection of anti-hIgG1 or **E)** anti-scFv C8 antibodies in serial dilutions of mouse sera from day 3 or day 18 post transfer by ELISA. **F)-G)** 2 x 10^{6} CD45.1^{+} T cells expressing an S-CAR with either human (n=3, back boxes) or murine IgG1 spacer domains (n=4, grey triangles) were transferred into CD45.2^{+} HBV-transgenic mice. **F)** Numbers of transferred, CD45.1^{+}

cells per µl peripheral blood. **G)** Detection of anti-hIgG1 or anti-scFv C8 antibodies in 1:200 diluted mouse sera from day 26 by ELISA. A, D, E: Data are given as mean values ± SD. B, C, F, G: Data points represent individual animals, mean values are indicated (in B, C and F ± SD). ns = not significant, ** = $p<0.01$ (Mann Whitney test).

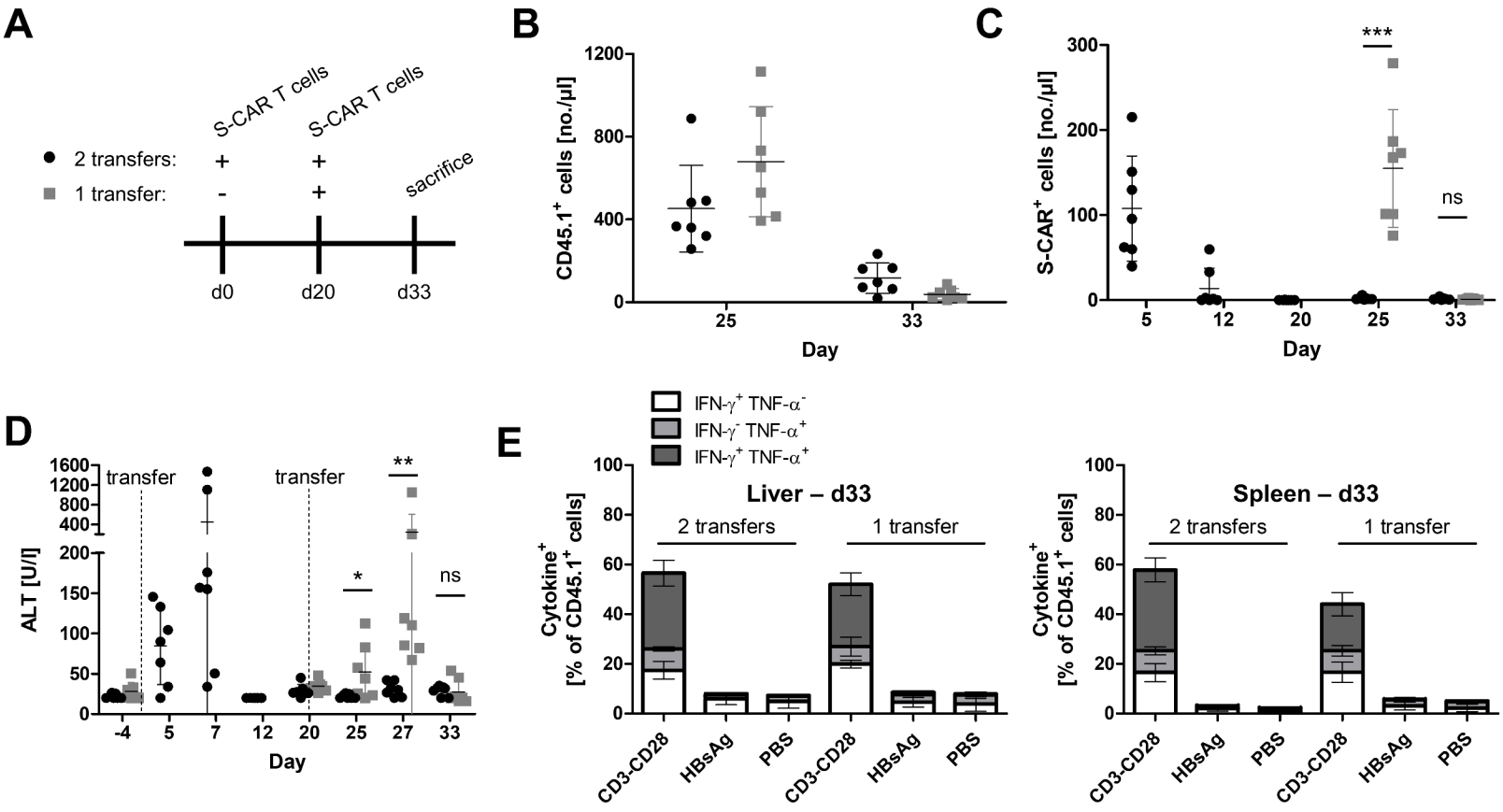
Figure 4: S-CAR T-cell engraftment in irradiated mice. A) Scheme of the experimental procedure. AAV-HBV-infected $CD45.2^+$ wildtype mice were injected with 1 x 10^6 $CD45.1^+$ $S\text{-}CAR^+/EGFRt^+$ or mock T cells per animal one day after sublethal total body irradiation (S-CAR = black boxes, mock = open circles), or without prior irradiation (S-CAR = grey triangles) (n=4 per group). **B)** Exemplary flow cytometry plot of (transferred) $CD45.1^+$ and (endogenous) $CD45.1^-$ $CD8^+$ T cells in peripheral blood on day 28. **C)** The amount of $S\text{-}CAR^+$ or $EGFRt^+$ T cells per µl peripheral blood was determined by flow cytometry at indicated time points. **D)** Amount of (endogenous) $CD45.1^-$ $CD19^+$ B cells (left panel) or $CD8^+$ T cells (right panel) in peripheral blood. Arrows mark time point of irradiation. **E)** Count of $S\text{-}CAR^+$ or $EGFRt^+$ cells in liver and spleen on day 140. **F)** Phenotype of $CD8^+$ T-cell subsets of transferred $CD45.1^+$ T cells in irradiated mice determined by flow cytometry after staining for CD62L and CD127: effector ($CD62L^-$ $CD127^-$), effector memory ($CD62L^-$ $CD127^+$), intermediate ($CD62L^+$ $CD127^-$), naïve / central memory ($CD62L^+$ $CD127^+$). **G)** Expression of exhaustion markers (PD-1, CTLA-4, Tim-3) on $CD45.1^+$ lymphocytes isolated from liver and spleen of irradiated mice on day 140. **H)** *Ex vivo* functionality of S-CAR T cells of irradiated mice determined by overnight culture on plate-bound HBsAg or PBS as control. ICS for IFN-γ and TNF-α. **I)** Sera from days -8, 14 and 140 were analyzed by ELISA for anti-hIgG1 and anti-scFv C8 antibodies. C, D, F, H: Data are given as mean values ± SD. E, G, I: Data points represent

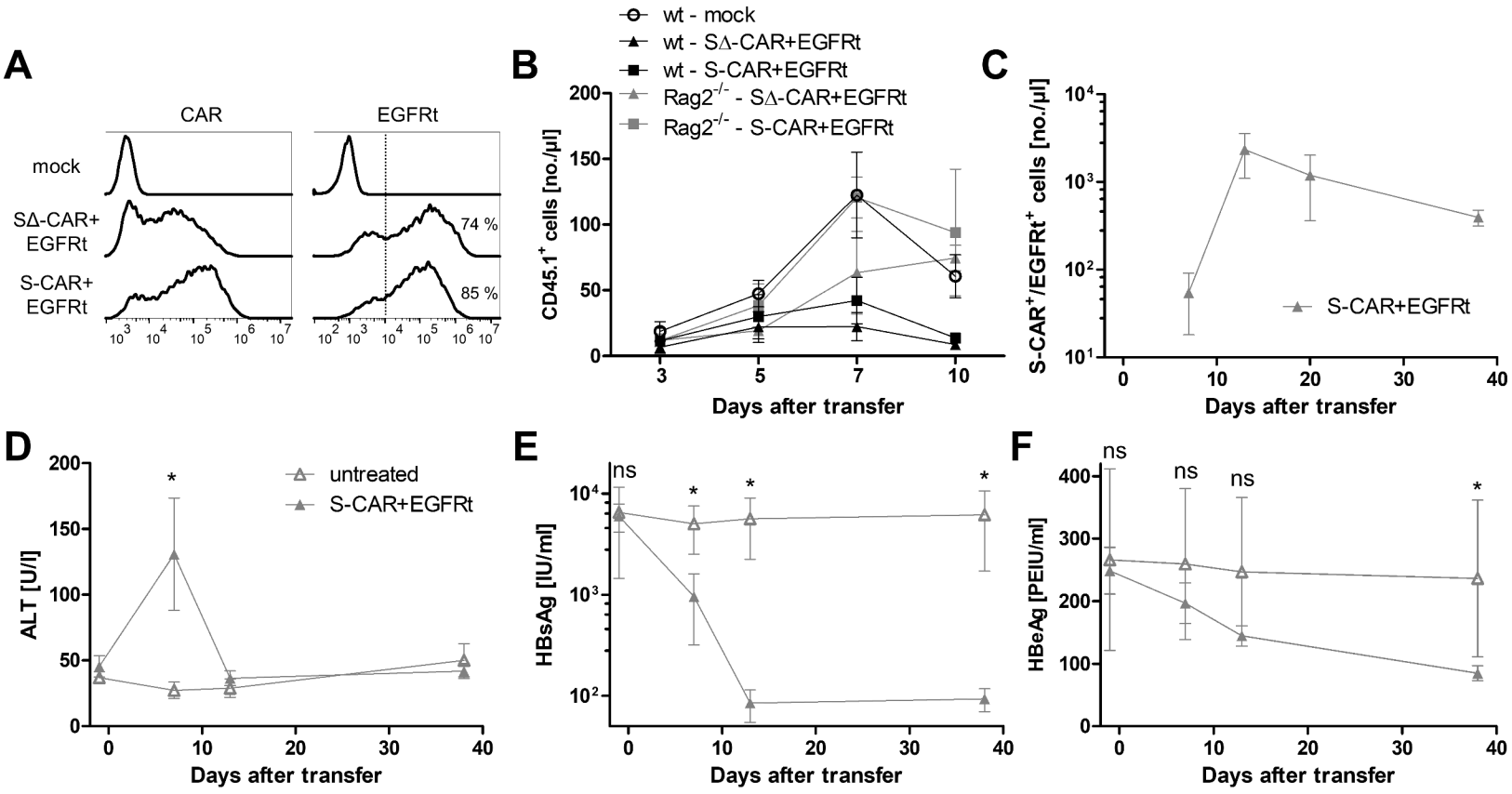
individual animals, mean values ± SD are indicated. Dotted line represents the background determined in mock T cell-treated mice. * = p<0.05 (Mann Whitney test).

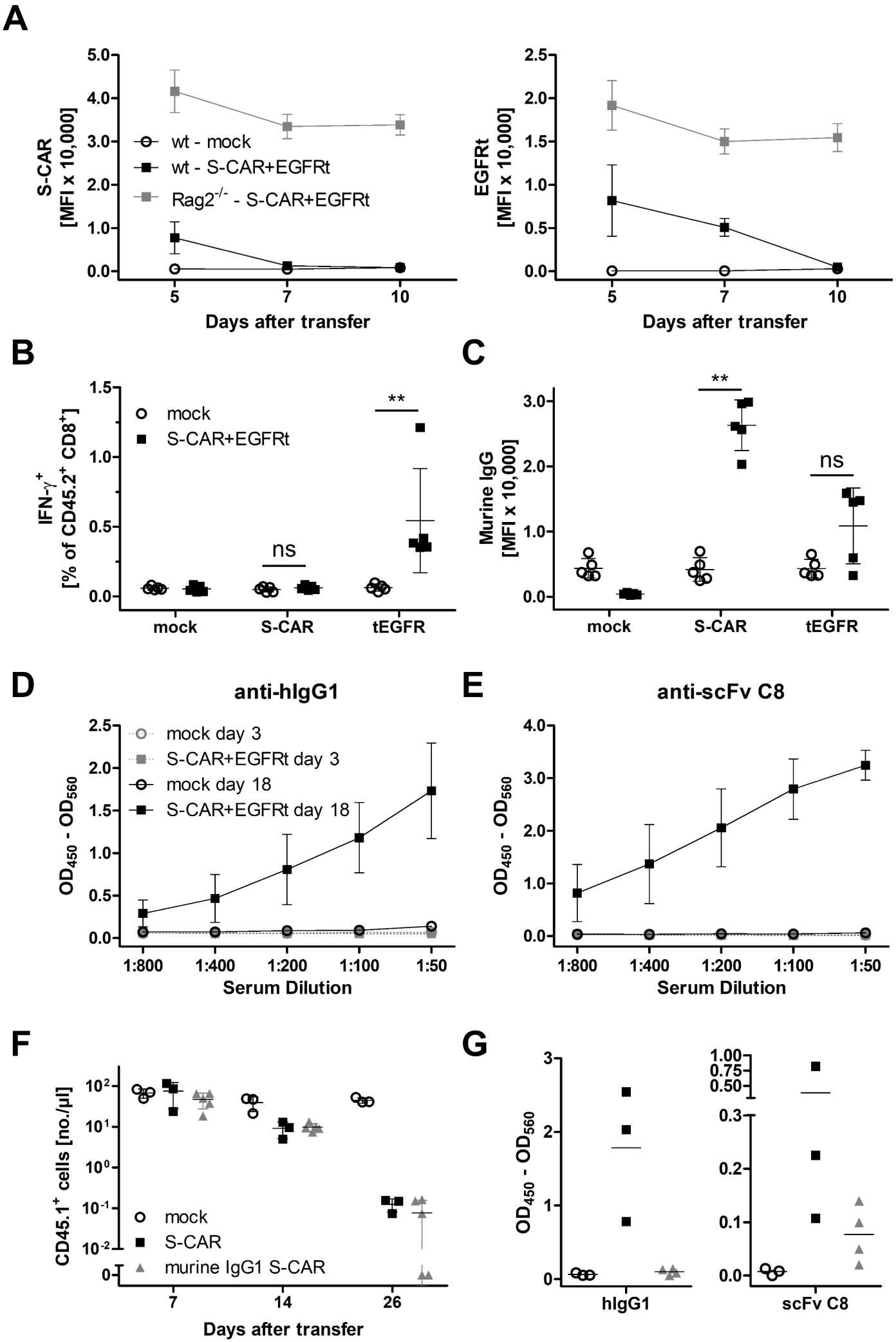
Figure 5: Antiviral effect of S-CAR T cells in irradiated mice. Identical experimental set-up as in Figure 4. **A)** ALT activity and **B)** HBsAg levels in serum over time. **C)** Inverse correlation of HBsAg and anti-HBsAg antibody concentrations in a single mouse that underwent spontaneous seroconversion. This animal was excluded from B). **D)** HBeAg levels in serum over time**. E)** Intrahepatic AAV- and HBV-DNA copies/cell were determined by qPCR and normalized to cell numbers using the single copy gene *PRNP*. Individual animals are indicated relative to the mean value determined in mock treated animals (set to 100 %). A, B, D: Data are given as mean values ± SD. E: Data points represent individual animals, mean values ± SD are indicated. ns = not significant, * = p<0.05 (Mann Whitney test).

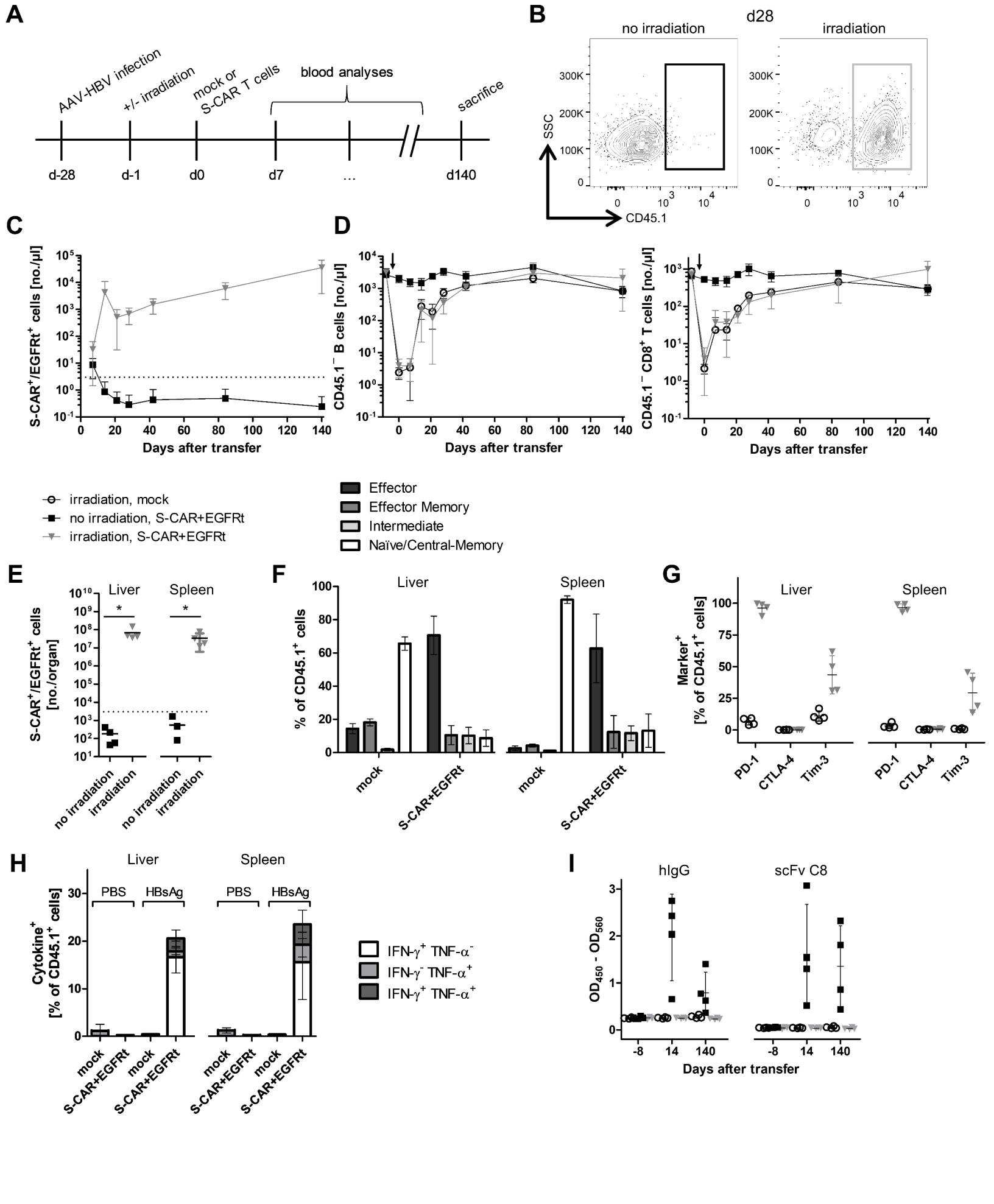
Figure 6: S-CAR T-cell persistence and antiviral effect after tolerization of immunocompetent mice. A) Scheme of the experimental procedure. AAV-HBV-infected CD45.2$^+$ wildtype mice underwent total body irradiation one day prior to the transfer of 5 x 10^6 non-functional, CD45.1$^+$ SΔ-CAR$^+$/EGFR$^+$ T cells per animal (n=5 per group). 3 months later mice were injected with 3 x 10^6 functional CD45.1$^+$/CD45.2$^+$ mock (open circles) or S-CAR$^+$ and EGFRt$^+$ T cells per animal (=day 0). Mice that received S-CAR$^+$/EGFRt$^+$ T cells were either only irradiated (= irradiation, black squares) or were irradiated and received SΔ-CAR$^+$/EGFRt$^+$ T cells (= tolerization, grey triangles). Mice that received mock T cells were not pretreated. Mice were sacrificed 110 days after transfer of functional S-CAR T cells. **B)** Sera

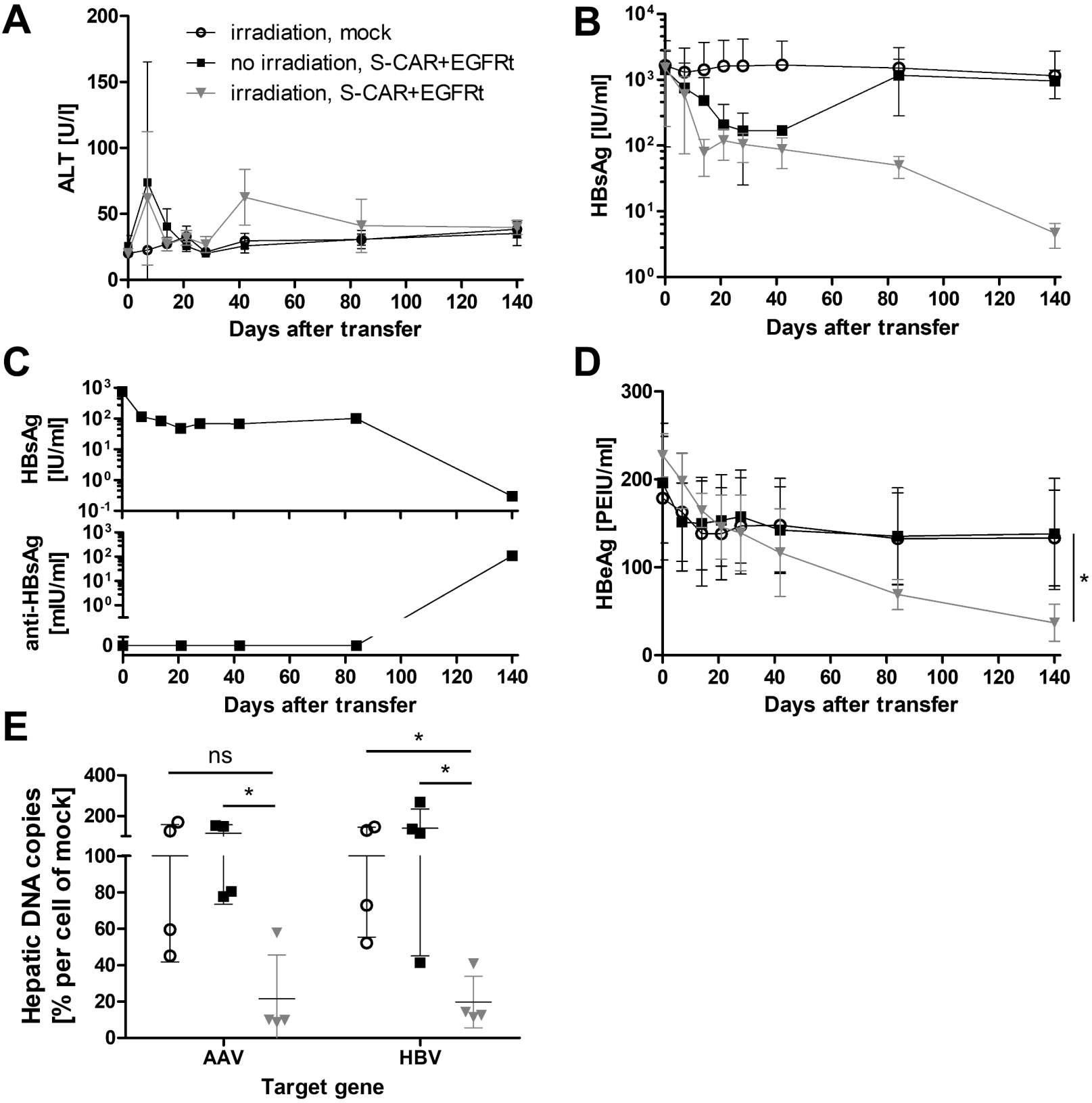
from day 27 were analyzed by ELISA for anti-hIgG1 and anti-scFv C8 antibodies. **C)** Splenocytes isolated at the end of the experiment were co-cultured with EGFRt-expressing target cells before ICS. IFN-γ expression of endogenous $CD45.1^-$ $CD8^+$ T cells upon antigen encounter is shown**. D)** Numbers of $CD45.1^+$/$CD45.2^+$ S-CAR^+ or $EGFRt^+$ T cells per µl peripheral blood determined at indicated time points. Dotted line represents the background determined in mock T cell-treated mice. **E)** ALT activity, **F)** HBsAg and **G)** HBeAg levels in serum measured over time. **H)** Intrahepatic AAV- and HBV-DNA copies/cell determined by qPCR normalized to the cellular single copy gene *PRNP*. Values are shown relative to the mean value determined in mock treated mice. D-G: Data are given as mean values ± SD. B, C, H: Data points represent individual animals, mean values ± SD are indicated. ns = not significant, * = $p<0.05$, ** = $p<0.01$ (Mann Whitney test).

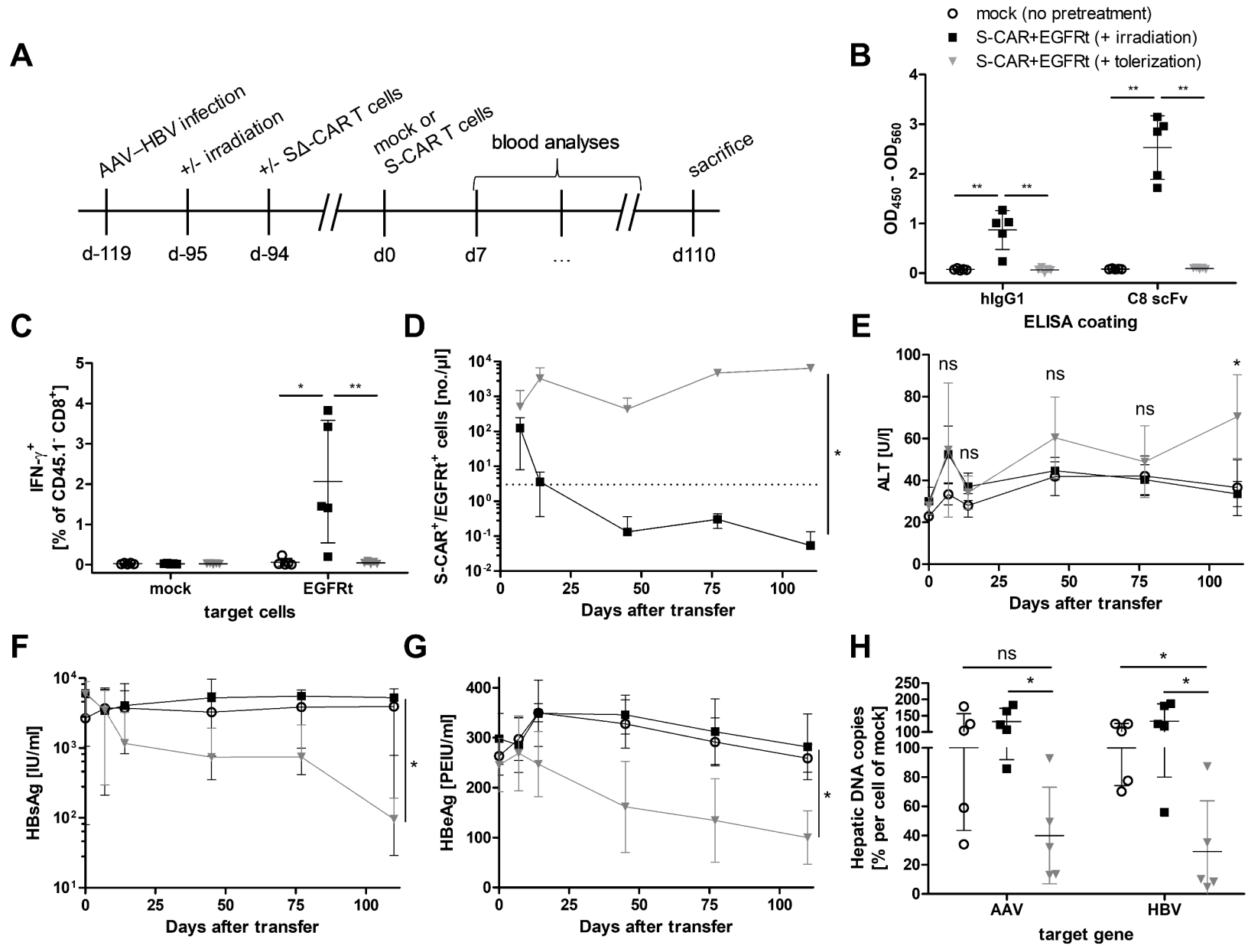

A
S-CAR T cells
S-CAR T cells
2 transfers:
1 transfer:
+
+
-
+
sacrifice
d0
d20
d33
B
CD45.1+ cells [no./µl]
1200
800
400
0
25
33
Day
C
S-CAR+ cells [no./µl]
300
200
100
0
***
ns
5
12
20
25
33
Day
D
ALT [U/l]
1600
1200
800
400
200
150
100
50
0
transfer
transfer
**
*
ns
-4
5
7
12
20
25
27
33
Day
E
IFN-γ+ TNF-α-
IFN-γ- TNF-α+
IFN-γ+ TNF-α+
Liver – d33
Spleen – d33
2 transfers
1 transfer
Cytokine+ [% of CD45.1+ cells]
100
80
60
40
20
0
CD3-CD28
HBsAg
PBS
CD3-CD28
HBsAg
PBS


A
CAR
EGFRt
mock
SΔ-CAR+ EGFRt
S-CAR+ EGFRt
74 %
85 %
B
wt - mock
wt - SΔ-CAR+EGFRt
wt - S-CAR+EGFRt
Rag2-/- - SΔ-CAR+EGFRt
Rag2-/- - S-CAR+EGFRt
CD45.1+ cells [no./µl]
Days after transfer
C
S-CAR+/EGFRt+ cells [no./µl]
S-CAR+EGFRt
Days after transfer
D
untreated
S-CAR+EGFRt
ALT [U/l]
Days after transfer
E
ns
HBsAg [IU/ml]
Days after transfer
F
ns
HBeAg [PEIU/ml]
Days after transfer


A
S-CAR [MFI x 10,000]
wt - mock
wt - S-CAR+EGFRt
Rag2-/- - S-CAR+EGFRt
Days after transfer
EGFRt [MFI x 10,000]
Days after transfer
B
IFN-γ+ [% of CD45.2+ CD8+]
mock
S-CAR+EGFRt
**
ns
mock
S-CAR
tEGFR
C
Murine IgG [MFI x 10,000]
**
ns
mock
S-CAR
tEGFR
D
anti-hIgG1
OD450 - OD560
mock day 3
S-CAR+EGFRt day 3
mock day 18
S-CAR+EGFRt day 18
1:800
1:400
1:200
1:100
1:50
Serum Dilution
E
anti-scFv C8
OD450 - OD560
1:800
1:400
1:200
1:100
1:50
Serum Dilution
F
CD45.1+ cells [no./µl]
mock
S-CAR
murine IgG1 S-CAR
Days after transfer
G
OD450 - OD560
hIgG1
scFv C8


A
AAV-HBV infection
+/- irradiation
mock or S-CAR T cells
blood analyses
sacrifice
d-28
d-1
d0
d7
...
d140
B
d28
no irradiation
irradiation
SSC
CD45.1
C
S-CAR+/EGFRt+ cells [no./µl]
Days after transfer
D
CD45.1− B cells [no./µl]
CD45.1− CD8+ T cells [no./µl]
Days after transfer
irradiation, mock
no irradiation, S-CAR+EGFRt
irradiation, S-CAR+EGFRt
Effector
Effector Memory
Intermediate
Naïve/Central-Memory
E
S-CAR+/EGFRt+ cells [no./organ]
Liver
Spleen
no irradiation
irradiation
F
% of CD45.1+ cells
mock
S-CAR+EGFRt
G
Marker+ [% of CD45.1+ cells]
PD-1
CTLA-4
Tim-3
H
Cytokine+ [% of CD45.1+ cells]
PBS
HBsAg
IFN-γ+ TNF-α−
IFN-γ− TNF-α+
IFN-γ+ TNF-α+
I
hIgG
scFv C8
OD450 - OD560
Days after transfer


A
irradiation, mock
no irradiation, S-CAR+EGFRt
irradiation, S-CAR+EGFRt
ALT [U/l]
Days after transfer
B
HBsAg [IU/ml]
Days after transfer
C
HBsAg [IU/ml]
anti-HBsAg [mIU/ml]
Days after transfer
D
HBeAg [PEIU/ml]
Days after transfer
*
E
Hepatic DNA copies [% per cell of mock]
ns
*
*
*
AAV
HBV
Target gene


A
AAV–HBV infection
+/- irradiation
+/- SΔ-CAR T cells
mock or
S-CAR T cells
blood analyses
sacrifice
d-119
d-95
d-94
d0
d7
…
d110
B
mock (no pretreatment)
S-CAR+EGFRt (+ irradiation)
S-CAR+EGFRt (+ tolerization)
OD450 - OD560
hIgG1
C8 scFv
ELISA coating
C
IFN-γ+ [% of CD45.1- CD8+]
mock
EGFRt
target cells
D
S-CAR+/EGFRt+ cells [no./µl]
Days after transfer
E
ALT [U/l]
ns
Days after transfer
F
HBsAg [IU/ml]
Days after transfer
G
HBeAg [PEIU/ml]
Days after transfer
H
Hepatic DNA copies [% per cell of mock]
AAV
HBV
target gene