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Dynamic, Helminth-Induced Immune Modulation Influences the Outcome of Acute and Chronic Hepatitis B Virus Infection

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Background. Chronic hepatitis B develops more frequently in countries with high prevalence of helminth infections. The crosstalk between these 2 major liver-residing pathogens, *Schistosoma mansoni* and hepatitis B virus (HBV), is barely understood.

Methods. We used state-of-the-art models for both acute and chronic HBV infection to study the pathogen-crosstalk during the different immune phases of schistosome infection.

Results. Although liver pathology caused by schistosome infection was not affected by either acute or chronic HBV infection, *S mansoni* infection influenced HBV infection outcomes in a phase-dependent manner. Interferon (IFN)-γ secreting, HBV- and schistosome-specific CD8 T cells acted in synergy to reduce HBV-induced pathology during the T_H1 phase and chronic phase of schistosomiasis. Consequently, HBV was completely rescued in IFN-γ-deficient or in T_H2 phase coinfected mice demonstrating the key role of this cytokine. It is interesting to note that secondary helminth infection on the basis of persistent (chronic) HBV infection increased HBV-specific T-cell frequency and resulted in suppression of virus replication but failed to fully restore T-cell function and eliminate HBV.

Conclusions. Thus, schistosome-induced IFN-γ had a prominent antiviral effect that outcompeted immunosuppressive effects of T_H2 cytokines, whereas HBV coinfection did not alter schistosome pathogenicity.

Keywords. coinfection; hepatitis B; immunomodulation; liver; *Schistosoma mansoni*.

Epidemiological studies report that chronic hepatitis B virus (HBV) infection is more frequent in developing countries where coinfections with helminths, such as *Schistosoma mansoni*, are common [1, 2]. More than 250 million humans are infected with schistosomes and/or HBV [3]. Morbidity related to these 2 pathogens mainly results from immunopathology in the host's liver. Although HBV pathology is mainly mediated by cytotoxic T and natural killer cells [4], *S mansoni* induces granulomatous, CD4 T cell-mediated responses [5].

Due to coevolution with their hosts, helminths have multiple mechanisms to evade and/or modulate the host's immune response, yielding an immunosuppressive environment required for the parasite's survival [5, 6]. Antischistosome immunity evolves via 3 consecutive, immunologically distinct phases: migrating larvae instigate an initial proinflammatory T_H1 response that is consequently suppressed by strong T_H2 immune responses elicited by tissue-dwelling eggs released by adult worms. Eventually, a long-term regulatory phase ensues in which regulatory elements such as interleukin (IL)-10, regulatory T cells (Treg), and regulatory B cells dominate [7–10].

As in chronic HBV infection, strong and sustained virus-specific CD8 and CD4 immune responses fail [11], the immune-regulatory capacities of helminths could contribute to HBV persistence in coinfection, and schistosome-triggered immunopathology in the liver might even aggravate that of HBV.

We and others [12, 13] have shown that concomitant schistosome infection aggravated liver disease in hepatitis C virus-infected Egyptian patients [14]. Nevertheless, epidemiological studies addressing the relationship between *S mansoni* and HBV remain controversial. Although some studies indicated that schistosome coinfection resulted in worsening of liver disease, others found no influence on liver pathology [1, 2, 15–17]. In previous experiments, acute schistosome infection has been studied in HBV-transgenic mice and resulted in suppressed replication of HBV from the transgene in an interferon

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(IFN)-γ-dependent manner [18]. However, due to the transgenic nature of the model, conclusions could not be drawn on HBV persistence and its consequences for liver pathology during coinfection. Furthermore, HBV infection during the different phases of schistosome infection has never been investigated. Because mice cannot be infected with HBV, we used novel mouse models in which HBV genomes are transferred into hepatocytes using liver-directed adenoviral or adenovirus-associated virus (AAV) vectors. These models—in contrast to transgenic mice—allowed us to monitor (1) immune-mediated control of HBV gene expression and replication as well as (2) elimination of HBV-replicating cells by cytotoxic T-cell responses.

We hypothesized that the time point of initial infection by either pathogen and the concurrent, dynamic immune responses elicited by schistosomes influence the severity and outcome of HBV infection in endemic areas. Thus, 2 relevant coinfection scenarios were simulated experimentally. The first is acute HBV acquired during adolescence on top of pre-existing schistosome infection [19, 20]: thus, we used an adenovirus-mediated genome transfer of replication-competent HBV genomes [21, 22] during each distinct phase of schistosomiasis. The second scenario is HBV chronicity after vertical transmission from mother to child before the first schistosome infection [3, 19]. Thus, we infected mice with AAV containing an HBV genome (AAV-HBV), which initiates persistent infection, HBV replication [23, 24], and consecutively infected these with schistosomes.

MATERIAL AND METHODS

Animals

C57BL/6J and BALB/c mice were purchased from Harlan Winkelmann GmbH, and C57BL/6J IFN-$\gamma^{-/-}$ mice were from Jackson Laboratory (Charles River Laboratories). C57BL/6J DEREG mice [25] were bred and housed at our institute under specific pathogen-free conditions.

Ethics Statement

Animal protocols were approved/performed in accordance with the government authorities of Bezirksregierung von Oberbayern (license no. AZ. 55.2.1.54-2532-112-13). Our animal care and use protocol are in accordance with national and European Union guidelines 2010/63.

Schistosome Infection

Female C57BL/6J and BALB/c mice were infected with a Brazilian strain of *S mansoni* with 180 cercariae/mouse (snail strain-*Biomphalaria glabrata*) as previously described [7, 8]. Infection was assessed by egg burden as described before [8]. Paraffin-embedded sections from the left liver lobe of each mouse were stained with Masson's blue to microscopically (Axioskop; Zeiss) determine granuloma sizes as described previously [8].

Acute and Chronic Hepatitis B Virus Infection

Human adenoviral vectors (serotype 5) transferring replication-competent 1.3-fold overlength HBV genomes (genotype D, subtype awy) were produced as described [21] and administered intravenously (10^9 IU/mL adenoviral HBV [AdHBV]). An empty adenoviral vector lacking any transgene (Ad-Empty; 10^9 IU/mL) served as control. Regulatory T-cell depletion was performed by intraperitoneal injection of 1 µg of diphtheria toxin (Merck KGaA) into DEREG mice on 2 consecutive days post-viral infection. For persistent HBV infection, HBV replication was initiated via intravenous injection of 3×10^{10} viral genomes AAV-HBV, an AAV (serotype 8) carrying a replication-competent 1.2-fold overlength HBV genome (genotype D, subtype awy) [24] in C57BL/6J mice.

Serological and Biochemical Analysis

Hepatitis B surface antigen (HBsAg) and hepatitis B early antigen (HBeAg) in serum were quantified by using AXSYM-assays following the manufacturer's instructions (Abbott Laboratories). Serum alanine aminotransferase (ALT) activity was determined using bioreaction stripes on a Reflovet Plus Reader (Roche Diagnostics).

Immunohistochemistry

For virus detection, paraffin-embedded liver tissue samples were stained with an antibody [26] against HBcAg (Diagnostic Biosystems). Major histocompatibility complex class II (MHC-II) (NBP1-43312; Novus Biologicals), Clec4f (AF2784-SP; R&D Systems), CD8 (14-0808-82; Invitrogen), and B220 (clone RA3-6B2; BD Biosciences) were stained using the BOND-MAX (Leica Biosystems). All slides were scanned using Aperio ScanScope-AT2 (Leica Aperio Technologies) and analyzed using the Aperio ImageScope Viewer.

Viral Deoxyribonucleic Acid Quantification

Deoxyribonucleic acid (DNA) from liver tissue was isolated with a DNeasy Blood & Tissue Kit (QIAGEN) according to the manufacturer's instructions. Adenoviral and HBV genomes were detected in comparison to a murine noncoding region by real-time polymerase chain reaction (PCR) using the SYBR Green I Master Mix (Roche). The relative quantification was performed on a Light Cycler 480 II using the second derivative algorithm (Roche). The corresponding primers are listed in Supplementary Table 1.

In Vitro Restimulation and Flow Cytometry

Erythrocyte-depleted cell suspensions from individual mesenteric lymph nodes, spleens, and liver-associated lymphocytes (LAL) were prepared, stained with ethidium monoazide bromide (Invitrogen) and either fluorochrome-labeled antibodies (Supplementary Table 2) or HBV-multimers. After antigen-specific restimulation, in vitro intracellular cytokine staining was performed. For details, see Supplementary Tables.

Quantitative Real-Time Polymerase Chain Reaction

Ribonucleic acid from liver tissues was extracted using the RNeasy Plus Universal Kit (QIAGEN) followed by reverse transcription with M-MLV (Invitrogen) according to the manufacturer's instructions. Quantitative real-time PCR for MHC-I and MHC-II was normalized to glyceraldehyde 3-phosphate dehydrogenase and Hypoxanthine-guanine phosphoribosyltransferase (HPRT) using TaqMan universal Probe library sets (Roche) (Supplementary Table 3).

Statistics

Statistical tests were performed with PRISM 5.01 (GraphPad Software Inc.). D'Agostino and Pearson omnibus normality tests were performed, and parametrically distributed data were analyzed with unpaired *t* test (2 groups). Mann-Whitney *U* test was used for non-parametric data. For more than 2 groups, one-way analysis of variance test was conducted, and if data were non-parametric, a Kruskal-Wallis test with a confidence interval of 95% was used. Results with $P < .05$ were considered significant.

RESULTS

Dynamics of Schistosome-Mediated Immune Responses

Mouse strains and housing conditions have a strong influence on the quality of immune responses especially in chronic infection models. We previously evaluated 3 distinct T_H1, T_H2, and chronic phases elicited during schistosomiasis in BALB/c mice [27]. Because we hypothesized that these phases would have an effect on coinfection, we evaluated these dynamical immune responses in infected C57BL/6J mice to define the optimal time points for coinfection studies. *Schistosoma mansoni* egg antigen (SEA)-specific IFN-γ gradually increased from week 4 onwards ("T_H1 phase") until it was replaced by T_H2 cytokines such as IL-4 and IL-10 ("T_H2 phase") beginning at 7.5 to 8 weeks postinfection (Figure 1A). Surprisingly and in contrast to BALB/c mice [27], at 16 and 22 weeks, IL-10 responses in C57BL/6J mice persisted but IFN-γ levels rose again (Figure 1A). Consequently, we performed HBV coinfection in the "T_H1 phase" at week 5, in the "T_H2 phase" at week 8, and in the "chronic phase" at week 16.

Acute Hepatitis B Virus Infection Acquired During the T_H1 Phase of Schistosome Infection Results in Suppressed Viral Replication

Because mice are not naturally susceptible to HBV [28, 29], replication-competent HBV genomes were transferred by liver-directed adenoviral and AAV vectors [21]. Adenoviral transfer of HBV genomes induces an "acute, self-limiting" HBV infection of murine hepatocytes, with production of infectious HBV virions and results in immunological control of HBV replication [30]. To elucidate whether the T_H1-prone milieu elicited by migrating schistosomes systemically and locally in the liver interferes with HBV replication, mice were infected with AdHBV 4 weeks postschistosome infection when systemic IFN-γ levels are just becoming detectable. Alanine aminotransferase activity and HBV antigen levels were comparable between schistosome-coinfected and HBV-monoinfected animals from day 3 to 21 postinfection at equal AdHBV transduction rate (Supplementary Figure S1A–C) as were the number and localization of HBcAg-positive hepatocytes at day 3. However, 3 weeks later, ie, at 7 weeks postschistosome infection, HBV replication (Supplementary Figure S1C) as well as numbers of HBcAg-positive hepatocytes (Supplementary Figure S1D and E) were reduced in coinfected animals. These results indicated that T_H1 cytokines such as IFN-γ could contribute to viral clearance.

Therefore, we infected animals with AdHBV at week 5, at the peak of IFN-γ secretion during acute schistosomiasis (Figure 1A). Serum levels of HBsAg and HBeAg were significantly lower at 3 days post-HBV infection, in *S mansoni*-coinfected compared with AdHBV-monoinfected animals. This tendency continued throughout the course of infection (Figure 1B, left). Significantly lower numbers of HBV genomes but comparable amounts of adenoviral DNA were detected in livers of coinfected mice, indicating a specific effect on HBV gene expression and replication rather than on genome transfer (Figure 1B, right).

To segregate bystander from HBV-specific immunity, we analyzed IFN-γ secretion from splenocytes upon antigen-specific, SEA-dependent and antigen-independent stimulation. It is interesting to note that, whereas IFN-γ release after antigen-specific stimulation was comparable in schistosome-monoinfected and AdHBV-coinfected groups, antigen-independent stimulation resulted in strongly enhanced IFN-γ secretion only in the coinfected mice (Figure 1C, left). Hepatitis B virus-specific immunity determined in LAL and splenocytes pulsed with an HBV-core (HBc) peptide pool was enhanced. Frequencies of virus-specific CD8 IFN-γ^+ T cells in livers of coinfected mice were significantly higher in comparison to AdHBV-monoinfected animals (Figure 1C, right) and remained comparable in the spleen because antigen-specific cells homed to the liver. The increase of virus-specific T cells correlated with elevated ALT activity in coinfected animals, showing that control of HBV replication came at the cost of hepatocyte damage (Figure 1D). These results indicated that helminth infection did not influence efficacy of AdHBV infection of hepatocytes but boosted the induction of virus-specific T cells via IFN-γ, resulting in HBV reduction.

T_H2 Phase of Schistosome Infection Does Not Alter Acute Hepatitis B Virus Infection

The ensuing T_H2 phase of helminth infection is initiated, when eggs are released by the fecund females and cause granulomatous CD4 T cell-mediated immune responses in the liver and intestine. During this period, IFN-γ is almost completely abolished from the circulation (Figure 1A).

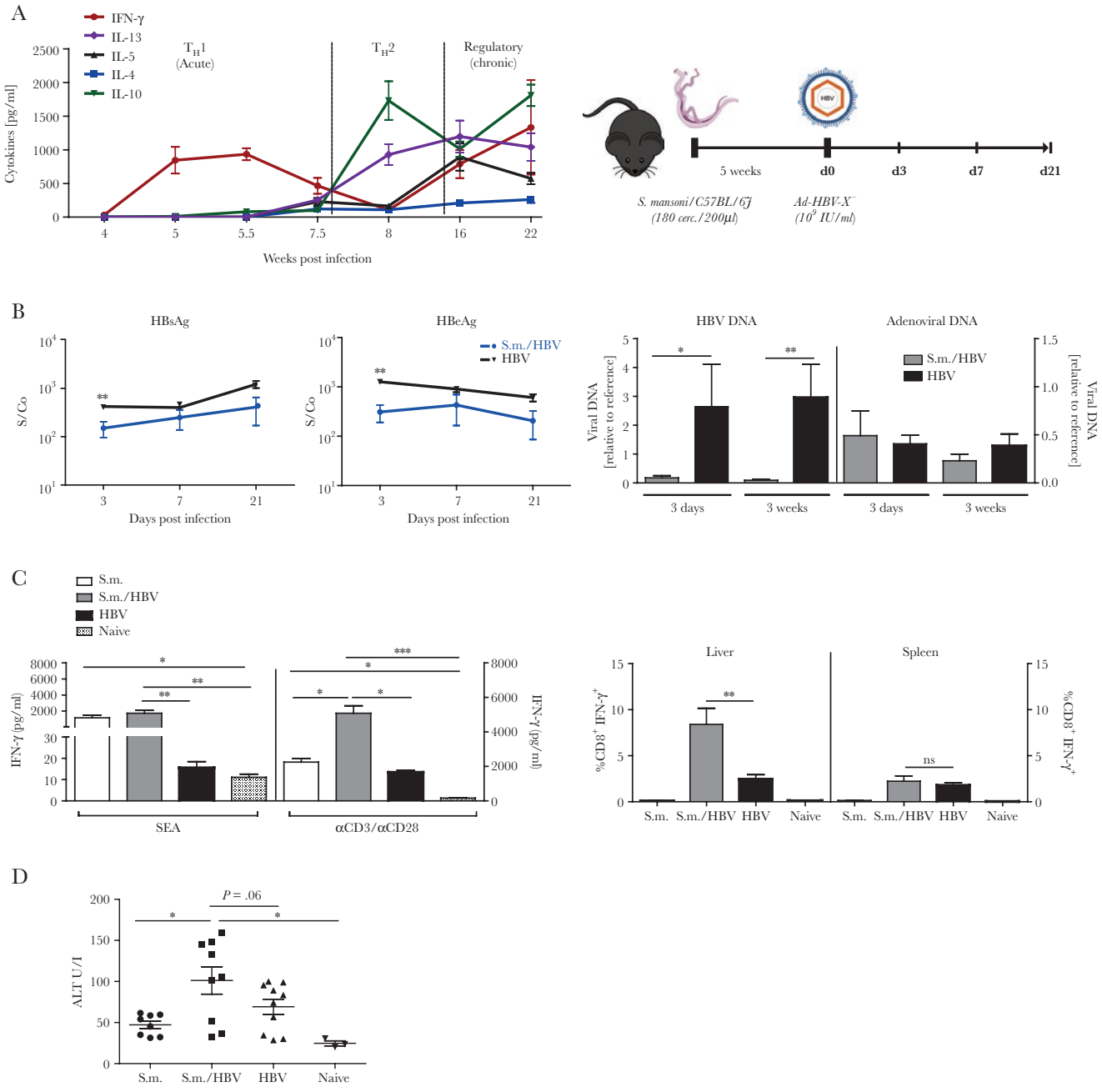

Figure 1. Acute adenoviral hepatitis B virus (AdHBV) infection during the T_H1 phase of schistosome infection. (A) Time course of *Schistosoma mansoni* infection in C57BL/6J mice. At indicated time points, mice were sacrificed, splenocytes were restimulated with S *mansoni* egg antigen (SEA), and cytokine-secretion was measured by enzyme-linked immunosorbent assay. (Right) Timeline of infection in the Th1 phase. (B–D) Mice were coinfected with AdHBV 5 weeks posthelminth infection. (B) Serum viral antigens were determined 3, 7, and 21 days after AdHBV infection (left), and HBV and adenoviral genomes in liver after 3 and 21 days (right). (C) SEA-specific and antigen-independent interferon (IFN)-γ secretion (left). Frequencies of HBV core-specific $CD8^+$ IFN-γ^+ T cells in liver and spleen (right). (A–C) Mean ± standard error of the mean is shown (Sm, n = 8; Sm/HBV, n = 8; HBV, n = 10; wild-type, n = 3). (D) Serum alanine aminotransferase (ALT) 3 weeks post-HBV infection of individual mice. Statistics were performed using Mann-Whitney *U* test (B and C) or analysis of variance and Bonferroni's multiple comparison (D) (*, $P < .05$; **, $P < .01$; ***, $P < .001$). Data are representative of 3 independent experiments. DNA, deoxyribonucleic acid; HBeAg, hepatitis B early antigen; HBsAg, hepatitis B surface antigen; IL, interleukin; ns, not significant.

Thus, this could result in contrasting effects on HBV replication and immunity. Indeed, mice infected with AdHBV at 8 weeks postschistosome infection showed similar serum viral antigen levels, viral loads, and ALT levels when compared with AdHBV-monoinfected animals (Figure 2A–C). Accordingly, frequencies of liver-resident schistosome-specific as well as HBV-specific CD8 IFN-γ^+ T cells remained comparable (Figure 2D) implying that helminth-induced T_H2 cytokines did not influence antiviral immunity and viral replication.

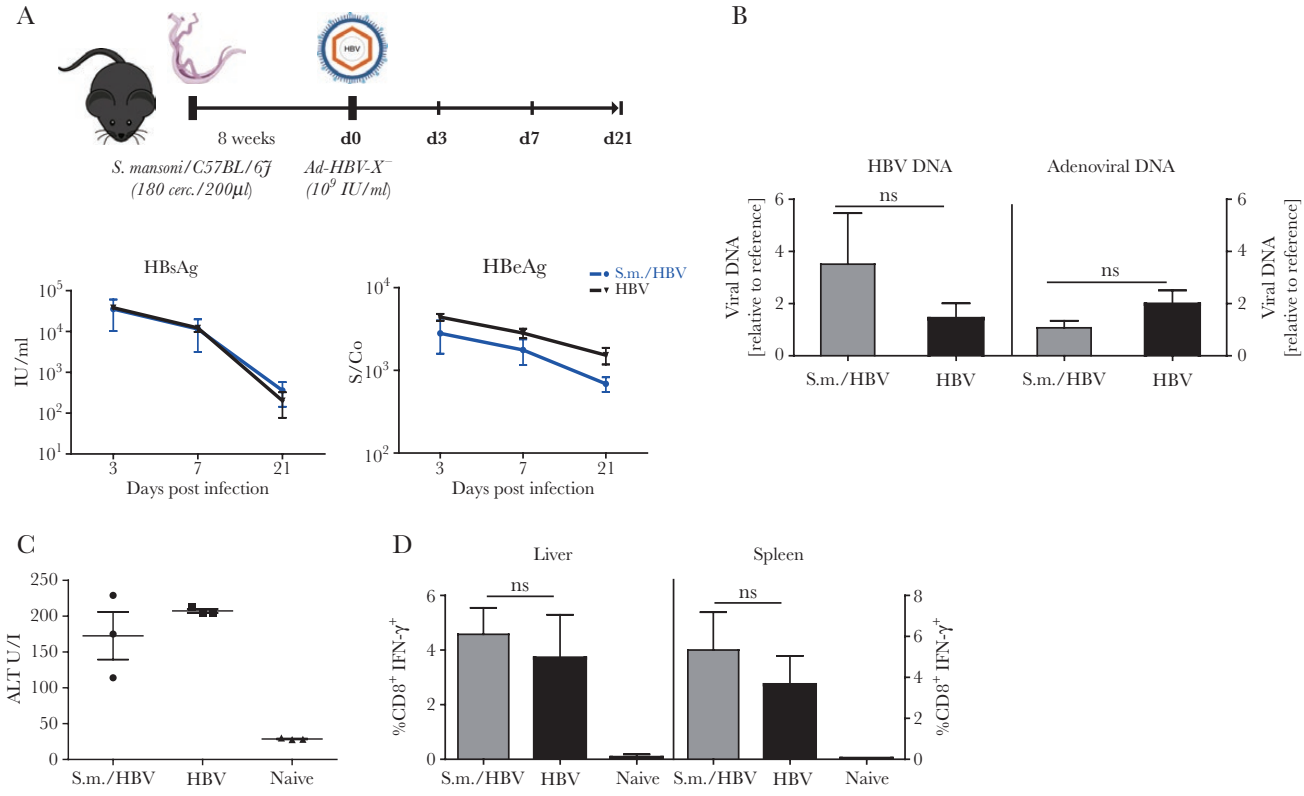

Figure 2. Establishment of acute adenoviral hepatitis B virus (AdHBV) infection during the T_H2 phase of schistosome infection. (A) Timeline of infection and serum viral antigen levels in C57BL/6J mice infected with AdHBV 8 weeks post-*Schistosoma mansoni* infection. (B) Relative quantification of adenoviral and HBV genomes in mouse liver lysates by quantitative polymerase chain reaction, and (C) serum alanine aminotransferase (ALT) activity at day 21 post-AdHBV infection. (D) Frequencies of $CD8^+$ interferon (IFN)-γ^+ T cells upon restimulation with HBV-core peptides. Mean ± standard error of the mean is given (Sm/HBV, n = 3; HBV, n = 3; wild-type, n = 3). No statistical differences after performing Mann-Whitney *U* test (A–C) or Kruskal-Wallis and Dunn's testing (D). Data are representative of 2 independent experiments. DNA, deoxyribonucleic acid; HBeAg, hepatitis B early antigen; HBsAg, hepatitis B surface antigen; ns, not significant.

During the Chronic Phase of Schistosomiasis Liver Damage Is Mitigated but Hepatitis B Virus Replication Remains Suppressed

Eventually, we investigated the outcome of acute HBV infection during the chronic phase of schistosomiasis where besides regulatory responses, IFN-γ increased again (Figure 1A). Mice infected with AdHBV at this time point showed significantly decreased levels of viral antigens and HBV DNA in the liver of coinfected compared with AdHBV-monoinfected mice (Figure 3A), whereas equal adenoviral DNA proved comparable and successful HBV infection (Figure 3A, right).

Because IFN-γ seemed to outcompete the immunosuppressive effects of coexisting T_H2-type cytokines, we analyzed the cytokine milieu in more detail. We were surprised to find that schistosome-infected animals produced higher levels of IFN-γ and IL-10 than AdHBV-monoinfected mice (Figure 3B). Hepatitis B virus-specific IFN-γ^+ CD8 T cells within the liver were significantly increased in coinfected mice (Figure 3C, left), whereas ALT activity reflecting liver damage was significantly lower in *S mansoni*-coinfected animals (Figure 3C, right). This may be attributed to previously reported increased levels of Treg during experimental HBV-infection that potentially control HBV-specific cytotoxic T-cell activity and thus liver inflammation [31]. To test this, we took advantage of DEREG mice in which Treg can be depleted by diphtheria toxin injection. Indeed, depletion of Treg 1 week after AdHBV infection in chronically *S mansoni*-infected DEREG mice resulted in increased serum ALT levels (as expected [31]) in HBV monoinfection but also in *S mansoni* coinfection (Figure 3D). Taken together, these results indicate that during the chronic phase of schistosome infection, antiviral properties of schistosome-induced IFN-γ outcompeted immunosuppressive effects of coexisting T_H2-type cytokines while Treg control of cytotoxic T cells prevented liver damage.

Hepatitis B Virus Infection Has No Impact on *Schistosoma mansoni* Disease Outcome

Because a virus coinfection might also have an impact on schistosome infection, we analyzed parasite egg burden, granuloma size, and schistosome-specific immune responses in mice infected with *S mansoni* only, coinfected with an "empty" adenoviral vector (Ad-Empty) or simultaneously infected with either AdHBV or with AAV-HBV that persists and induces chronic HBV infection [24]. Neither acute nor chronic HBV infection influenced egg burden, granuloma

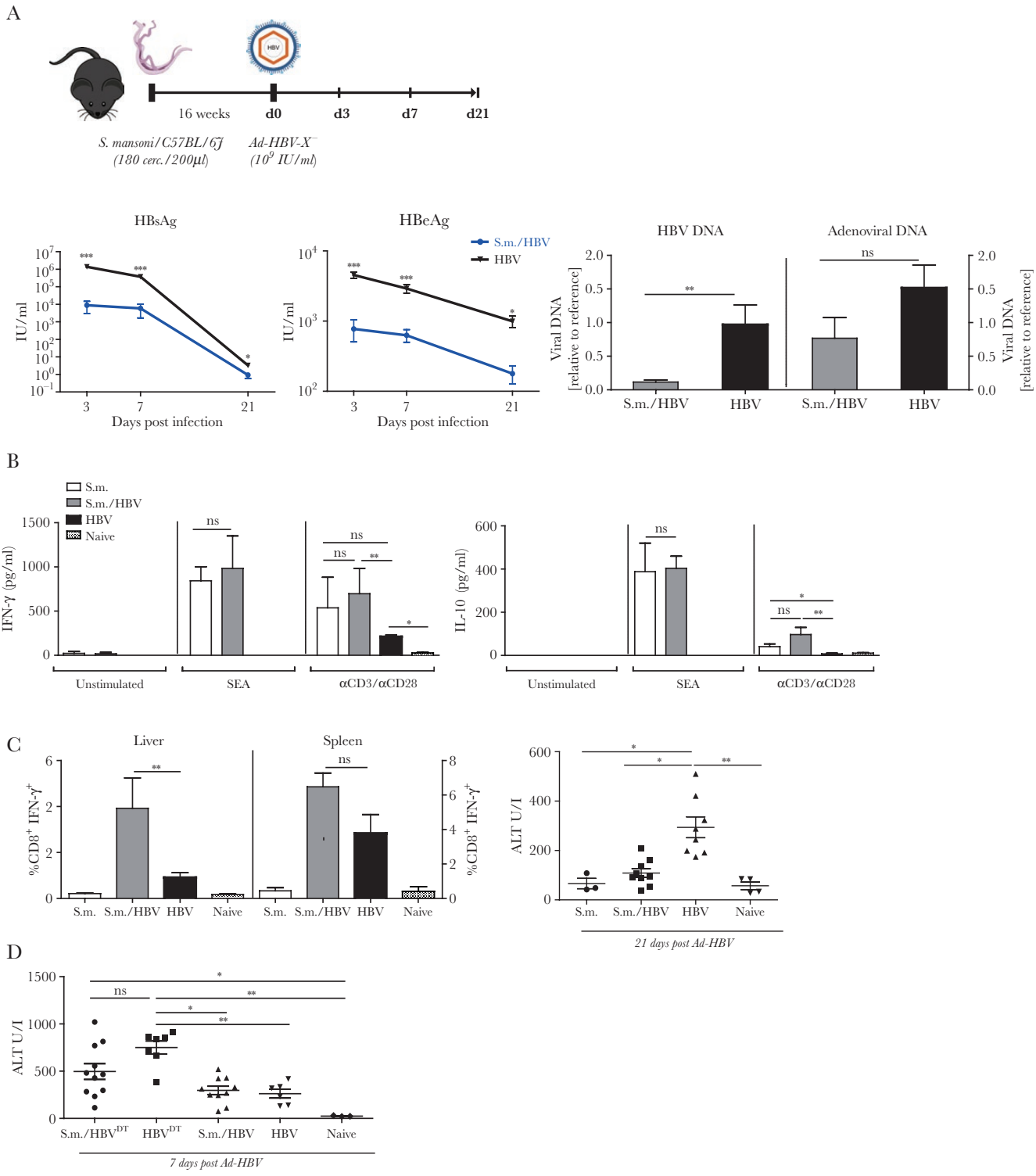

Figure 3. Acute adenoviral hepatitis B virus (AdHBV) infection during the chronic phase of schistosomiasis. (A) Timeline of infection and serum viral antigens in C57BL/6J mice infected with AdHBV 16 weeks after *Schistosoma mansoni* infection (left). Relative quantification of adenoviral and HBV genomes in liver 21 days after AdHBV (right). (B) *Schistosoma mansoni* egg antigen (SEA)- and antigen-independent interferon (IFN)-γ and interleukin (IL)-10 secretion by mesenteric lymph nodes and (C) frequencies of $CD8^+$ IFN-γ^+ T cells detected in liver and spleen (left) as well as serum alanine aminotransferase (ALT) 21 days after AdHBV infection (right). (D) Serum ALT of chronically schistosome-infected C57BL/6J and DEREG mice (DT) 7 days post-AdHBV infection and injection of diphtheria toxin. Mean ± standard error of the mean is given (A–C) (Sm, n = 3; Sm/HBV, n = 9; HBV, n = 8; wild-type [WT], n = 3), (D) (Sm/HBV^{DT}, n = 11; HBV^{DT}, n = 7; Sm/HBV, n = 10; HBV, n = 6; WT, n = 3). Statistical analysis was performed using Mann-Whitney *U* test (A and C) or Kruskal-Wallis and Dunn's test (B and D) (*, $P < .05$; **, $P < .01$; ***, $P < .001$). (A–C) Data are representative of 3 independent experiments. (D) Data are representative of 2 independent experiments. DNA, deoxyribonucleic acid; HBeAg, hepatitis B early antigen; HBsAg, hepatitis B surface antigen; ns, not significant.

size, or immune responses as parameters of schistosome infection severity (Figure 4A–C). Furthermore, we could not detect any impact on these parameters upon administration of the empty adenoviral vector, lacking HBV DNA (Ad-Empty) (Figure S2A–D).

Schistosome-Induced Interferon-γ Suppresses Hepatitis B Virus Replication

Because our results indicated that schistosome-induced IFN-γ was a key player in HBV control, we addressed its role in noncytolytic control of HBV and in stimulating antiviral

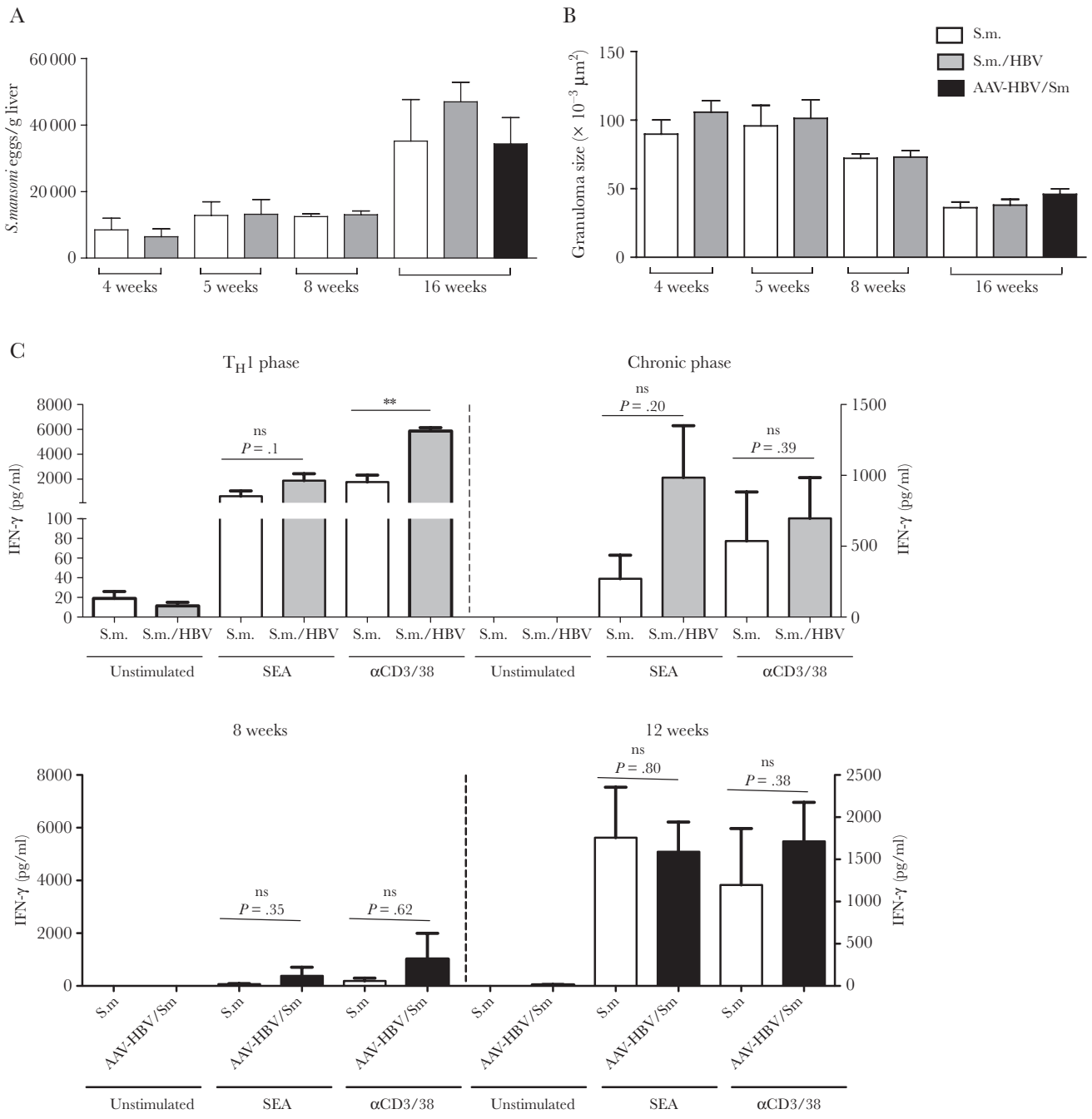

Figure 4. Impact of acute and chronic hepatitis B virus (HBV) infections on *Schistosoma mansoni* (S.m) disease outcome. (A and B) Mice were either infected with *S mansoni* only or simultaneously infected with adenoviral HBV causing self-limited HBV infection (S.m./HBV) in the 4th, 5th, 8th, and 16th week of schistosomiasis and sacrificed 21 days later. Otherwise, mice were infected with adenovirus-associated virus (AAV)-HBV (leading to persistent infection) 2 weeks before schistosome infection (AAV-HBV/S.m.) and sacrificed in the 16th week of schistosomiasis. (A) Egg count and (B) granuloma sizes were evaluated within the liver. (C) *Schistosoma mansoni* egg antigen (SEA)- or antigen-independent interferon (IFN)-γ secretion in splenocytes (left) or mesenteric lymph nodes (right) was determined. Mean ± standard error of the mean is given (Sm, n = 3; Sm/HBV, n = 4; HBV, n = 4; wild-type, n = 3). No statistical differences after Kruskal-Wallis and Dunn's testing. Data are representative of 3 independent experiments. ns, not significant.

immunity. After demonstrating that parasite development and dynamics of T_H2 responses in schistosome-monoinfected C57BL/6 IFN-$\gamma^{-/-}$mice is comparable to wild-type animals (Supplementary Figure S3A–C), we monitored HBV replication during monoinfection and coinfection of IFN-$\gamma^{-/-}$ mice. Unlike wild-type mice, IFN-$\gamma^{-/-}$ mice failed to suppress HBV replication in coinfected animals, and circulating virus antigen as well as HBV viremia were comparable to those of monoinfected groups (Figure 5A–C).

To further confirm the central role of IFN-γ, we analyzed the outcome in coinfected BALB/c mice during the chronic phase of schistosomiasis where only very little (if any) IFN-γ is detected [27]. Upon AdHBV infection, schistosome-infected BALB/c mice indeed controlled HBV replication to a lesser extent and had significantly elevated serum HBsAg levels (Supplementary Figure S4A and B).

To investigate whether IFN-γ could enhance priming of virus-specific CD8 T cells by modifying antigen presentation, we analyzed MHC-I and MHC-II expression in the livers of infected mice and found an upregulation of both MHCs particularly in schistosome-infected groups throughout all immune phases (Figure 6A–D). We further confirmed increased MHC-II protein expression within liver sections (Figure 6E and F). Additional characterization of the microenvironment revealed increased B cells and $CD8^+$ cells, whereas Kupffer cell (Clec4f) frequencies remained unchanged (Supplementary Figure S6). In summary, these data point towards a noncytolytic control of HBV by schistosome-induced IFN-γ accumulating within the liver and indicate that an upregulation of MHC expression enhanced T-cell activity.

Concomitant Schistosome Infection Breaks Immune Tolerance in Mice With Persistent Hepatitis B Virus Infection

Another clinically important coinfection scenario arises when chronically HBV-infected individuals who failed to mount antiviral T-cell responses [11] acquire secondary schistosomiasis. Thus, we infected mice with AAV-HBV that induces chronic infection with persistent HBV replication over many months [24]. After 2 weeks, when stable HBV replication was establishing, mice were superinfected with schistosomes. This led to reduced HBV antigen expression and replication, as soon as schistosomes-induced IFN-γ production begins, but not to clearance of the AAV-HBV infection (Figure 7A). Splenocytes responded vigorously with IFN-γ secretion to SEA as well as an antigen-unspecific stimulus (Figure 7B).

Concerning functionality and phenotype of HBV-specific T-cells, we observed significantly higher frequencies of HBc-specific CD8 T cells in liver and spleen of *S mansoni* superinfected mice (Figure 7C). Fifty percent of HBV-specific T cells expressed PD-1, however at lower frequency (Figure 7C). Functionally, however, no HBV-specific CD8 IFN-γ responses were detected in LAL or splenocytes as expected in this model (Figure 7D, left). Upon 12-*O*-tetradecanoyl-phorbol-13-acetate (PMA)/ionomycin stimulation as control, the liver CD8 T cells from the 2 schistosome-infected groups nevertheless responded with IFN-γ secretion, whereas circulating CD8 T cells did not (Figure 7D, right). It is interesting to note that comparable numbers of granzyme B ($GrzB^+$) cells were detected in these groups irrespective of whether stimulation was HBV-specific (Figure 7E), not present (Supplementary Figure S5A), or schistosome antigen-specific (Supplementary Figure S5B), indicating that schistosome infection induced GrzB in T cells in a nonantigen-specific fashion.

In summary, schistosome infection during persistent HBV infection led to an increase of HBV-specific T cells in the liver that remain dysfunctional. Thus, suppression of HBV replication was mediated by bystander effects such as IFN-γ from schistosome-specific T cells recruited to the liver or by a schistosome-induced cytotoxic effector function. Overall, schistosome-induced T-cell activation led to control of HBV replication in the liver but was not able to eliminate AAV HBV and "cure" HBV infection.

DISCUSSION

The crosstalk of *S mansoni* and HBV and its effect on hepatic damage during coinfection remains elusive mainly due to lack of appropriate experimental HBV infection models [1, 2, 16, 32]. In this study, we used state-of-the-art models for both acute and chronic HBV infection to study the crosstalk during different phases of schistosome infection. Taken together, neither concomitant acute, self-limiting, nor chronic, persistent HBV replication influenced the severity or course of schistosome infection. However, HBV was suppressed during the T_H1 and chronic phases of helminth infection. Liver damage was accompanied by an infiltration of IFN-γ-secreting, HBV-specific and schistosome-specific CD8 T cells during the T_H1 phase. In chronic schistosome infection, immune-mediated liver damage was mitigated by increasing numbers of Treg. A rescue of HBV replication in IFN-γ-deficient mice or in mice coinfected with schistosomes during the T_H2 phase lacking IFN-γ secretion demonstrated the key role of this cytokine in controlling HBV. A secondary helminth superinfection on the basis of persistent HBV infection restored exhausted antiviral T-cell immunity and resulted in—at least partial–control of virus replication.

Intrahepatic IFN-γ is known to accelerate priming and/or proliferation of virus-specific cytotoxic T cells [33] and to interfere with noncytolytic HBV replication [18]. In our coinfection model, IFN-γ from CD4 and CD8 T cells as well as bystander cells activated in the liver during schistosomiasis [34] enhanced cytolytic as well as noncytolytic antiviral immunity. Its role was confirmed by the failure of coinfected IFN-γ-deficient mice to suppress viral replication. Because mice, in contrast to humans, do not establish HBV covalently closed circular DNA (cccDNA), T cell-derived IFN-γ and tumor necrosis factor-α

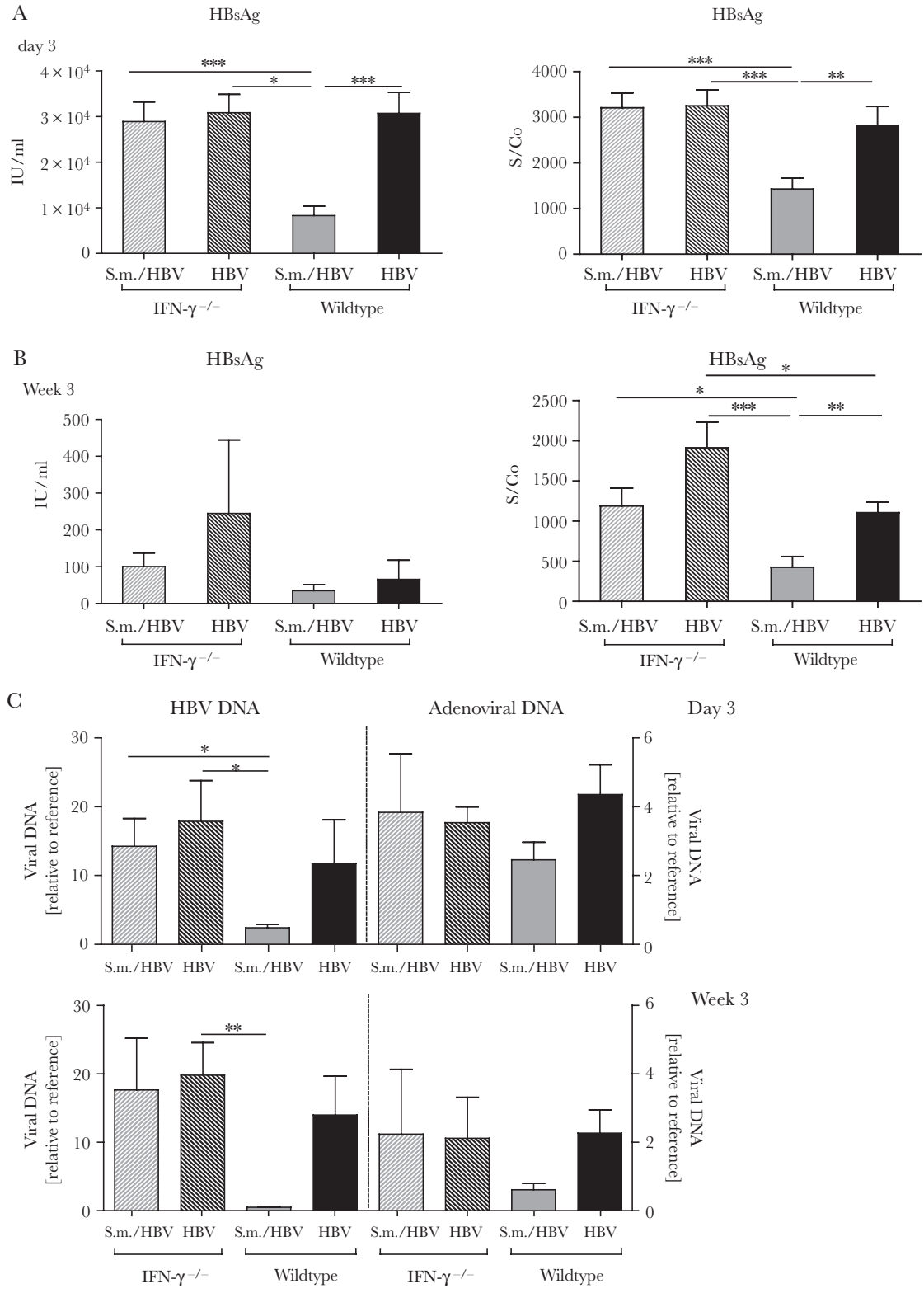

Figure 5. Coinfection of interferon (IFN)-$\gamma^{-/-}$ mice with hepatitis B virus (HBV) and *Schistosoma mansoni*. C57BL/6J- and IFN-γ-deficient C57BL/6J mice were infected with adenoviral HBV (AdHBV) 5 weeks after *S mansoni* infection. Hepatitis B surface antigen (HBsAg) and hepatitis B early antigen (HBeAg) in mouse serum were determined (A) 3 days and (B) 21 days after AdHBV infection. Relative quantification of adenoviral and HBV genomes in mouse liver after (C) 3 days and 3 weeks. Mean ± standard error of the mean is given (Sm/HBV IFNgKO, n = 4; HBV IFNgKO, n = 4; Sm IFNgKO, n = 3; Sm/HBV, n = 4; HBV, n = 4; Sm, n = 3; wild-type, n = 3). For statistics, analysis of variance and Bonferroni's multiple comparison test (A and B) or Kruskal-Wallis and Dunn's test (C) were performed (*, $P < .05$; **, $P < .01$; ***, $P < .001$). Data are representative of 2 independent experiments. DNA, deoxyribonucleic acid.

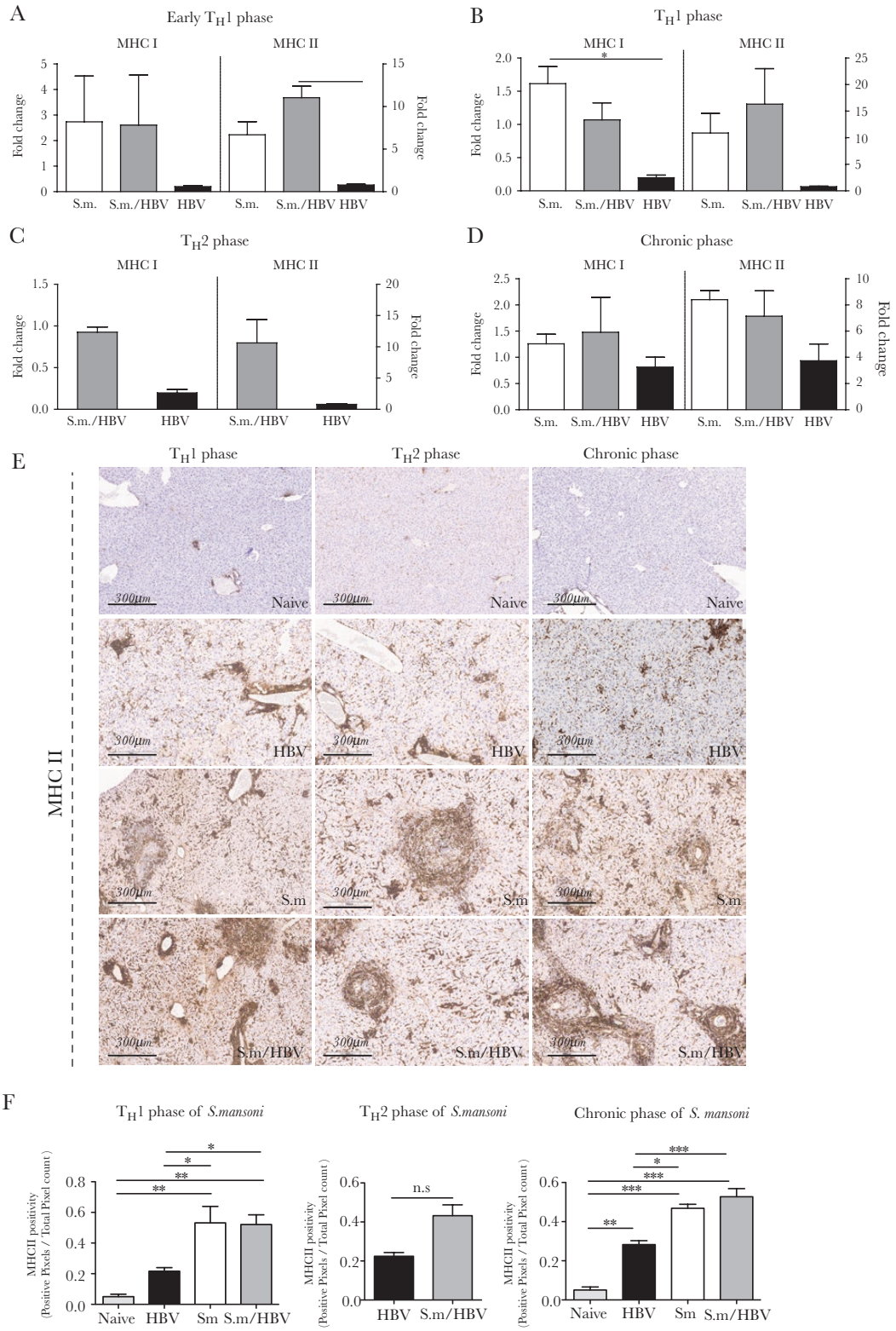

Figure 6. Effect of schistosome infection on major histocompatibility complex class (MHC)-I and MHC-II expression in liver cells. (A–D) Quantitative real-time polymerase chain reaction for murine MHC-I and MHC-II in livers of indicated animal groups 21 days after adenoviral hepatitis B virus (HBV) infection during distinct immune phases of schistosome infection ([A] early T_H1 phase, 4 weeks schistosome infection; [B] T_H1 phase, 5 weeks schistosome infection; [C] T_H2 phase, 8 weeks schistosome infection; [D] chronic phase, 16 weeks schistosome infection; n ≥ 3). (E and F) Immunohistochemistry of MHC-II in liver tissues of all groups during distinct immune phases of schistosome infection. The positive pixel count algorithm (Aperio ImageScope) was used to measure MHC-II expression. Positive pixels were compared with total number of pixels for quantification. Mean ± standard error of the mean is shown (Sm, n = 3; Sm/HBV, n = 5; HBV, n = 4; wild-type, n = 3). Statistical differences were determined by Kruskal-Wallis and Dunn's test (*, $P < .05$; **, $P < .01$; ***, $P < .001$). Data are representative of 3 independent experiments. ns, not significant.

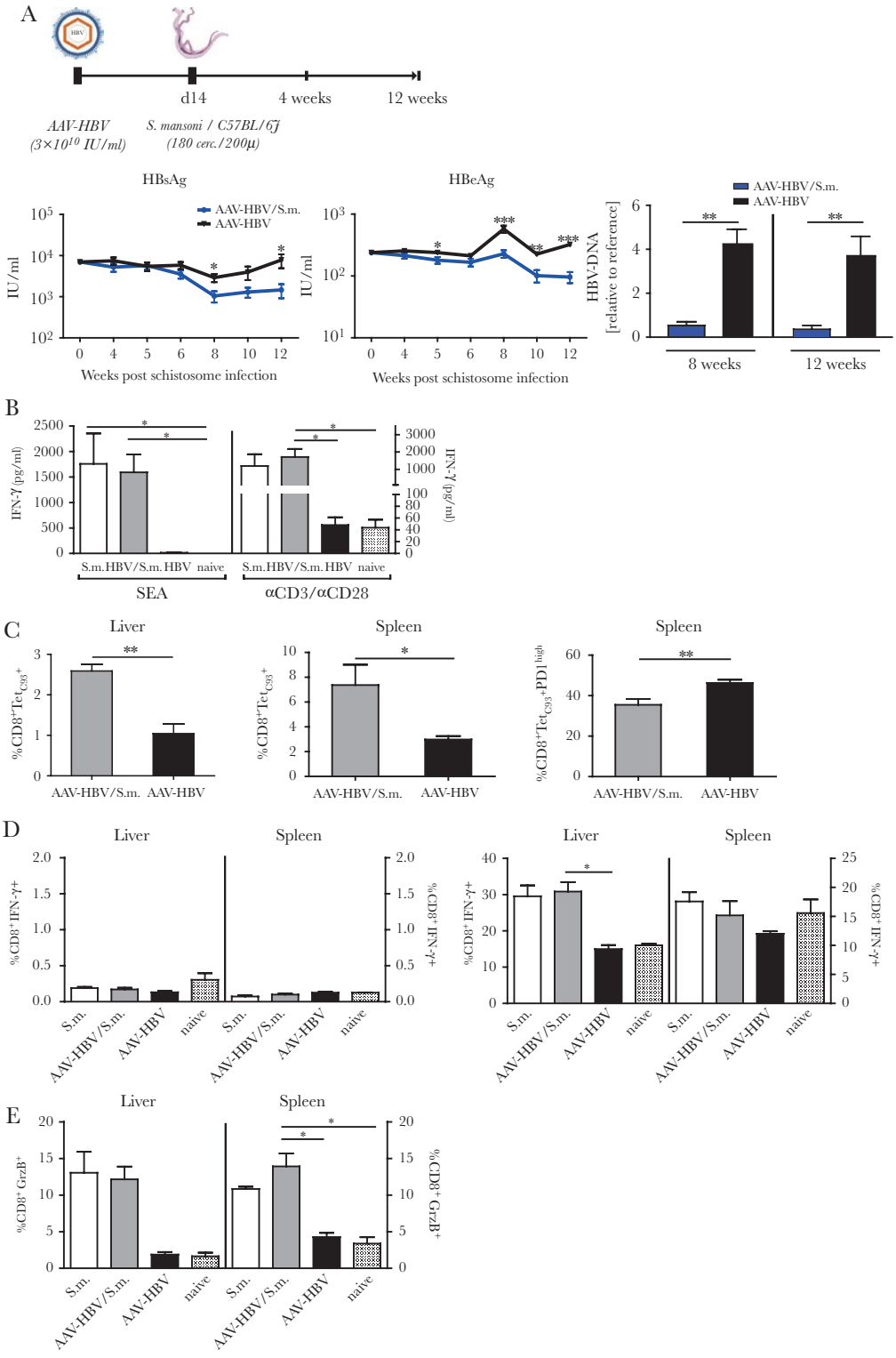

Figure 7. Schistosome infection in mice with persistent hepatitis B virus (HBV) infection. (A) Timeline of infection: serum viral antigens (hepatitis B surface antigen [HBsAg], left; hepatitis B early antigen [HBeAg], middle) were determined at indicated time points. Relative quantification of HBV genomes by polymerase chain reaction in livers 8 and 12 weeks after schistosome infection (right). (B–E) Splenocytes and liver-associated lymphocytes were isolated 12 weeks after schistosome infection. (B) Interferon (IFN)-γ secretion of splenocytes stimulated with *Schistosoma mansoni* egg antigen (SEA)- or αCD3/αCD28-coated beads. (C) Frequencies of HBV-tetramer-positive $CD8^+$ T cells including PD-1 expression were determined by flow cytometry. (D) Frequencies of $CD8^+IFN\text{-}\gamma^+$ T cells in liver and spleen after stimulation with HBV-core (HBc) peptide pool (left) and upon 12-*O*-tetradecanoyl-phorbol-13-acetate (PMA)/ionomycin stimulation (right). (E) Frequencies of $CD8^+GrzB^+$ T cells after stimulation with HBc peptide pool were determined by flow cytometry. Mean ± standard error of the mean is shown (Sm, n = 4; adenovirus-associated virus [AAV]-HBV/Sm, n = 6; AAV-HBV, n = 6; wild-type, n = 3). Statistical analysis was done using Mann-Whitney *U* test (A and C) or Kruskal-Wallis and Dunn's test (B, D, and E) (*, $P < .05$; **, $P < .01$; ***, $P < .001$). Data are representative of 2 independent experiments.

very likely will have even broader effects in humans: they will also reduce levels of HBV cccDNA by inducing deamination and subsequent cccDNA decay without the destruction of liver cells [35], and they limit HBV spread by inducing the expression of cell-intrinsic restriction factors [35–37].

It is interesting to note that, during the chronic phase of schistosome infection, cytotoxicity and liver damage were mitigated despite increased T-cell priming—an effect that was reverted when Treg that are induced by both, *S mansoni* and HBV [31], were depleted. This indicated that during chronic schistosomiasis, on the one hand, T-cell cytotoxicity is controlled by Treg while noncytolytic T-cell function suppressing HBV mediated by IFN-γ remains active. On the other hand, the reduction HBV-antigen expression by IFNg prevents recognition of infected hepatocytes by T cells, supporting rather than limiting viral persistence. Thus, chronic *S mansoni* infection supports HBV persistence at different levels.

Accordingly, HBV was not eliminated when we evaluated the influence of schistosome infection on chronic HBV infection using the AAV-HBV model. Hepatitis B virus replication was suppressed during the T_H1 phase of *S mansoni* infection and remained so when the disease progressed to T_H2 phase in accordance with the findings that schistosome-induced T_H2-type cytokines do not outcompete the antiviral effects of initial IFN-γ. In contrast to a previous study in HBV-transgenic mice that do not allow clearance of HBV infection [18], in our model HBV persistently replicates from an episomal AAV-HBV template that establishes a cccDNA [38] and allows noncytolytic as well as cytolytic control of HBV. However, we did not observe a clearance but strong suppression of HBV in AAV-HBV-infected mice. Helminth infection apparently only partially restored antiviral immunity by high levels of IFN-γ released from schistosome-specific effector T cells revitalizing exhausted T cells, for example, by preventing PD-1-mediated T-cell exhaustion [39]. This did not suffice to completely control or clear HBV.

Finally, HBV infection or replication had no impact on helminth-induced immunopathology and disease outcome. In contrast, coinfection with lymphocytic choriomeningitis virus, a strong T_H1 inducer, resulted in a suppressive effect on T_H2-type cytokines and immunopathology of schistosomiasis [40]. Apparently, HBV is an immunologically "stealth" [41] pathogen, and epidemiological studies have failed to report any alteration of schistosome infection due to viral coinfection [1, 2, 16, 42].

CONCLUSIONS

Continuous exposure in endemic areas to schistosomes result in high reinfection rates despite mass drug administration [43] and thus possibly lead to repetitive IFN-γ flares systemically and in the liver [44] alongside a high prevalence of chronic infection [5, 45]. Unexpectedly and in contrast to HCV [12–14], our study reveals that schistosomiasis and HBV interact rather subtly concerning liver disease outcome. Schistosome-induced immune responses might even be beneficial to control HBV to some extent but are not sufficient to clear the infection.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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Author contributions. C. U. P. d. C. designed and supervised the study and wrote and edited the manuscript; U. P. designed and supervised the study and wrote and edited the manuscript; E. L.-V. designed the study, performed the experiments, analyzed the data, and wrote the manuscript; S. B. performed experiments, analyzed the data, and edited the manuscript; A. M. performed experiments; J. F. performed experiments and provided support for data interpretation; A. D. K. performed experiments and provided support for data interpretation; M. R. performed experiments; S. R. S. performed experiments; K. S. performed experiments and provided support for data interpretation; M. H. performed experiments and provided support for data interpretation.

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