TECHNISCHE UNIVERSITÄT MÜNCHEN

Lehrstuhl für Biochemische Pflanzenpathologie

The Influence of regulatory T cells and other Immunoregulators on the Course of Hepatitis B Virus Infection

Leonhard Stross

Vollständiger Abdruck der von der Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften

genehmigten Dissertation.

Vorsitzender: Univ.-Prof. Dr. S. Scherer

Prüfer der Dissertation:

1. Univ.-Prof. Dr. J. Durner
2. Univ.-Prof. Dr. U. Protzer

Die Dissertation wurde am 12.05.2011 bei der Technischen Universität München eingereicht und durch die Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt am 26.09.2011 angenommen.

Diese Dissertation wurde durch die Friedrich-Naumann-Stiftung für die Freiheit mit einem Promotionsstipendium gefördert.

Table of Contents

ABBREVIATIONS

Ad	Adenoviral vector
ALT	Alanine aminotransferase
APC (fluorescent dye)	Allophycocyanin
APC	Antigen-presenting cell
BFA	Brefeldin A
CBA	Cytometric bead array
cccDNA	Covalently closed circular DNA
CD	Cluster of differentiation
CM	Central memory T cell
CP	Cytoplasm
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte antigen 4
Cy7	Cyanin 7
d(x)	day(x) post infection
DC	Dendritic cell
DEREG	Depletion of regulatory T cells
DNA	Deoxyribinucleic acid
DTX	*Diphteria* toxin
EBV	Eppstein-Barr virus
Eff	Effector T cell
EM	Effector-memory T cell
EMA	Ethidium monoazide bromide
ER	Endoplasmatic reticulum
FACS	Fluorescence assigned cell sorting
FCS	Fetal calf serum
Foxp3	Forkhead-box protein 3
FSC	Forward scatter channel
(e)GFP	(enhanced) Green fluorescent protein
GITR	Glucocorticoid induced TNFR related protein
HBc	Hepatitis B virus core protein
HBeAg	Hepatitis B e antigen
HBs	Hepatitis B virus small surface protein
HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HIV	Human immunodeficiency viruss
ICS	Intracellular cytokine staining
IFN	Interferon
IL	Interleukin
i.p.	Intraperitoneal
i.u.	Infectious units
i.v.	Intravenous
k/o	Knock-out
L	Ligand
LAG-3	Lymphocyte activation gene 3
LAL	Liver-associated lymphocyte
LCMV	Lymphocytic choriomeningitis virus
LSEC	Liver-sinusoidal endothelial cell
МΦ	Macrophage

MCMV	Mouse cytomegalovirus
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
MOI	Multiplicity of infection
mRNA	Messenger RNA
MVA	Modified vaccinia virus Ankara
N	Nucleus
NK	Natural killer
OVA	Ovalbumin
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PerCP	Peridininchlorophyll protein
pgRNA	Pregenomic RNA
PRR	Pathogen recognition receptor
qPCR	Quantitative PCR
rcDNA	relaxed circular DNA
RNA	Ribonucleic acid
RT-PCR	Reverse transcription PCR
s.c.	Single cells
SCC	Sideward scatter channel
TCR	T cell receptor
TGF	Transforming growth factor
Th	T helper
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
wt	Wild-type

Abstract

Hepatitis B is one of the most important infectious diseases worldwide and a major global health problem. About one billion of today's population was in contact with the hepatitis B virus (HBV), and between 300 and 400 million people are chronically infected. Even though an effective prophylactic vaccine is available, the world community was not able to contain the disease. Therapies for chronic HBV infection are in most cases ineffective. Thus, there is an urgent need for new approaches towards an effective therapy. But immunological mechanisms involved in the clearance of HBV infection are still not completely understood. Various cell populations may be involved in regulating the immune response and deciding about the outcome of infection. In this study, the role of $Foxp3^+$ $CD4^+$ regulatory T cells (Tregs), the immunoregulatory cytokine interleukin-10 (IL10) and $CD4^+$ effector T cells in HBV control was examined. An adenoviral vector transferring a 1.3 fold overlength HBV genome (AdHBV) to initiate HBV replication in mice was used. In the first part of the study, DEREG mice, a transgenic mouse strain in which $Foxp3^+$ Tregs can be depleted transiently and very specifically by injection of *diphtheria* toxin (DTX), were used to study the impact of Tregs on HBV immune responses. Tregs were depleted either before or during infection. Liver damage and virological parameters (HBeAg, HBsAg, HBV titers) were determined. Liver-associated lymphocytes and splenocytes were isolated and stimulated with HBV core and S-protein peptide pools and analysed by intracellular cytokine staining (ICS) and flow cytometry. Enhanced immune responses were only observed when Tregs were depleted early after infection. Treg-depleted mice showed a more severe liver damage, better controlled HBV-replication, had lower serum virus titers and secreted less HBeAg and HBsAg. Tregs decreased the influx of T cells and macrophages into the liver and diminished T cell mediated cytokine responses. By this, Treg reduce immune-mediated liver damage early after infection but at the cost of prolonging clearance of the infection. In the second part of this study, the role of Treg-derived IL10 in acute liver-inflammation was examined. Using DEREG mice and IL10-reporter mice, it was observed that Tregs enhance IL10 secretion in the liver during acute liver inflammation. Surprisingly, hepatic Tregs were not the main producers of IL10, but instead, infiltrating $CD8^+$ T cells and macrophages. Loss of Treg-derived IL10 seemed to abolish IL10 secretion by these cells, resulting in markedly increased liver damage. It was also observed that HBc induces IL10 secretion by macrophages, not HBs. In the third part of this study, the influence of effector $CD4^+$ T cells on the outcome of HBV infection was analyzed. Depletion of $CD4^+$ T cells in AdHBV-infected C57BL/6 mice lead to persistence of HBsAg, but not HBeAg. CD4-depleted mice failed to mount B cell responses against the

virus, and no liver inflammation was established. Even though $CD4^+$ T cells repopulated the liver after some time, no T cell responses were induced against HBV, resulting in markedly delayed clearance of the virus. However, replication of HBV was suppressed in the late phase of infection, independently of T cells. To address the possible use of CD4-depleted, AdHBV-infected mice as a new small-animal model of chronic HBV infection, different CD4-depletion strategies and their influence on HBV infection were compared. Alltogether, this study provides new and detailed insight into the complex interplay between immunoregulatory mechanisms and HBV infection. It demonstrates that Tregs and $CD4^+$ T cells are master regulators of anti-HBV immune responses, deciding about the outcome of infection. The increased knowledge about virus-host interactions provided by this study will enable new approaches for immunotherapy.

Zusammenfassung

Hepatitis B ist eine der weltweit wichtigsten Infektionskrankheiten und eines der größten Probleme für die weltweite Gesundheit. Etwa eine Milliarde der heute lebenden Menschen hatten Kontakt mit dem Hepatitis B Virus (HBV), und zwischen 300 und 400 Millionen Menschen sind chronisch infiziert. Trotz eines verfügbaren, effektiven prophylaktischen Impfstoffs ist es der Weltgemeinschaft bisher nicht gelungen, die Krankheit einzudämmen. Die Therapien für chronische Heptitis B sind of ineffektiv. Es besteht also ein dringender Bedarf für neue Behandlungsoptionen. Jedoch sind die immunologischen Mechanismen, die an der Eliminierung des Virus mitwirken, nicht komplett verstanden. Verschiedene Zellpopulationen können in die Steuerung der Immunantwort gegen HBV involviert sein und über den Ausgang der Infektion entscheiden. In dieser Arbeit wurde die Rolle der $CD4^+$ $Foxp3^+$ regulatorischen T Zellen (Tregs), des immunoregulatorischen Zytokins Interleukin-10 (IL10) sowie der $CD4^+$ Effektor T Zellen für die Kontrolle von HBV untersucht. Dafür wurde ein adenoviraler Vektor (AdHBV) genutzt, der ein 1.3-faches Überlängen-HBV-Genom transferiert, um HBV-Replikation in Mäusen zu induzieren. Im ersten Teil dieser Arbeit wurden DEREG-Mäuse, ein transgener Mausstamm, in dem Tregs durch Injektion von *diphteria*-Toxin (DTX) vorübergehend und hochspezifisch depletiert werden können, infiziert, um den Einfluss von Tregs auf die HBV Immunantworten zu definieren. Tregs wurden entweder vor oder während der Infektion depletiert. Der Leberschaden sowie virologische Parameter (HBeAg, HBsAg, HBV Titer) wurden gemessen. LALs und

Splenozyten wurden isoliert, mit HBV core-Protein- und HBV S-Protein-Peptidpools stimuliert, und mittels intrazellulärer Zytokinfärbung (ICS) und Durchflusszytometrie analysiert. Nur wenn Tregs kurz nach der Infektion depletiert wurden, wurden erhöhte Immunantworten gemessen. Treg-depletierte Mäuse zeigten einen höheren Leberschaden und niedrigerer HBV-Titer, kontrollierten die HBV-Replikation besser und sekretierten weniger HBeAg und HBsAg. Tregs reduzierten den Einstrom von T Zellen und Makrophagen in die Leber und milderten T Zell vermittelte Zytokinantworten. Auf diese Weise minimieren Tregs den immunvermittelten Leberschaden, aber um den Preis einer verzögerten Eliminierung des Virus. Im zweiten Teil dieser Arbeit wurde die Rolle des von Tregs produzierten IL10 auf die akute Leberentzündung untersucht. Durch die Verwendung von DEREG- und IL10-Reportermäusen konnte gezeigt werden, dass Tregs die IL10-Produktion in der Leber während einer akuten Leberentzündung erhöhen. Erstaunlicherweise waren hepatische Tregs jedoch nicht die hauptsächlichen Produzenten von IL10 in der Leber, sondern infiltrierende $CD8^+$ T Zellen sowie Makrophagen. Ein Verlust des von Tregs produzierten IL10 verhinderte die IL10-Sekretion durch diese genannten Zellen, was zu einem deutlich erhöhten Leberschaden führte. Es wurde weiterhin festgestellt, dass HBc IL10-Sekretion in Makrophagen induziert, nicht HBs. Im dritten Teil dieser Arbeit wurde der Einfluss von $CD4^+$ Effektor T Zellen auf den Ausgang der HBV Infektion analysiert. Depletion von $CD4^+$ T Zellen in AdHBV-infizierten C57BL/6 Mäusen führte zur Persistenz von HBsAg, aber nicht HBeAg. CD4-depletierte Mäuse entwickelten keine B Zell-Antwort gegen HBV und auch keine Leberentzündung. Trotz der späteren Wiederbesiedlung der Leber durch $CD4^+$ T Zellen wurde keine T Zell Antwort gegen das Virus induziert, was zu einer deutlich verzögerten Eliminierung des Virus führte. Allerdings wurde die HBV-Replikation in der späten Phase der Infektion auch T Zell-unabhängig unterdrückt. Um den möglichen Nutzen CD4-depletierter, AdHBV-infizierter Mäuse als neues Kleintiermodell der chronischen Hepatitis B zu bestimmen, wurden verschiedene Depletionsstrategien und ihr Einfluss auf den Ausgang der Infektion verglichen. Zusammengenommen ermöglicht diese Arbeit neue und detaillierte Einsichten in die komplexen Wechselwirkungen zwischen immunregulatorischen Mechanismen und der HBV Infektion. Die Ergebnisse dieser Arbeit belegen, dass Tregs und $CD4^+$ T Zellen zentrale Steuereinheiten der gegen HBV gerichteten Immunantworten sind und maßgeblich über den Ausgang einer HBV Infektion entscheiden. Das durch diese Arbeit vergrößerte Wissen über die Virus-Wirt-Interaktion wird neue Ansätze für Immuntherapein ermöglichen.

1 Introduction

1.1. Hepatitis B virus infection

Hepatitis B is a major global health problem caused by the human hepatitis B virus (HBV). Up to 10% of adults acutely infected with this virus develop a chronic infection, correlating with T cell dysfunction and hyporesponsiveness (Rehermann & Nascimbeni 2005). Today, about 350 million people worldwide suffer from chronic hepatitis B, and 1-2 million die every year as a result of infection, mainly from liver malignancies like cirrhosis and hepatocellular carcinoma (HCC) (Rehermann & Nascimbeni 2005; Lavanchy 2004). The disease is mainly transferred by blood, blood products and sexual intercourse. But also bad hygienic conditions and re-use of contaminated chirurgical instruments or syringes – for example for drug abuse – promote spreading of the disease. Especially countries of the third world and under-developed regions are endemic areas (Fig.1). In these areas, the virus is often conferred from mother to child, resulting in chronic infection of the newborn in 90% of cases (Fig. 2) (Sirma, Funk & Will, in "Hepatitis B: Infektion – Therapie – Prophylaxe"). A prophylactic vaccine is available, but it does not induce viral clearance in chronically infected patients. Therapies for chronic HBV infection include the application of conventional and pegylated interferon (Lin et al. 1999), Lamivudin (Liaw et al. 2004), the nucleotide analogon Adefovir (Marcellin et al. 2003) or the nucleoside analogon Entecavir (Lai et al. 2002). Additional substances, e.g. Tenofovir or Telbivudin are also in use (Heintges, in "Hepatitis B: Infektion – Therapie – Prophylaxe"). But these therapies often fail to cure the disease. Also, resistant HBV mutants can be selected over time under therapy with nucleoside or nucleotide analoga (Heintges, in "Hepatitis B: Infektion – Therapie – Prophylaxe"). As a result, there is an urgent need for new immunoTherapieutic approaches, but due to the lack of model systems and appropriate patient material, the knowledge about possible mechanisms leading to HBV persistence in the host is limited. Therefore, much effort is needed to elucidate the immunological interplay between virus and host.

1.2. The hepatitis B virus (HBV)

HBV belongs to the family of *hepadnaviridae* (*hepa*titis-*DNA*-viruses). Related viruses were found in rodents, birds and primates, e.g. woodchucks, ducks and chimpanzees (Baumert, Barth & Blum, 2005). The major characteristics of hepadnaviruses are their hepatotropism, strict species-specificity, their capacity to persist and to induce a chronic

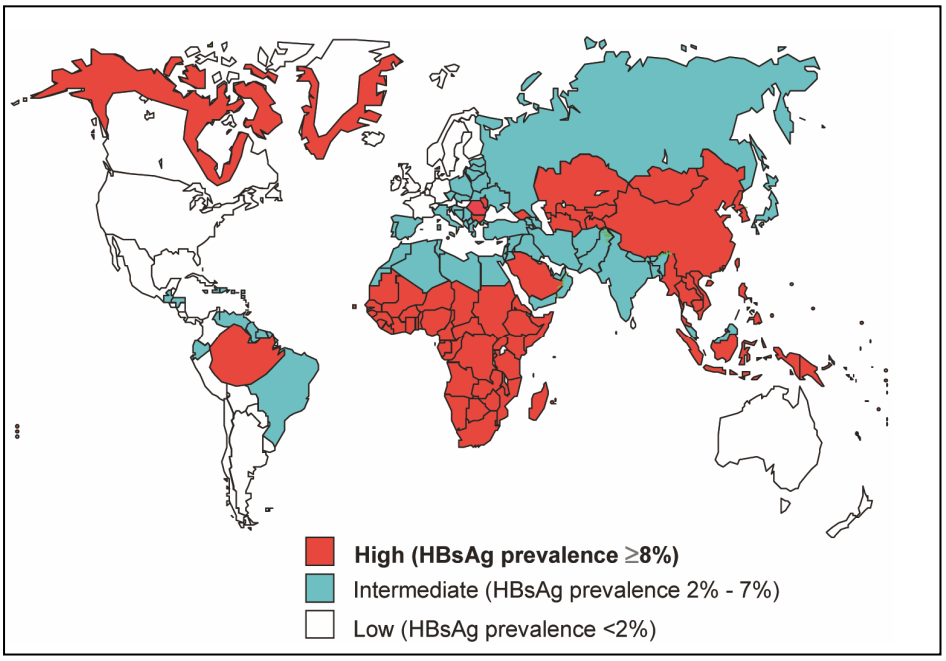


Fig. 1: Geographical distribution of HBV endemicity. Taken from: Introduction of hepatitis B vaccine into childhood immunization services. WHO, 2001.

infection, a similar mode of replication including a reverse-transcription step, and similar genome-structure and –organization (Ganem & Schneider, 2001).

Three different forms of virus particles can be discriminated in the sera of infected individuals (Sirma, Funk & Will, in "Hepatitis B: Infektion – Therapie – Prophylaxe"): First the infectious virions or Dane-particles, which consist of an icosahedral nucleocapsid containing the genome and a lipid-membrane with embedded viral surface proteins. The majority of nucleocapsids is built up from 240 units of core-protein (HBcAg or HBc). Second, empty spherical and filamentous particles (subviral particles) that consists only of lipid-membranes and surface proteins (Fig.3). There are three viral surface proteins: The large L-protein, the medium M-protein and the small S-protein. Together, they are often referred to as HBsAg.

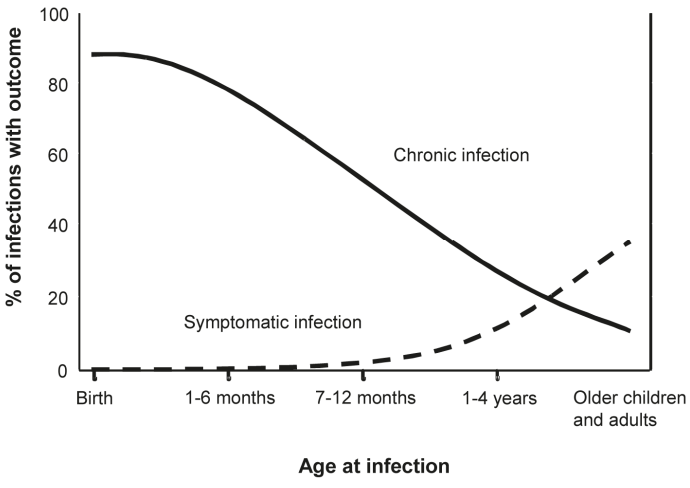


Fig. 2: Outcome of hepatitis B virus infection by age at infection.
Taken from: Introduction of hepatitis B vaccine into childhood immunization services. WHO, 2001.

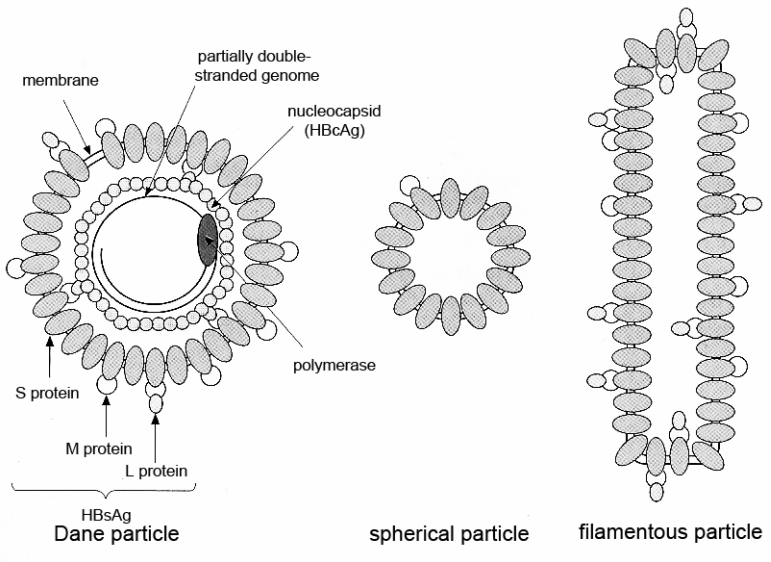


Fig. 3: The three different HBV particle entities. The schematic depiction shows the infectious, enveloped virion **(left)** and the two forms of uninfectious, empty subviral particles, the spherical **(middle)** and the filamentous **(right)** particle. Taken and modified from: Sirma H, Funk A and Will H. Molekulare Virologie von Hepatitis-B- und –D-Viren. In: Hepatitis B. Infektion – Therapie – Prophylaxe. Georg Thieme Verlag, 2006. Chapter 1.5, p.4.

The HBV genome has a quite unique structure (Sirma, Funk & Will, in "Hepatitis B: Infektion – Therapie – Prophylaxe"). It is partially double-stranded with an about 3200 bp negative strand and a shorter positive strand. The viral polymerase is bound to one end of the genome. The genome organization is very compact; there are four overlapping open reading frames (ORF) and all nucleotides have coding function. The first ORF encodes the three related forms of the HBsAg, the second the core-protein and its secreted form, HBeAg. The third ORF encodes the polymerase and the last one the HBV X-protein, which seems to have regulatory and carcinogenic properties.

According to different antigenic determinants of HBsAg, HBV can be classified into different serological subtypes. The original first described serological subtypes were adw, adr, ayw and ayr. But this classification does not reflect the phylogenetic relationship between HBV genomes, therefore, HBV-genotypes were later on characterized according to similarity of their genomes (Norder et al. 2004). The resulting genotypes A – H have geographical prevalences; in Europe, genotypes A and D are dominant. It is unclear, how the genotype or serological subtypes influences the course of infection, but it is possible that differences in amino acid sequences can influence the immunogenicity of HBV proteins or the efficiency of antiviral drugs, and it was suggested that different genotypes result in different strengths of disease symptoms (Kao et al., 2000).

1.3. The viral life cycle

The cellular receptor responsible for HBV-binding on and entry into hepatocytes is still not characterized, as well as the early steps following binding of the virus on the host cell (Nassal 1999). It is known however, that the viral large surface-protein (L-protein) mediates binding

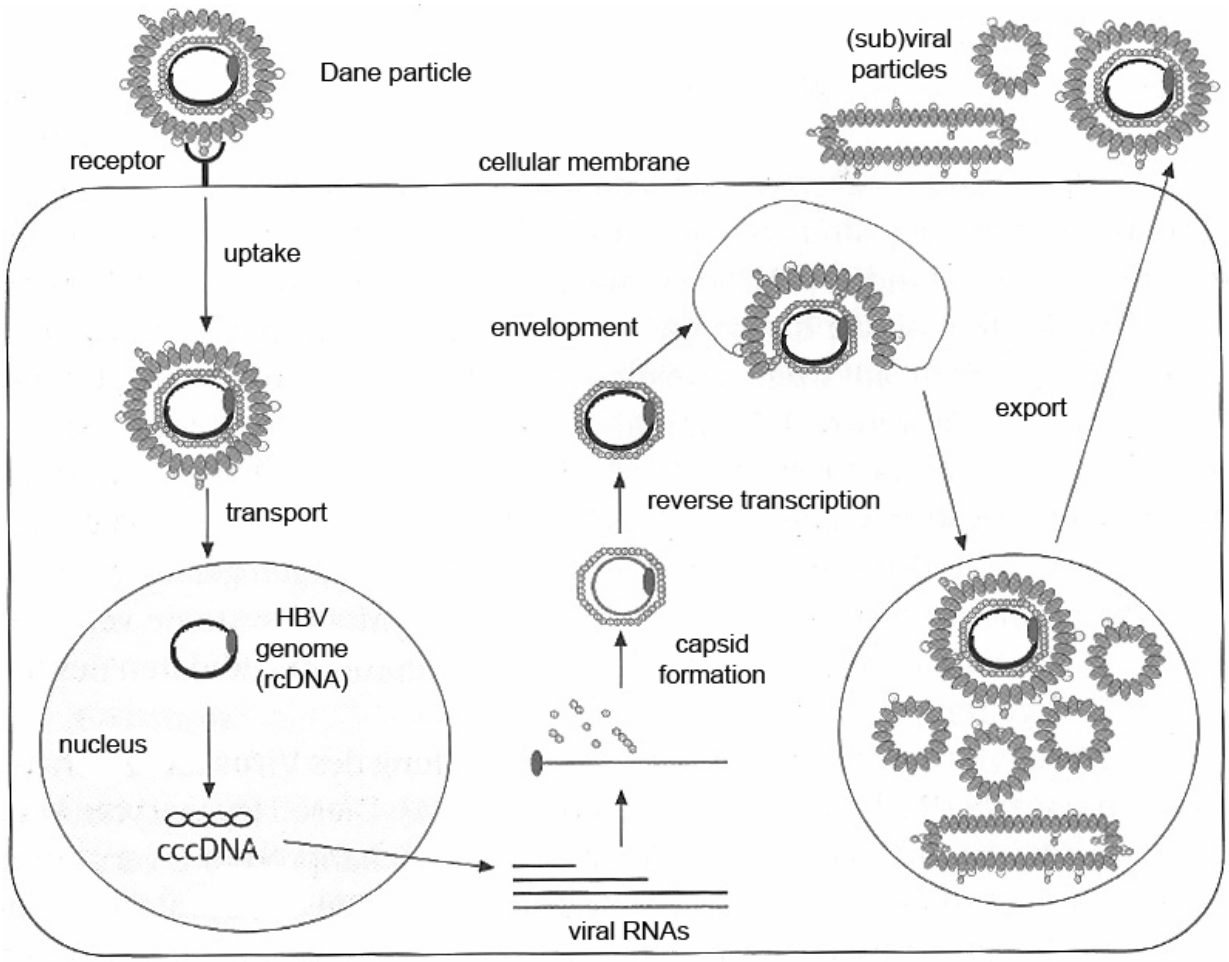


Fig. 4: Schematic depiction of the HBV life cycle. Taken and modified from: Sirma H, Funk A and Will H. Molekulare Virologie von Hepatitis-B- und –D-Viren. In: Hepatitis B. Infektion – Therapie – Prophylaxe. Georg Thieme Verlag, 2006. Chapter 1.6.1, p.12.

of the viral particle to the yet unknown receptor on the hepatocyte-surface. After entry, the viral genomes are actively transported into the nucleus of the cell, and a productive infection is established (Ganem & Schneider, 2001). The usually relaxed-circular HBV-DNA (rcDNA) is then transformed into a covalently closed circular DNA (cccDNA), which serves as highly stable "mini-chromosome" and is usually not integrated into the host genome (Bock et al. 2001). Only a small fraction of the viral DNA is integrated into the cellular DNA and looses its replicative capacity, but may cause malignant transformation of the infected cell.

The cccDNA serves as transcription template for cellular polymerases, and different forms of viral RNAs are produced and exported from the nucleus. One of the largest RNAs (pregenomic or pgRNA) serves as template for reverse transcription into viral genomes by the viral polymerase and additionally encodes the core-protein and viral polymerase. Another large RNA encodes the precursor protein for HBeAg, PreC. This RNA is found in a multitude of spliced variants with unknown function (Sommer et al. 2000). Another viral RNA encodes the L-protein, while the most abundant RNA serves as template for the translation of M- and

S-protein. The RNA encoding the viral X-protein is still unknown (Sirma, Funk & Will, in "Hepatitis B: Infektion – Therapie – Prophylaxe").

The synthesized viral surface proteins are integrated into the endoplasmatic reticulum and either form empty subviral particles or interact with viral capsids to form enveloped Dane-particles. Autonomously, core-proteins interact with each other and with pgRNA to form viral capsid. Once encapsidated, reverse transcription of the RNA into rcDNA-genomes takes place (J Summers & Mason 1982). After interaction with surface proteins, the enveloped particles are transported to the cell surface and released (Ganem & Schneider, 2001). Some mature capsids take an alternative route and are transported back to the nucleus, releasing the viral DNA and filling up the cccDNA pool (Zhang et al. 2003). The viral life cycle is schematically depicted in Fig. 4.

1.4. Immunopathogenesis of HBV infection

HBV is a non-cytopathic virus and does not lyse the host cell during its life cycle (Dienes & Uta Drebber 2010). The tissue damage and the liver inflammation are instead mediated by the host's immune system, trying to eliminate the virus by inhibiting virus-replication and killing infected hepatocytes. The success of these mechanisms decides if the virus is eliminated (acute, self-limiting infection) or persists (chronic infection).

After entry into the host, the virus infects hepatocytes and starts to replicate there. How it reaches and enters the hepatocytes is still unclear. The first line of defense reaction to HBV infection is the innate immunity. First, infected cells as well as local dendritic cells (DCs) produce IFNα, which in turn induces several other genes that hamper viral replication and help to degrade viral DNA (Banchereau & Steinman 1998). Furthermore, IFNα activates other cells of the innate immunity, e.g. natural killer cells (NK cells) and monocytes/macrophages, resulting in an early drop of viral load(Webster et al. 2004). But if IFNα is induced and plays a major role in the early control of HBV infection is still controversial (Antonio Bertoletti & Gehring 2006). However, the innate immune system alone is not sufficient to clear the virus. Instead, the successful induction of an efficient adaptive immune response seems to decide about the outcome of infection (Thimme et al. 2003). After uptake of virus particles and proteins, DCs with their potent costimulatory capacity migrate from the periphery to local lymphatic tissues and present the processed viral antigens to naïve, antigen-specific $CD4^+$ and $CD8^+$ T cells. Also, other cells in the liver, e.g. macrophages, monocytes and liver sinusoidal endothelial cells (LSEC), are able to present viral antigens to T cells, contributing to the induction of an adaptive immune response. After activation, HBV-specific T cells proliferate

and accumulate at the site of infection, exerting their effector function. $CD8^+$ cytotoxic T cells (CTLs) are supposed to lyse infected hepatocytes directly by utilizing perforin and granzymes (Whitmire 2011), but some data questions the importance of $CD8^+$ secreted perforin/granzyme, the enzymes that usually confer cytotoxicity (Kafrouni et al. 2001; Dienes & Uta Drebber 2010). But it was suggested that $CD8^+$ T cells, together with monocytes/macrophages, mediate apoptosis of infected hepatocytes by secretion of tumor necrosis factor alpha (TNFα) (Sitia et al. 2004). Furthermore, both HBV-specific $CD8^+$ and $CD4^+$ T cells secrete interferon gamma (IFNγ), thus inhibiting HBV replication (D. M. Brown et al. 2010).

B cells are also activated by DCs in cooperation with $CD4^+$ T cells, and differentiate into HBV-specific B memory and plasma cells (Whitmire 2011). They produce antibodies specific for the different viral proteins. Some of these antibodies, namely anti-HBc, anti-HBeAg and anti-HBs, are useful diagnostic markers (Böcher, in "Hepatitis B: Infektion – Therapie – Prophylaxe"). Since anti-HBs forms immune-complexes with secreted HBs-protein, they are often only detectable with special assays as long as viral surface proteins are secreted in high amounts (Maruyama T 1993). The neutralizing antibodies play an important role in the immune response against HBV, since binding of free virions prevents them from infecting new cells, while binding of viral proteins on the surface of infected cells targets them for immune mediated lysis. Thus, the reservoir for HBV replication can be reduced. If the adaptive immune response is strong and multispecific, HBV infection can be spontaneously resolved after an acute, often clinically inapparent course of disease (Böcher, in "Hepatitis B: Infektion – Therapie – Prophylaxe"). This is the usual course in adults (Fig. 2). But the immune system generally fails to eliminate every copy of the virus, instead, HBV persists without detectable replication and protein-secretion in most individuals with a resolved hepatitis B (Böcher, in "Hepatitis B: Infektion – Therapie – Prophylaxe").

In a certain percentage of HBV infections, most often in newborn children, the immune system fails to suppress and clear the virus (Fig. 2). In that case, a chronic-persistent infection is the result. Studies provide evidence that chronically infected patients are not able to launch efficient $CD8^+$ and $CD4^+$ T cell responses, essential for viral clearance (Livingston et al. 1999; Webster et al. 2004). The cause for this specific lack of cellular immunity is still elusive. Negative selection of HBV-specific T cells, immunological ignorance or anergy – as a result of continuous exposure to viral antigens – and lacking activation of innate immunity may lead to T cell hyporesponsiveness (Kazuhiro Kakimi et al. 2002). Furthermore, the liver provides an inherently tolerizing immunological environment (Thomson & Knolle 2010). It

traps and eliminates activated $CD8^+$ T cells (Thomson & Knolle 2010). Antigen presenting cells in the liver, i.e. hepatocytes and non-parenchymal liver cells, including hepatic DCs, have cell intrinsic mechanisms to maintain a tolerogenic phenotype and are subject to anti-inflammatory mediators. The resulting imbalance between immune responses and viral replication results in chronic inflammatory processes in the liver, which can lead to liver cirrhosis and HCC. There are different forms of chronic HBV, some with weak immunological antiviral activity, causing low viral replication and moderate liver damage, others with nearly no antiviral immune responses, resulting in uncontrolled high HBV replication, but no liver damage (Webster et al. 2004).

1.5. $CD4^+$ effector T cells

$CD4^+$ T cells are a major cell population of the adaptive immunity and serve several important immunological functions. In case of viral infection, they promote activation and proliferation of virus specific cytotoxic $CD8^+$ T cells (Williams & Bevan 2007), attract them to the infected tissue (Lane et al. 2000) and enhance the formation of $CD8^+$ memory T cells (Whitmire 2011). Also, they perpetuate antiviral $CD8^+$ T cell responses by secretion of IL21 (Elsaesser & Sauer 2009). $CD4^+$ T cells also play a central role in the induction of antiviral humoral immune responses, as they induce B cells to differentiate into plasma cells and B memory cells and influence the kind of antibody that is produced (Whitmire 2011). $CD4^+$ T cells have been shown to be essential for induction of $CD8^+$ T cell responses, since they improve the capacity of antigen presenting cells (APCs) to present viral antigens to effector T cells (Whitmire 2011). Besides the indirect functions described above, $CD4^+$ T cells also have direct antiviral abilities. Firstly, they can secrete antiviral cytokines (e.g. IFNγ) to suppress replication of viruses in infected cells (Whitmire 2011). Secondly, cytolytic $CD4^+$ T cells have recently been identified (Jellison et al. 2005; Yates et al. 2007). In contrast to cytotoxic $CD8^+$ T cells, that utilize perforin and granzymes to lyse infected target cells, it was observed that $CD4^+$ T cells rely mostly on FAS-L and TRAIL to drive infected cells into caspase-dependent apoptosis (Whitmire 2011). But other experiments have shown that cytotoxic $CD4^+$ T cells use mainly perforin/granzyme, only in an IL2-limited environment they switch to usage of Fas-L (Brown et al. 2010). However, it was demonstrated that the protective function of cytotoxic $CD4^+$ T cells mediated by direct killing is not as effective as that of $CD8^+$ CTLs (Jellison et al. 2005). Thus, the role of this newly characterized $CD4^+$ effector cell population needs further investigation.

$CD4^+$ T cells are induced upon recognition of antigens presented in an MHCII-context (Whitmire 2011). While $CD8^+$ T cells recognize antigens with a length of 8-10 amino acids, $CD4^+$ T cells recognize fragments of 12-15 amino acids. Only professional antigen presenting cells that express MHCII molecules on their surface can mount $CD4^+$ T cell responses: DCs, monocytes/macrophages, B cells and some specialized cell types in certain tissues, e.g. LSECs (Whitmire 2011; Knolle & Gerken 2000). These cells present extracellular material that they have endocytosed, degraded and processed, e.g. viral particles and proteins or viral debris from lysed cells (necrotic/apoptotic cells). This results in an enhanced sensitivity of $CD4^+$ T cells to foreign antigens in comparison to $CD8^+$ T cells, since they can be induced by cells that are not infected themselves and by non-replicating viral material (Whitmire 2011). The strength of a $CD4^+$ T cell response depends largely on amount and duration of viral antigens, and presence of the antigenic stimulus has been shown to be essential during the initial expansion of the $CD4^+$ T cell response (Obst et al. 2005; Ravkov & Williams 2010). On the other hand, prolonged and continuous presence of high quantities of antigen can be deleterious to $CD4^+$ T cell responses and result in functional decline and exhaustion, as recently shown (Han et al. 2010).

A characteristic feature of $CD4^+$ T cells is their functional diversity, apparent by the large number of defined subpopulations (Table 1). The $CD4^+$ subsets characterized firstly were the Th1 and Th2 subsets. Th1 cells can be found in lymphoid tissues but they also migrate to peripheral sites (Whitmire 2011). They produce pro-inflammatory cytokines like IFNγ, thereby inducing inflammation, suppressing viral replication, recruiting $CD8^+$ T cells and macrophages to sites of infection and enhancing antigen presentation by APCs. Additionally, they secrete IL2 to induce and support the proliferation of effector cells (Whitmire 2011). Th2 cells, on the other hand, are mainly responsible for the induction of antibody responses (Whitmire 2011; Zhou et al. 2009). Many other $CD4^+$ subpopulations have been defined in recent years (Zhou et al. 2009; Murphy & Stockinger 2010), some with immunosuppressive function (Tr1, Th3, Th22, $Foxp3^+$ Tregs), some with proinflammatory function (Th17, Th21). It has been shown however, that the fate of a $CD4^+$ T cell belonging to a certain subpopulation is not always firmly determined, but instead that CD4-differentiation is flexible and reversible. Th2 cells for example can switch to Th1 phenotype when they are exposed to a cytokines expressed during viral infection (Hegazy et al. 2010). Also, Foxp3-negative $CD4^+$ can be converted into $Foxp3^+$ Tregs under certain conditions (Miyara & Sakaguchi 2007).

Due to the mechanisms of activation, the functional diversity and phenotypical flexibility, it is difficult for viruses to evade $CD4^+$ T cell responses, but some viruses have evolved

subpopulation	secreted cytokines	supposed function
Th1	IFNγ, TNFα, IL2	inflammation, T cell and macrophage activation, cytotoxicity
Th2	IL10, TGFβ, IL4	induction of antibody response
Th0	Th1+Th2 phenotype	not defined
Th3	TGFβ	suppression of immunity against ingested antigens
Th17	IL17	inflammation, microbial and antiviral immunity
Th21	IL21	maintaining $CD8^+$ T cell responses
Th22	IL22	tissue protection and regeneration
Tr1	IL10	immunosuppression
$Foxp3^+$ Treg	IL10, TGFβ	immunosuppression

Table 1: Important $CD4^+$ T cell subpopulations. Indicated are the name of the subpopulation **(left)**, the mainly secreted cytokines **(middle)**, and the supposed function **(right)**. This list does claim neither completeness nor finality, but exemplarily depicts the subpopulations considered being most important by the author.

mechanisms to accomplish that: Viruses (e.g. EBV, MCMV) suppress immune responses by producing viral IL10-homologues (Kanai et al. 2007; Kotenko et al. 2000), others specifically
target DCs for $CD8^+$ mediated lysis (Zuniga et al. 2008) or downregulate MHCII expression (Hegde et al. 2002).

A lot of effort has been taken to elucidate the role of $CD4^+$ T cells in HBV infection. While the significance of $CD4^+$ T cells for the induction of antiviral T cell responses is still somewhat controversial (Castellino & Germain, 2006), evidence was found that early $CD4^+$ T cell responses decide about viral clearance and outcome of infection and serve as master regulators of the adaptive immune response to HBV (P. L. Yang et al. 2010). In another study it was observed that $CD4^+$ T cells are possibly required for HBV clearance and prevention of viral persistence (Asabe et al. 2009). A nucleocapsid-specific $CD4^+$ T cell response has been shown to be important for HBV control (Bertoletti & Gehring 2006), while HBsAg induces $CD4^+$ T cells only weakly in natural HBV infection (Böcher et al. 1999). The interesting

finding that induction of $CD4^+$ T cells by HBsAg in natural HBV infection is weak, while HBsAg elicits strong $CD4^+$ T cell responses upon vaccination implies differences in antigen-presentation (Bertoletti & Gehring 2006). Other HBV proteins, the polymerase and X-protein, have not yet been examined for their immunogenicity towards $CD4^+$ T cells (Bertoletti & Gehring 2006).

Summarized, $CD4^+$ T cells serve a variety of important immunological purposes. With their highly flexible functional diversity, they constitute a major junction between immunologic protection and immunopathogenesis in viral infection, and they play a major role for the outcome of HBV infection.

1.6. Regulatory T cells

$CD4^+Foxp3^+$ regulatory T cells (Tregs) were originally shown to be a specialized T cell subpopulation actively suppressing pathological and physiological immune responses for maintaining immunological tolerance (Sakaguchi 2004; Sakaguchi 2005). First, this T cell subset was characterized by the constitutive surface expression of the interleukin-2 receptor alpha (CD25) (Taguchi & Takahashi 1996; McHugh & Shevach 2002). In the following years, however, it became clear that CD25 is also expressed on activated conventional effector T cells, limiting the value of data gained by CD25-depletion experiments (Sutmuller et al. 2001). In the following, the transcription factor forkhead-box-protein 3 (Foxp3) was identified to control Treg-function, providing the best available marker for Tregs in mice and men (Fontenot et al. 2003; Fontenot et al. 2005; Hori et al. 2003). Foxp3 controls development and function of Tregs (Sakaguchi 2004). $CD4^+$ Tregs arise out of two sources: *Natural* Tregs mature physiologically in the thymus as a fully functional population, while certain antigenic stimuli lead to the transformation of naïve effector T cells into so-called *adaptive* Tregs (Miyara & Sakaguchi 2007). In mice, 5-10% of $CD4^+$ PBMC are $Foxp3^+$ Tregs (Haeryfar et al. 2005). Studies in different settings (viral and bacterial infections, tumor models, autoimmunity) demonstrated that Tregs suppress proliferation, cytokine production and cytotoxic activity of naïve and antigen-specific $CD4^+$ and $CD8^+$ effector T cells (Billerbeck et al. 2007) and are able to interfere with the activity of antigen-presenting cells (APCs) as well as B cells (von Boehmer 2005). In general, Treg-depleted mice showed 2-3-fold enhanced effector T cell responses and eliminate viruses faster (Rouse et al. 2006). With their immunosuppressive capacity, Tregs were shown to counteract T cell hyperactivation, mitigate chronic inflammatory reactions and maintain tolerance against self-antigens (Rouse et al. 2006). The mechanisms of Treg-mediated immunosuppression are still mostly unknown,

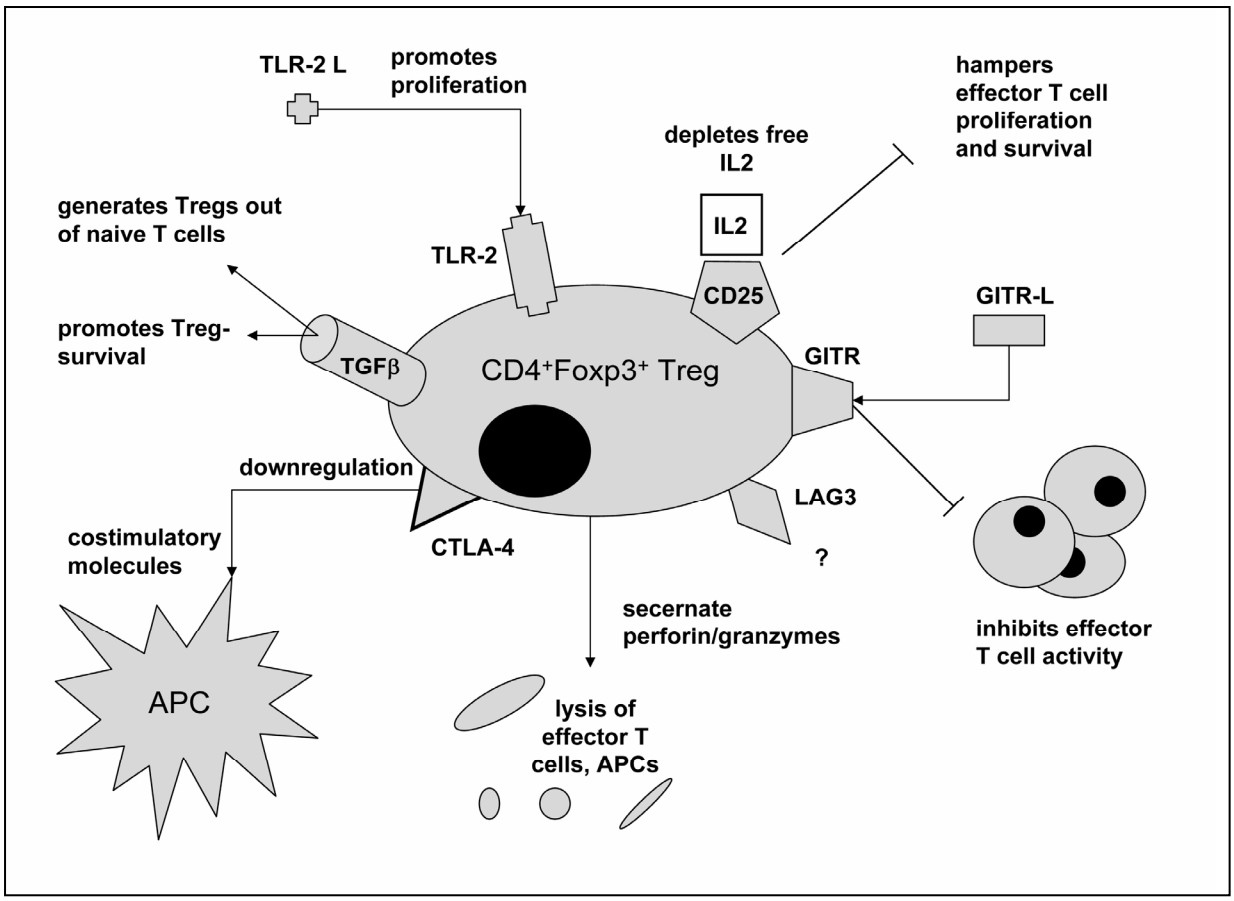


Fig. 5: Pathways of Treg-activity. Depicted are known molecular pathways by which regulatory T cells exert their immunosuppressive function. This schematic figure was designed according to information cited in the chapter "Regulatory T cells". GITR-L: GITR-ligand; TLR-2 L: TLR-2 ligand.

but several studies have recently tried to elucidate some of the pathways Tregs use to communicate with and influence their environment (Miyara & Sakaguchi 2007; Grossman et al. 2004). Tregs utilize different signalling-pathways and receptor/ligand interactions to exert their function (Fig. 5). The most important molecules which have been observed to play a role in Treg-function are cytotoxic T-lymphocyte antigen 4 (CTLA-4), transforming growth factor β (TGFβ), glucocorticoid-induced TNFR (tumor necrosis factor receptor) related protein (GITR), lymphocyte activation gene 3 (LAG-3), CD25, Toll-like Receptor (TLR)-2 and -8 (Miyara & Sakaguchi 2007). Additionally, Tregs can rely on perforin and granzymes to lyse activated effector T cells (Grossman et al. 2004) and can secrete immunoregulatory cytokines (Miyara & Sakaguchi 2007). The question if Tregs act antigen-specifically or not still remains open (Billerbeck et al. 2007). In recent studies, three theories how viruses can activate Tregs were published (Rouse et al. 2006). The first theory describes a phenomenon called "molecular mimicry", meaning that shared determinants between viral and self-antigens cause Treg-activation. The second theory claims that Tregs are activated in an antigen-unspecific fashion by activation-mediators like cytokines and/or TLR-ligands. In contrast, the third

theory hypothesizes that Tregs are activated antigen-specifically when they recognize their antigen. Also, it might well be that all of these factors contribute to Treg-activation. Since many questions concerning Treg-activation and –activity remain open, systemic studies are needed to determine the impact of Tregs on different infections and immunological mechanisms.

1.7. Macrophages (MΦ)

Macrophages are mononuclear phagocytes constituting an essential part of the innate immune system of man and mouse. In mice, they are clearly characterized by the surface expression of F4/80, a stable antigen uniquely found on macrophages, but not on other leukocytes (Austyn & Gordon 1981; Hume et al. 1983). In general, macrophages possess phagocytic capability and serve as a first line of defense between parenchymal cells and pathogens entering the organism via the bloodstream. In the liver, resident macrophages (Kupffer cells) make up between 5 and 10% of the LAL-population. They are associated with LSECs, capture pathogens from the sinusoidal lumen and act as suppressors of viral replication in case of hepatic infection (Lang et al. 2010). It was shown that after depletion of Kupffer cells by chlodronate liposomes, replication of LCMV as well as spreading of the virus from hepatocyte to hepatocyte was significantly enhanced (Lang et al. 2010).

The essential function of macrophages in the innate immune system is highlighted by their functional diversity: In general, macrophages can be divided into two distinct phenotypes, classical M1 macrophages and alternative M2 macrophages. M1 macrophages act pro-inflammatory by secretion of cytokines and chemokines, e.g. TNFα and MIP-1β, support TH1-responses, contribute to tissue destruction and exhibit high phagocytic and antigen-presenting capacity (Verreck et al. 2006; Gordon 2003). M2 macrophages secrete anti-inflammatory cytokines, especially IL10, promote tissue repair and tumor progression, and are characterized by a downmodulated expression of MHCII and costimulatory molecules (Verreck et al. 2006; Gordon 2003). Activation of macrophages by pathogens is conferred by a broad collection of pattern recognition receptors (PRRs), e.g. Toll-like receptors (TLRs), RIG-I-like helicases (RLH/RLRs) or intracellular nuclear oligomerization domain (NOD) like receptors (Mondelli et al. 2010).

1.8. Dendritic Cells (DCs)

Dendritic cells constitute a cell population with central function in antiviral immunity. As professional APCs, they are indispensable for the formation of effective adaptive immune

responses (Banchereau et al. 2000). Similar to macrophages and NK cells, DCs can be efficiently activated by pattern recognition receptors (TLRs, RIG-I-like helicases, NOD-like receptors) (Mondelli et al. 2010). In the liver, DCs are part of the reticulo-endothelial system, together with Kupffer cells and LSECs, clearing antigens, metabolic products and toxins from the bloodstream by endocytosis (Thomson & Knolle 2010). In a healthy state, human hepatic DCs have tolerogenic properties (Rastellini et al. 2010; A. Thomson 1999; Bamboat et al. 2009), secreting anti-inflammatory mediators (e.g. IL10, IL27, prostaglandin E_2) upon activation (Bamboat et al. 2009; Xia et al. 2011; Chen et al. 2009). But inflammatory stimuli can result in transformation of tolerogenic DCs into immune-stimulatory ones (Thomson & Knolle 2010). In that case, MHCII and costimulatory molecules on the DC surface are upregulated and they promote T cell and NK cell activation and proliferation by secretion of cytokines, e.g. IL12, IL6, IFNα (Kingham et al. 2007; O'Connell et al. 2003). Their role in HBV infection is still only insufficiently examined. It was suggested that dysfunction of DCs might be a factor promoting the establishment of chronic HBV infection, but studies have found only minimal stimulatory defects in chronic hepatitis B (Wang et al. 2001; van der Molen et al. 2004). But as hepatic DCs can exert tolerogenic and proinflammatory functions, they are certainly crucial mediators of hepatic immune response (Thomson & Knolle 2010).

1.9. NK/NK-T cells

NK cells, representing one of the main effector cell populations involved in innate immune responses, are large granular lymphocytes containing high amounts of granules filled with perforin and granzyme (Mondelli et al. 2010). With these enzymes, they can directly kill virus-infected cells (Mondelli et al. 2010). Another means by which they exert their antiviral function is the production of cytokines, e.g. IFNγ and TNFα (Mondelli et al. 2010). Much like macrophages and DCs, NK cells can be activated rapidly by TLRs, RIG-I-like helicases and NOD-like receptors. Healthy cells are protected from NK-mediated unspecific lysis by signals inhibiting NK cell function. But loss or downregulation of MHCI molecules caused by viral infection lifts these inhibitory signals, so that NK cells can be activated and exert their immunological function (Kärre et al. 1986). The role of NK cells in HBV infection is still poorly understood, but work in chimpanzees has provided evidence that NK cells could be responsible for initial control of HBV replication (Bertoletti & Gehring 2006). But another study in NK1.1 knock-out mice artificially HBV infected using plasmid injection contradicted that result and rejected the importance of NK cells for suppression of early HBV replication (Yang et al. 2010).

1.10. Interleukin-10

IL10 is a cytokine playing a prominent role in regulation of immune responses. Generally, it downregulates immune responses, reduces cytokine expression, interferes with the proliferation of T cells and influences the function of APCs (Blackburn & Wherry 2007). Mouse studies have demonstrated that a lack of IL10 results in increased immunopathology during viral infection (Lin et al. 1998), and in human HIV patients, IL10 hampered IL2-secretion of effector T cells (Elrefaei et al. 2007). Both in chronic and acute viral infection, absence of IL10 was shown to promote antiviral T cell responses, effector T cells (both CD4 and CD8) proliferated more strongly, their functionality was improved and the formation of memory T cell populations was more efficient (Brooks et al. 2008; Ejrnaes et al. 2006). In case of HBV infection, *Miyazoe et al.* observed that increased IL10-production correlates with more severe forms of chronic infection (Miyazoe et al. 2002). However, under certain circumstances, IL10 can also be stimulatory, activating NK cells and possibly promoting $CD8^+$ T cell mediated cytotoxicity (Shevach 2006).

Many of the cell populations involved in immunity can produce IL10: T cells, B cells, DCs and macrophages are able to produce the cytokine (Blackburn & Wherry 2007). For example, monocytes in HBV infected individuals secrete IL10 (Hyodo et al. 2004), and $CD8^+$ T cells from HIV infected patients have been shown to gain the ability to produce IL10 (Elrefaei et al. 2007). It also known that Tregs can exert their immunosuppressive activity by IL10 *in vivo*, but it is unclear how IL10 is induced in this cell subset (Saraiva & O'Garra 2010).

The regulation of IL10 is complex, and many aspects are still elusive. It is known, however, that IL10 expression is highly variable, and can differ in the same cell type according to the stimulus and its strength (Saraiva & O'Garra 2010). Some molecular mechanisms involved in IL10 production seem to be cell type specific, while common mechanisms also exist (Saraiva & O'Garra 2010). A negative feedback-loop protecting the organism from immune-mediated tissue damage is indicated by the fact that IL10 is often induced together with pro-inflammatory cytokines and in many different effector T cell subsets ($CD4^+$ as well as $CD8^+$) (Saraiva & O'Garra 2010). Additionally, it was demonstrated that IL10 has the ability to regulate its own production by triggering IL10 mRNA degradation (Brown et al. 1996).

Collectively, the questions how and when IL10 is induced, which cell types produce IL10 in a certain environment and situation, and if IL10 plays a role in the establishment of chronic infection, need further elucidation.

1.11. Depletion of Regulatory T cells (DEREG) mice

For this study, DEREG-mice ("**de**pletion of **reg**ulatory T cell" mice) were used, bacterial artificial chromosome (BAC)-transgenic mice on C57BL/6 background in which an enhanced GFP (eGFP)-human *diphtheria*-toxin receptor (hDTR) fusion protein is expressed under the control of the *foxp3*-locus (Lahl et al. 2007). In DEREG mice, $Foxp3^+$ Tregs can be depleted systemically and specifically, but only transiently by injecting *diphtheria*-toxin (DTX), which does not affect other cell populations lacking the receptor (Lahl et al. 2007). Additionally, Foxp3-expressing Tregs can easily be detected by GFP-fluorescence. *Lahl et al* demonstrated that DEREG mice and wt C57BL/6 mice show no phenotypical differences concerning Foxp3 expression levels, frequency and function of Tregs (Lahl et al. 2007).

1.12. Adeno-HBV (AdHBV)

Since HBV cannot infect murine hepatocytes, an adenoviral vector transferring a 1.3-fold overlenght HBV-genome (AdHBV) into the mouse liver across the species barrier was used in this study (Sprinzl et al. 2001). The replication-competent HBV-genome was inserted into the E1 region of an adenovirus type 5 genome with deleted E1 and E3 regions (Fig. 6). Application of AdHBV allows efficient and dose-dependent HBV-replication in various cells of different species (Sprinzl et al. 2001). Following HBV-genome transfer, infectious HBV virions are also efficiently produced and secreted by murine hepatocytes *in vivo*. Infection with AdHBV induces an acute, self-limiting hepatitis B infection in mice, without persistent circulation of adenovirus in the bloodstream (Sprinzl et al. 2001; von Freyend et al. 2011). This vector system has various advantages (Sprinzl et al. 2001): As already

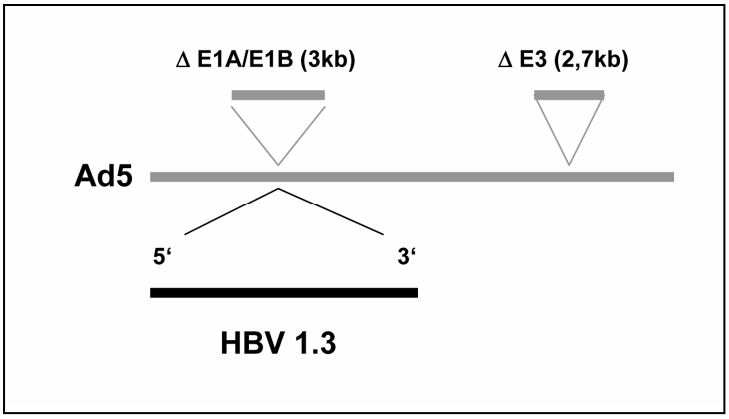


Fig. 6: The recombinant adenovirus genome used to generate AdHBV. Taken and modified from Sprinzl MF, Oberwinkler H, Schaller H and Protzer U. Transfer of Hepatitis B Virus Genome by Adenovirus Vectors into Cultured Cells and Mice: Crossing the Species Barrier. J Virol 2001; 75(11): 5108-5118.

stated, it allows to induce HBV replication in a broad range of cell types and species from an extrachromosomal template as in the natural infection. Secondly, it allows to control the strength of infection by varying the dose of the inoculum. Thirdly, adenoviruses are hepatotropic viruses targeting hepatocytes very efficiently upon intravenous injection. Thus, AdHBV is a valuable tool to study HBV infection in well-defined model organisms.

2 Materials and Methods

2.1. Materials

2.1.1. Reagents and solutions

RPMI 1640 medium	Cambrex, Taufkirchen, Germany
Williams medium E	
FCS, heat-inactivated	Biochrom AG, Berlin, Germany
Trypan blue	Gibco, Invitrogen
Bicoll, Ficoll separating solution, density 1.077g/mL	Biochrom AG
Brefeldin A, 5mg/mL in DMSO	Sigma-Aldrich, Taufkirchen, Germany
Ethidium monoazide bromide (EMA)	Molecular Probes, Invitrogen, Karlsruhe, Germany
FACS buffer	1% BSA, 0.09% Na Azide in PBS
Perm/Wash Solution	Becton Dickinson, Heidelberg, Germany
Cytofix/Cytoperm reagent	Becton Dickinson
1x PBS, pH 7.4	0.14% M NaCl, 2.7 mM KCl, 3.2 mM Na_2HPO_4, 1.5 mM KH_2PO_4 in H_2O
Virus dialysis buffer, pH 8.0	137 mM NaCl, 5 mM KCl, 10 mM Tris, 1 mM $MgCl_2 \bullet 6H_2O$ in H_2O
Collagenase Type IV	Worthington, Lakewood, USA
diphteria toxin (DTX)	Merck, Darmstadt, Germany
SYBR Green I Master Mix	Roche Diagnostics, Mannheim, Germany
TRIzol reagent	Invitrogen, Carlsbad, USA

2.1.2. Primers for PCR

HBV1745+	5’-GTTGCC-CGTTTGTCCTCTAATTC-3’
HBV1844-	5’-GGAGGGATACATAGAGGTT-CCTTGA-3’
Ad156+	5' TAAGCGACGGATGTGG 3'
Ad388-	5' CCACGTAAACGGTCAAAG 3'
CD4+	5’-AGCTCAACAATACTTTGACC-3’
CD4-	5’-CCCAGAAAGCCGAAGGA-3’
CD8+	5’-GGATTGGACTTCGCCTG-3’
CD8-	5’-CAAGTATGCTTTGTGTCAAAGA-3’

IFNg+	5’-ATGGTGACATGA-AAATCCTG-3’
IFNg-	5’-GTGGACCACTCGGATGA-3’
TNFa+	5’-ACG-TCGTAGCAAACCAC-3’
TNFa-	5’-AGATAGCAAATCGGCTG-3’
IL10+	5' GCTGGACAACATACTGC 3'
IL10-	5' GCATCCTGAGGGTCTT 3'
GAPDH+	5’-ACCAACTGCTTAGCCC-3’
GAPDH-	5’-CCACGACGGACACATT-3’

2.1.3. Antibodies and tetramers

Antibody	Manufacturer	Concentration for use
anti-mCD3 V500	eBioscience, San Diego, USA	1:50
anti-mCD4 eFluor450	eBioscience	1:100
anti-mCD8 APC-eFluor780	eBioscience	1:200
anti-mNK1.1 PerCO-Cy5.5	eBioscience	1:200
anti-mNK1.1 APC	eBioscience	1:200
anti-mIFNγ PE	eBioscience	1:300
anti-mTNFα PE-Cy7	eBioscience	1:200
anti-mIL2 APC	eBioscience	1:200
anti-mFoxp3 Alexa Fluor647	eBioscience	1:100
anti-mCD62L PE-Cy7	eBioscience	1:200
anti-mCD127 APC	eBioscience	1:100
anti-m33D1 (DC marker) PE	eBioscience	1:300
anti-mF4/80 Antigen APC	eBioscience	1:200
anti-mMHC Class II eFluor450	eBioscience	1:300
anti-mCD137 PE	eBioscience	1:200
RmCD4-2 (anti-CD4)	E. Kremmer	500 or 250 µg per mouse
anti-human CD154	E. Kremmer	500 µg per mouse
tetramer HBV C93-100	D. H. Busch	1 µL per sample
tetramer HBV S190-197	D. H. Busch	1 µL per sample

2.1.4. Peptides and full proteins

HBc peptide library, genotype D, spanning the whole protein sequence. 15mer peptides overlapping by 11, manufactured by Thinkpeptides, Oxford, UK. Divided into three pools:

- HBcP1: peptides 1-15
- HBcP2: peptides 16-30
- HBcP3: peptides 31-43

HBs peptide library, genotype D, spanning the whole protein sequence. 15mer peptides overlapping by 11, kindly provided by D. H. Busch, Institute of Medical Microbiology, Immunology and Hygiene, TU München. Divided into three pools:

- HBsP1: peptides 1-18
- HBsP2: peptides 19-36
- HBsP3: peptides 37-54

A8R	kindly provided by Ingo Drexler, Institute of Virology, Helmholtz Zentrum München/TU München
bGal	kindly provided by Ingo Drexler
SIINFEKL	JPT, Berlin, Germany
Full HBV core protein	kindly provided by P. Pumpens and A. Dishlers, Latvian Biomedical Research and Study Centre, Riga, Latvia
Full HBs protein	kindly provided by Rhein Biotech, Düsseldorf, Germany
Ovalbumin (OVA)	kindly provided by Ingo Drexler

Peptides and peptide pools were used 2 µg/mL in 200 µL sample volume.

2.1.5. Kits

Super Script III First-Strand Synthesis Super Mix Kit	Invitrogen
DNeasy Blood & Tissue Kit	Qiagen, Hilden, Germany
CBA Mouse/Rat Soluble Protein Master Buffer Kit	Becton Dickinson

2.1.6. Consumables

96-well plates, V- and flat-bottom	Corning, New York, USA
Eppendorf tubes, 1.5 mL and 2.0 mL	Eppendorf, Hamburg, Germany
FACS tubes	Bio-Rad, Munich, Germany
Falcon tubes, 15 mL and 50 mL	BD Pharmingen, Hamburg, Germany
Gloves	Kimberley-Clark, Mainz, Germany
Pipettes, 5 mL, 10 mL, 25 mL	Greiner, Nürtingen, Germany
SW40 polyallomer vials	Beranek Laborgeräte

2.1.7. Laboratory equipment

Equipment	Model	Manufacturer
Centrifuge	Rotanta 400R Micro 200R	Heraeus, Hanau, Germany
Ultracentrifuge		Beckmann
SW40 rotor		Beckmann
Flow Cytometer	FACSCantoII	Beckton Dickinson
Light Cycler for qRT-PCR	Light Cycler 480 II	Roche Diagnostics
Freezer -20°C	Premium	Liebherr, Biberach, Germany
Freezer -80°C	VIP-Series	Sanyo, Pfaffenhofen, Germany
Fridge 4°C	Profi-Line	Liebherr
CO2 Incubator	CB 150	Binder, Tuttlingen, Germany
Microscope	Telaval 31	Carl Zeiss, Oberkochen, Germany
Confocal Microscope	Fluoview FV101	Olympus, Hamburg, Germany
Haematocytometer	Neubauer chamber	Karl Hect KG, Sondheim, Germany
Vortexer	Lab Dancer	IKA, Staufen, Germany
Laminar Flow	Hera Safe ClassII, Type A7B3	Heraeus
Pipettes	Pipetman P10-1000	Gilson, Middleton, USA
Multi-channel pipette	Pipetman Ultra	Gilson
Pipettus	accu-jet pro	Brand, Wertheim, Germany

2.1.8. Software

FacsDIVA	Becton Dickinson
FlowJo v8.5.3	Treestar, Ashland, USA
Graphpad Prism 4	Graph Pad Software, San Diego, USA
Spice v4.1.5	kindly provided by Mario Roederer, NIH, USA
Pestle v5.0.1	kindly provided by Mario Roederer
MS Office	Microsoft, Redmond, USA

2.2. Production of adenoviral vector stocks

2.2.1. Growth of AdHBV and AdHBV k/o

HEK293 cells were seeded into 12 175 cm^2 cell culture flasks. When the cells reached 80-90% confluency, each cell culture flask of HEK293 cells was infected with $1x10^8$ infectious AdHBV or AdHBV k/o particles (MOI 3-5). As soon as the vast majority of cells showed cytopathic effect (approximately 48h post infection), they were harvested together with the complete culture medium. The harvested cell suspensions were collected in 50 mL Falcon tubes and centrifuged at 4000 rpm for 5 min. The supernatant was discarded except 48 mL, in which the cell pellets were then pooled together. To release the virus from the harvested cells, the collected harvest was subjected to three freeze/thaw cycles using a -80°C freezer and a 37°C water bath. After a centrifugation step (4000 rpm, 10 min), the virus-containing supernatant was used for CsCl gradient centrifugation to purify the virus (see below).

2.2.2. Purification of adenoviruses by CsCl-gradient ultracentrifugation

26.8 g CsCl were added to the 48 mL virus solution and thoroughly mixed until the CsCl dissolved. This solution was portioned into 6 ultracentrifugation tubes (SW-40 polyallomer vials) in equal fractions and balanced. The solution in each tube was then covered with mineral oil until 1 mm below the edge and balanced a second time. Ultra-centrifugation was performed in SW40 rotor at 30000 rpm for 48 h at 15°C. After the centrifugation, the mineral oil was pipetted off and discarded. In the CsCl-solution, three white bands were normally found: Two thin upper bands containing defective virus particles, and a thick, lower band containing the infectious virus particles. The two upper bands were completely discarded, while the lower band was removed from the tube in 2 mL volume. The collected purified virus from all 6 tubes was pooled and then subjected to dialysis for further use (see below).

2.2.3. AdHBV (k/o) dialysis

In order to remove the neurotoxic CsCl, the virus solution was injected into 12 mL dialysis chambers. The chamber was then transferred into 1 L of virus dialysis buffer and stirred gently at room temperature. The buffer was replaced after 1h and 3h. After at least six hours of dialysis, the virus solution was removed from the dialysis chamber using a 10 mL syringe and aliquoted in 1 mL aliquots. The aliquots were stored at -80°C.

2.2.4. Titration of AdHBV (k/o)

The amount of infectious particles in the produced virus stock was estimated by assessment of cytopathic effect in infected HEK293 cells. HEK293 cells were seeded into 12 well plates to be 80-90% confluent at time of infection, corresponding to approximately $1x10^6$ cells/well. Each well contained 1 mL medium. One frozen aliquot of the virus stock was thawed and a dilution series was estanlished as indicated in the following: 100 µL, 30 µL, 10 µL and 3 µL of the viral stock solution were added to the wells in the top row of the plate and gently dispensed. 100 µL culture medium of each well were transferred to the well below (middle row), corresponding to a virus solution volume of 1 µL, 0.3 µL, 0.1 µL and 0.03 µL. The bottom row of the plate served as uninfected control. The medium was mixed again, and the plate was incubated for 48h at 37°C. After 48h post infection, the cytopathic effect was exmined. The well of cells infected with the least amount of virus that induced total cell detachment was used for titer calculation of the viral stock. The calculation of virus titer was based on the following assumptions: Firstly, each well contained approximately $1x10^6$ HEK293 cells; secondly, an MOI of 3-5 induces detachment of a HEK293 cell after 48h; thus, the lowest volume of viral stock solution that induced total cell detachment after 48h contained a MOI of approx. 3-5 infectious viral particles (infectious units) per cell.

100 µL	$5x10^7$ i.u./mL	1 µL	$5x10^9$ i.u./mL
30 µL	$2x10^8$ i.u./mL	0.3 µL	$2x10^{10}$ i.u./mL
10 µL	$5x10^8$ i.u./mL	0.1 µL	$5x10^{10}$ i.u./mL
3 µL	$2x10^9$ i.u./mL	0.03 µL	$2x10^{11}$ i.u./mL

2.3. Animal experiments

All animal experiments were approved by the local authorities and animals received human care in accordance to the National Institutes of Health guidelines. For all experiments, 8 week old female mice were used. All mice were kept under specific pathogen free (SPF) conditions

following the institutional guidelines of the animal facility, Helmholtz Zentrum München, Neuherberg.

mouse strain	obtained from:
DEREG	in-house breeding
C57BL/6	Charles Rivers
IL10 reporter mice	Hicham Bouabe Max-von-Pettenkofer Institute LMU, Munich
$IL10^{flx/flx}$CD4Cre+ mice	Axel Roers Institute for Immunology, TU Dresden

The DEREG mice were originally obtained from Tim Sparwasser, Institute of Infection Immunology, TWINCORE, Centre for Experimental and Clinical Infection Research, who kindly gave permission to start a new in-house breeding in the animal facility of the Helmholtz Zentrum München, Neuherberg.

2.3.1. Screening of DEREG mice

Between 25% and 50% of the female offspring in the DEREG mouse breeding show a transgene-positive phenotype, indicated by eGFP-expression of regulatory T cells. Therefore, the offspring of each breeding pair had to be screened for GFP-positive regulatory T cells in order to gain mice for experiments and for further breeding. DEREG-mice were bled at the tail vein. Five to 10 µL blood were dissolved in 50 µL heparin solution (heparin 1:5 in cold PBS) and stored on ice. Lysis of erythrocytes (2.3.8.) was performed, cells washed 2x with 200 µL FACS-buffer. Thereafter, cells were stained with anti-mCD4 APC 1:200 as described below (2.5.2.). After 2x washing of the stained PBMCs with 200 µL FACS-buffer, flow cytometry was performed to detect the $CD4^+eGFP^+$ regulatory T cells.

2.3.2. Depletion of $CD4^+Foxp3^+$regulatory T cells in DEREG mice

For Treg-depletion, DEREG mice received daily injections of 1 µg *diphtheria* toxin i.p. on three subsequent days.

2.3.3. Depletion of $CD4^+$ T cells in C57BL/6 mice

Mice received either one single injection of 500 µg RmCD4-2 antibody (anti-CD4) i.p. one or seven day(s) prior infection, or repeated injections of 250 µg RmCD4-2 i.v. one day prior infection and every 10 days thereafter. As a control, mice received injections of anti-human CD154 (anti-hCD154) in a similar fashion. Both antibodies were isotype rat IgG2b.

2.3.4. Intravenous injection

Intravenous injection was performed into the tail vain. The mice were warmed under a infrared light to dilate the veins. One mouse was then placed in a mouse holder and the tail was disinfected with 70% ethanol. A 1 mL syringe containing the material to be injected was carefully applied into the lumen of the vein; successful insertion of the syringe into the vein was confirmed if no resistance was felt when pushing the plunger. The material (virus or depletion antibody RmCD4-2) was injected quickly in a total volume of 200 or 250 µl. After removal of the syringe, the tail was disinfected again and pressure was applied to the site of injection until the bleeding stopped.

2.3.5. Intraperitoneal injection

The mouse was taken firmly at its neck, the tail hold firmly. A 1 mL syringe containing the material to be injected (*diphtheria* toxin or depletion antibody RmCD4-2) was carefully inserted into the intraperitoneal lumen, followed by injection. Up to 500 µL volume can be applied easily by this method.

2.3.6. Retro-orbital blood collection

To obtain larger volumes of blood (e.g. 0.5 mL for serological testing), the peri-orbital sinus of the mouse was used as a source of venous blood. For this procedure, mice had to be anaesthetized; per g body weight (mouse) 100 µL of anaesthethic solution (1 mL Ketanest (Ketavet; 100 mg/mL), 0.25 mL Rompun (2%) and 8.75 mL 0.9% NaCl) were applied intraperitonially. The mouse was restrained in one hand and a microhematocrit tube was applied into the lateral canthus of the eye in an angle of about 60° to the side of the head. The tube was quickly rotated to score the sinus. Once the sinus was ruptured, blood was collected. As soon as the tube was withdrawn, the bleeding stopped.

2.3.7. Euthanazation of mice

Mice were killed using carbon dioxide for collection of whole-blood and organs. If only spleen and/or liver and no blood needed to be harvested, mice were killed by cervical dislocation.

2.3.8. Isolation of splenocytes

Spleens were explanted under semi-sterile conditions and transferred into 50 mL Falcon-tubes containing 5 mL RPMI1640/10% FCS medium. The tubes were stored on ice. The spleens were then grinded on a grid and repeatedly washed with medium to separate the cells from crude constituents (e.g. fat) and afterwards flushed through 100 micrometer cell strainers with a total of 15 mL medium for each spleen. The resulting suspensions were centrifuged at 300xg and 4°C for 5 min. The supernatant was discarded, each pellet resuspended in 3 mL TAC buffer and transferred instantly into a 37°C water bath, were they were incubated under continuous shaking for 2 min. By this step, lysis of erythrocytes contained in the pellets was achieved. Following this, the suspensions were diluted with 40 mL RPMI1640/1% FCS medium, inverted several times and poured through cell strainers again. A centrifugation followed as above. The resulting pellets were resuspended in 3 mL RPMI1640/10% FCS medium each. Of every suspension, samples were taken to count the live cells in a 1:40 dilution using trypanblue and a Neubauer counting chamber. After counting, the suspensions were adjusted to 2 x 10^6 cells/mL using RPMI1640/10% FCS medium.

2.3.9. Isolation of liver-associated lymphocytes

Liver-associated lymphocytes (LALs) were prepared after PBS-perfusion of livers. Livers were minced through 100 µm cell strainers and washed with RPMI1640/10% FCS medium. After centrifugation at 300x g for 5 min at 4°C, the cell pellets were suspended in 50 mL collagenase-medium (Williams Med. E + 70 µL 2.5 M $CaCl_2$ + 220 U/mL collagenase Type IV) and digested for 20 min at 37°C. The cell suspension was then layered onto 8 mL Biocoll separating solution and centrifuged at 300x g for 17 min at 4°C without breaks. While hepatocytes were discarded, the cells contained in the supernatant were washed three times with 50 mL RPMI1640 medium. The washed cells were suspended in RPMI1640/10% FCS medium, counted using Neubauer counting chambers and used for further analysis.

2.3.10. Immunohistochemistry

Liver tissue samples were fixed in 4% buffered formalin for 48h and then transferred into 1x PBS. Mouse livers slices were fixed in 10% buffered formalin and embedded in paraffin. 8µm tissue sections were stained with CD3 or HBV core protein-specific antibody (Diagnostic Biosystems, Pleasanton, CA, USA). Semiquantitative analysis of stained sections was performed by counting localization, intensity, distribution and percentage of positive cell staining throughout the whole tissue specimen.

2.3.11. Serological analysis

Liver damage was assessed by measuring alaninaminotransferase (ALT) in 32 µL murine serum using a Reflovet® Plus reader (Roche Diagnostics, Mannheim, Germany). HBsAg, HBeAg and antibodies (anti-HBs and anti-core) were quantified in 1:20 dilutions (diluted in 1x PBS) of murine serum using AXSYM^{TM} assays (Abbott Laboratories, Abbott Park, IL, USA).

2.4. Molecular biology

2.4.1. Isolation of whole-liver RNA

Liver-tissue samples (~ 3x3 mm) were subjected to RNA-isolation using TRIzol according to manufacturer's instructions.

2.4.2. cDNA-synthesis

750 ng RNA were used for cDNA-synthesis with the Super Script III First-Strand Synthesis Super Mix according to manufacturer's instructions.

2.4.3. DNA-isolation

DNA from liver tissue samples (~3x3 mm) or 50 µL murine serum was performed using the DNeasy Blood & Tissue Kit according to manufacturer's instructions.

2.4.4. Real-time PCR

Real-time PCR was performed on a Light Cycler^{TM} 480 II using the SYBR Green I Master Mix. Immune marker gene transcripts were analyzed relative to GAPDH transcripts using exon-exon spanning primers and normalized to expression levels in liver tissue of naïve mice. For quantification of HBV genomes in murine serum or liver tissue, HBV DNA was

quantified using an external plasmid standard that contained a HBV-DNA sequence. Samples were prepared as follows:

DNA template	2 µL
PCR master mix	5 µL
forward primer	0.5 µL
reversed primer	0.5 µL
H_2O	2 µL

The running parameters for the PCR reaction were the same for both immune marker gene detection and viral titer quantification:

initial denaturation	95°C	300 sec
cyclic denaturation	95°C	15 sec
annealing	60°C	5 sec
elongation	72°C	15 sec
melting curve	65°C – 95°C	0.1°C/sec
cooling	40°C	30 sec

Cyclic denaturation, annealing and elongation were repeated for 45 cycles.

2.4.5. PCR efficiency

In theory, every cycle of the PCR reaction doubles the amount of DNA (efficiency = 100%). But in most cases, the real efficiency is less than 100%. Small differences in efficiency can strongly influence the actually generated amount of PCR product and therefore the data gained by the reaction. Thus, the real efficiency of a certain PCR reaction needs to be determined in order to obtain realistic results. For this purpose, a dilution series of DNA containing the gene of interest (ideally a plasmid sample with known copy number) had to be tested with the respective primer pair.

2.5. Immunological Assays

2.5.1. Stimulation of LALs and splenocytes for intracellular cytokine staining

LALs or splenocytes were stimulated in the presence of brefeldin A (BFA) for 5h with peptide pools derived from an HBV-core- and an HBs-peptide library, genotype D. To assess

CD137-expression, LALs were stimulated for 24h with full HBV core protein or HBs protein or ovalbumine (OVA). Stimulation was performed in 96-well flat-bottom plates in an incubator at 37°C and 5% CO_2. The number of samples depended on the number of mice (responders) and peptides to be tested:

responders x peptides = number of wells

Additional wells were filled to be stained as single colours to subtract signal overlap in the later analysis.

2.5.2. Intracellular cytokine staining (ICS)

After this incubation, the content of the wells was transferred to a 96-well-V-bottom plate and centrifuged at 300 x g and 4°C for 2 min. The supernatant was discarded and the pellets resuspended in 100 µL FACS buffer each + ethidium monoazide bromide (EMA, 1:2000) as a marker for discrimination of live/dead cells. The plate was then exposed to light for 20 min on ice to allow EMA staining reaction. The wells were then filled up to 180 µL volume with FACS buffer, followed by a centrifugation as described above, the supernatant was discarded and the pellets washed two times with 180 µL FACS buffer (2.5x wash). To avoid undesired antibody-binding by F_C-receptors (e.g. on macrophages), cells were resuspended in 50 µL F_C-blocking solution (F_C-antibody diluted 1:100 in FACS buffer) and incubated for 20 min in the dark on ice. Afterwards, the pellets were washed 2.5x and resuspended in 50 µL/well antibody solution (surface staining antibodies diluted in FACS buffer). The single colour samples were resuspended in 50 µL FACS buffer and 0.1 µL of the respective staining antibody added.

The plate was incubated for 25 min in the dark on ice. After that the plate was washed 2.5x with 180 µL FACS buffer. Then the pellets were resuspended in 100 µL Cytofix/Cytoperm each, while the single colours were transferred directly into FACS-tubes prepared with 150 µL FACS buffer. The plate with the remaining wells was incubated in the dark at 4°C for 15 min. Following this incubation, the cells were washed 1.5x with 1x Perm and resuspended in 50 µL/well intracellular antibody solution (intracellular staining antibodies diluted in 1x Perm/Wash). An incubation for 30 min on ice in the dark followed, then 2.5x wash with 1x Perm/Wash. At last, the pellets were taken up in 150 µL 2% PFA and transferred to FACS tubes prepared with 150 microL FACS buffer. It was then possible to store the stained cells

for up to five days at 4°C. This staining procedure was followed by flow cytometrical analysis. Flow cytometry was performed on a FACSCantoII™.

2.5.3. Tetramer staining

As for intracellular cytokine staining, freshly isolated splenocytes or LALs were transferred into a 96-well V-bottom plate. The complete staining procedure was performed on ice. First, the tetramers HBc C93-100 and HBs S190-197 were coupled to the dye PE. Per sample, 1 µL tetramer and 1 µL PE were added to 8 µL FACS buffer and incubated for 20 min in the dark. Afterwards, cells were centrifuged at 300 x g for 2 min at 4°C. Cells were then resuspended in 50 µL F_C-blocking solution (F_C-antibody 1:100 in FACS buffer) and incubated for 20 min in the dark. After a 2.5x wash with FACS buffer, 10 µL tetramer solution and 30 µL FACS buffer were added per well and gently mixed. The plate was incubated for 20 min in the dark. After this tetramer staining, cells could be stained with surface marker antibodies as described above. Following a 2.5x wash with FACS buffer, cells were transferred into FACS tubes in 180 µL volume and flow cytometry followed directly.

2.5.4. Cytometric Bead Array (CBA)

The assay was performed using the BD CBA capture beads for murine IFNγ, IL10, IL21, MIP-1α, MIP-1β, TNFα and the BD CBA Mouse/Rat Soluble Protein Master Buffer Kit according to manufacturer's instructions.

2.5.5. IL10 reporter reaction using IL10 reporter mice

The IL10 reporter reaction was performed using freshly isolated LALs or splenocytes from IL10 reporter mice, according to instructions by Hicham Bouabe, Max-von-Pettenkofer Institut, LMU München. Since the details of this assay are confidential, the reader is advised to contact Hicham Bouabe for further information.

2.5.6. Flow cytometry

Flow cytometry allows for analysis of cells on a single cell level. By aspiration through a capillary and hydrodynamic focusing, cells successively enter a detection channel, where they pass through a set of laser beams. Analysis of cell size, granularity and protein expression is based on the forward light scatter (FSC), the sideward light scatter (SSC) and the emission of light by laser-activated fluorochromes, respectively. The usage of several lasers and different fluorochromes with distinct emission spectra allows for the simultaneous analysis of a variety

of different markers. For analysis of cellular protein expression primary antibodies conjugated with a fluorochrome or detected by a labeled, species-specific secondary antibodies can be used. Analysis is possible for proteins expressed at the cell surface, as well as for intracellular proteins after permeabilization and fixation of the cell. The optical readout from analyzed cells is converted to digital information in a detector system and can be visualized and analyzed using specific software such as FACS-Diva or FlowJo. Since emission spectra of some fluorochromes show partial overlaps, each experiment contains samples stained only with a single colour to be able to define cells that are truly positive and to adjust instrument settings in order to subtract signal overlaps for each detection channel.

2.5.7. Confocal microscopy

For confocal microscopy, LALs were freshly isolated. $2x10^5$ cells were resuspended in 1 mL RPMI1640/10% FCS medium and transferred into a microscopy dish. Life-cell imaging was performed on a Fluoview FV101^{TM}. Cells were visualized using phase-contrast and eGFP-mediated fluorescence was detected.

2.5.8. Adenovirus-Neutralisation Assay

To detect antibodies against the applied adenoviral vector, $5x10^7$ i.u. AdHBV in 10 µL volume were added to 10 µL murine serum and incubated for 10 min at room temperature. Afterwards, the mixture was added to 10^6 HEK 293 cells in a 12-well plate. 20 µL PBS added to 10^6 HEK 293 cells served as negative control, $5x10^7$ i.u. AdHBV mixed with 10 µL virus dialysis buffer as positive control. After 48h incubation at 37°C, cells were examined for cytopathic effect due to adenovirus infection. All cells showed cytopathic effect if no adenovirus was neutralised by specific antibodies, if all of the virus was neutralised, cells remained healthy. This is a purely qualitative assay (antibody-positive versus antibody-negative) that allows no quantification of the antibody concentration.

2.6. Statistical analysis

Data are shown as mean with standard deviation of the mean. Results were analyzed using Student's t-test. A P-value of ≤ 0.05 was considered as significant.

3 Results

Depletion of Tregs is efficient in liver and spleen of AdHBV-infected DEREG mice. To study Treg function, DEREG mice were injected intraperitoneally with DTX before they were infected intravenously with AdHBV (Fig. 7A). As expected, Tregs, which can easily be detected by their GFP fluorescence in DEREG mice (.Fig. 8A,B), were depleted specifically and systemically in AdHBV infected DEREG mice by intraperitoneal injection of DTX (Fig. 7B, Fig. 8A). First, Treg-frequencies in AdHBV-infected mice and efficiency of Treg-depletion in liver and spleen over time were determined. AdHBV-infection induced a rapid increase of Treg-frequencies in the liver (Fig. 7C, left panel) while Treg-frequencies in the spleen remained largely unchanged (Fig. 7C, right panel). Treg-increase to 6 – 9 % of total liver $CD4^+$ T cells, which may be either due to novel recruitment or local expansion of Tregs in the liver shortly after infection, remained detectable for more than two months after infection.

Three consecutive intraperitoneal injections of DTX reduced intrahepatic Treg-frequencies to undetectable levels (Fig. 7B, C). Between d3 and d7 post infection (p.i.), Tregs started to repopulate the liver, but the frequency remained reduced compared to non-depleted mice even at later stages of infection. Newly generated Tregs lost GFP-expression, indicating selection for transgene-negative Tregs due to DTX-application (Fig. 8C). In the spleen, Treg-frequency increased to more than 8% of total $CD4^+$ T cells two weeks after infection after initial depletion, but normalized again thereafter (Fig. 7C). This demonstrates that activation of Treg responses after AdHBV-infection is mostly restricted to the liver.

To determine if Tregs exert their activity in the liver equally during the course of infection or if it is restricted to a certain phase of infection, Tregs were depleted either before infection or on d7, d14 or d37 after infection (Fig. 9A). Seven days after 1^{st} depletion, mice were sacrificed and levels of IFNγ, TNFα, CD8 and CD4 mRNA were determined by qPCR using whole-liver cDNA (Fig. 9B). When Tregs were depleted at the time point of infection, levels of CD8, IFNγ, TNFα and CD4 expression were increased 11-, 13-, 6- and 4-fold indicating an increased influx of antivirally active $CD8^+$, but also $CD4^+$ T cells in the absence of Tregs (Fig. 9B). When Tregs were depleted at later time points, the increase was less pronounced, suggesting that Tregs exert their maximal suppressive effect in the liver during the very early phase of HBV infection. Therefore, it was chosen to deplete Tregs directly at the onset of infection for the following experiments.

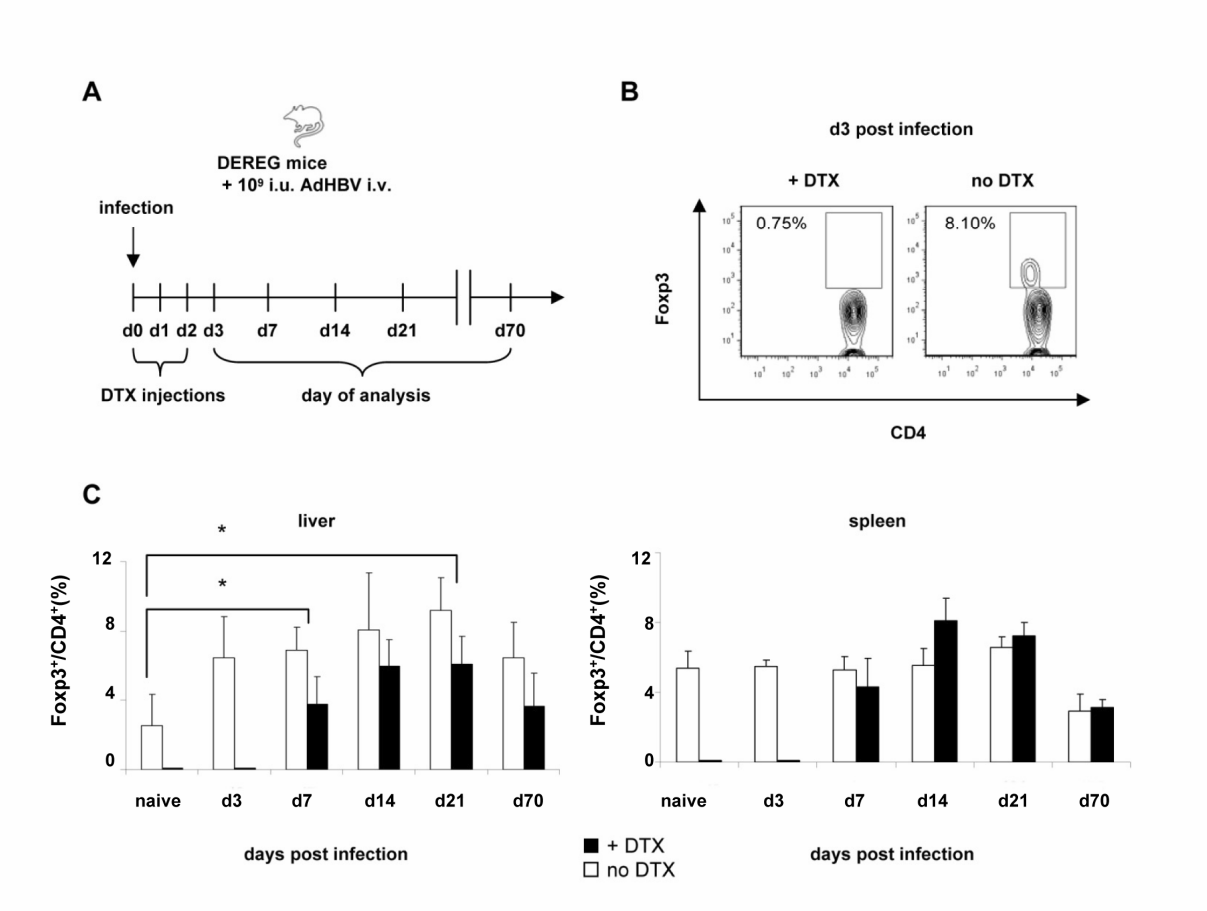


Fig. 7. Analysis of Treg-frequency and depletion efficiency in AdHBV-infected DEREG mice. DEREG mice (n = 3) were injected intraperitoneally with 1 µg of DTX, followed by intravenous injection of 109 i.u. AdHBV. The DTX injections were repeated on day 1 and day 2 post infection. Control groups received no DTX. **(A)** Experimental scheme. **(B)** Representative flow cytometry plots of CD4+Foxp3+ LALs of Treg-depleted and non-depleted mice at d3. **(C)** At indicated time points, LALs **(left panel)** and splenocytes **(right panel)** were isolated and Treg-frequencies analyzed by staining of CD4 and Foxp3 followed by flow-cytometrical analysis. * $P < 0.05$. DTX: diphtheria toxin; LALs: liver associated lymphocytes.

Tregs suppress immune control of HBV. To define the impact of Tregs on the establishment and course of HBV infection, infection parameters (HBeAg, HBsAg, peripheral and intrahepatic titers) were followed in Treg-depleted and non-depleted AdHBV-infected DEREG mice. Although mice were infected with comparable efficiency (indicated by equal levels of HBeAg up to day 7 p.i.), HBeAg and HBsAg were cleared significantly faster from the serum of Treg-depleted animals (Fig. 10A,B). Strikingly, HBV titers in the peripheral blood were reduced by ~90% if Tregs were depleted and HBV viremia was completely cleared (Fig. 10C).

To quantify HBV replication in hepatocytes, it was determined how many HBV copies per AdHBV genome were produced. In non-treated DEREG mice, increasing HBV replication

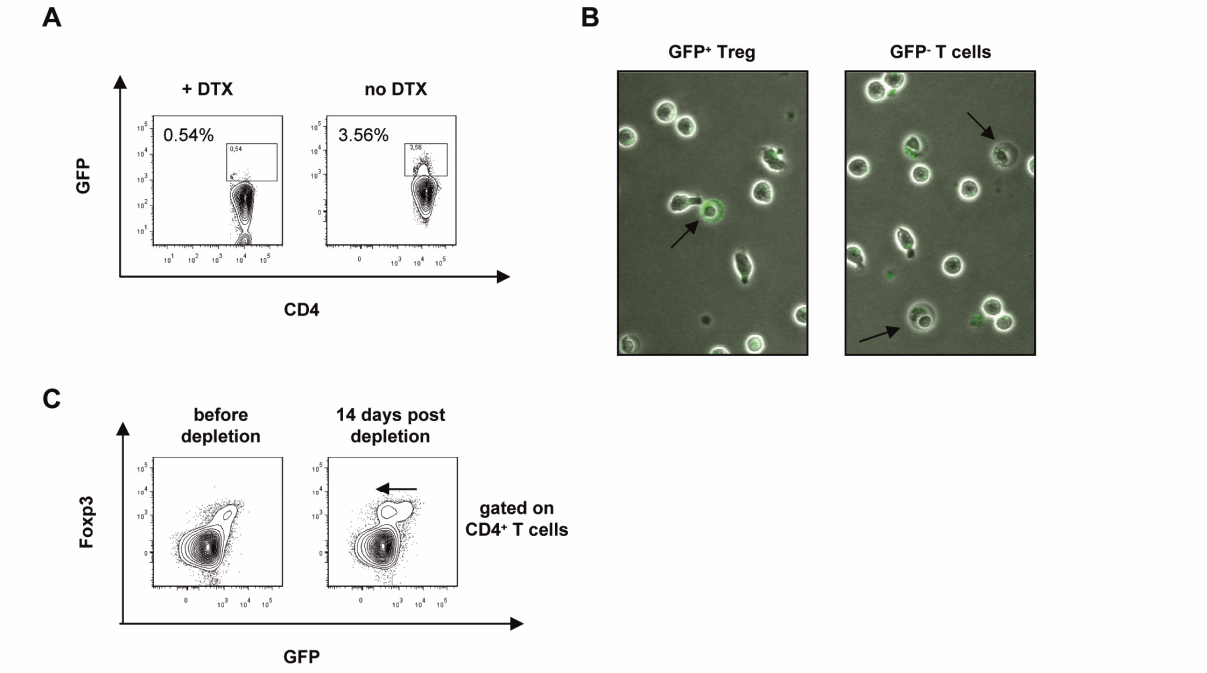


Fig. 8. GFP-expression of Tregs in DEREG mice. **(A)** DTX was applied in DEREG mice on three consecutive days as described earlier. Control groups received no DTX. Representative flow cytometry plots of LALs show GFP expressing Tregs. **(B)** Confocal microscopy of DEREG LALs reveals GFP+ Tregs and GFP- effector T cells. **(C)** Representative flow cytometry plots showing Foxp3-GFP coexpression in Tregs of DEREG mice **(left plot)** and development of a Foxp3+GFP- Treg-population after depletion (population shift marked by arrow). GFP: green fluorescent protein.

from d7 to 21 was found, which was controlled after d21. In Treg-depleted animals, however, only minimal HBV replication was detected (Fig. 10D). Together, these results suggest that Tregs support an immunological environment favoring HBV persistence by inhibiting antiviral immune responses.

Tregs influence HBV-specific B cell responses. Production of antibodies against HBs (anti-HBs), finally leading to seroconversion from HBsAg to anti-HBs, is a hallmark of HBV elimination. Thereby, the immune system may prevent the virus from spreading via the blood stream. In the first weeks of infection, no anti-HBs in the serum of AdHBV-infected mice was detected, certainly due to the overweight of secreted HBsAg, which forms immune complexes with secreted HBs-specific antibodies, thereby making them undetectable by standard assays. From d44 onwards, an anti-HBs response was detected, which was significantly increased after initial Treg-depletion (Table 2, upper panel). This provides evidence that Tregs exert an inhibitory effect on the formation of an HBV-specific antibody response. Also, HBc-specific antibodies (anti-HBc) were measurable in the late phase of infection (Table 2, middle panel).

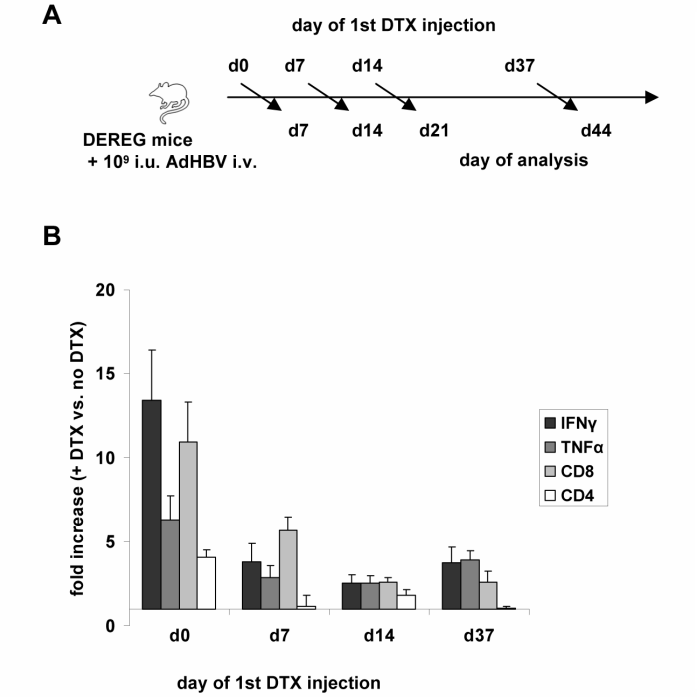


Fig. 9. Strength of Treg-mediated immune suppression at different time points during HBV-infection.
(A) DEREG mice (n = 3) were AdHBV-infected and received DTX-injections i.p. at different time points after infection as described in the experimental scheme. DTX was applied on three consecutive days. Control groups received no DTX. Seven days after the first DTX-injection, mice were sacrificed, liver tissue samples taken and whole-liver RNA extracted. After cDNA-synthesis, qPCR for several immune marker genes was performed. **(B)** qPCR results showing the increase of relative gene expression due to Treg-depletion. Measurement of gene expression was performed relative to GAPDH.

Antibodies against the adenoviral vector were detected from d21 post infection onwards in both Treg-depleted and non-depleted animals, but due to the merely qualitative nature of the adenovirus-neutralisation assay used for detection of those antibodies, no statement concerning differences in the strength of the anti-adeno response can be made. These data suggest that Tregs mitigate, but do not prevent, HBV specific B cell responses, thereby supporting HBV persistence.

Tregs alleviate liver damage early after infection. One characteristic of acute HBV infection is inflammatory liver disease, which can be detected by elevated ALT activity in the serum of the infected individual. Around d7 p.i., ALT activity peaked in AdHBV-infected mice, and remained elevated until d21 (Fig. 11A). Interestingly, Treg-depleted mice showed 2-fold higher ALT activity on d7, but reduced liver damage on d21, indicating a stronger, but slightly shorter liver inflammation after initial Treg-depletion. In control DEREG mice infected with an empty adenoviral control vector (AdHBV k/o), no significant increase of ALT activity in the serum was detected (Fig. 12A).

To investigate if Tregs become activated by AdHBV infection or consecutive liver inflammation, LALs from livers of AdHBV-infected mice were isolated and stimulated *in vitro* with recombinant HBc, HBs or ovalbumin (OVA) or left unstimulated for 24h, and expression of the activation marker CD137 (Wehler et al. 2008; Wölfl et al. 2008) was

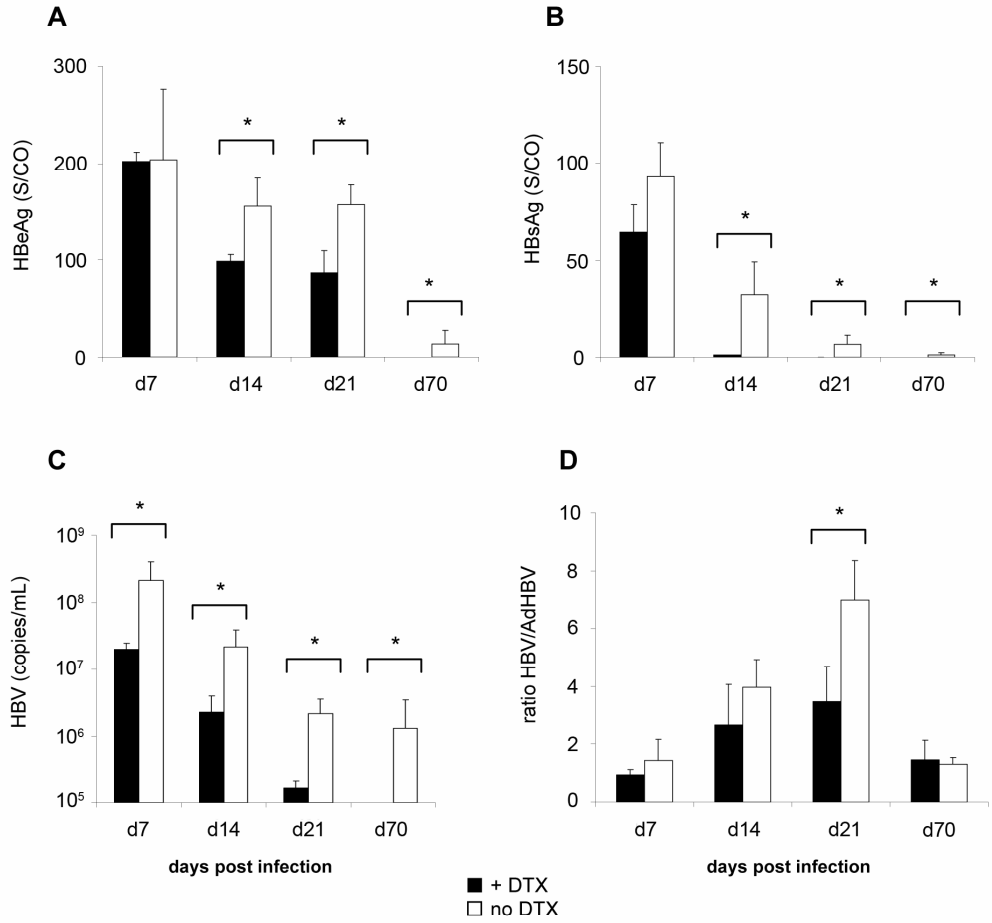


Fig. 10. Viremia in AdHBV-infected DEREG mice. DEREG mice (n = 3) were injected intraperitoneally with 1 µg of DTX, followed by intravenous injection of 10^9 i.u. AdHBV. The DTX injections were repeated on day 1 and day 2 post infection. Control groups received no DTX. At indicated time points, mice were bled and **(A)** HBeAg and **(B)** HBsAg were measured in 1:20 serum dilutions. **(C)** Whole serum DNA was extracted and HBV serum titers were determined by PCR. **(D)** Titers of HBV and the adenoviral vector were quantified in whole-liver DNA using qPCR. The ratio of HBV-copies to AdHBV-copies represents HBV replication in infected hepatocytes. * $P < 0.05$.

analyzed by flow cytometry. In the liver, the highest numbers of $CD137^+$ activated Tregs were found on d7 when liver damage was most pronounced. In parallel to ceasing liver damage, numbers of activated Tregs normalized (Fig. 11B), whereas in the spleen, numbers of $CD137^+$ Tregs remained stable over time (data not shown). The fact that no differences between Tregs stimulated with HBV-proteins, the control protein OVA and cells without stimulus were found implies that Tregs were either already activated *in vivo* prior to isolation of LALs or that Tregs are activated in an antigen-unspecific manner.

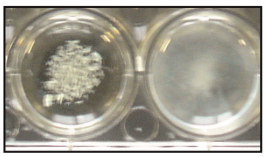
To determine which cells may contribute to liver damage by killing infected hepatocytes, the immune cell population in the liver on day 7 at the peak of liver inflammation was analyzed using flow cytometry. As expected, significantly more LALs were found in the liver of AdHBV-infected mice than in naïve control mice (Fig. 11C and Fig. 12B). Lymphocyte populations in the AdHBV-infected liver consisted mainly of $CD8^+$ T cells (Fig. 11C), but

anti-HBs	d3	d7	d21	d44	d70
+ DTX	negative	negative	negative	++	+++
no DTX	negative	negative	negative	+/-	++

anti-HBc	d3	d7	d21	d44	d70
+ DTX	negative	negative	negative	positive	positive
no DTX	negative	negative	negative	positive	positive

anti-Ad	d3	d7	d21	d44	d70
+ DTX	negative	negative	positive	positive	positive
no DTX	negative	negative	positive	positive	positive

adenovirus-neutralisation assay



negative **positive**

Table 2. Antibody responses in AdHBV infected DEREG mice. DEREG mice were Treg-depleted and AdHBV-infected as described earlier. At the indicated time points, mice were bled and anti-HBs **(upper panel)** and anti-HBc **(middle panel)** were quantified using AXSYM assays with 1:20 diluted murine serum. Antibodies against adenovirus **(lower panel)** were detected by adenovirus-neutralisation assay. The picture below shows representative examples of the adenovirus-neutralisation assay. +/-: anti-HBs < 10 IU/l; +: 10-100 IU/l; ++: 101-500 IU/l; +++: >500 IU/l.

CD4+ T cells and NK cells were also increased. Infection with AdHBV k/o resulted only in a slight increase of intrahepatic $CD8^+$ T cell numbers (Fig. 12B). In Treg-depleted mice, the total amount of liver-associated $CD8^+$ and $CD4^+$ T cells was more than 2-fold higher than in non-depleted mice, while the amount of $NK1.1^+$ (NK and NK-T) cells was not significantly influenced. Immunohistochemical analysis of AdHBV-infected liver tissue at the peak of liver inflammation confirmed that significantly more $CD3^+$ T cells infiltrated the liver after initial Treg-depletion (Fig. 11D, upper panel). This lead to markedly reduced amounts of HBc^+ hepatocytes in the liver in the later stages of infection (Fig. 11D, lower panel). Since cytoplasmatic HBc is a marker for HBV replication while nuclear HBc persists, the histological analysis confirmed that HBV replication is more efficiently suppressed in the absence of Tregs (Fig. 11D, lower panel). In AdHBV k/o infected liver tissue, only single CD3+ cells could be detected, and HBV core protein was totally absent (Fig. 12C). These findings imply that Tregs are activated and mitigate liver damage, but at the same time

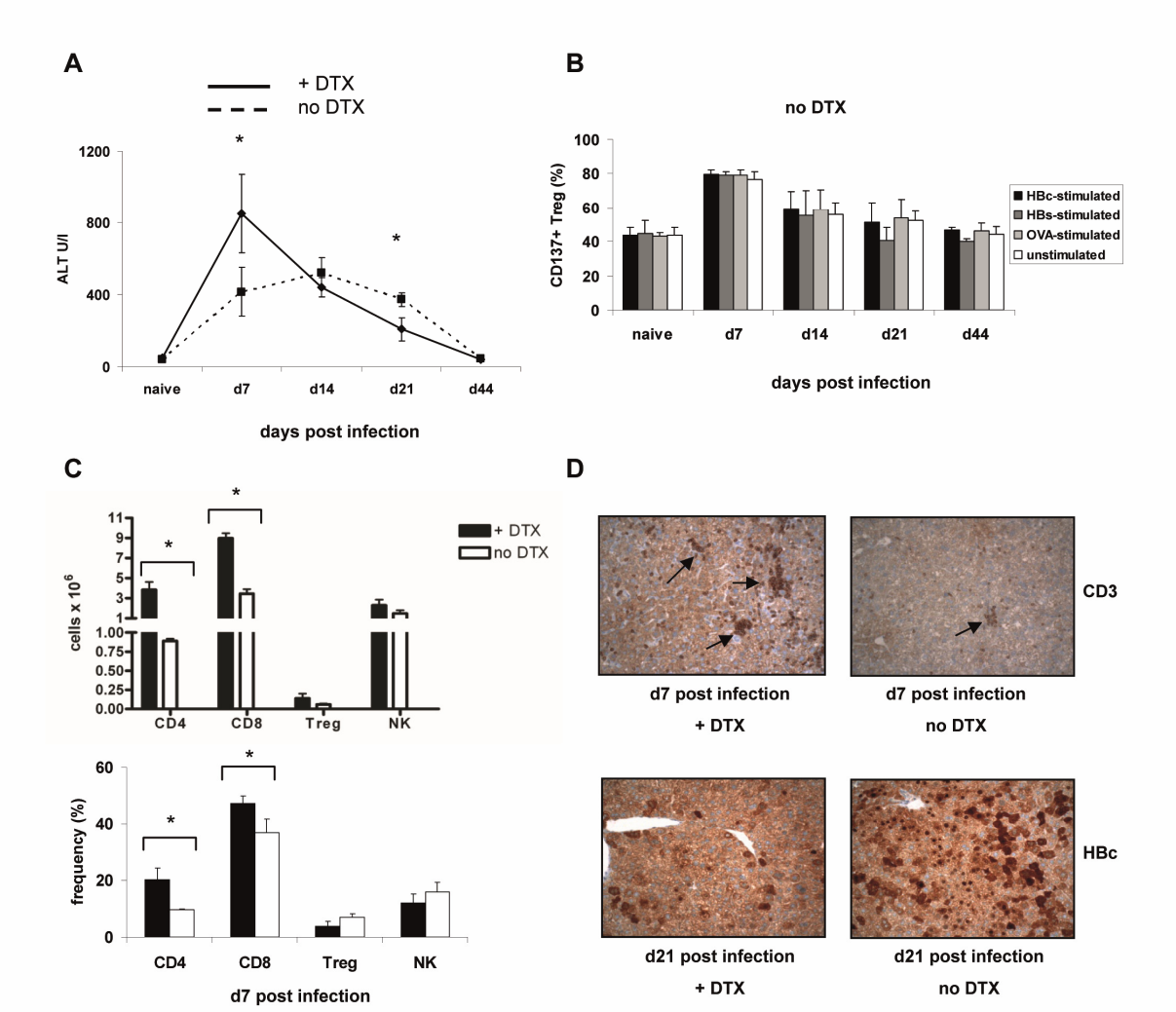


Fig. 11. Impact of Tregs on liver inflammation. (A,B) DEREG mice were Treg-depleted and AdHBV-infected as described earlier. **(A)** Mice were bled at the indicated time points and ALT-concentration in serum was determined. **(B)** LALs were stimulated with HBc, HBs or OVA for 24h and stained with antibodies for CD4, Foxp3 and CD137. Percentages of Tregs expressing surface-CD137 are shown. **(C)** LALs were isolated on d7 post infection and stained with antibodies for CD3, CD4. Foxp3, CD8 and NK1.1. Shown are total numbers **(upper panel)** and frequencies **(lower panel)** of the respective cell types. **(D)** Representative immunohistochemical stainings of CD3 on d7 post infection and for HBc on d21 post infection in liver tissue (magnification 200x). Black arrows mark foci of infiltrating T cells. * $P < 0.05$. HBc: HBV core protein; HBs: HBV S protein; OVA: Ovalbumin.

prolong clearance of infected hepatocytes.

Tregs delay the HBV-specific CD8$^+$ T cell cytokine response in the liver and prevent the development of polyfunctional HBV-specific CD8$^+$ T cells. To characterize the role of Tregs in the regulation of the antiviral CD8$^+$ T cell response in more detail, LALs were isolated from AdHBV-infected, Treg-depleted and non-depleted DEREG mice. The HBV-specific IFNγ-, IL2- and TNFα-production of CD8$^+$ T cells was monitored using intracellular cytokine staining during the course of infection. Since responses against an HBc derived peptide pool were readily detected, while cytokine responses above background level against HBs derived peptides pools could hardly be detected (data not shown), analysis was

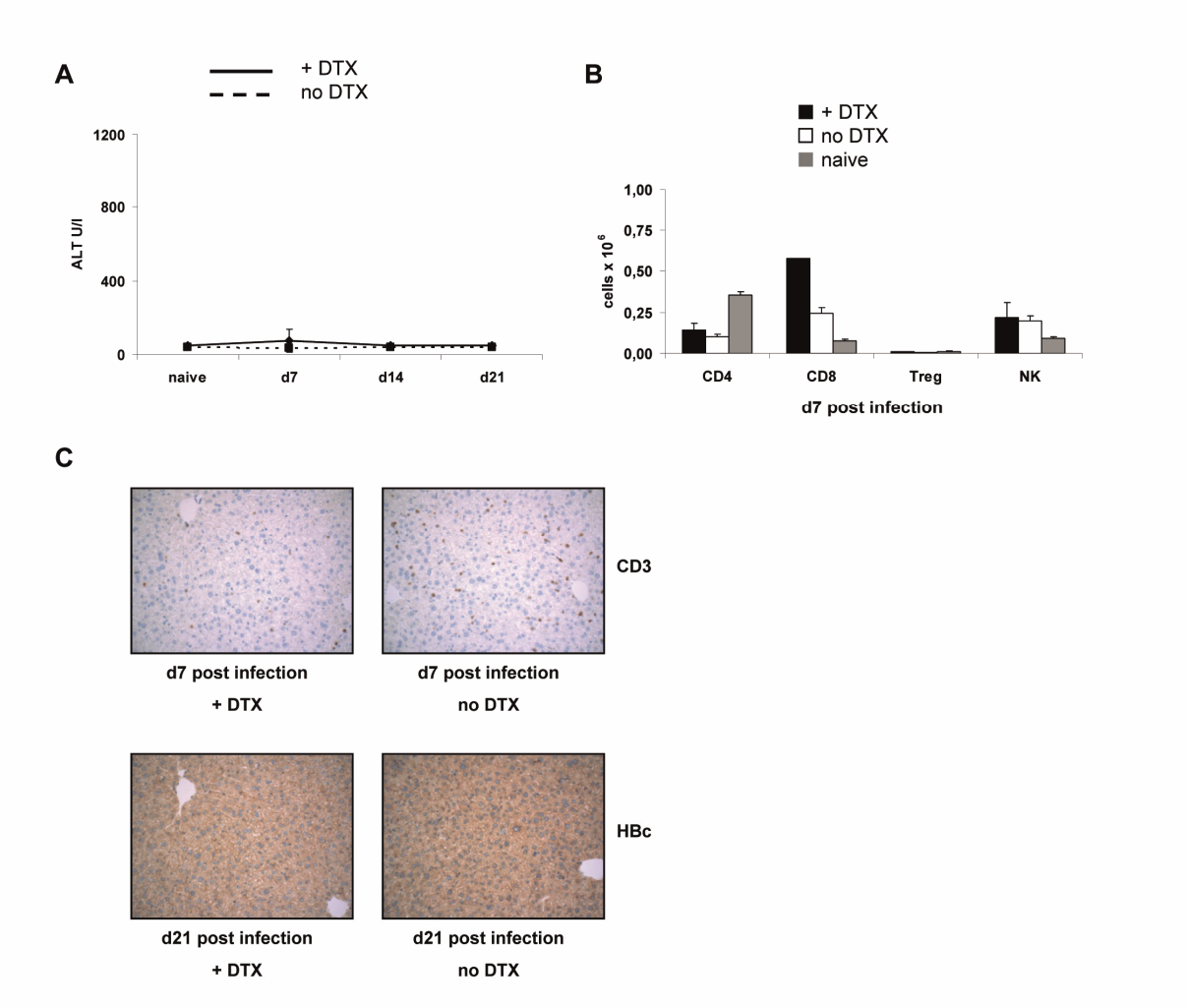


Fig. 12. Analysis of liver inflammation in DEREG mice infected with an empty adenoviral control vector. DEREG mice (n = 3) were infected with 1 x 109 i.u. AdHBV knock-out i.v. and Treg-depleted as described earlier. **(A)** Mice were bled at the indicated time points and ALT-concentration was measured in the serum. **(B)** LALs were isolated on d7 post infection and stained with antibodies to CD3, CD4, CD8, Foxp3 and NK1.1. Shown are total numbers of cells (x 106) in AdHBV k/o infected and naïve DEREG mice. **(C)** Representative immunohistochemical stainings for CD3 on d7 post infection and for HBc on d21 post infection in liver tissue (magnification 200x).

focussed on HBc specific $CD8^+$ T cell responses. On d7 and d21, Treg-depleted mice exhibited a significantly increased IFNγ-response (Fig. 10A). Interestingly, the overall frequency of IFNγ-producing $CD8^+$ T cells was low at the peak of liver inflammation, but increased up to ~6% of total $CD8^+$ T cells on d70 (Fig. 13A). TNFα was produced by a large proportion of $CD8^+$ T cells, but only at d7 at the peak of liver inflammation, and Treg-depletion resulted in an enhanced response (Fig. 13B). This points to a direct correlation between liver damage and TNFα secreted by $CD8^+$ T cells migrating into the infected liver (Fig. 11A, C). To a remarkable extent, $CD8^+$ T cells secreted TNFα also upon stimulation with a negative control peptide (Fig. 14B, C). This corresponds with studies demonstrating that acute liver inflammation is mediated by antigen-unspecific T cells and other mononuclear cells infiltrating the liver upon attraction by a small population of HBV-specific T cells (Sitia et al. 2004; Kakimi et al. 2001; Bertoletti & Maini 2000). An IFNγ background that increased

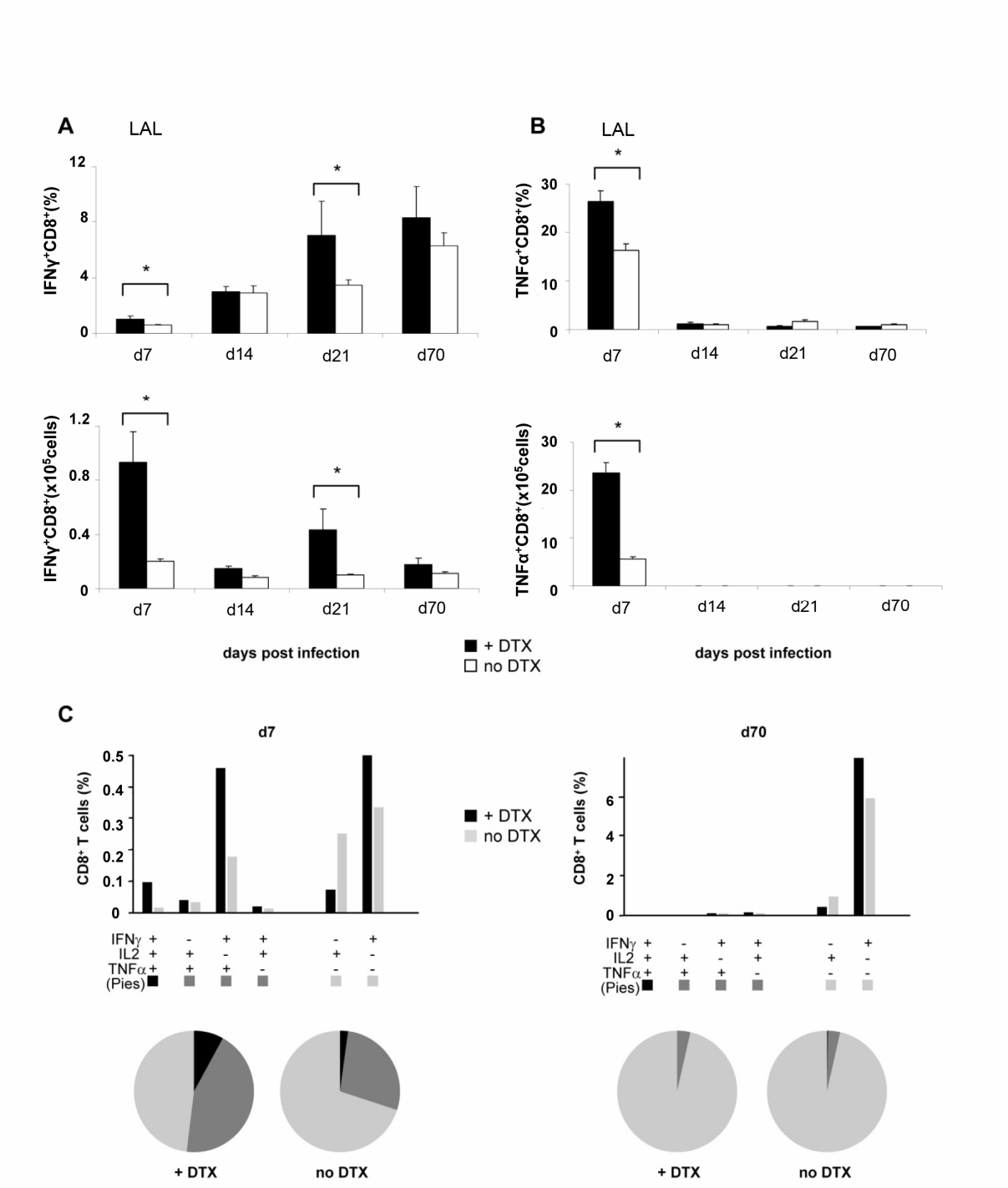


Fig. 13. Dissection of HBV-specific CD8+ T cell cytokine responses and polyfunctionality in AdHBV-infected DEREG mice. DEREG mice were Treg-depleted and AdHBV-infected as described earlier. At indicated time points, LALs were isolated and stimulated with HBc derived peptide pools. After 5h, LALs were stained with antibodies for CD3, CD8, IFNγ, TNFα and IL2. **(A)** IFNγ- and **(B)** TNFα-responses of CD8+ T cells were quantified. Shown are frequencies **(upper row**) and total numbers **(lower row)** of cytokine-secreting cells. **(C)** Spice-analysis of HBV-specific CD8+ T cells depicting polyfunctional, bifunctional and monofunctional IFNγ-responses as well as IL2-response at d7 **(left panel)** and d70 **(right panel)** post infection. T cells producing only TNFα or no cytokines were ignored in this analysis. * $P < 0.05$.

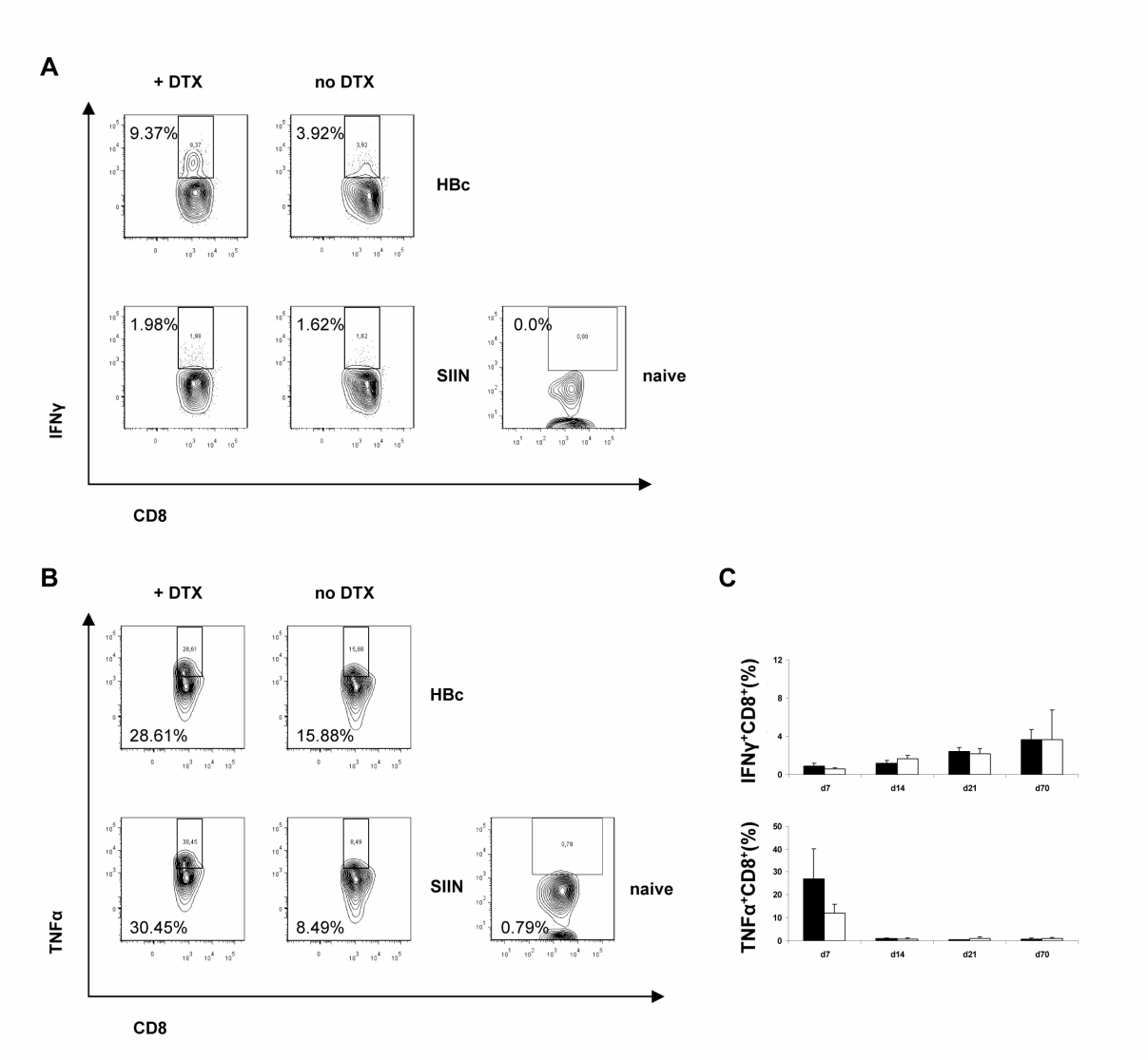


Fig. 14. Background of IFNγ- and TNFα-staining. DEREG mice were infected with AdHBV and Treg-depleted as described earlier. LALs were isolated and stimulated *ex vivo*. Representative flow cytometry plots showing the CD8+ T cell mediated intrahepatic **(A)** IFNγ-response on d21 post infection and **(B)** TNFα-response on d7 post infection after 5h stimulation with HBc derived peptide pools **(upper row)** or the negative control peptide SIINFEKL (SIIN) **(lower row)**. To determine the unspecific binding of the used staining antibodies, IFNγ- and TNFα-staining was performed using LALs of naive DEREG mice after 5h incubation with BFA **(A, B, right row)**. **(C)** IFNγ- **(upper panel)** and TNFα-response **(lower panel)** after 5h stimulation with SIINFEKL over the whole course of infection. BFA: brefeldin A.

over time was also detected (Fig. 14A, C), probably due to *in vivo* activated HBV-specific T cells and APCs already loaded with HBV-derived peptides upon isolation.

Since polyfunctional T cells are regarded the most important effector cells for limiting viral infections (Seder et al. 2008), the functional properties of the IFNγ-secreting $CD8^+$ T cells were determined by discriminating monofunctional ($IFN\gamma^+IL2^-TNF\alpha^-$) cells from bifunctional ($IFN\gamma^+IL2^+TNF\alpha^-$ or $IFN\gamma^+IL2^-TNF\alpha^+$) and polyfunctional ($IFN\gamma^+IL2^+TNF\alpha^+$) cells (Fig. 13C). At the peak of liver inflammation (d7), a large proportion of IFNγ-secreting cells produced also TNFα while bifunctional cells producing IL2 were detected only in minor quantities (Fig. 13C, left panel). Only a small fraction of IFNγ-secreting cells was polyfunctional. Strikingly, Treg-depleted mice exhibited a significantly higher proportion of

polyfunctional and bifunctional CD8$^+$ T cells. On d70, nearly all effector T cells reacting to HBV-derived peptides produced only IFNγ (Fig. 13C, right panel), irrespective if initial Treg-depletion was performed or not. As an additional aspect, Treg-depletion lead to reduced IL2-secretion by CD8$^+$ T cells (Fig. 13C).

In summary, these experiments provide evidence that in the early phase of infection Tregs suppress the secretion of the antiviral cytokines IFNγ and TNFα by HBV-specific T cells and reduce functionality of effector T cells.

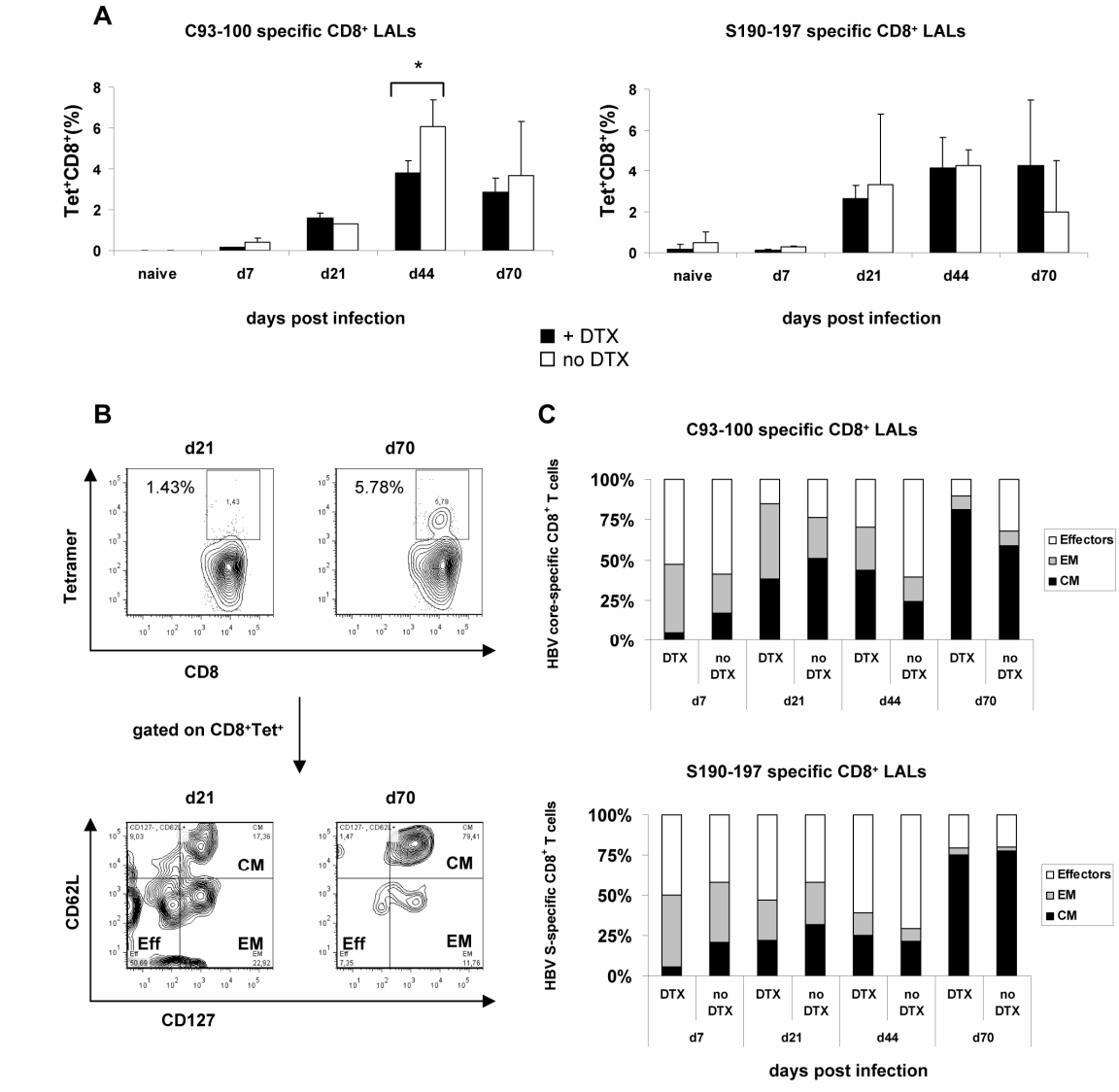


Fig. 15. Influence of Tregs on the development of HBV-specific CD8+ memory T cells. DEREG mice were Treg-depleted and AdHBV-infected as described earlier. Directly after isolation, LALs were stained with HBc (C93-100)- and HBs (S190-197)-tetramers and antibodies for CD3, CD8, CD62L and CD127. Staining background was determined by tetramer staining of CD8+ T cells in naïve Dereg mice. **(A)** The frequencies of C93-100 specific **(left)** and S190-197 specific **(right)** CD8+ T cells were determined. **(B)** Representative flow cytometry plots depicting the gating strategy for the discrimination of effector and memory T cell subpopulations. **(C)** Distribution of effector and memory subpopulations within the C93-100 specific **(upper panel)** and S190-197 specific **(lower panel)** CD8+ T cell populations. CM: central memory T cells; EM: effector memory T cells; Eff/Effectors: effector T cells.

Tregs do not influence development of long-term HBV-specific T cell memory. To investigate whether Tregs have influence on the establishment of long-term HBV immunity, the intrahepatic frequency of HBV-specific $CD8^+$ T cells was examined during the course of infection using tetramer staining. Memory and effector T cells were discriminated by their differential expression of CD127 and CD62L. Tetramer staining revealed HBV core (C93-100)- and S-protein (S190-197) specific $CD8^+$ T cell populations in the liver, which increased slowly from below 0.5% on d7 to 2 – 4% on d70 (Fig. 15A). No significant differences were found comparing Treg-depleted and non-depleted mice except a reduced frequency of C93-100 specific $CD8^+$ T cells on d44 in Treg-depleted mice.

Since long-lived central memory T cells express the survival factor CD127 and the homing receptor CD62L, they can be easily differentiated from effector T cells, which do not express these surface molecules (Parish & Kaech 2009; Obar & Lefrançois 2010) (Fig. 15B). While on d7 post infection most HBV-specific $CD8^+$ T cells displayed effector and effector memory phenotype, a growing population of HBV C93-100- (Fig. 15C, upper panel) and S190-197-specific (Fig. 15C, lower panel) central memory T cells emerged between d7 and d44. In the late phase of infection (d70), 70 – 90% of HBV-specific $CD8^+$ T cells in the liver were of central memory phenotype. Hereby, depletion of Tregs during the early phase of infection did not influence establishment of long-term HBV-specific immunological memory. Additionally, the data clearly show that central memory T cells do not only reside in lymphoid tissues, but also in the liver.

Tregs postpone the migration of macrophages and DCs into the infected liver and suppress the cytokine response of NK/NK-T cells. The first line of defense against viral infections is the innate immune system, in which macrophages, dendritic cells (DCs) and natural killer (NK) or natural killer T (NK-T) cells play a prominent role. Release of cytokines and other mediators by these cells induce inflammation and help to suppress viral replication. Priming capacity of professional antigen presenting cells (i.e. macrophages, DCs) decides about the development of a profound adaptive immune response. Thus, it was of interest if Tregs exert their suppressive effects also on macrophages, DCs and NK/NK-T cells. To analyse this, intrahepatic leukocytes were isolated and $F4/80^+$ macrophages, DC $marker^+MHCII^+$ DCs and $NK1.1^+$ cells (which comprise NK and NK-T cells) were quantified by flow cytometry at different time points during the course of infection. Additionally, cytokine responses of all three cell types were analyzed.

A pronounced influx of macrophages into the liver shortly after infection, which was significantly delayed by Tregs, was detected (Fig. 16A, left). While macrophages produced

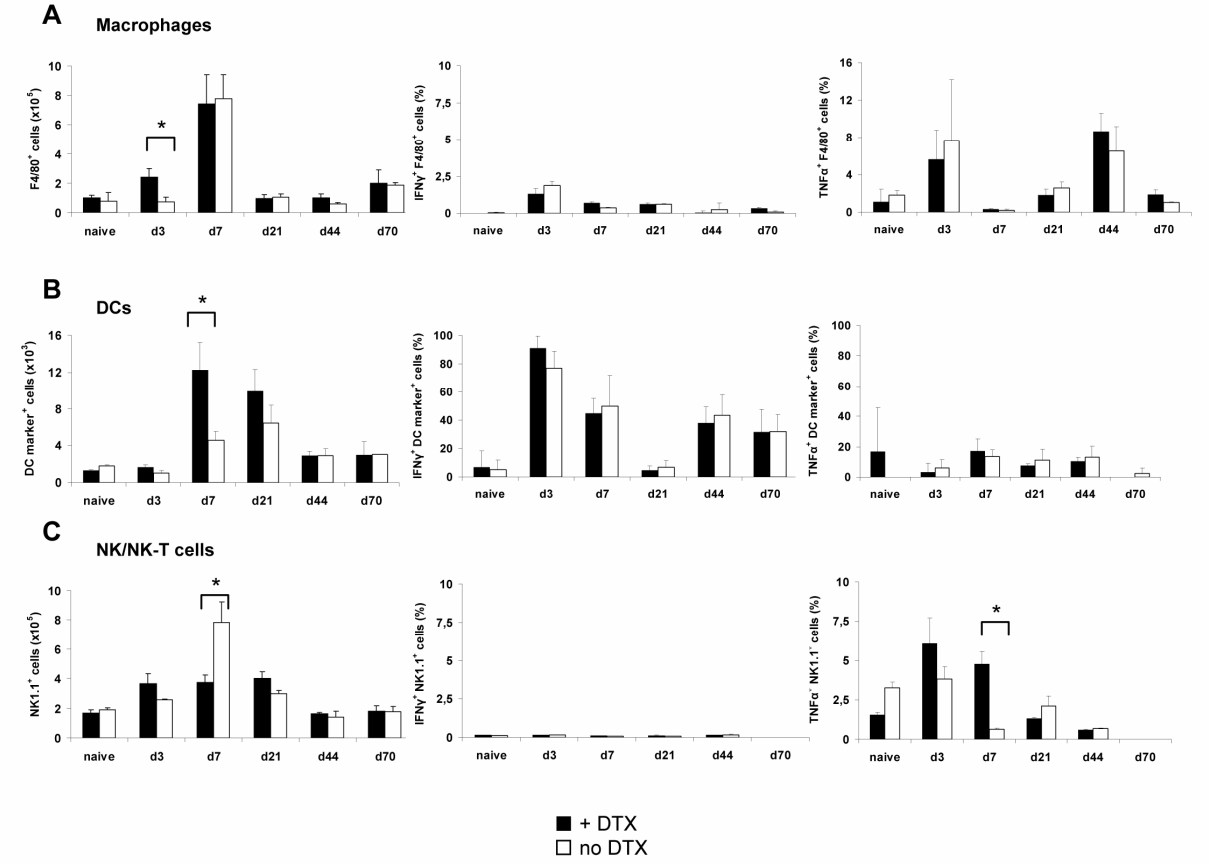


Fig. 16. Treg-mediated regulation of early innate responses in AdHBV-infected DEREG mice. DEREG mice were Treg-depleted and AdHBV-infected as described earlier. At the indicated time points, LALs were isolated and cultured in the presence of BFA. After 5h, cells were stained with antibodies to CD3, F4/80, DC marker (33D1), NK1.1, IFNγ and TNFα. **(A)** Absolute numbers **(left)**, IFNγ-secretion **(middle)** and TNFα-secretion **(right)** of F4/80+ macrophages. **(B)** The respective values for DCs and **(C)** NK/NKT cells. * $P < 0.05$. BFA: brefeldin A.

only low amounts of IFNγ (Fig. 16A, middle), they secreted TNFα (Fig. 16A, right) in the very early and late phase of infection. None of these cytokine responses was altered by Treg-depletion. Only low numbers of intrahepatic DCs were found, producing mainly IFNγ (Fig. 16B). Intrahepatic DCs increased during liver inflammation from d7 to d21 (Fig. 16B, left). Treg-depletion increased the numbers of DCs in the liver at d7, but did not influence cytokine production at all. In contrast, Tregs seemed to enhance recruitment of $NK1.1^+$ cells into the liver at d7 (Fig. 16C). However, hepatic $NK1.1^+$ NK/NK-T cells seemed not to secrete IFNγ at all (Fig. 16C, middle), but rather TNFα (Fig. 16C, right). Hereby, Tregs negatively influenced TNFα secretion at d7.

Taken together, it was found that Tregs delay the influx of macrophages and DCs into the liver during the early phase of AdHBV infection, but have no effect on the cytokine production of those cell types. On the other hand, recruitment of $NK1.1^+$ cells into the liver

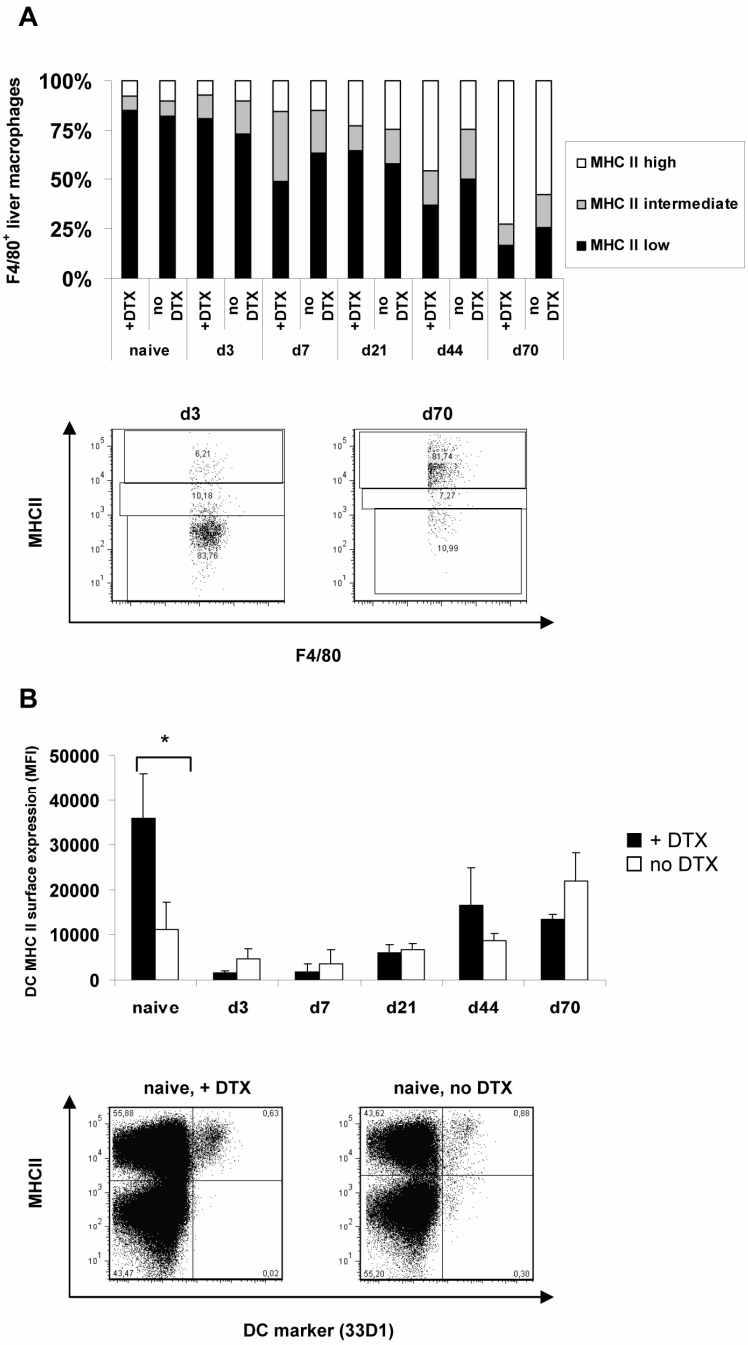


Fig. 17. MHC II expression profile of liver macrophages and DCs. DEREG mice were Treg-depleted and AdHBV-infected as described earlier. At the indicated time points, LALs were isolated and stained with antibodies to CD3, F4/80, DC marker (33D1) and MHC II. **(A)** Differentiation of macrophages into MHCII^{high} and MHCII^{low} subsets during the course of infection. Below, two representative flow cytometry plots depicting MHCII-expression of liver macro-phages on d3 **(left)** and d70 **(right)** in non-depleted DEREG-mice. **(B)** Strenght of surface expression of MHCII on intra-hepatic DCs, shown as mean fluorescence intensity (MFI). Below, two representative FACS-plots depicting the MHCII ex-pression of intrahepatic DCs in naïve DEREG mice after Treg-depletion **(left)** and without depletion **(right)**. * $P < 0.05$.

seems to be enhanced by Tregs, suggesting that Tregs may regulate innate immune responses more subtle and diverse than adaptive immunity.

Tregs inhibit the phenotypical shift from immunosuppressive M2 to proinflammatory M1 macrophages, but have no influence on MHCII expression of DCs during HBV infection. Macrophages exist in two main phenotypes, the MHCII^{low}, anti-inflammatory M2 and the MHCII^{high}, phagocytic, proinflammatory M1 macrophages. By flow-cytometrical analysis of

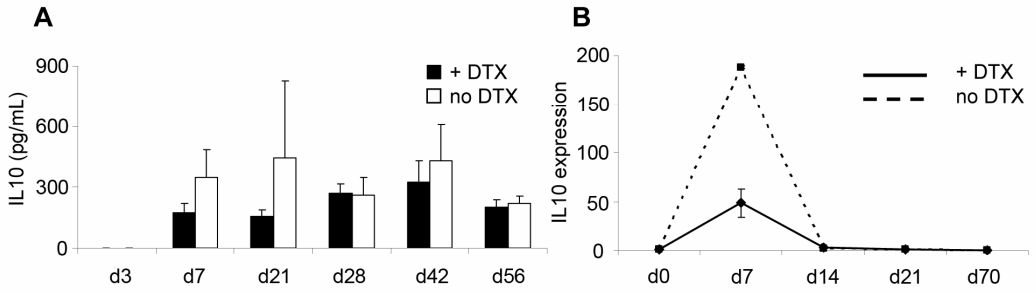


Fig. 18. Analysis of peripheral and intrahepatic IL10 response in AdHBV infected DEREG mice. DEREG mice were Treg-depleted and AdHBV-infected as described earlier. **(A)** At the indicated time points, mice were bled and IL10 concentration in the serum was measured using cytometric bead array. **(B)** Liver samples were taken at the indicated time points, whole-liver RNA extracted and IL10 transcripts quantified relative to GAPDH by qPCR.

MHCII expression, both forms can easily be differentiated (Fig. 17A). Interestingly, F4/80^{+} intrahepatic macrophages were mainly MHCII^{low} at the onset and during the early phase of infection, but their phenotype changed until the late phase of infection, when the vast majority of intrahepatic macrophages was of MHCII^{high} phenotype (Fig. 17A). Tregs seem to inhibit this phenotypical shift, since a significantly larger proportion of intrahepatic macrophages was of MHCII^{high} phenotype in the late phase of infection in Treg-depleted DEREG mice. In naive mice, Treg-depletion lead to a marked increase of MHCII surface expression on intrahepatic DCs, indicating that Tregs reduce their capability to present exogenous antigens to T cells (Fig. 17B). In AdHBV-infected mice however, the MHCII surface expression dropped shortly after infection, and increased again slowly during the course of infection. Here, Tregs did not exhibit any significant effect. This result suggests that Tregs possess the ability to hamper the presentation of exogenous antigen by intrahepatic DCs, but in a setting of liver infection, this suppressive mechanisms seems to be inactive or blocked.

Tregs are not the main producers of IL10, but induce the expression of IL10 by other cells during acute liver inflammation. IL10 is known to be an immunosuppressive cytokine and is supposed to be one key mechanism Tregs use to regulate immune responses. Therefore, IL10 was quantified in the serum of AdHBV-infected DEREG mice at different time points during the course of infection using cytometric bead array (CBA). While no IL10 was detectable at d3 post infection, high amounts of IL10 were measurable during and after liver inflammation, exceeding 300 pg/mL (Fig. 18A). After initial Treg-depletion, IL10-concentrations in the serum were reduced compared to non-depleted mice at d7 and d21 post infection, even though not significantly due to high standard deviations. To examine the IL10-response by a second method, whole-liver RNA was extracted from AdHBV-infected

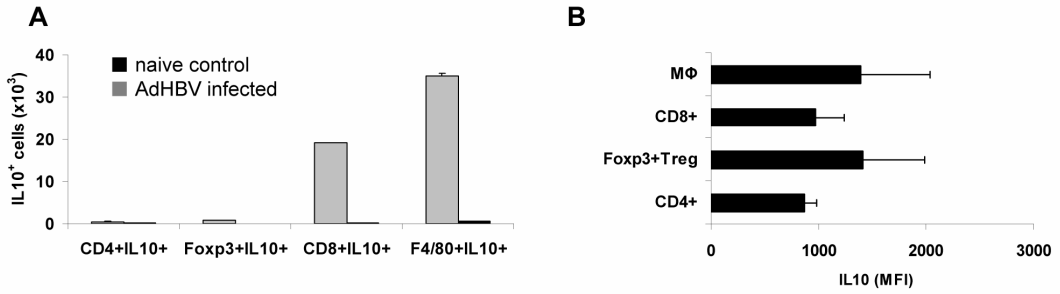


Fig. 19. Analysis of the intrahepatic IL10 response in AdHBV infected mice at the peak of liver inflammation. IL10 reporter mice were AdHBV-infected as described earlier. At d7 post infection, LALs were isolated and stained with antibodies to CD3, CD4, CD8, F4/80 and Foxp3 after a 90 min incubation with the IL10-reporter-substrate. **(A)** Absolute numbers of IL10-secreting cells from AdHBV-infected (grey bars) and naïve (black bars) IL10-reporter mice. **(B)** MFI of the respective IL10-signals, representing the amount of produced IL10. MFI: Mean fluorescence intensity.

DEREG mice, followed by cDNA-synthesis and detection of IL10 transcripts by qPCR. IL10 transcripts peaked during acute liver inflammation (d7) (Fig. 18B), with IL10 expression increased 187fold. Strikingly, this increase of intrahepatic IL10 expression was markedly reduced (38fold) when Tregs were depleted initially. To clarify if this loss of IL10 expression was merely due to the depletion of IL10-secreting Tregs, it had to be determined exactly which cell types produce IL10 in the liver during acute liver inflammation. Since intracellular cytokine staining of IL10 is ineffective and qPCR, ELISA and CBA do not allow to conclude which cells produce the detected cytokine, IL10-reporter mice were used to detect IL10-producing intrahepatic cells *ex vivo*. In these mice, IL10-secreting cells produce an enzyme that catalyzes cleavage of an added substrate, which leads to emission of a signal detectable in the Pacific Blue channel of a flow cytometer. Thereby, IL10 producing cells can be quantified using flow cytometry without the need for IL10-specific staining antibodies. At day 7 post infection, intrahepatic $F4/80^+$ macrophages and, strikingly, $CD8^+$ T cells were the main IL10-secreting cell types (Fig. 19A). This confirmed that intrahepatic macrophages are mostly of immunosuppressive M2 phenotype during acute liver inflammation (Fig. 17A). Intrahepatic Tregs also produced IL10, but their numbers were very low (< 1000 $IL10^+Foxp3^+$ Tregs/liver). The amount of IL10-producing CD4 effector T cells was nearly undetectable (< 500 $IL10^+CD4^+$ T cells/liver). The mean fluorescence intensity (MFI) of IL10 signals revealed that all cell types produced nearly equal amounts of IL10 per cell (Fig. 19B). Collectively, this suggests that Tregs are not the main producers of IL10 in the liver, but that Tregs regulate the IL10-production of other cell types, possibly via the small amount of Treg-derived IL10. To prove this, $IL10^{flx/flx}CD4Cre^+$ mice, in which $CD4^+$ T cells lack the ability to produce IL10, were infected with AdHBV as described and compared with Treg-depleted

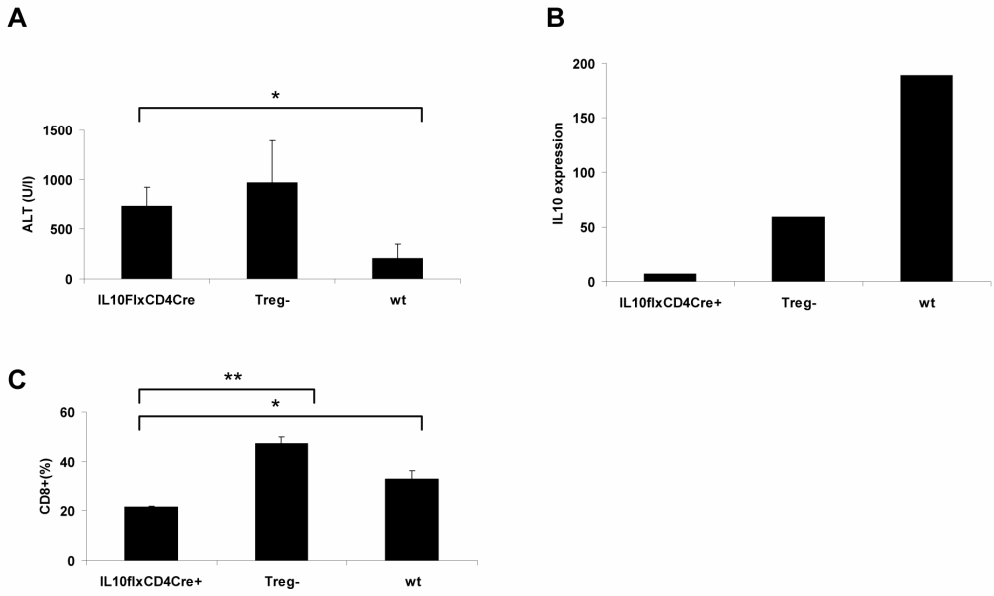


Fig. 20. Changes in liver inflammation and intrahepatic IL10-secretion upon lack of CD4⁺-derived IL10 . IL10$^{flx/flx}$CD4Cre^{+} mice, Treg-depleted DEREG mice (Treg-) and C57BL/6 mice (wt) were AdHBV-infected as described earlier. At d7 post infection, mice were sacrificed. **(A)** ALT-concentrations in the serum. **(B)** Liver samples were taken at the indicated time points, whole-liver RNA extracted and IL10 transcripts quantified relative to GAPDH by qPCR. **(C)** LALs were isolated and stained with antibodies for CD3 and CD8 to quantify liver-infiltrating CD8^{+} T cells. * P < 0.05; ** P < 0.01

DEREG mice (Treg-) and C57BL/6 mice (wt) on d7. Strikingly, IL10$^{flx/flx}$CD4Cre^{+} mice exhibited a significantly increased liver damage compared to wt-control mice, reaching concentrations similar to that of Treg-depleted DEREG mice (Fig. 20A). Using qPCR, it was revealed that the increase of IL10 transcript in liver tissue was markedly less pronounced in IL10$^{flx/flx}$CD4Cre^{+} mice (7-fold) compared with wt-mice (189-fold) and even Treg-depleted DEREG mice (59-fold) (Fig. 20B). In contrast to that, IL10$^{flx/flx}$CD4Cre^{+} mice had the smallest frequency of intrahepatic CD8^{+} T cells on d7 (22%), while Treg-depleted DEREG mice and also wt-mice showed higher frequencies (47% and 33%, respectively) (Fig. 20C). Together, this indicates that CD4^{+} derived IL10 induces IL10 secretion by other major intrahepatic cell subsets (possibly macrophages, CD8^{+} T cells), thereby alleviating liver damage. IL10 itself does not seem to reduce frequencies of CD8^{+} T cells infiltrating the infected liver.

HBc and not HBs induces IL10 secretion by macrophages. Since HBsAg is shed by HBV-infected hepatocytes in large excess (Seeger & Mason 2000), the question arose if this shedding of HBsAg is a viral defense mechanism to escape the immune response by flooding the blood circulation with “decoy antigen” and by exhausting HBV-specific T cells through continuous stimulation. This idea was strengthened by the fact that no significant HBsAg-

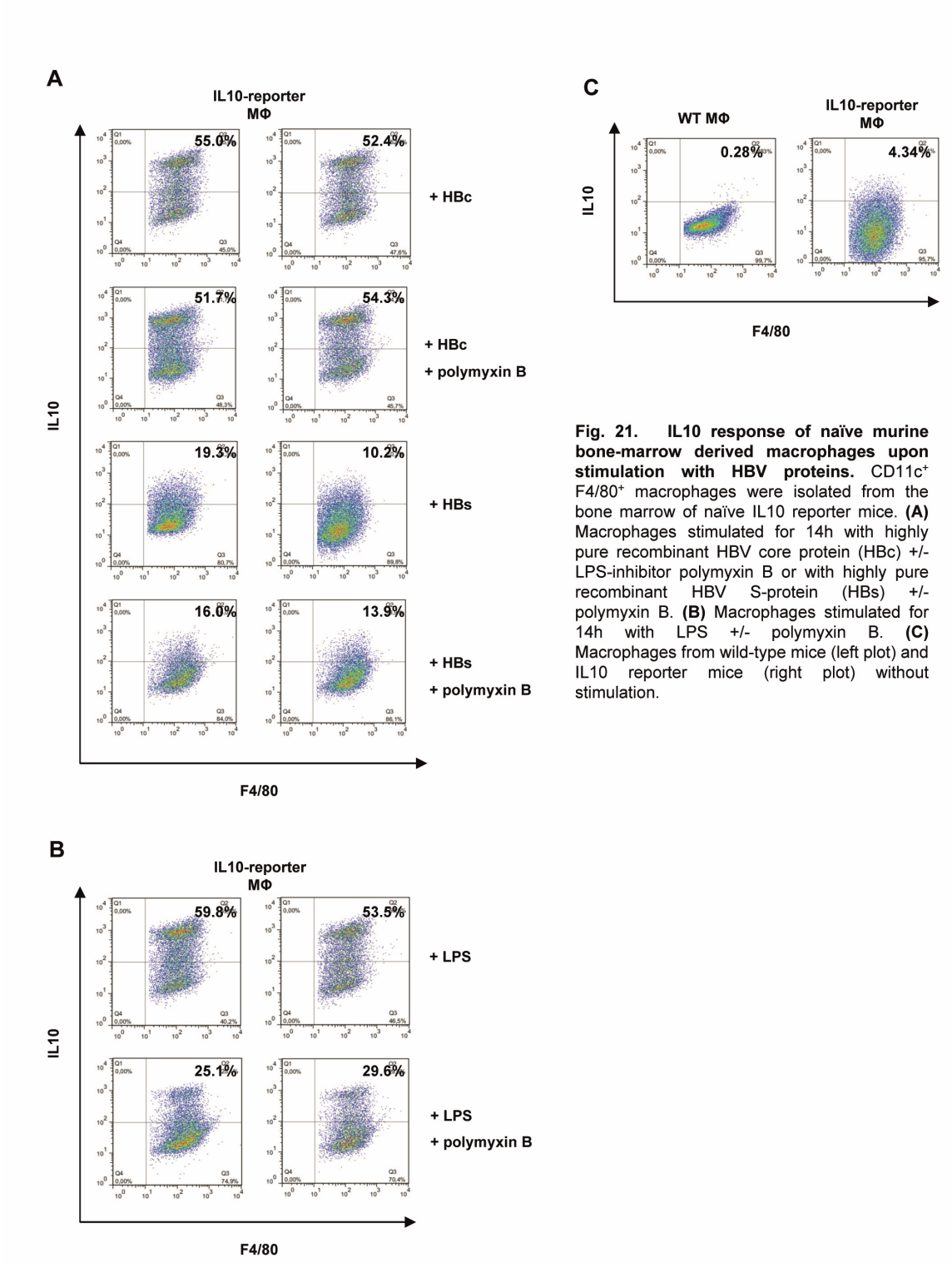


Fig. 21. IL10 response of naïve murine bone-marrow derived macrophages upon stimulation with HBV proteins. $CD11c^+$ $F4/80^+$ macrophages were isolated from the bone marrow of naïve IL10 reporter mice. **(A)** Macrophages stimulated for 14h with highly pure recombinant HBV core protein (HBc) +/- LPS-inhibitor polymyxin B or with highly pure recombinant HBV S-protein (HBs) +/- polymyxin B. **(B)** Macrophages stimulated for 14h with LPS +/- polymyxin B. **(C)** Macrophages from wild-type mice (left plot) and IL10 reporter mice (right plot) without stimulation.

specific T cell response was detectable in AdHBV-infected mice. Furthermore, HBsAg could have immunosuppressive effects itself, possibly by induction of anti-inflammatory cytokines. To address this question, $CD11c^+F4/80^+$ macrophages were isolated from bone-marrow of IL10-reporter mice and stimulated for 14h with 10 µg/mL highly pure recombinant HBc or HBs. To exclude unspecific stimulation due to LPS remnants in the protein solution, the LPS-inhibitor polymyxin B was added (20 µg/mL). Stimulation with LPS (500 ng/mL) served as

positive control. Stimulation with HBV core protein induced IL10-secretion in more than 50% of the stimulated macrophages, while HBsAg induced IL10-secretion in only 10-20% of the stimulated cells (Fig. 21A). Stimulation in the presence of polymyxin B demonstrated that this IL10-induction was not due to LPS remnants in the purified protein solution (Fig. 21A). The positive control stimulation with 500 ng/mL LPS also induced IL10 in more than 50% of the stimulated macrophages (Fig. 21B). The background IL10-secretion of isolated macrophages after 14h was 4% (Fig. 21C). These results provide evidence that HBV core protein – possibly released by apoptotic infected hepatocytes – induces IL10 secretion, while HBsAg seems to be a low-immunogenic protein, neither inducing strong pro- nor anti-inflammatory cytokine responses.

Depletion of CD4$^+$ T cells in liver and spleen of AdHBV-infected C57BL/6 mice. It has already been demonstrated that CD4$^+$ T cell help is required to mount effective HBV-specific T cell responses (Whitmire 2011). Additionally, CD4$^+$ T cells play an important regulatory role by secretion of pro- or anti-inflammatory cytokines and chemokines. To clearly define the regulatory role of CD4$^+$ T cells in HBV infection, the course of infection was studied in CD4-depleted, AdHBV-infected C57BL/6 mice. For complete and systemic depletion of CD4$^+$ T cells, female, 8 week old C57BL/6 mice received one intraperitoneal injection of 500

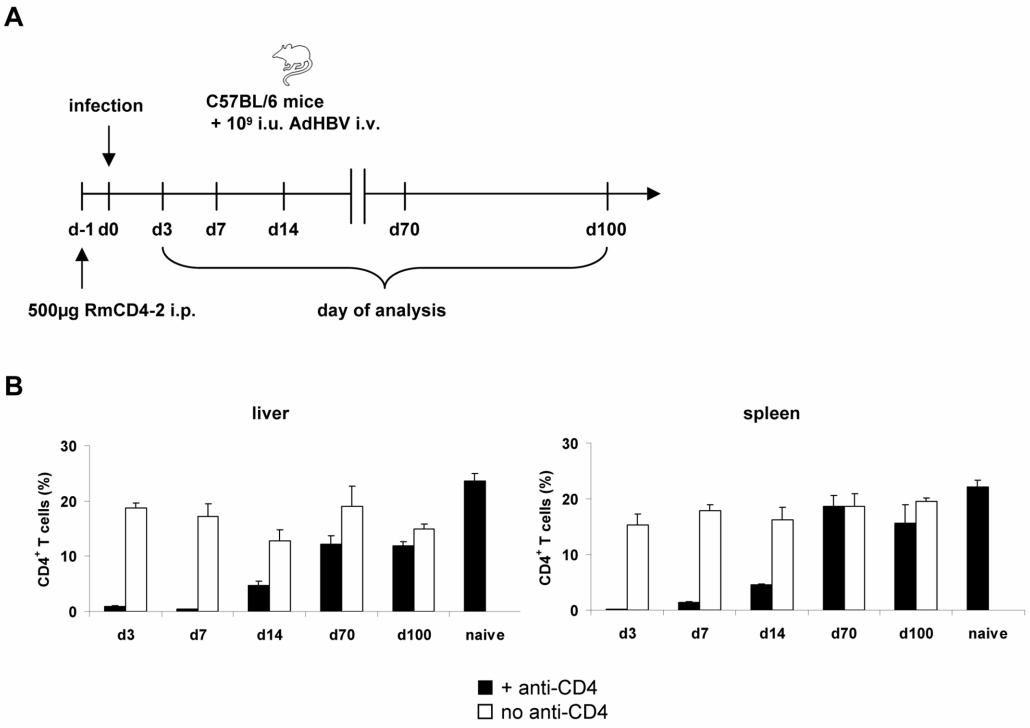


Fig. 22. CD4$^+$ T cell frequency in AdHBV-infected BL6 mice with or without CD4-depletion. Female 8 week old C57BL/6 mice were AdHBV-infected as described earlier and received one intraperitoneal injection of 500 µg anti-CD4 one day prior infection. Non-depleted mice served as controls. **(A)** Experimental scheme. **(B)** On the time points indicated, mice were sacrificed, liver and spleen explanted and splenocytes/LALs isolated. CD4$^+$ T cell frequencies were analyzed using flow cytometry after staining with antibodies to CD3 and CD4.

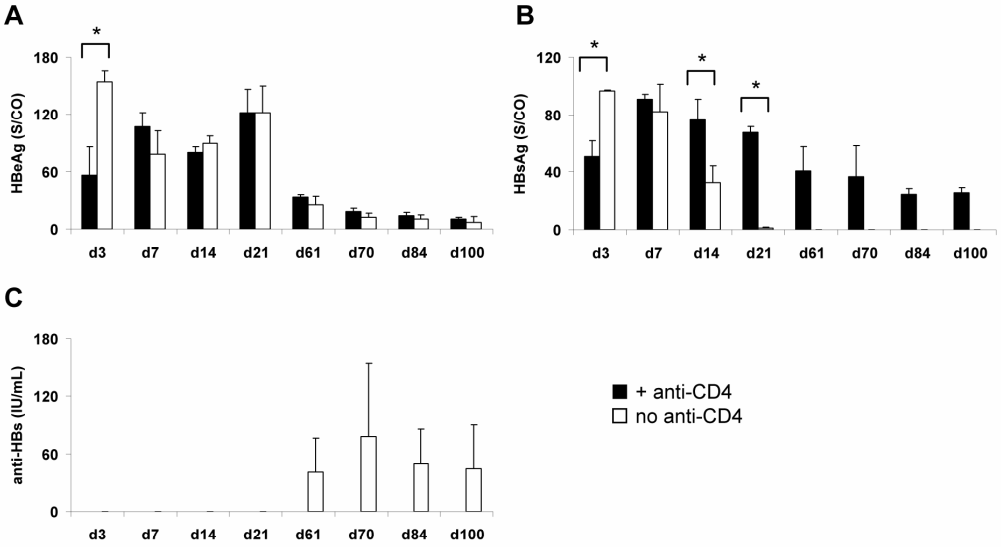


Fig. 23. Serological markers of HBV infection in CD4-depleted, AdHBV-infected mice. Female 8 week old C57BL/6 mice were AdHBV-infected and CD4-depleted as described earlier. At the indicated time points, mice were bled and **(A)** HBeAg, **(B)** HBsAg and **(C)** antibodies against HBsAg (anti-HBs) were quantified in 1:20 serum dilutions. * $P < 0.05$.

µg CD4-antibody (anti-CD4) one day prior infection. AdHBV-infection was performed as described before (Fig. 22A). CD4-depletion proved to be highly efficient in both liver and spleen, reducing CD4-frequencies below 1% (Fig. 22B). Between d7 and d14 p.i., $CD4^+$ T cells started to repopulate the liver.

$CD4^+$ T cells are required for the induction of anti-HBs and clearance of HBsAg, but not HBeAg. To monitor the influence of $CD4^+$ T cells on HBV clearance, AdHBV-infected, CD4-depleted mice were bled at different time points during infection and serological markers HBeAg, HBsAg and anti-HBs were quantified. After an initial phase of strong HBeAg secretion (d3 to d21), HBeAg decreased slowly between d21 and d100 (Fig. 23A). No significant differences between CD4-depleted mice and non-depleted controls were found, except on d3, when CD4-depleted mice exhibited far lower HBeAg concentrations in the serum than the control mice. HBsAg however was cleared completely in non-depleted control mice after d21, while it persisted in initially CD4-depleted mice (Fig. 23B). Like HBeAg, HBsAg was also markedly reduced on d3 in CD4-depleted animals compared to non-depleted controls. As expected, anti-HBs was measurable in the serum of AdHBV-infected, non-depleted mice in the late phase of infection, but it was totally absent in mice that received CD4-depletion (Fig. 23C). Even though $CD4^+$ T cell frequency regenerates between d14 and d70 (Fig. 22B), no anti-HBs was detectable even on d100. This suggests that $CD4^+$ T cell help is required in the very early phase of infection for mounting of an anti-HBs response and thus

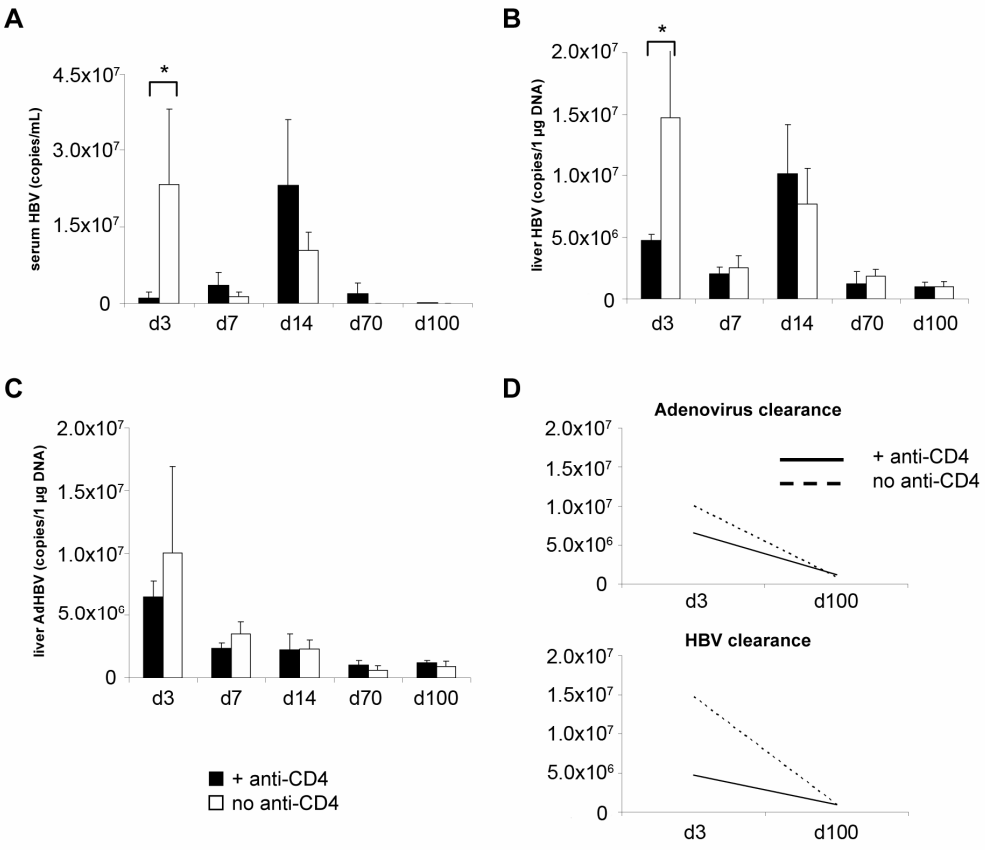
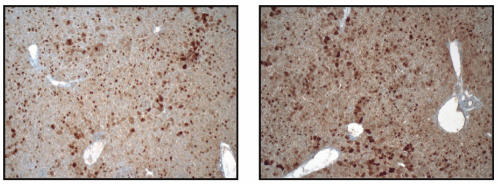


Fig. 24. Viral titers in CD4-depleted, AdHBV-infected mice. Female 8 week old C57BL/6 mice were AdHBV-infected and CD4-depleted as described earlier. **(A)** At the indicated time points, mice were bled and HBV copies were quantified in whole serum DNA using qPCR. **(B,C)** Whole-liver DNA was extracted at the indicated time points and **(B)** intrahepatic HBV titers and **(C)** intrahepatic adenoviral vector copies were quantified. **(D)** Clearance of the adenoviral vector **(upper panel)** and HBV **(lower panel)** from d3 to d100 depicted as linear slope. * $P < 0.05$.

clearance of HBsAg. In the late phase of infection, $CD4^+$ T cells seemed to have lost the ability to induce an anti-HBs response. Furthermore, the results show clearly that $CD4^+$ T cells have no importance for the diminution of HBeAg.

$CD4^+$ T cells promote suppression of HBV replication and clearance of infected hepatocytes. The data gained from HBV antigen quantification were confirmed by analysis of viral titers in the periphery and in liver tissue using qPCR. HBV was cleared from the circulation of non-depleted mice in the late phase of infection, while it persisted until d100 in CD4-depleted mice (Fig. 24A). In the liver, however, non-depleted as well as CD4-depleted mice reduced HBV as well as the adenoviral vector over time (Fig. 24B, C). On d3, CD4-depleted mice exhibited lower viral titers (HBV as well as adenoviral vector) than non-depleted mice, suggesting some immunological event reducing viral titers unspecifically upon CD4-depletion during the first 3 days after infection. Immunohistochemical staining of HBc in liver tissue of infected mice also confirmed a reduced amount of infected hepatocytes in

HBc+ cells (%)	d3	d7	d14	d70	d100
+ anti-CD4	N: 60; CP: 20	N: 60; CP: 10	N: 70; CP: 30	N: 30; CP: 10	N: 20; CP: s.c.
no depletion	N: 70; CP: 30	N: 70; CP: 40	N: 40; CP: s.c.	N: 15; CP: s.c.	N: 20; CP: s.c.



+ anti-CD4 no anti-CD4

d7 post infection

Table 3. Immunohistochemical analysis of HBc in liver tissue sections of CD4-depleted, AdHBV-infected mice. Female 8 week old C57/BL6 mice were AdHBV-infected and CD4-depleted as described earlier. Immunohistochemical staining of HBV core protein was performed at the indicated time points in parrafin-embedded liver tissue. Representative stainings of HBV core protein are shown below. N: nucleus; CP: cytoplasm; s.c.: single cells.

CD4-depleted mice in the early phase (Table 3, d3 and d7). While the highly stable nuclear form of HBc was reduced in CD4-depleted mice in the early phase, it was increased from d14 to d70, but declined to levels comparable to those in non-depleted mice on d100 (Table 3). The cytoplasmatic form of HBc, a marker for HBV replication, behaved similar. Alltogether, these data indicate that initial application of anti-CD4 leads to early suppression of HBV replication and even clearance of a certain percentage of the viral inoculum, furthermore promotes enhanced HBV-replication between d14 and d70, but not in the very late phase (d100), and affects clearance of infected hepatocytes. In the very late phase (d100), however, no difference between CD4-depleted and non-depleted mice was detected. Taking into account that part of the viral inoculum was eliminated very early after infection (d3) in CD4-depleted mice, this suggests that clearance of infected hepatocytes was not as efficient as in non-depleted mice (Fig 24D).

CD4$^+$ T cells induce migration of leukocytes into HBV-infected liver tissue. In order to clarify if CD4$^+$ T cells play a role for the induction of acute liver inflammation, C57BL/6 mice were AdHBV-infected and CD4-depleted as described before. On d7, LALs were isolated and absolute numbers of intrahepatic CD4$^+$ and CD8$^+$ T cells, Tregs and NK cells were determined using flow cytometry. Furthermore, ALT concentrations were quantified as a marker for liver damage. As expected, no CD4$^+$ T cells and thus also no Tregs were detected in livers of CD4-depleted mice, while both cell types were found in high numbers in livers of non-depleted control mice (Fig. 25A). While in those control mice, CD8$^+$ T cells and NK cells

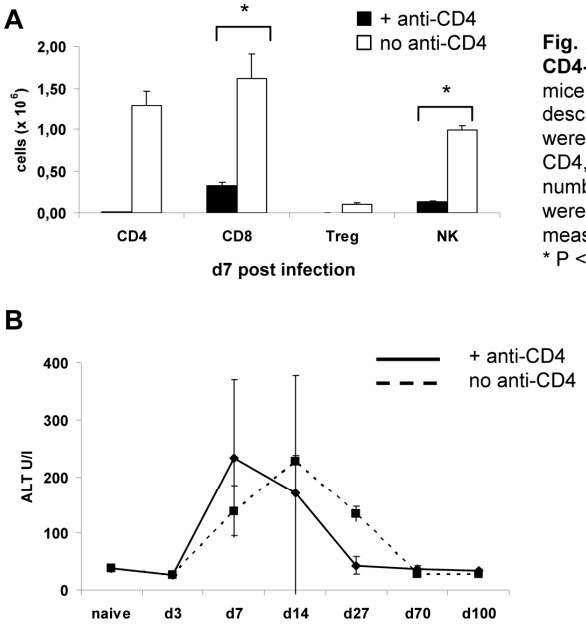


Fig. 25. Liver inflammation in AdHBV-infected, CD4-depleted mice. Female 8 week old C57BL/6 mice were AdHBV-infected and CD4-depleted as described earlier. **(A)** On d7 post infection, LALs were isolated and stained with antibodies for CD3, CD4, Foxp3, CD8 and NK1.1. Shown are total numbers. **(B)** At the indicated time points, mice were bled and liver damage was determined by measuring ALT-concentration in murine serum.
* $P < 0.05$.

infiltrated the liver in large amounts, only few of these cells were observed in livers of CD4-depleted mice, suggesting that $CD4^+$ T cells provide stimuli early after infection that attract $CD8^+$ T cells and other immune cells to the infected liver tissue. In contrast to this observation, both groups of mice showed increased ALT-levels on d7 and d14 (Fig. 25B), the only difference being the high standard deviations in the CD4-depleted group. The cause for the inconsistent ALT-levels in CD4-depleted mice remains obscure.

$CD4^+$ T cells induce an intrahepatic HBV-specific cytokine response by $CD8^+$ T cells early after infection. Since $CD4^+$ T cells are known to provide help for the induction of $CD8^+$ T cells, it was analyzed if CD4-depletion prior to infection influences the establishment of an HBV-specific $CD8^+$ T cell response in the liver of AdHBV-infected mice. For this purpose, LALs of CD4-depleted and non-depleted control mice were stimulated *ex vivo* with the HBV-derived peptide pool HBcP3 or an irrelevant control peptide (bGal) and stained with antibodies for CD3, CD8 and IFNγ, followed by flow cytometrical analysis. In non-depleted control mice, an HBc specific $CD8^+$ T cell IFNγ-response was indeed detected from d14 onwards (Fig. 26A). In mice that received CD4-depletion, however, no such response could be found until d100. One cause for this $CD8^+$ T cell dysfunction might be exhaustion due to the prolonged presence of viral antigens, i.e. HBsAg (Wherry et al. 2003; Zhou et al. 2004). Thus, splenocytes and LALs of AdHBV-infected, CD4-depleted and non-depleted control mice were stained for CD3, CD8, IL2, TNFα, IFNγ and the activation/exhaustion marker PD1 on d100. T cells that express PD1 on their surface, but do not secrete cytokines anymore can

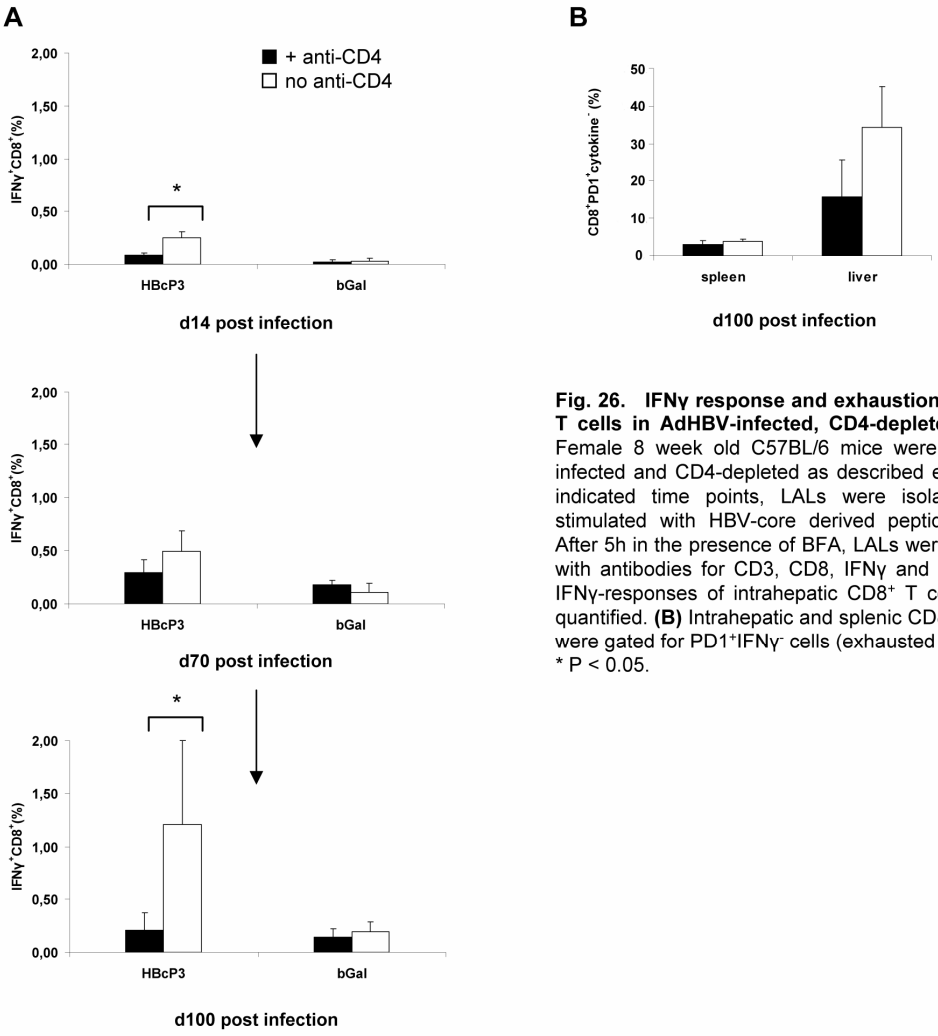


Fig. 26. IFNγ response and exhaustion of CD8⁺ T cells in AdHBV-infected, CD4-depleted mice. Female 8 week old C57BL/6 mice were AdHBV-infected and CD4-depleted as described earlier. At indicated time points, LALs were isolated and stimulated with HBV-core derived peptide pools. After 5h in the presence of BFA, LALs were stained with antibodies for CD3, CD8, IFNγ and PD1. **(A)** IFNγ-responses of intrahepatic CD8⁺ T cells were quantified. **(B)** Intrahepatic and splenic CD8⁺ T cells were gated for PD1⁺IFNγ⁻ cells (exhausted T cells). * $P < 0.05$.

be considered exhausted T cells (Barber et al. 2006). Unexpectedly, we found a very prominent population of exhausted $CD8^+$ T cells ($PD1^+cytokine^-$) in the liver of AdHBV infected control mice (34.28±10.29% of total $CD8^+$ T cells), but not in the spleen (3.85±0.51% of total $CD8^+$ T cells) (Fig. 26B). After initial CD4-depletion, the frequency of exhausted T cells in the liver was markedly, even though insignificantly, reduced (15.73±10.07% of total $CD8^+$ T cells), ruling out that enhanced exhaustion causes $CD8^+$ T cell dysfunction in AdHBV-infected mice after CD4-depletion. The reduced amount of exhausted T cells after CD4-depletion implies that less $CD8^+$ T cells were activated in the early phase of infection, and thus, less $CD8^+$ T cells are subject to exhaustion over time. Summarized, this provides evidence that the presence of $CD4^+$ T cells is necessary during the very early phase of HBV infection to induce an HBV-specific, $CD8^+$ T cell cytokine response in the liver. Even though $CD4^+$ T cells repopulate the liver two weeks after infection, they seem to loose the ability to induce an antiviral $CD8^+$ T cell cytokine response.

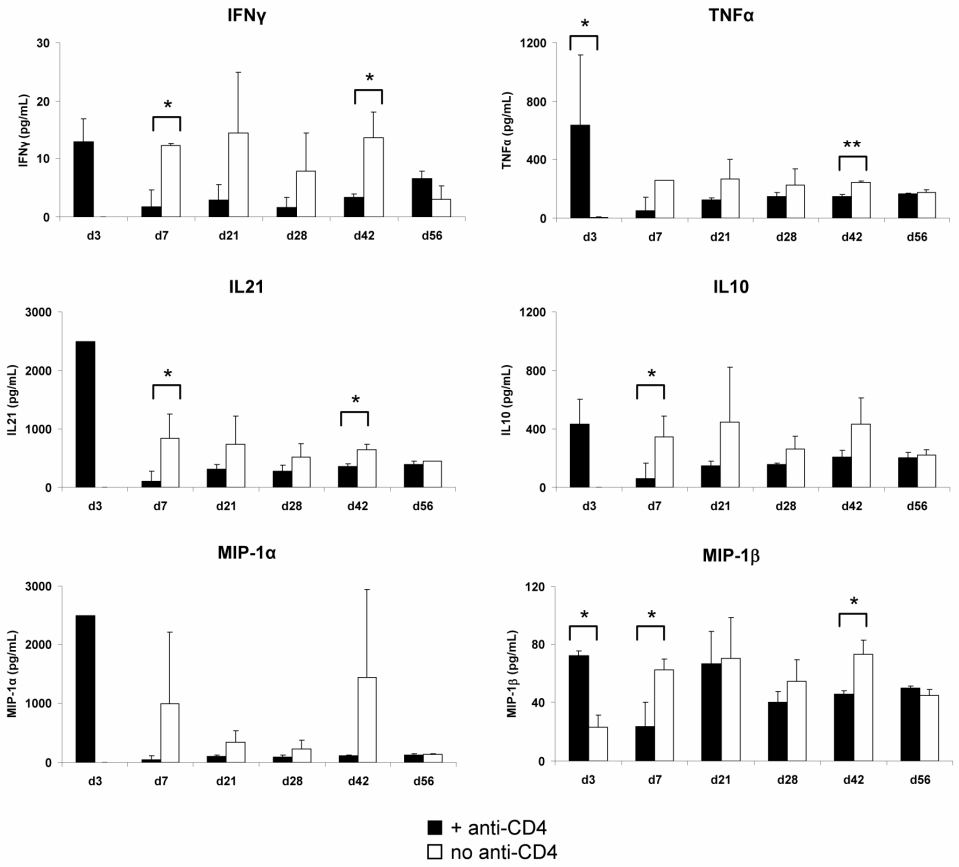


Fig. 27. Serum levels of cytokines and chemokines secreted in AdHBV-infected, CD4-depleted mice. Female 8 week old C57BL/6 mice were AdHBV-infected and CD4-depleted as described earlier. At the indicated time points, mice were bled and the cytokines IFNγ, TNFα, IL21, IL10 as well as the chemokines MIP-1α and MIP-1β were quantified in the serum by cytometric bead array analysis. Please note that 2500 pg/mL is the maximum concentration measurable by this method.
* $P < 0.05$; ** $P < 0.01$.

CD4$^+$ T cells are required for cytokine and chemokine secretion early after infection. Many cytokines are involved in the immune response against viruses, and chemokines are important for the induction of inflammation as well as activation and attraction of immune cells to the site of infection. To define the influence of CD4$^+$ T cells on systemic cytokine and chemokine secretion, peripheral concentrations of IFNγ, TNFα, IL21, IL10 and the chemokines MIP-1α and MIP-1β were quantified in the serum of AdHBV-infected, CD4-depleted and non-depleted control mice using cytometric bead arrays. Surprisingly, all analyzed cytokines and chemokines were massively secreted in CD4-depleted mice on d3, but were totally absent in non-depleted mice at that time point (Fig. 27). This points to a strong, systemic immune activation upon application of anti-CD4. On d7, this response was already faded. From d7 onwards, the non-depleted control mice showed upregulation of all measured cytokines and chemokines, proving immune activation and acute inflammation. In CD4-depleted mice,

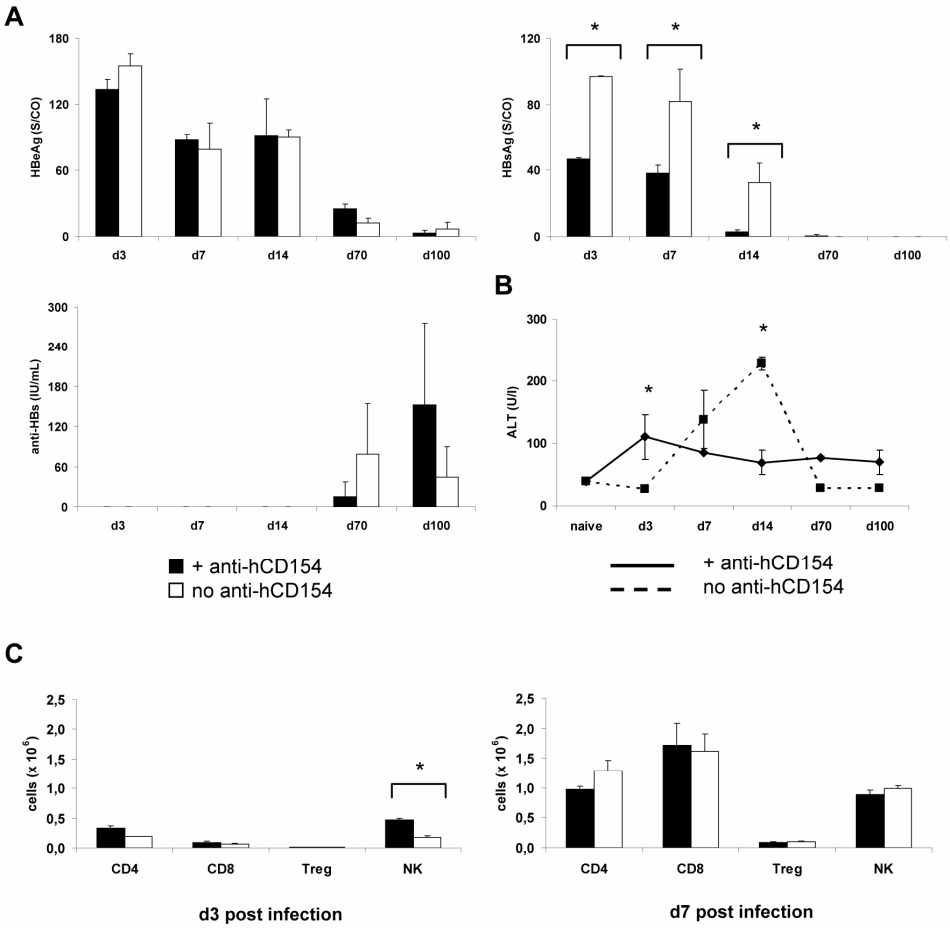


Fig. 28. Analysis of AdHBV-infection in mice receiving an anti-human CD154 isotype control antibody. Female 8 week old C57BL/6 mice were AdHBV-infected as described earlier and received a single dose of 500 µg anti-human CD154 (rat IgG_2a) i.p. one day prior infection. **(A)** At the indicated time points, mice were bled and HBeAg, HBsAg and anti-HBs were quantified in 1:20 serum dilutions. **(B)** Liver damage was determined by measuring ALT-concentration in murine serum. **(C)** On day 3 **(left panel)** and day 7 **(right panel)** mice were sacrificed, LALs isolated and stained with antibodies to CD3, CD4, CD8, Foxp3 and NK1.1, followed by flow cytometrical analysis. Shown are total cell numbers. * $P < 0.05$.

cytokine secretion was markedly reduced compared to the control mice until d56, and only MIP-1β reached comparable concentrations earlier (d21). Thus, it was confirmed that $CD4^+$ T cells are required in the early phase of infection to mount an immune response and inflammation, and that they mediate secretion of a broad bandwidth of cytokines and chemokines.

Application of rat IgG_2b does not trigger anti-inflammatory signalling, but induces a strong, systemic innate immune response immediately after application. In order to exclude that binding of the depletion antibody anti-CD4 to immunosuppressive F_C-receptors induces the observed immunological hyporesponsiveness in CD4-depleted mice, an irrelevant isotype control antibody, rat IgG_2b anti-human CD154 (anti-hCD154), was injected intraperitoneally in mice one day prior infection (500µg/mouse). The course of infection was followed

measuring HBsAg, HBeAg, anti-HBs and ALT on d3, d7, d14, d70 and d100 as well as by characterization of liver-infiltrating immune cells at d3 and d7 p.i. Very early after infection (d3), mice treated with anti-hCD154 showed decreased concentrations of HBeAg and HBsAg compared to untreated control mice, suggesting an early suppression of viral replication as observed in the CD4-depleted mice (Fig. 28A). But in contrast to mice receiving anti-CD4, mice treated with anti-hCD154 cleared HBsAg rapidly and developed anti-HBs (Fig. 28A). Also, these mice exhibited significantly increased ALT-concentrations already on d3 (110±35.9 U/L vs. 27.3±2.3 U/L in untreated mice), and ALT-levels remained elevated even until d100 (Fig. 28B). Numbers of $CD4^+$ and $CD8^+$ T cells were not elevated in the liver on d3, but significantly more NK cells were present ($0{,}47x10^6 \pm 0{,}019x10^6$ NK cells vs. $0{,}18x10^6 \pm 0{,}017x10^6$ NK cells in untreated controls), suggesting that NK cells are activated by antibody-treatment, kill virus-infected hepatocytes and reduce viremia (Fig. 28C, left panel). On d7, both the anti-hCD154 treated mice and the untreated control mice exhibited a marked influx of T cells in the liver and NK cell numbers were also elevated in both groups (Fig. 28C, right panel). Collectively, these data provide evidence that the application of the CD4-depletion antibody induces an unspecific innate immune response after application, causing reduced viremia in the early phase of infection. Additionally, it was demonstrated that the lack of inflammation and antiviral immune responses observed in CD4-depleted mice is indeed a result of the lack of $CD4^+$ T cells early after infection and not due to binding of immunosuppressive F_C-receptors by the depletion antibody.

To avoid this undesired innate response induced by the antibody-application, and to possibly increase viremia and viral persistence, two new CD4-depletion schemes were used (Fig. 29). One group of mice ("CD4- repeated") received 250 µg anti-CD4 i.v. one day prior infection and then every 10 days, with the intention to cause continuous CD4-depletion over at least 70 days and to avoid innate immune activation due to lower dose and circumvention of intraperitoneal macrophages by intravenous application of the antibody; the other group ("CD4- 1x") received 500 µg anti-CD4 i.p. 7 days prior infection, so that the innate immune response could fade before infection.

Both continuous and initial CD4-depletion diminish liver inflammation and result in HBsAg-, but not HBeAg-persistence. Assessment of liver damage by measuring ALT-concentrations in the peripheral blood revealed only slightly increased ALT-concentrations in both CD4-depleted groups, while non-depleted mice exhibited more pronounced liver damage (Fig. 30A). On d3, both CD4-depleted groups had HBeAg and HBsAg levels comparable to those of non-depleted mice, suggesting that the new application protocols circumvented

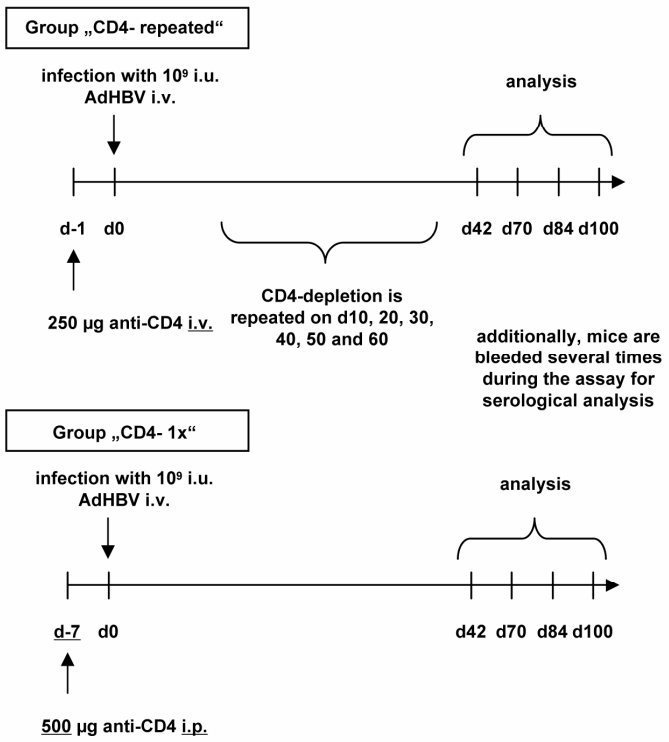


Fig. 29. Experimental setting for the comparison of continuous and initial CD4-depletion in AdHBV-infected mice. Female 8 week old C57BL/6 mice were AdHBV-infected as described earlier. One group received repeated injections of 250 µg anti-CD4 i.v. **(upper panel)**, while a second group received a single injection of 500 µg anti-CD4 i.p. one week prior infection **(lower panel)**. Non-depleted, AdHBV-infected mice served as controls.

activation of innate immune cells (Fig. 30B). But CD4-depletion had again no effect on HBeAg clearance, while HBsAg persisted until d100 after both types of CD4-depletion. Interestingly, mice receiving repeated intravenous applications of anti-CD4 seemed to suppress HBsAg secretion during the first two weeks after infection. Additionally, antibody responses against HBc, HBs and the adenoviral vector were analyzed using AXSYM assays (anti-HBs, anti-HBc) and an adenovirus neutralization assay (anti-Ad). As expected, non-depleted control mice developed antibody responses against HBc, HBs and the adenoviral vector (Table 4). Antibodies against the adenoviral vector were detectable from d42 onwards (Table 4, lower panel). Also, HBc-antibodies were first measurable on d42, but only very weakly. On d70, all mice were again anti-core negative, but from d84 onwards, all non-depleted mice showed stable secretion of HBc-antibodies (Table 4, upper panel). An anti-HBs response was detected comparably late after infection in this assay, not until d84 (Table 4, middle panel). This late anti-HBs secretion might be a result of low HBsAg expression in non-depleted mice infected with this certain AdHBV-stock (Fig. 30B). The CD4-depleted mice receiving continuous CD4-depletion were totally negative for all three antibody responses, while one out of three mice receiving only one initial CD4-depletion showed a late and weak antibody response against HBc (d100) and the adenoviral vector (d84). Taken together, it was confirmed that $CD4^+$ T cell presence in the early phase of infection is

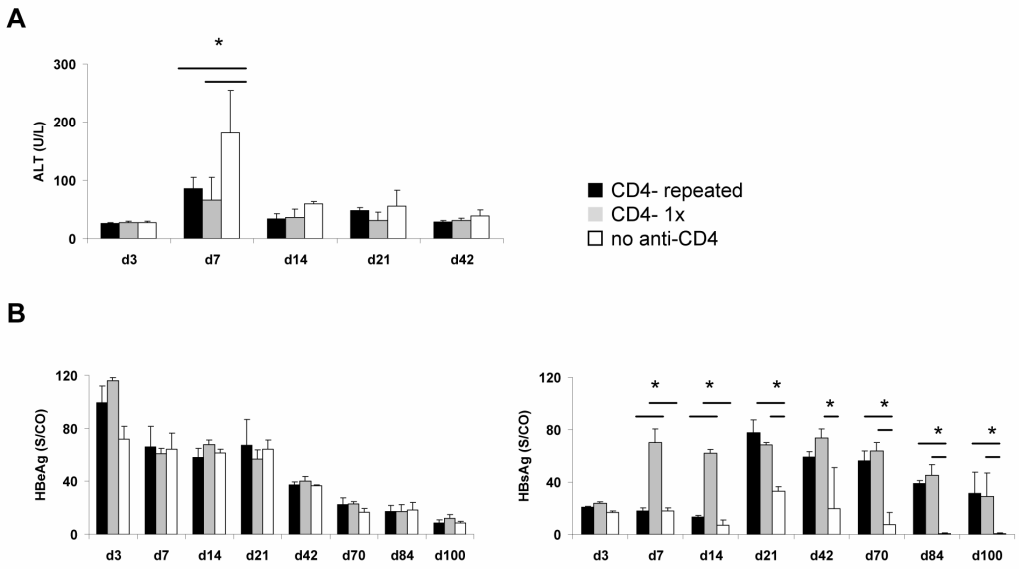


Fig. 30. Liver inflammation and serological HBV markers in AdHBV-infected mice under different CD4-depletion regimens. Female 8 week old C57BL/6 mice were AdHBV-infected and either continuously or one-time CD4-depleted as described earlier. **(A)** At the indicated time points, mice were bled and ALT-concentrations in the serum measured. **(B)** Furthermore, HBeAg **(left panel)** and HBsAg **(right panel)** were monitored over the whole course of infection in 1:20 serum dilutions. * $P < 0.05$.

necessary to induce antibody responses against viruses infecting the liver (HBV as well as adenovirus) and for liver inflammation. Again, HBsAg persisted for at least 100 days in CD4-depleted mice irrespective if CD4-depletion was performed only once initially or repeatedly, while HBeAg-clearance was not influenced by the loss of $CD4^+$ T cells.

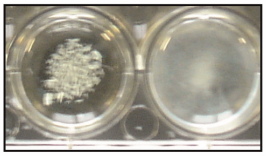
Both depletion protocols cause $CD8^+$ T cell inactivity in the late phase of infection. To confirm that CD4-depletion leads to loss of antiviral $CD8^+$ T cell function in the late phase of AdHBV-infection, IFNγ-secretion of $CD8^+$ T cells was monitored after stimulation of LALs with HBV-derived peptide pools HBsP3, HBcP3 or a poxvirus-derived control peptide (A8R) *ex vivo* on d42, d70, d84 and d100 post infection, followed by intracellular cytokine staining and flow cytometrical analysis. In non-depleted control mice, HBV-specific IFNγ-secretion was observed on d42, d84 and d100, but unexpectedly not on d70 post infection (Fig. 31). In mice receiving continuous CD4-depletion, no HBV-specific IFNγ-secretion was detected, except some response on d70. But this response was not significantly higher than the background response against A8R ($P=0.34$). Also mice receiving one initial CD4-depletion exhibited no HBV-specific $CD8^+$ IFNγ response on d42, d70 and d84. On d100, no data about HBV-specific cytokine secretion is available for this group of mice, since they exhibited a strong, HBV-unspecific IFNγ-production with high standard deviation (Fig. 31). Probably, these mice received an undesired inflammatory stimulus, i.e. another infection, or the LALs were stimulated unspecifically during preparation or stimulation, i.e. through bacterial

anti-HBs	d7	d21	d42	d70	d84	d100
CD4- repeated	negative	negative	negative	negative	negative	negative
CD4- 1x	negative	negative	negative	negative	negative	negative
control	negative	negative	negative	negative	+	+

anti-HBc	d7	d21	d42	d70	d84	d100
CD4- repeated	negative	negative	negative	negative	negative	negative
CD4- 1x	negative	negative	negative	negative	negative	+/-
control	negative	negative	+/-	negative	++	++

anti-Ad	d7	d21	d42	d70	d84	d100
CD4- repeated	negative	negative	negative	negative	negative	negative
CD4- 1x	negative	negative	negative	negative	**1 mouse positive**	negative
control	negative	negative	**positive**	**positive**	**positive**	**positive**

adenovirus-neutralisation assay



negative **positive**

Table 4. Antibody responses in CD4-depleted, AdHBV infected mice. Female 8 week old C57BL/6 mice were AdHBV-infected and CD4-depleted as described earlier. At the indicated time points, mice were bled and anti-HBs **(upper panel)**, anti-HBc **(middle panel)** were quantified using AXSYM assays with 1:20 diluted serum. Anti-Ad **(lower panel)** was detected by adenovirus-neutralisation assay. The picture below shows representative examples of the adenovirus-neutralisation assay. +/-: anti-HBs < 10 IU/l; +: 10-100 IU/l; ++: 101-500 IU/l; +++: >500 IU/l.

contamination. Additionally, exhaustion of $CD8^+$ T cells was assessed by surface PD1-staining. Significantly lower frequencies of exhausted ($PD1^+cytokine^-$) $CD8^+$ T cells were only found on d70 in the liver of CD4-depleted mice compared to non-depleted control mice (CD4-repeated: 20.55±6.61% of $CD8^+$ T cells; CD4-1x: 13.61±7.68%; control: 42.6±9.59%), irrespective of the depletion protocol used (Fig. 32A, left panel). Overall, mice receiving only one initial CD4-depletion had the lowest frequency of exhausted $CD8^+$ T cells, but the differences to the other two groups were – except on d70 – insignificant. That the difference in T cell exhaustion between CD4-depleted and non-depleted AdHBV-infected mice could only be confirmed on d70 in this assay might be a result of the comparably low antigen secretion of the virus stock used in this experiment (Fig. 30B). Thus, also the $CD8^+$ T cells in the non-depleted mice received less stimulation during infection and therefore did not exhaust

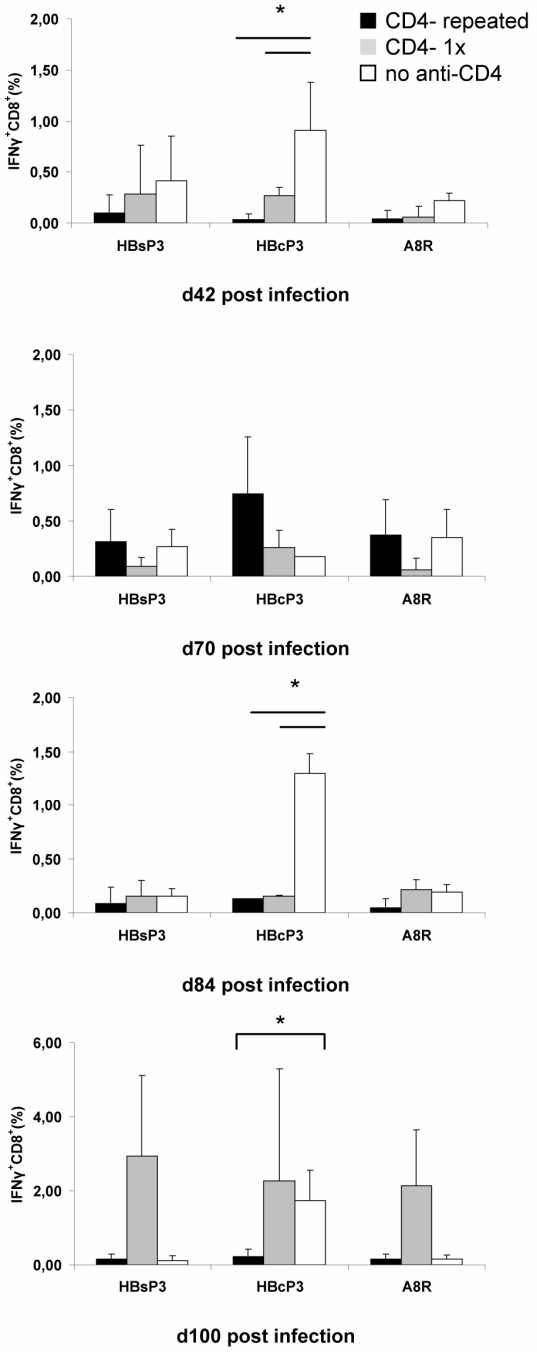


Fig. 31. IFNγ response of intrahepatic CD8+ T cells in AdHBV-infected, CD4-depleted mice. Female 8 week old C57BL/6 mice were AdHBV-infected and CD4-depleted as described earlier. At indicated time points, LALs were isolated and stimulated with HBc derived peptide pools. After 5h in the presence of BFA, LALs were stained with antibodies for CD3, CD8 and IFNγ. IFNγ-responses of intrahepatic CD8+ T cells were quantified.
* P < 0.05.

to the extent observed in the first CD4-depletion experiment (Fig. 26B). But since nearly undetectable amounts of exhausted $CD8^+$ T cells were found in the spleen of CD4-depleted and non-depleted mice (Fig. 32A, right panel), it was confirmed here that exhausted effector T cells accumulate in the liver of AdHBV-infected mice.

Another cause for the $CD8^+$ T cell inactivity after CD4-depletion might be increased immunosuppression by Tregs, possibly through fast regeneration of depleted Tregs or through enhanced suppressive activity of newly generated Tregs. To address this, the frequency of $CD4^+Foxp3^+$ Tregs was monitored in the liver of CD4-depleted and non-depleted, AdHBV-infected mice using flow cytometry. In non-depleted control mice, the intrahepatic Treg-frequency declined from 7.44±3.38% of $CD4^+$ T cells (d42) to values around 5% (d84 and d100) (Fig 32B, left panel.). A peak of Treg-frequency was detected on d70 (16.52±3.87% of $CD4^+$ T cells). Interestingly, significantly increased Treg-frequencies (compared to non-depleted controls) were found on d84 and d100 in mice receiving continuous CD4-depletion (d84: 11.39±2.35% vs. 4.87±0.07%; d100: 11.24±2.69% vs. 5.56±1.21%), and the mice receiving one initial CD4-depletion had Treg-frequencies higher than the non-depleted controls on d100 (12.68±1.99% vs. 5.56±1.21%), too (Fig. 32B, left panel). In

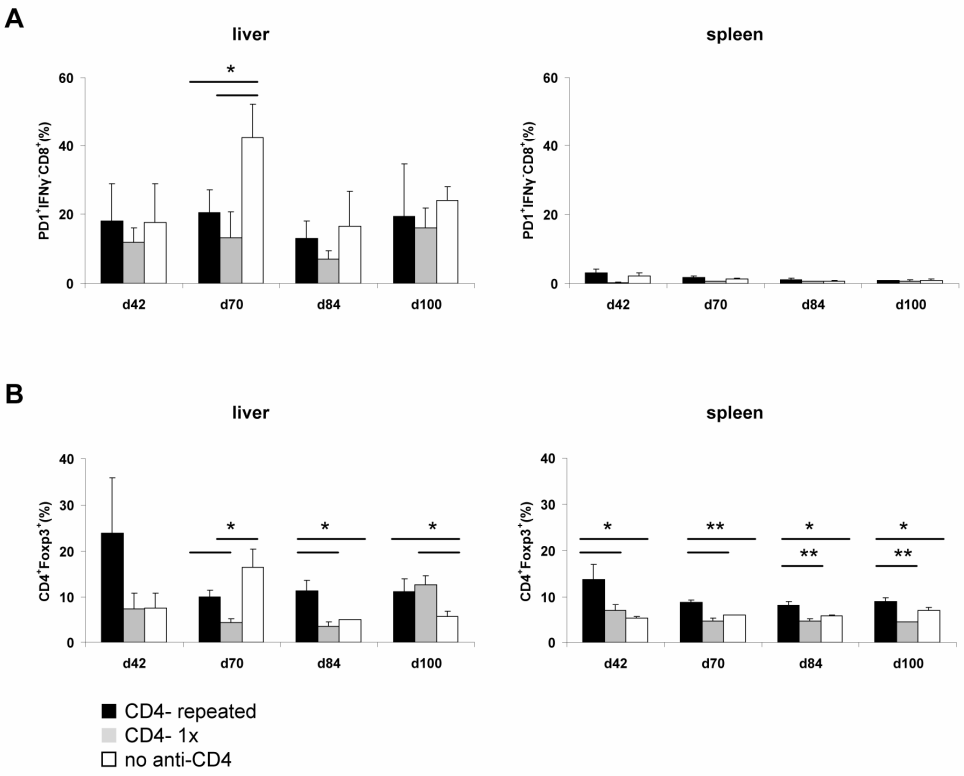


Fig. 32. T cell exhaustion and Treg-frequencies in CD4-depleted, AdHBV infected mice. Female 8 week old C57BL/6 mice were AdHBV-infected and CD4-depleted as described earlier. **(A)** At indicated time points, LALs and splenocytes were isolated and stimulated with HBc derived peptide pools. After 5h in the presence of BFA, LALs were stained with antibodies for CD3, CD8, IFNγ and PD1. Intrahepatic **(left panel)** and splenic **(right panel)** CD8+ T cells were gated for PD1+IFNγ- cells (exhausted T cells). **(B)** At indicated time points, LALs and splenocytes were isolated and stained with antibodies to CD3, CD4 and Foxp3 without prior stimulation. Frequencies of $CD4^+Foxp3^+$ Tregs in liver **(left panel)** and spleen **(right panel)** are shown. * $P < 0.05$; ** $P < 0.01$.

the spleen, mice receiving continuous CD4-depletion exhibited increased Treg-frequencies compared to non-depleted control mice, but not mice receiving one single CD4-depletion (Fig. 32B, right panel). This result implies that CD4-depletion in a setting of HBV-infection leads to enhanced migration of Tregs into the infected liver late after infection. It might well be that Tregs are more resistant to depletion by anti-CD4 antibodies than other $CD4^+$ cell subpopulations, so that repeated application of the depletion antibody positively selects for Tregs. In any case, the enhanced occurrence of intrahepatic Tregs in CD4-depleted mice might contribute to the observed $CD8^+$ T cell inactivity. Summarized, these data confirm that $CD4^+$ T cell help is required in the early phase of infection to mount a long-lasting HBV-specific $CD8^+$ T cell cytokine response.

Viral clearance is less efficient upon both continuous and initial CD4-depletion. To clarify if continuous and/or initial CD4-depletion and the resulting $CD8^+$ T cell inactivity cause increased replication of HBV in hepatocytes of AdHBV-infected mice during the late

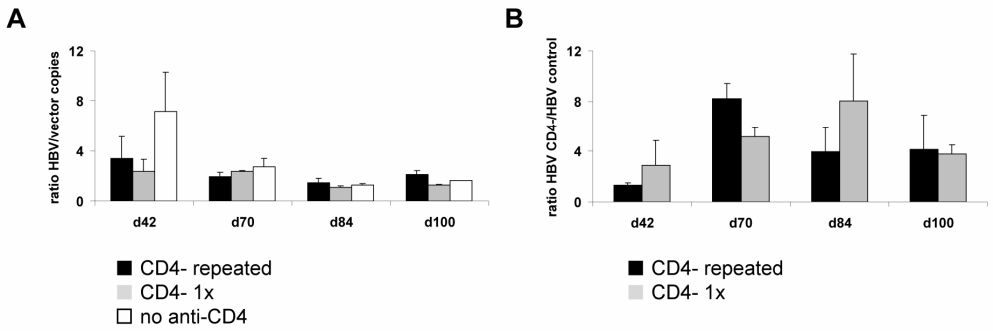


Fig. 33. HBV replication in hepatocytes of CD4-depleted, AdHBV infected mice. Female 8 week old C57BL/6 mice were AdHBV-infected and CD4-depleted as described earlier. Whole-liver DNA was extracted at the indicated time points and HBV copies and adenoviral vector copies were quantified using qPCR. **(A)** HBV replication shown as ratio of HBV copies to adenoviral vector copies. **(B)** The ratio of HBV copies in continuously CD4-depleted mice **(black bars)** and initially CD4-depleted mice **(grey bars)** to HBV copies in non-depleted mice was calculated to show differences in intrahepatic HBV titers. * $P < 0.05$.

phase of infection, whole-liver DNA was extracted from liver tissue of AdHBV-infected mice with or without CD4-depletion on d42, d70, d84 and d100. Intrahepatic titers of HBV and the adenoviral vector were quantified using qPCR. The ratio of HBV copies to adenoviral vector copies was used as a measure of HBV replication. HBV still replicated in all tested mice during the late phase of infection, but with decreasing efficiency (Fig. 33A). From d70 onwards, only weak HBV replication was detected (ratio HBV/adenoviral vector < 3 in all groups), with no significant differences between control mice and CD4-depleted mice, irrespective of the applied depletion scheme. This indicates that the immune system is able to suppress HBV replication despite $CD8^+$ T cell inactivity resulting from CD4-depletion, suggesting an alternative, $CD8^+$ T cell independent mechanism of virus control in the late phase of infection.

Suppression of viral replication does not necessarily coincide with lysis of infected hepatocytes and clearance of the virus. Therefore, CD4-depletion might mitigate viral clearance, in spite of efficient suppression of HBV replication. Comparison of intrahepatic HBV titers in CD4-depleted and non-depleted mice revealed that at all examined time points during the late phase of infection, CD4-depleted mice had a significantly higher intrahepatic viral load that non-depleted control mice (Fig. 33B). In the very late phase of infection (d100), both CD4-depleted groups exhibited a 4fold higher intrahepatic HBV load than non-depleted control mice. Collectively, these results provide evidence that CD4-depletion results in reduced HBV clearance, probably due to abolition of liver inflammation and HBV-specific $CD8^+$ T cell response, but does not influence suppression of HBV replication in the late phase of infection.

4 Discussion

4.1. The role of $CD4^+Foxp3^+$ regulatory T cells for the course of HBV infection

Despite the medical importance of HBV, not much is known about the interaction of the virus and the immune system of its host. Especially the factors regulating the antiviral immune response and thereby deciding the outcome of the infection remain obscure. Since the liver is an environment favouring tolerance (Thomson & Knolle 2010) and because of the significant number of cases in which ineffective immune responses result in chronic HBV infection, it has been theorized that regulatory $CD4^+Foxp^+$ T cells might play an important role in regulating the antiviral responses of the immune system in the liver (Billerbeck et al. 2007).

But since studies investigating the role of peripheral Tregs in the setting of HBV infection failed to come up with decisive results and due to the lack of patient liver samples and appropriate small animal models, no conclusive results revealing the interplay between intrahepatic Tregs, HBV and antiviral immune response were gained yet. This study – making use of the novel combination of two well-established model systems – documents for the first time in detail the regulatory effects Tregs impose on the intrahepatic T cell response, native immunity and B cell response in acute HBV infection.

In line with previous studies (Xu et al. 2006; Yang et al. 2007), increased Treg-frequencies in the liver during HBV-infection were found here, and a maximum of Treg-mediated immune suppression in the first week of infection. Tregs diminish liver damage by limiting the influx of immune cells into the liver, especially that of TNFα-secreting $CD8^+$ T cells, which possibly mediate hepatocyte apoptosis. Several other studies have reported that in case of HBV-infection, CTLs exert their antiviral activity through noncytolytic mechanisms, e.g. production of cytokines (Guidotti et al. 1996; Guidotti & Chisari 2001; McClary et al. 2000). The fact that a large amount of antigen-unspecific, TNFα-secreting, $CD8^+$ T cells infiltrated the liver on d7, correlating with liver damage, which by far outnumbered the amount of intrahepatic HBV- specific $CD8^+$ T cells, goes well in line with data from other groups (Sitia et al. 2004; Maini et al. 2000; Kakimi et al. 2001; Bertoletti & Maini 2000), who provided evidence that liver damage during acute liver inflammation is mediated by antigen-unspecific T cells and mononuclear cells attracted to the infected liver tissue by a small population of virus-specific T cells. Additionally, it is known that virally infected hepatocytes are resistant to perforin/granzyme mediated killing (Dienes & Drebber 2010; Kafrouni et al. 2001), supporting the hypothesis that TNFα mediates apoptosis of HBV-infected hepatocytes. Besides limiting antigen-unspecific TNFα-secretion, Tregs also reduced IFNγ-production by

HBV-specific effector T cells. This study shows that the Treg-suppressed IFNγ-secretion was mainly HBc-specific. It was already demonstrated that apoptosis of HBV-infected hepatocytes releases large amounts of viral capsids (Arzberger et al. 2010), thus explaining the upcoming of a strong, HBc-specific T cell response in the liver, which is in turn exposed to Treg-mediated immune suppression. This Treg-mediated immune suppression in the liver resulted in increased viremia and a delayed clearance of the virus. It is known that hepatocytes are extremely sensitive to cytokine-mediated control of viral replication (Guidotti & Chisari 2001), pointing out the functional importance of IFNγ-suppression by Tregs. The depletion of Tregs lead to markedly reduced HBV replication, as shown by intrahepatic HBV titers and immunohistochemical staining of cytoplasmatic HBc. The limitation of effector T cell functionality by Tregs observed in this study might play an important role for favouring HBV replication, since polyfunctional T cell responses are described as being efficient in suppressing viruses, while monofunctional T cell responses often fail to control viral replication (Seder et al. 2008). The functional importance of polyfunctional T cells is highlighted by data showing a correlation between polyfunctional $CD8^+$ and $CD4^+$ T cells with long-term nonprogression of HIV infection and increased proliferation, cytotoxicity and cytokine production of polyfunctional T cells (Seder et al. 2008). Long-term protection on the other hand was not influenced by Tregs, since no suppressive effects on the development of an HBV-specific memory T cell pool were detected. The HBsAg/anti-HBs seroconversion was delayed by Tregs, but happened nonetheless. Anti-HBc and anti-Ad were detected irrespective if Tregs were depleted or not.

Strikingly, Tregs showed antigen-unspecific CD137 surface expression, contradicting results of *Koay et al*. and *Kondo et al*., who claimed to have detected HBc-specific Tregs in chronic HBV-carriers (Koay et al. 2011; Kondo et al. 2006). Since the mechanism of Treg-activation is certainly highly important for an improved understanding of immune regulation, systematic studies are needed to resolve the ongoing debate about Treg-specificity. CD137-expression of Tregs correlated with liver damage, strongly indicating that Tregs are activated during the peak of liver inflammation to limit tissue damage and prevent the host from suffering dangerous immune-mediated hepatocyte destruction. While the HBV-specific $CD8^+$ T cell population developed slowly during the course of infection, Tregs were maximally activated early after infection (d7), strengthening the interpretation that Tregs are activated by general inflammatory stimuli (e.g. cytokines, TLRs), not by antigen-specific T cell receptor binding of HBV-epitopes. Nevertheless, this does not exclude the development of HBV-specific Treg-populations, but it relativizes the importance of antigen-specificity for Treg-mediated

immunosuppression. Summarized, Tregs might be a link between innate and adaptive immunity, combining antigen-specific and unspecific mechanisms of immune regulation.

For the first time, this study presents data on the influence of Tregs on innate immune cells in the HBV-infected liver. A diverse pattern of Treg-influence was found: On the one hand, cytokine responses of macrophages and DCs were not altered, but their recruitment to the infected liver tissue was delayed. While it was shown here that Tregs can reduce the antigen-presenting ability of intrahepatic DCs by reducing MHCII surface expression, this effect does not seem to play a significant role in HBV-infected mice. On the other hand, Tregs seemed to enhance NK cell recruitment, while alleviating their antiviral activity. Additionally, intrahepatic macrophages changed their phenotype during the course of infection, from immunosuppressive M2-macrophages that might assist in limiting tissue damage by secretion of IL10, to pro-inflammatory, TNFα-secreting and antigen-presenting M1-macrophages (Verreck et al. 2006; Gordon 2003). The latter might help to sustain T cell responses through production of pro-inflammatory cytokines and presentation of HBV-antigens to effector T cells, and to finally clear the virus by inducing TNFα-mediated apoptosis in infected hepatocytes. Interestingly, Tregs delayed the phenotypical shift. This implies an additional, indirect mechanism by which Tregs can mitigate antiviral immune responses: Keeping immune cells with different functional phenotypes in a more anti-inflammatory state. By influencing intrahepatic macrophages in that manner, Tregs can on the one hand reduce pro-inflammatory cytokine levels and activation of effector T cells in the liver, and on the other hand increase anti-inflammatory cytokine production, thereby promoting tissue protection and regeneration. Obviously, this is a very efficient way of immune regulation, since it shifts immune homeostasis towards tissue protection in several ways, just by manipulation of one immunological key cell type of the liver.

It is important to notice that macrophages seem to play an important role during the clearance phase of HBV-infection, since they produced TNFα in large numbers during the late phase of infection. Since it is clearly shown here that effector T cells are merely monofunctional during this stage of the disease, it might well be that clearance of HBV is not only achieved by the activity of CTLs, but that macrophages play a prominent role in this process as well.

Mouse studies suggested that Treg-depletion can improve the protective effect of DNA-vaccination against certain viruses, including HBV (Toka et al. 2004; Furuichi et al. 2005), and the results presented here verify that Treg-depletion accelerates viral clearance. This might lead to the conclusion that depletion of regulatory T cells could serve as a valuable tool for immunotherapy, but several hurdles remain. First of all, there is no specific marker

exclusively expressed on human Tregs, which could be used for depletion. CD25, which has been used extensively for depletion studies, is now known to be expressed also on conventional activated effector T cells, and Foxp3 as well is not expressed in all subtypes of human regulatory T cells. Furthermore, due to its nature as a transcription factor, it is not accessible for depletion antibodies. Secondly, depletion of Tregs might cause extensive side effects in patients, especially increased immune-mediated tissue damage or autoimmune reactions. Thirdly, it is questionable if Tregs can indeed enhance the protective effect of vaccination, since no influence of Tregs on central memory T cells was detected here.

Taken together, this study proves that Tregs suppress HBV-specific immune responses as well as certain aspects of the innate immune response in the early phase of acute HBV infection. It was confirmed that Tregs are recruited to the liver when activated effector T cells infiltrate the infected tissue (Bochtler et al. 2008). They mitigate antiviral effector activity, cytokine production and cytotoxicity, without affecting long-term immune memory. Thereby they reduce immune-mediated tissue damage, but at the cost of delaying HBV-clearance. The question was raised weather Tregs in HBV-infection play a positive role by protecting the host from excessive liver damage or a negative role by suppressing the antiviral immune response (Alatrakchi & Koziel 2009). According to the data presented here, there is no clear answer to this question. It is evident that Tregs mediate a subtle balance between effective immune response and tissue protection. This complex and seemingly paradox function with beneficial and detrimental aspects seems to be a key role of Tregs, since it was already suggested by other viral infection models (Suvas et al. 2003; Suvas et al. 2004). Thus, the results of this study substantiate that intrahepatic Tregs have a crucial influence on the outcome of HBV infection, strengthening the idea that chronic HBV infection might result from Treg-hyperactivity or other Treg-mediated regulatory malfunction. It was speculated that some pathogens may exploit Treg-activity to facilitate their spread or persistence (Belkaid & Rouse 2005; Mills 2004), and at least it is demonstrated here that HBV profits from Treg-mediated immune suppression. Further experiments are needed to investigate if the virus can shift immune-homeostasis towards tolerance by directly manipulating Treg-activity.

4.2. The role of Treg-derived interleukin-10 in acute liver inflammation

Interleukin-10 is an anti-inflammatory cytokine that is supposed to have an important function in regulating inflammatory events and might be one of the major mechanisms by which Tregs exert their suppressive function (Saraiva & O'Garra 2010). Therefore, it was one of the key questions in this study if liver-resident Tregs secrete IL10 during HBV infection and what

effect intrahepatic IL10-secretion has on liver inflammation and infection. It was shown here that HBV-infected, but Treg-depleted mice have reduced IL10 serum-levels during the early infection phase compared with non-depleted, HBV-infected mice. Unfortunately, this difference was not significant. But qPCR revealed that the marked increase of intrahepatic IL10-transcripts is significantly lower (nearly 5-fold) if Tregs are depleted. From d14 onwards, no significant increases of IL10-transcripts were detectable, indicating that IL10 secretion during HBV infection is limited to acute liver inflammation. The mitigated increase of IL10-transcripts after Treg-depletion could have been interpreted as evidence for a strong IL10-secretion by intrahepatic Tregs during acute liver inflammation, however, qPCR does not allow to speculate on the origins of the detected transcripts. Therefore, IL10 reporter mice were used in which IL10-secreting cells can directly be measured due to a colour reaction. Strikingly, it was proven here that Tregs are not the main producers of IL10. Less than 1000 cells per liver were IL10-secreting Tregs. In contrast, the major amount of IL10 was produced by F4/80$^+$ intrahepatic macrophages (more than 35.000 cells per liver) and CD8$^+$ T cells (20.000 cells per liver). *Hyodo et al* already demonstrated that peripheral blood monocytes produce IL10 in HBV-infected individuals (Hyodo et al. 2004). The number of IL10-secreting CD4$^+$ effector T cells was insignificant (less than 500 cells per liver). It might seem contradictory that CD8$^+$ T cells infiltrating the liver and correlating directly with liver damage were shown to be one of the main IL10-secreting cell populations as well. But it was shown in HIV-infected patients that effector CD8$^+$ T cells can gain the ability to produce IL10, thereby suppressing pro-inflammatory responses (Elrefaei et al. 2007), and that strong pro-inflammatory stimuli (e.g. T cell receptor triggering by high amounts of antigen) can induce IL10-production in a broad variety of immune effector cell (Gabryšová et al. 2009; Blackburn & Wherry 2007). This represents a negative-feedback control of immune responses, preventing excessive immunopathology.

In this study, all IL10-secreting cell populations produced nearly the same amount of IL10 per cell. The fact that the majority of IL10 in the acutely inflamed liver is not produced by Tregs, but that Treg-depletion leads to a 5-fold reduction of intrahepatic IL10-expression, implies a strong, regulative function for the small amount of Treg-derived IL10. Upon a strong inflammatory stimulus as in acute HBV infection, intrahepatic Tregs might produce a small amount IL10, thereby inducing IL10-secretion in liver-residing macrophages and infiltrating CD8$^+$ T cells. In consequence, liver damage can be alleviated and the infected host is protected from excessive immune-mediated tissue destruction. This hypothesis was proven here by the use of IL10$^{flx/flx}$CD4Cre$^+$ mice, in which CD4-positive cells (including CD4$^+$

Tregs) lack the ability to produce IL10. Upon AdHBV-infection, these mice developed a strong liver inflammation and liver damage significantly exceeding that of infected wild-type mice. ALT-levels reached a concentration comparable to Treg-depleted, AdHBV-infected DEREG mice. Furthermore, intrahepatic IL10-transcripts were also markedly lower than in wild-type mice, and even lower as in Treg-depleted mice. Considering that the number of intrahepatic, IL10-secreting $CD4^+$ effector T cells is very low, even much lower than the number of IL10-secreting Tregs, it can be concluded that this massive effect was caused mainly by the lack of Treg-derived IL10. This strengthens the hypothesis that IL10 derived from intrahepatic Tregs induces IL10-secretion in macrophages and infiltrating $CD8^+$ T cells. In the absence of Treg-derived IL10, macrophages and $CD8^+$ T cells possibly produce significantly less IL10, leading to stronger liver inflammation. Strikingly, the lack of IL10 did not result in increased intrahepatic $CD8^+$ T cell frequency; in contrast, $IL10^{flx/flx}CD4Cre^+$ mice showed even a lower frequency of intrahepatic $CD8^+$ T cells than wild-type mice, while Treg-depleted mice had, as expected, markedly more $CD8^+$ T cells infiltrating the liver. This obviously means that the increased liver damage observed in mice lacking Treg-mediated IL10-secretion is not a result of more T cells infiltrating the liver. It might well be that a lack of IL10 increases the cytotoxicity of T cells and NK cells, and that TNFα is more efficient in inducing hepatocyte-apoptosis in a IL10-negative environment. More experiments are needed to deeply investigate these interactions.

In addition to the examination of IL10 origin and its effects on liver inflammation, it was of interest here which viral antigen induces IL10-secretion in the main cell population secreting IL10 in the liver, macrophages. Here, macrophages exhibited pronounced secretion of IL10 upon stimulation with HBc. Stimulation with HBs also induced IL10 secretion in macrophages, but on a significantly lower level. The ability to induce IL10 correlates with the ability to induce pro-inflammatory cytokines in T cells, since strong HBc-specific, but no significant HBs-specific $CD8^+$ T cell IFNγ-responses were detected in this study. Summarized, this implies that in a natural infection-setting, HBc is the major immunogenic protein inducing strong adaptive and innate immune responses, while HBsAg seems to be a rather low-immunogenic protein. HBsAg may therefore be considered to be a protective "shield" hiding the immunogenic HBV capsid from the immune system to avoid strong immune activation. It was already shown by other studies that HBsAg-specific $CD8^+$ T cells do not react to fluctuations in the level of HBV-DNA in patients, implying tolerance (Bertoletti & Gehring 2006). Taken together, HBsAg, which is secreted in large excess over whole virions as subviral particles (Seeger & Mason 2000), might serve three functions: It

promotes functional exhaustion and deletion of virus-specific T cells (Wherry et al. 2003; Zhou et al. 2004), it serves as "decoy antigen" and redirects cellular and humoral immune responses to empty particles, and it hides highly immunogenic parts of the virion from recognition by the immune system.

4.3. The role of $CD4^+$ T cells for the course of HBV-infection: Towards a new model of chronic hepatitis B virus infection.

It is known that $CD4^+$ T cells are important effector cells for the immunological defense against viruses (Whitmire 2011). It has been described by many studies that $CD4^+$ T cells are important for the development of effective T and B cell responses (Whitmire 2011), that they influence the proliferation and activation of $CD8^+$ T cells (Williams & Bevan 2007), induce inflammation and attract antiviral immune cells to the site of infection (Lane et al. 2000). Additionally, they produce a wide variety of cytokines themselves and can act as cytotoxic cells (Jellison et al. 2005; Yates et al. 2007). Their importance is underlined by the complex network of $CD4^+$ T cell subpopulations, that fulfill many different functions. Since it was hypothesized that $CD4^+$ T cells must also have significant influence on the outcome and course of HBV-infection, many research groups have attempted to define the role $CD4^+$ T cells play in this infectious setting. It was indeed shown that $CD4^+$ T cells have an important effector function in the immune response against HBV (Yang et al. 2010; Asabe et al. 2009), but since the data were gained in a variety of models (chimpanzees, woodchuck, human patient PBMC, knock-out mice) and more or less artificial settings (natural acute or chronic infection, infection through high-pressure plasmid-injection, HBV-transgenic mice), the results are difficult to combine. This study aimed at describing the role of $CD4^+$ T cells for the course of HBV-infection in detail and to analyze which parts of the antiviral immune response are influenced by them. Therefore, AdHBV-infected mice were CD4-depleted using different protocols and virological as well as immunological parameters were determined in comparison to non-depleted, AdHBV-infected mice.

As expected, CD4-depletion was highly efficient and resulted in a rapid, systemic, but transient loss of the complete $CD4^+$ T cell population, with CD4-counts recovering between d7 and d70 after depletion. Thus, an infectious setting could be established with a lack of $CD4^+$ T cell help at the onset of infection, but with a complete set of immune cells in the late phase. Surprisingly, even though $CD4^+$ T cells repopulated the liver, HBsAg persisted until at least d100 on a reduced, but stable level in mice receiving CD4-depletion, and no antibody response, neither against HBV proteins nor against the adenoviral vector, was mounted. This

was expected since it is a known fact that $CD4^+$ T cells activate B cells, promoting B cell differentiation into plasma cells (Whitmire 2011). In contrast to HBsAg, HBeAg secretion was suppressed irrespective if $CD4^+$ T cells were present at the onset of infection or not. It was shown here that the persisting HBsAg did not consist solely of non-infectious, subviral particles, but that virions containing HBV-genomes persisted as well. HBV-replication, even though slightly enhanced between d14 and d70 upon initial CD4-depletion, was suppressed in the late phase of infection with or without initial $CD4^+$ T cell help, corresponding to the reduction of HBeAg secretion. However, clearance of HBV and the adenoviral vector was markedly less efficient if $CD4^+$ T cells were depleted. This suggests that $CD4^+$ T cell help is needed to promote clearance of infected hepatocytes, but that HBV replication and HBeAg secretion are suppressed by a CD4-independent mechanism in the late phase of infection. Studies using CD4-knock/out mice transduced with an HBV-plasmid or CD4-depleted, HBV-infected chimpanzees also found intrahepatic persistence of the virus (Yang et al. 2010; Asabe et al. 2009). Without initial $CD4^+$ T cell help, only mild liver damage was detected, and only very few $CD8^+$ T cells and NK cells infiltrated the liver tissue, providing evidence that $CD4^+$ T cells are responsible for the induction of liver inflammation and the characteristic infiltration of HBV-infected liver tissue by $CD8^+$ T cells and monocytes. This goes well in line with other studies reporting that $CD4^+$ T cells mediate the homing of effector cells (e.g. $CD8^+$ T cells, monocytes) to peripheral sites of viral infection (Lane et al. 2000; Whitmire 2011). A second cause contributing to the alleviated liver damage might be the lack of cytotoxic $CD4^+$ T cells, since it was shown by several groups that some $CD4^+$ T cells possess cytolytic capacity, even though it is weak compared to that of $CD8^+$ T cells (Jellison et al. 2005; Yates et al. 2007). But since it was demonstrated that $CD4^+$ T cells, in contrast to $CD8^+$ T cells, primarily utilize Fas-L/Fas interaction and not perforin/granzyme to induce apoptosis in infected cells (Whitmire 2011), they might play an important role in the liver, since HBV-infected hepatocytes are resistant to perforin/granzyme (Dienes & Drebber 2010; Kafrouni et al. 2001).

$CD8^+$ T cells remained ignorant towards HBV upon initial CD4-depletion, even in the very late phase of infection, demonstrating that $CD4^+$ T cell help is necessary early after infection for the formation of a HBV-specific, $CD8^+$ T cell mediated IFNγ-response. No HBc-specific IFNγ-response was found during the whole course of infection. In CD4-knock/out mice transduced with an HBV-replicating plasmid, no HBc-specific $CD8^+$ T cells were found either, supporting this observation (Yang et al. 2010). However, the role of $CD4^+$ T cells in acute viral infection is controversial (Guidotti et al. 1996), since several groups claim that

$CD4^+$ T cells are only necessary for generation of an antiviral $CD8^+$ memory pool, not for antiviral effector T cell responses (Weiser et al. 1983; Abougergi et al. 2009). But the results presented here are supported by data demonstrating that $CD4^+$ T cells are strictly required in the early phase of HBV-infection for mounting of an effective antiviral intrahepatic $CD8^+$ T cell response(Asabe et al. 2009), and that initial $CD4^+$ T cell responses are indispensable for clearance of acute HCV infection (Young et al. 1989).

Additionally, it was shown here that the serum-levels of other cytokines and chemokines important for antiviral immune responses (TNFα, IL21, IL10, MIP-1α, MIP-1β) were also markedly reduced in CD4-depleted, HBV-infected mice compared to non-depleted controls. This lack of cytokine and chemokine production can explain many of the aspects stated above: MIP-1α and MIP-1β are chemotactic and proinflammatory chemokines (Maurer & von Stebut 2004), and their reduction might lead to reduced liver infiltration by T cells and monocytes, thereby also reducing the intrahepatic TNFα secretion and liver inflammation. Less TNFα directly results in reduced liver damage, since TNFα is an important factor driving HBV-infected hepatocytes into apoptosis. IL21 is known to be required for sustaining antiviral $CD8^+$ T cell responses during long-lasting infections (Elsaesser & Sauer 2009), thus lacking or reduced secretion of IL21 might contribute to the inactivity of $CD8^+$ T cells observed in CD4-depleted mice. The very mild liver inflammation observed in these mice might in turn be the cause for the reduced IL10 secretion, since IL10 is a strictly regulated, anti-inflammatory cytokine, whose secretion correlates with the strength of inflammation and tissue damage. Also, it is an interesting aspect that a large proportion of intrahepatic $CD8^+$ T cells (20-40%) during the late phase of HBV-infection (d70 and later) showed PD1 surface expression accompanied by lacking cytokine secretion, representing the status of functional exhaustion (Barber et al. 2006). In the splenic $CD8^+$ T cell population, only a small fraction of cells were exhausted (< 5%). This implies that the pronounced secretion of viral antigens by HBV-infected hepatocytes leads to exhaustion of HBV-specific effector T cells that infiltrated the liver or that exhausted T cells in general accumulate in the liver to become eliminated. This hypothesis is supported by results connecting functional decline and changed tissue distribution of antiviral T cells with persistence of viral antigens (Wherry et al. 2003; Zhou et al. 2004). However, mice receiving initial or repeated CD4-depletion showed a tendency – even though mostly not significant – to be exhausted less often. The interpretation here is that $CD8^+$ T cells in CD4-depleted mice do not only lack stimuli sustaining their antiviral functionality but that they need $CD4^+$ T cell help to become activated and primed in the first place. As a result, less $CD8^+$ T cells are responding to HBV and therefore, less $CD8^+$ T cells

undergo functional exhaustion, even though HBsAg and the virus itself persist longer and in higher amounts after CD4-depletion.

It could be hypothesized that the observed effects do not or not only originate from the lack of $CD4^+$ T cells, but instead that the antibody used for depletion binds to immunosuppressive F_c-receptors, thereby inducing strong anti-inflammatory signals that suppress HBV-specific immune responses. But this was excluded here by application of an irrelevant isotype control antibody targeting human CD154, whose application induced not the downregulation of the anti-HBV responses observed upon CD4-depletion, but in contrast lead to very early liver damage, markedly reduced HBsAg secretion followed by HBsAg/anti-HBs seroconversion and a normal course of HBV clearance. On d3, NK cells accumulated in the liver after application of anti-hCD154, while on d7, T cells infiltrated the liver tissue with the same magnitude as in non-treated mice. Collectively, this suggests a systemic activation of the innate immune system caused by application of rat IgG_2b antibodies.

Strikingly, application of the depletion antibody lead to rapid secretion of high amounts of proinflammatory cytokines and chemokines, some of them (IL21, MIP-1α) exceeding the maximal detection limit of the cytometric bead array assay. Since this pronounced cytokine response was detected in the serum on d4 post depletion (d3 post infection), but was faded already on d8 p.d. (d7 p.i.), it could not originate from the adaptive immune system, confirming a strong, systemic activation of the innate immune system. Part of the viral inoculum was eliminated by this innate response, manifested by reduced AdHBV- and HBV-titers and lower peripheral concentrations of HBeAg and HBsAg.

To circumvent the undesired, unspecific innate immune activation by the depletion antibody, two different application protocols for the CD4-depletion antibody were tested in this study. Firstly, intravenous injection of 250µg anti-CD4 on d-1 and repeated on d10, 20, 30, 40, 50 and 60 post infection. The aim behind this application strategy was to minimize innate immune activation by application of a lower dose and circumvention of proinflammatory intraperitoneal macrophages using the intravenous route, and to examine if a continuous state of CD4-depletion until the late phase of infection increases HBV-replication and –persistence compared to one initial, transient CD4-depletion.

Secondly, the one-time intitial CD4-depletion protocol was modified by applying 500µg anti-CD4 one week instead of one day prior infection, thereby letting the innate immune response fade before injection of the virus. Fig. 22B shows that CD4-counts are still at the detection limit one week after antibody application.

Comparing both depletion strategies with non-depleted mice, it was shown here that there were no significant differences in the course of infection between the two groups of differently CD4-depleted mice. Both groups showed a very mild liver damage compared to untreated mice, cleared HBeAg while HBsAg persisted until d100. One minor difference was a suppressed secretion of HBsAg in repeatedly CD4-depleted mice on d7 and d14, indicating some immunological activity suppressing HBsAg expression (cytokines) or removing it from the circulation (phagocytosis by macrophages), until tolerance for the depletion antibody was achieved. Since HBeAg secretion was not affected and HBsAg secretion was restored on d21, this difference can be considered to be marginal.

Both CD4-depletion protocols lead to abolition of antiviral B cell responses, no anti-HBc, anti-HBs or anti-Ad could be detected in those mice. It was noticeable, however, that the HBs antibody response was comparably late and weak in the untreated control group, probably due to the low HBsAg secretion of the AdHBV stock used in this assay. Nevertheless, the difference in anti-HBs production between CD4-depleted and untreated mice was obvious. It should be noted that the lack of antibody responses made the repeated application of the depletion antibody possible, otherwise, a B cell response against anti-CD4 would certainly have developed. Also, the HBV-specific IFNγ response of intrahepatic $CD8^+$ T cells was missing in CD4-depleted mice, irrespective of the CD4-depletion protocol used. Finally, HBV replication was suppressed in both CD4-depleted groups as well as in untreated mice, but HBV titers in liver tissue were markedly higher compared to non-depleted mice.

The only pronounced difference between the two differentially CD4-depleted groups of mice was the increased Treg-frequencies in the spleen and in the liver of repeatedly CD4-depleted mice compared to untreated mice. Initially CD4-depleted mice showed increased Treg frequencies only in the liver and only on d100, and this increase was accompanied by an unspecific IFNγ production by intrahepatic $CD8^+$ T cells and might therefore be a consequence of an undesired infection or another inflammatory event. Thus, it can be concluded that only repeated CD4-depletion leads to increased Treg-frequencies in liver and spleen, but without changing the course of infection in comparison to only initially depleted mice. It might well be that $CD4^+Foxp3^+$ Tregs are more resistant to anti-CD4 application and are in consequence selected by repeated application of that depletion antibody. This might either be a result of less surface expression of the CD4-protein compared to $CD4^+$ effector T cells, faster proliferation of Tregs caused by their high expression of the high-affinity IL2-receptor α-chain (CD25) or of the immunosuppressive capacity of Tregs, making them more robust to destruction by the immune system after binding of the depletion antibody.

Taken together, both depletion protocols have circumvented the activation of the innate immune system by the depletion antibody and have caused similar changes in the course of HBV infection. Since repeated CD4-depletion has not changed HBV replication or other infection parameters compared with one initial depletion, the latter protocol should be used in future experiments. It requires less depletion-antibody and only one injection, therefore being more cost- and time-efficient. Additionally, it is a less artificial experimental setting, since $CD4^+$ T cell counts reach normal levels again on d70, therefore providing a full repertoire of immune cells in the late phase of infection.

Summarized, this study has given insight into the regulatory role of $CD4^+$ T cells in HBV-infection. It was proven that $CD4^+$ T cells are necessary in the early phase of infection (d1-7) to induce liver inflammation, to attract $CD8^+$ T cells, additional $CD4^+$ T cells and monocytes into the infected liver, and to mount antiviral B cell and T cell responses. They massively influence pro-inflammatory cytokine and chemokine responses, either directly by producing them themselves or by stimulating other immune cells to secrete them. Additionally, it was shown here that a large proportion of intrahepatic $CD8^+$ T cells exhausts during HBV infection in the late phase of infection. Initial, but transient CD4-depletion leads to persistence of HBsAg, and viral clearance is significantly delayed. Strikingly, the adaptive and humoral immunity is not activated at later time points, even though $CD4^+$ T cells proliferate again shortly after depletion. This clearly demonstrates that $CD4^+$ T cells need to perform their pro-inflammatory, regulatory role at the onset of infection – at later stages, they are not able anymore to induce efficient immune responses. The HBsAg persistence and delayed HBV clearance together with the inactivity of the adaptive immunity and B cells suggest to use initially CD4-depleted, AdHBV-infected mice as a new model of chronic HBV infection. Upon CD4-depletion, HBV persists significantly longer than in non-depleted animals, which correlates with observations in HBV- and HCV-infected, CD4-depleted chimpanzees, where the virus also persists (Asabe et al. 2009; Grakoui et al. 2003). In natural chronic HBV infection, the adaptive immune system also fails to efficiently clear the virus, and HBV-specific T cell responses are weak. Also, other animal models of chronic HBV infection, e.g. neonatally infected woodchucks, share the characteristic immunological dysfunctions (lack of TNFα and IFNγ responses, failure to mount antiviral T cell responses) observed in CD4-depleted, AdHBV, infected mice (Menne et al. 2002). Furthermore, chronic HBV infection is usually associated with a lack of HBs/anti-HBs seroconversion. Therefore, this mouse-model meets the characteristic features of chronic HBV infection; CD4-depletion as well as AdHBV-infection are easy to perform and can be applied to any mouse strain. The model

would also allow to study new immunotherapeutic approaches to reactivate tolerant or inactive T and B cells and induce immune responses able to clear the persisting virus. The only drawback of this experimental model is the inability of HBV to infect wt mouse hepatocytes by itself. Therefore, the virus can not spread in mice, and the adenoviral vector, from which HBV is replicated, will inevitably be cleared from the liver. Even though it has proven to be not strongly immunogenic due to lack of transcription of adenoviral proteins, it will be eliminated slowly from the liver by the natural death and replacement of hepatocytes. Because of that, HBV infection will also end over time, even without efficient immune responses.

Another interesting result here is that HBeAg and HBV are cleared even without efficient T cell responses. This implies that some other factor, probably cells of the innate immune system, can compensate the T cell inactivity or even have a more prominent role than T cells in the clearance of HBV during the late phase of infection.

5 Conclusion

This study provides detailed insight into the mechanisms involved in the antiviral immune response against HBV. For the first time, the complex interplay between regulatory and effector cells of the adaptive and innate immunity was examined in the liver of an acutely HBV-infected organism. Taken together, the data collected here results in the following model: Shortly after HBV infection, innate immune cells (macrophages, NK cells, DCs) and $CD4^+$ effector T cells are activated in the liver. Macrophages and NK cells secrete TNFα, DCs IFNγ, thus providing first pro-inflammatory stimuli. At the same time, $CD4^+$ effector T cells start to produce additional pro-inflammatory cytokines and chemokines to attract and stimulate $CD8^+$ T cells and macrophages from the periphery. These infiltrating T cells and macrophages provide large amounts of TNFα, inducing apoptosis in HBV-infected hepatocytes. Together, the broad bandwith of cytokines establish an inflammatory milieu, which suppresses HBV replication, kills infected hepatocytes, releases immunogenic HBV core protein and leads to the induction of a long-term, HBV-specific immune response mediated by $CD8^+$ T cells. Furthermore, B cells are induced to differentiate into memory B cells and plasma cells, secreting neutralizing HBV-antibodies.

But at the same time, regulatory T cells react to the established liver inflammation by secreting small amounts of IL10, which in turn induce IL10-production in infiltrating $CD8^+$ T cells and macrophages. Also, Tregs reduce proliferation and influx of T cells and monocytes/macrophages into the liver and mitigate T cell mediated TNFα-secretion. As a result, the liver inflammation and the accompanying immune mediated tissue damage are limited. Following acute liver inflammation, Tregs suppress antiviral T cell and B cell responses, while $CD4^+$ T cells possibly secrete cytokines sustaining $CD8^+$ T cell mediated, antiviral responses. Furthermore, macrophages undergo a phenotypical shift from anti-inflammatory M2 macrophages to M1 macrophages, which secrete antiviral cytokines and upregulate MHCII-expression to support antiviral T cell activity. Tregs also delay this phenotypical shift, while they do not influence the development of HBV-specific memory T cells. During the course of infection, the continuous secretion of HBV antigens possibly leads to functional exhaustion of intrahepatic $CD8^+$ T cells, which secrete only IFNγ in the late stage of HBV infection. The limited functionality of antiviral effector T cells, which is also enforced by Tregs, relativizes the importance of $CD8^+$ T cells for the clearance of HBV. Since macrophages are mostly of M1 phenotype in the late stage of infection and produce TNFα, this study implies an important role for these cells in the final clearance of HBV. Future

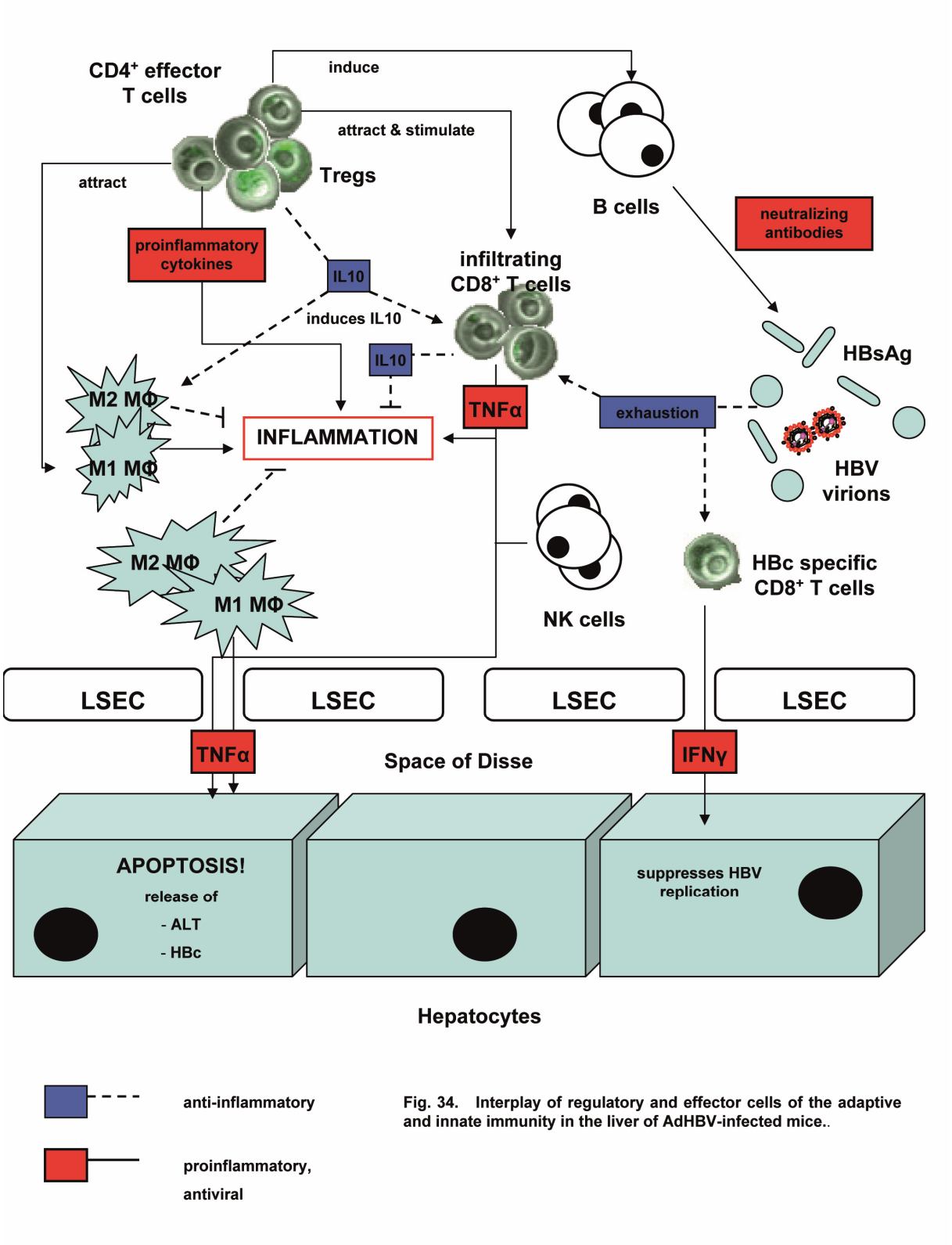


Fig. 34. Interplay of regulatory and effector cells of the adaptive and innate immunity in the liver of AdHBV-infected mice..

studies should thoroughly examine the role of intrahepatic macrophages for the course of HBV infection. Fig. 34 schematically depicts the collected results.

The increased understanding of intrahepatic immunological mechanisms involved in HBV clearance will support the development of new therapeutic approaches. Manipulation of Tregs, $CD4^+$ effector T cells and macrophages might provide additional instruments for anti-

HBV therapy. Also, dissecting the regulatory network of intrahepatic immune responses will help to discover the factors leading to HBV chronicity. Finally, CD4-depleted, AdHBV-infected mice are a novel small-animal model for persistent HBV-infection, providing a helpful tool for HBV-research.

6 References

Abougergi, M.S. et al., 2009. Fas and TNFR1, But Not Cytolytic Granule-Dependent Mechanisms, Mediate Clearance of Murine Liver Adenoviral Infection. *Infection*, 41(1), pp.97-105.

Alatrakchi, N. & Koziel, M., 2009. Regulatory T cells and viral liver disease. *Journal of viral hepatitis*, 16(4), pp.223-9.

Arzberger, Silke, Hösel, M. & Protzer, Ulrike, 2010. Apoptosis of hepatitis B virus-infected hepatocytes prevents release of infectious virus. *Journal of virology*, 84(22), pp.11994-2001.

Asabe, S. et al., 2009. The size of the viral inoculum contributes to the outcome of hepatitis B virus infection. *Journal of virology*, 83(19), pp.9652-62.

Austyn, J.M. & Gordon, S, 1981. F4/80, a monoclonal antibody directed specifically against the mouse macrophage. *European journal of immunology*, 11(10), pp.805-15.

Bamboat, Z.M. et al., 2009. Human liver dendritic cells promote T cell hyporesponsiveness. *Journal of immunology (Baltimore, Md. : 1950)*, 182(4), pp.1901-11.

Banchereau, J. et al., 2000. Immunobiology of dendritic cells. *Annual review of immunology*, 18, pp.767-811. 75.

Banchereau, J. & Steinman, R.M., 1998. Dendritic cells and the control of immunity. *Nature*, 392(6673), pp.245-52.

Barber, D.L. et al., 2006. Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature*, 439(7077), pp.682-7.

Baumert, T.F. et al., 2005. Genetic variants of hepatitis B virus and their clinical relevance. *Minerva Gastroenterol Dietol*, 51, pp.95-108.

Belkaid, Y. & Rouse, B.T., 2005. Natural regulatory T cells in infectious disease. *Nature immunology*, 6(4), pp.353-60.

Bertoletti, A & Maini, M.K., 2000. Protection or damage: a dual role for the virus-specific cytotoxic T lymphocyte response in hepatitis B and C infection? *Current opinion in immunology*, 12, pp.403-408.

Bertoletti, Antonio & Gehring, A.J., 2006. The immune response during hepatitis B virus infection. *The Journal of general virology*, 87(Pt 6), pp.1439-49.

Billerbeck, E., Bottler, T. & Thimme, R., 2007. Regulatory T cells in viral hepatitis. *World journal of gastroenterology : WJG*, 13(36), pp.4858-64.

Blackburn, S.D. & Wherry, E.J., 2007. IL-10, T cell exhaustion and viral persistence. *Trends in microbiology*, 15(4), pp.143-6.

Bochtler, P. et al., 2008. Local accumulation and activation of regulatory Foxp3+ CD4 T(R) cells accompanies the appearance of activated CD8 T cells in the liver. *Hepatology*, 48(6), pp.1954-63.

Bock, C.T. et al., 2001. Structural Organization of the Hepatitis B Virus Minichromosome. *J Mol Biol*, 307, pp.183-196.

Brooks, D.G. et al., 2008. Interleukin-10 determines viral clearance or persistence in vivo. *Nat Med*, 12(11), pp.1301-1309.

Brown, C.Y. et al., 1996. Differential regulation of the stability of cytokine mRNAs in lipopolysaccharide-activated blood monocytes in response to interleukin-10. *The Journal of biological chemistry*, 271(33), pp.20108-12.

Brown, D.M. et al., 2010. IL-2 and Antigen Dose Differentially Regulate Perforin- and FasL-Mediated Cytolytic Activity in Antigen Specific CD4+ T cells. *Cell*, 257, pp.69-79.

Böcher, W.O. et al., 1999. Kinetics of hepatitis B surface antigen-specific immune responses in acute and chronic hepatitis B or after HBs vaccination: stimulation of the in vitro antibody response by interferon gamma. *Hepatology*, 29(1), pp.238-44.

Böcher, W.O., 2006. Immunologie und Immunprophylaxe. In: Heintges, T., Haeussinger, D., eds. *Hepatitis B. Infektion - Therapie - Prophylaxe*. Stuttgart: Georg Thieme Verlag, pp. 54-73.

Chen, Y. et al., 2009. Distinct Response of Liver Myeloid Dendritic Cells to Endotoxin is Mediated by IL-27. *J Hepatol*, 51(3), pp.510-519.

Dienes, H.-P. & Drebber, Uta, 2010. Pathology of immune-mediated liver injury. *Digestive diseases (Basel, Switzerland)*, 28(1), pp.57-62.

Ejrnaes, M. et al., 2006. Resolution of a chronic viral infection after interleukin-10 receptor blockade. *The Journal of experimental medicine*, 203(11), pp.2461-72.

Elrefaei, M. et al., 2007. HIV-specific IL-10-positive CD8+ T cells suppress cytolysis and IL-2 production by CD8+ T cells. *Journal of immunology*, 178(5), pp.3265-71.

Fontenot, J.D., Gavin, M. a & Rudensky, A.Y., 2003. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nature immunology*, 4(4), pp.330-6.

Fontenot, J.D. et al., 2005. Regulatory T cell lineage specification by the forkhead transcription factor foxp3. *Immunity*, 22(3), pp.329-41.

Furuichi, Y. et al., 2005. Depletion of CD25+CD4+T cells (Tregs) enhances the HBV-specific CD8+ T cell response primed by DNA immunization. *World journal of gastroenterology : WJG*, 11(24), pp.3772-7.

Gabryšová, L. et al., 2009. Negative feedback control of the autoimmune response through antigen-induced differentiation of IL-10 – secreting Th1 cells. *Regulation*, 206(8), pp.1755-1767.

Ganem, D. & Schneider R.J., 2001. Hepadnaviridae: The viruses and their replication. In: Knipe, D.M., Howley, P.M., eds. *Fields Virology. Volume 2*. Philadelphia: Lippincott, Williams & Wilkins, pp. 2923-2969.

Gordon, Siamon, 2003. Alternative activation of macrophages. *Nature reviews. Immunology*, 3(1), pp.23-35.

Grakoui, A. et al., 2003. HCV persistence and immune evasion in the absence of memory T cell help. *Science*, 302(5645), pp.659-62.

Grossman, W.J. et al., 2004. Human T regulatory cells can use the perforin pathway to cause autologous target cell death. *Immunity*, 21(4), pp.589-601.

Guidotti, L G & Chisari, F V, 2001. Noncytolytic control of viral infections by the innate and adaptive immune response. *Annual review of immunology*, 19, pp.65-91.

Guidotti, L G et al., 1996. Intracellular inactivation of the hepatitis B virus by cytotoxic T lymphocytes. *Immunity*, 4(1), pp.25-36.

H. Elsaesser, K. Sauer, D.G.B., 2009. IL-21 is Required to Control Chronic Viral Infection. *Science*, 324(5934), pp.1569-1572.

Haeryfar, S.M.M. et al., 2005. Regulatory T cells suppress CD8+ T cell responses induced by direct priming and cross-priming and moderate immunodominance disparities. *Journal of immunology*, 174(6), pp.3344-51.

Han, S. et al., 2010. Role of antigen persistence and dose for CD4+ T-cell exhaustion and recovery. *Proceedings of the National Academy of Sciences of the United States of America*, 107(47), pp.20453-20458.

Hegazy, A.N. et al., 2010. Interferons direct Th2 cell reprogramming to generate a stable GATA-3(+)T-bet(+) cell subset with combined Th2 and Th1 cell functions. *Immunity*, 32(1), pp.116-28.

Hegde, N.R. et al., 2002. Inhibition of HLA-DR Assembly , Transport , and Loading by Human Cytomegalovirus Glycoprotein US3 : a Novel Mechanism for Evading Major Histocompatibility Complex Class II Antigen Presentation. *Journal of virology*, 76(21), pp.10929-10941.

Heintges, T., 2006. Therapie. In: Heintges, T., Haeussinger, D., eds. *Hepatitis B. Infektion - Therapie - Prophylaxe*. Stuttgart: Georg Thieme Verlag, pp. 138-171.

Hori, S., Nomura, T. & Sakaguchi, S., 2003. Control of regulatory T cell development by the transcription factor Foxp3. *Science*, 299(5609), pp.1057-61.

Hume, D.A. et al., 1983. The Mononuclear Phagocyte System of the Mouse defined by Immunohistochemical Localization of Antigen F4/80. *The Journal of experimental medicine*, 158(November), pp.1522-1536.

Hyodo, N., Nakamura, I. & Division, M.I., 2004. Hepatitis B core antigen stimulates interleukin-10 secretion by both T cells and monocytes from peripheral blood of patients with chronic hepatitis. *Clinical and Experimental Immunology*, pp.462-466.

Jellison, E.R., Kim, S.-K. & Welsh, R.M., 2005. Cutting Edge: MHC Class II-restricted Killing In Vivo during Viral Infection. *The Journal of Immunology*, 174, pp.614-618.

Kafrouni, M.I., Brown, G.R. & Thiele, D L, 2001. Virally infected hepatocytes are resistant to perforin-dependent CTL effector mechanisms. *Journal of immunology*, 167(3), pp.1566-74.

Kakimi, K et al., 2001. Blocking chemokine responsive to gamma-2/interferon (IFN)-gamma inducible protein and monokine induced by IFN-gamma activity in vivo reduces the pathogenetic but not the antiviral potential of hepatitis B virus-specific cytotoxic T lymphocytes. *The Journal of experimental medicine*, 194(12), pp.1755-66.

Kakimi, Kazuhiro et al., 2002. Immunogenicity and Tolerogenicity of Hepatitis B Virus Structural and Nonstructural Proteins : Implications for Immunotherapy of Persistent Viral Infections. *Journal of virology*, 76(17), pp.8609-8620.

Kanai, K. et al., 2007. The vIL-10 gene of the Epstein-Barr virus (EBV) is conserved in a stable manner except for a few point mutations in various EBV isolates. *Virus genes*, 35(3), pp.563-9.

Kao, J.H. et al., 2000. Hepatitis B genotypes correlate with clinical outcomes in patients with chronic hepatitis B. *Gastroenterology*, 118, pp.554-559.

Kingham, T.P. et al., 2007. Murine liver plasmacytoid dendritic cells become potent immunostimulatory cells after Flt-3 ligand expansion. *Hepatology*, 45(2), pp.445-54.

Knolle, P. & Gerken, G., 2000. Local control of the immune response in the liver. *Immunological reviews*, 174, pp.21-34.

Koay, L.-B. et al., 2011. Hepatitis B virus (HBV) core antigen-specific regulatory T cells confer sustained remission to anti-HBV therapy in chronic Hepatitis B with acute exacerbation. *Human immunology*, pp.1-12.

Kondo, Y. et al., 2006. Mechanism of T cell hyporesponsiveness to HBcAg is associated with regulatory T cells in chronic hepatitis B. *World journal of gastroenterology : WJG*, 12(27), pp.4310-7.

Kotenko, S.V. et al., 2000. Human cytomegalovirus harbors its own unique IL-10 homolog (cmvIL-10). *Proceedings of the National Academy of Sciences of the United States of America*, 97(4), pp.1695-700.

Kärre, K. et al., 1986. Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. *Nature*, 678, pp.675- 678.

Lahl, K. et al., 2007. Selective depletion of Foxp3+ regulatory T cells induces a scurfy-like disease. *The Journal of experimental medicine*, 204(1), pp.57-63.

Lai, C.-L. et al., 2002. Entecavir is superior to lamivudine in reducing hepatitis B virus DNA in patients with chronic hepatitis B infection. *Gastroenterology*, 123(6), pp.1831-8.

Lane, T.E. et al., 2000. A central role for CD4(+) T cells and RANTES in virus-induced central nervous system inflammation and demyelination. *Journal of virology*, 74(3), pp.1415-24.

Lang, P.A. et al., 2010. Tissue macrophages suppress viral replication and prevent severe immunopathology in an interferon-I-dependent manner in mice. *Hepatology*, 52(1), pp.25-32.

Lavanchy, D., 2004. Hepatitis B virus epidemiology, disease burden, treatment, and current and emerging prevention and control measures. *Journal of viral hepatitis*, 11(2), pp.97-107.

Liaw, Y.-F. et al., 2004. Lamivudine for patients with chronic hepatitis B and advanced liver disease. *The New England journal of medicine*, 351(15), pp.1521-31.

Lin, M.T. et al., 1998. The role of IL-10 in mouse hepatitis virus-induced demyelinating encephalomyelitis. *Virology*, 245(2), pp.270-80.

Lin, S.M. et al., 1999. Long-term beneficial effect of interferon therapy in patients with chronic hepatitis B virus infection. *Hepatology*, 29(3), pp.971-5.

Livingston, B.D. et al., 1999. Altered Helper T Lymphocyte Function Associated with Chronic Hepatitis B Virus Infection and its Role in Response to Therapeutic Vaccination in Humans. *The Journal of Immunology*, 162, pp.3088-3095.

Maini, M.K. et al., 2000. The role of virus-specific CD8(+) cells in liver damage and viral control during persistent hepatitis B virus infection. *The Journal of experimental medicine*, 191(8), pp.1269-80.

Marcellin, P. et al., 2003. Adefovir dipivoxil for the treatment of hepatitis B e antigen-positive chronic hepatitis B. *The New England journal of medicine*, 348(9), pp.808-16.

Maurer, M. & von Stebut, E., 2004. Macrophage inflammatory protein-1. *International Journal of Biochemistry*, 38(10), pp.1882-6.

McClary, H. et al., 2000. Relative sensitivity of hepatitis B virus and other hepatotropic viruses to the antiviral effects of cytokines. *Journal of virology*, 74(5), pp.2255-64.

McHugh, R.S. & Shevach, E.M., 2002. Cutting edge: depletion of CD4+CD25+ regulatory T cells is necessary, but not sufficient, for induction of organ-specific autoimmune disease. *Journal of immunology*, 168(12), pp.5979-83.

Menne, S. et al., 2002. Deficiencies in the Acute-Phase Cell-Mediated Immune Response to Viral Antigens Are Associated with Development of Chronic Woodchuck Hepatitis Virus Infection following Neonatal Inoculation. *Journal of virology*, 76(4), pp.1769-1780.

Mills, K.H.G., 2004. Regulatory T cells: friend or foe in immunity to infection? *Nature reviews. Immunology*, 4(11), pp.841-55.

Miyara, M. & Sakaguchi, S., 2007. Natural regulatory T cells: mechanisms of suppression. *Trends in molecular medicine*, 13(3), pp.108-16.

Miyazoe, S. et al., 2002. Influence of interleukin-10 gene promoter polymorphisms on disease progression in patients chronically infected with hepatitis B virus. *The American journal of gastroenterology*, 97(8), pp.2086-92.

Mondelli, M.U., Varchetta, S. & Oliviero, B., 2010. Natural killer cells in viral hepatitis: facts and controversies. *European journal of clinical investigation*, 40, pp.851-863.

Murphy, K.M. & Stockinger, B., 2010. Effector T cell plasticity: flexibility in the face of changing circumstances. *Nature immunology*, 11(8), pp.674-80.

Nassal, M., 1999. Hepatitis B virus replication: novel roles for virus-host interactions. *Intervirology*, 42(2-3), pp.100-16.

Norder, H. et al., 2004. Genetic diversity of hepatitis B virus strains derived worldwide: genotypes, subgenotypes, and HBsAg subtypes. *Intervirology*, 47(6), pp.289-309.

Obar, J.J. & Lefrançois, L., 2010. Memory CD8+ T cell differentiation. *Annals of the New York Academy of Sciences*, 1183, pp.251-66.

Obst, R. et al., 2005. Antigen persistence is required throughout the expansion phase of a CD4(+) T cell response. *The Journal of experimental medicine*, 201(10), pp.1555-65.

O'Connell, P.J. et al., 2003. Type-1 polarized nature of mouse liver CD8alpha- and CD8alpha+ dendritic cells: tissue-dependent differences offset CD8alpha-related dendritic cell heterogeneity. *European journal of immunology*, 33(7), pp.2007-13.

Parish, I.A. & Kaech, S.M., 2009. Diversity in CD8(+) T cell differentiation. *Current opinion in immunology*, 21(3), pp.291-7.

Rastellini, C. et al., 2010. Granulocyte/macrophgae colony-stimulating factor-stimulated hepatic dendritic cell progenitors prolong pancreatic islet allograft survival. *Transplantation*, 60(11), pp.1366-1370.

Ravkov, E.V. & Williams, M.A., 2010. The magnitude of CD4+ T cell recall response is controlled by the duration of the secondary stimulus. *The Journal of Immunology*, 183(4), pp.1-19.

Rehermann, B. & Nascimbeni, M., 2005. Immunology of hepatitis B virus and hepatitis C virus infection. *Nature reviews. Immunology*, 5(3), pp.215-29.

Rouse, B.T., Sarangi, P.P. & Suvas, S., 2006. Regulatory T cells in virus infections. *Immunological reviews*, 212, pp.272-86.

Sakaguchi, S., 2004. Naturally arising CD4+ regulatory t cells for immunologic self-tolerance and negative control of immune responses. *Annual review of immunology*, 22, pp.531-62.

Sakaguchi, S., 2005. Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in immunological tolerance to self and non-self. *Nature immunology*, 6(4), pp.345-52.

Saraiva, M. & O'Garra, A., 2010. The regulation of IL-10 production by immune cells. *Nature reviews. Immunology*, 10(3), pp.170-81.

Seder, R.A., Darrah, P.A. & Roederer, M., 2008. T-cell quality in memory and protection: implications for vaccine design. *Nature reviews. Immunology*, 8(4), pp.247-58.

Seeger, C. & Mason, W.S., 2000. Hepatitis B virus biology. *Microbiology and molecular biology reviews : MMBR*, 64(1), pp.51-68.

Shevach, E.M., 2006. From vanilla to 28 flavors: multiple varieties of T regulatory cells. *Immunity*, 25(2), pp.195-201.

Sirma, H., Funk, A., Will, H., 2006. Molekulare Virologie von Hepatitis-B- und -D-Viren. In: Heintges, T., Haeussinger, D., eds. *Hepatitis B. Infektion - Therapie - Prophylaxe*. Stuttgart: Georg Thieme Verlag, pp. 1-22.

Sitia, G. et al., 2004. MMPs are required for recruitment of antigen-nonspecific mononuclear cells into the liver by CTLs. *Journal of Clinical Investigation*, 113(8), p.1158–1167.

Sommer, G., van Bömmel, F. & Will, H., 2000. Genotype-specific synthesis and secretion of spliced hepatitis B virus genomes in hepatoma cells. *Virology*, 271(2), pp.371-81.

Sprinzl, M.F. et al., 2001. Transfer of Hepatitis B Virus Genome by Adenovirus Vectors into Cultured Cells and Mice : Crossing the Species Barrier. *Journal of virology*, 75(11), pp.5108-5118.

Summers, J & Mason, W.S., 1982. Replication of the genome of a hepatitis B--like virus by reverse transcription of an RNA intermediate. *Cell*, 29(2), pp.403-15.

Sutmuller, R.P. et al., 2001. Synergism of cytotoxic T lymphocyte-associated antigen 4 blockade and depletion of CD25(+) regulatory T cells in antitumor therapy reveals alternative pathways for suppression of autoreactive cytotoxic T lymphocyte responses. *The Journal of experimental medicine*, 194(6), pp.823-32.

Suvas, S. et al., 2004. CD4+CD25+ regulatory T cells control the severity of viral immunoinflammatory lesions. *Journal of immunology*, 172(7), pp.4123-32.

Suvas, S. et al., 2003. CD4+CD25+ T cells regulate virus-specific primary and memory CD8+ T cell responses. *The Journal of experimental medicine*, 198(6), pp.889-901.

Taguchi, O. & Takahashi, T., 1996. Administration of anti-interleukin-2 receptor alpha antibody in vivo induces localized autoimmune disease. *European journal of immunology*, 26(7), pp.1608-12.

Thimme, R. et al., 2003. CD8 + T Cells Mediate Viral Clearance and Disease Pathogenesis during Acute Hepatitis B Virus Infection. *Journal of virology*, 77(1), pp.68-76.

Thomson, A., 1999. Are dendritic cells the key to liver transplant tolerance? *Immunology Today*, 20(1), pp.27-32.

Thomson, A.W. & Knolle, P.A., 2010. Antigen-presenting cell function in the tolerogenic liver environment. *Nature Reviews Immunology*, 10(11), pp.753-766.

Toka, F.N., Suvas, S. & Rouse, B.T., 2004. CD4+ CD25+ T Cells Regulate Vaccine-Generated Primary and Memory CD8+ T-Cell Responses against Herpes Simplex Virus Type 1. *Journal of Virology*, 78(23), pp.13082-13089.

van der Molen, R.G. et al., 2004. Functional impairment of myeloid and plasmacytoid dendritic cells of patients with chronic hepatitis B. *Hepatology (Baltimore, Md.)*, 40(3), pp.738-46.

Verreck, F.A.W. et al., 2006. Phenotypic and functional profiling of human proinflammatory type-1 and anti-inflammatory type-2 macrophages in response to microbial antigens and IFN- γ - and CD40L-mediated costimulation. *Journal of Leukocyte Biology*, 79(February), pp.285-293.

von Boehmer, H., 2005. Mechanisms of suppression by suppressor T cells. *Nature immunology*, 6(4), pp.338-44.

von Freyend, M.J. et al., 2011. Sequential control of hepatitis B virus in a mouse model of acute, self-resolving hepatitis B. *Journal of viral hepatitis*, 18(3), pp.216-26.

Wang, F.S. et al., 2001. Dysfunction of peripheral blood dendritic cells from patients with chronic hepatitis B virus infection. *World journal of gastroenterology : WJG*, 7(4), pp.537-41.

Webster, G.J.M. et al., 2004. Longitudinal Analysis of CD8+ T Cells Specific for Structural and Nonstructural Hepatitis B Virus Proteins in Patients with Chronic Hepatitis B : Implications for Immunotherapy. *Journal of virology*, 78(11), pp.5707-5719.

Wehler, T.C. et al., 2008. Rapid identification and sorting of viable virus-reactive CD4(+) and CD8(+) T cells based on antigen-triggered CD137 expression. *Journal of immunological methods*, 339(1), pp.23-37.

Weiser, B. et al., 1983. Closed circular viral DNA and asymmetrical heterogeneous forms in livers from animals infected with ground squirrel hepatitis virus. *Journal of virology*, 48(1), pp.1-9.

Wherry, E.J. et al., 2003. Viral Persistence Alters CD8 T-Cell Immunodominance and Tissue Distribution and Results in Distinct Stages of Functional Impairment. *Journal of virology*, 77(8), pp.4911-4927.

Whitmire, J.K., 2011. Induction and function of virus-specific CD4+ T cell responses. *Virology*, 411(2), pp.216-28.

Williams, Matthew A & Bevan, M.J., 2007. Effector and memory CTL differentiation. *Annual review of immunology*, 25, pp.171-92.

Wölfl, M. et al., 2008. Use of CD137 to study the full repertoire of CD8+ T cells without the need to know epitope specificities. *Cytometry. Part A : the journal of the International Society for Analytical Cytology*, 73(11), pp.1043-9.

Xia, S. et al., 2011. Hepatic microenvironment programs hematopoietic progenitor differentiation into regulatory dendritic cells , maintaining liver tolerance. *Hematology*, 112(8), pp.3175-3185.

Xu, D. et al., 2006. Cells Actively Influence the Antiviral Immune Response and Disease Progression in Patients with Hepatitis B 1. *The Journal of Immunology*, 177, pp.739-747.

Yang, G. et al., 2007. Association of CD4+CD25+Foxp3+ regulatory T cells with chronic activity and viral clearance in patients with hepatitis B. *International immunology*, 19(2), pp.133-40.

Yang, P.L. et al., 2010. Immune effectors required for hepatitis B virus clearance. *Proceedings of the National Academy of Sciences of the United States of America*, 107(2), pp.798-802.

Yates, A. et al., 2007. Revisiting estimates of CTL killing rates in vivo. *PloS one*, 2(12), p.e1301.

Young, B.Y. et al., 1989. In Vivo Expression of Perforin by CD8+ Lymphocytes during an Acute Viral Infection. *The Journal of experimental medicine*, 169(June), pp.2159-2171.

Zhang, Y.-yuan et al., 2003. Single-cell analysis of covalently closed circular DNA copy numbers in a hepadnavirus-infected liver. *Proceedings of the National Academy of Sciences of the United States of America*, 100(21), pp.12372-12377.

Zhou, L., Chong, M.M.W. & Littman, D.R., 2009. Review Plasticity of CD4 + T Cell Lineage Differentiation. *Immunity*, 30(5), pp.646-655.

Zhou, S. et al., 2004. Differential Tissue-Specific Regulation of Antiviral CD8 + T-Cell Immune Responses during Chronic Viral Infection. *Journal of virology*, 78(7), pp.3578-3600.

Zuniga, E.I. et al., 2008. In vivo virus infection inhibits type I interferon production by plasmacytoid dendritic cells thereby facilitating opportunistic infections. *Cell Host Microbe*, 4(4), pp.374-386.

7 Acknowledgements

First, I would like to thank Prof. Dr. Ulrike Protzer for the opportunity to perform my PhD-thesis in her institute. I always felt supported, and I am very grateful for the possibility to work and learn independently, yet with continuous advise and help. I have learned a lot about HBV and liver immunology, and I have grown personally during the last three years.

My special gratitude goes to the Friedrich-Naumann-Stiftung für die Freiheit, especially Michael Gold, Nicole Jagodschinski and Prof. Dr. Sabine Stengel-Rutkowski, for financial and intellectual support.

I would like to thank my fellow group members, who provided assistance, motivation, and good atmosphere at work. My special thanks to Theresa Asen and Andrea Weicht for their help in the daily lab work. Also, I want to acknowledge the immune-monitoring group (Sarah Kutscher, Claudia Dembek, Simone Allgayer and Tanja Bauer), who always made me feel welcome in the "Schnecke". And of course I cannot leave out Georg Gasteiger and Simone Backes, to whom I am grateful for helpful discussions and advise. Furthermore, I want to mention Joachim Weidmann and Johannes Günther, who did excellent internships and were a great help in my extensive mouse assays.

I owe further thanks to Tim Sparwasser and Ingo Drexler for providing DEREG mice, Hicham Bouabe for providing IL10-reporter mice and insight into IL10 effects, Elisabeth Kremmer for producing large amounts of antibodies for me, Michaela Aichler and Irene Esposito for immunohistochemical analyses, and of course the very friendly team of the animal facility in Neuherberg. Also, I need to mention Albert Wesker – even though a fictitious character, he always reminded me that knowledge is power, and that it needs effort and determination to earn it.

Particularly, I would like to express my gratitude to Stephan Fischer for sharing this exciting time with me.

At last, and of utmost importance, I want to thank my family, Angelika, Thomas and Henrike Stross, for their permanent support, for their interest and their involvement. Without you, this work would not have been possible.

Thanks to everybody who contributed, in whatever way, to this work!