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HEPATOCYTE-DEPENDENT INDUCTION OF IMMUNOREGULATORY $CD4^+$ T CELLS

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1. Introduction

1.1. The liver as an immunological organ

The liver is a remarkable organ that plays an important role in metabolic and detoxification processes. Its functions include energy supply (uptake and release of amino acid and glucose, lipid metabolism, urea production), production and release of bile, degradation and excretion of metabolic products and xenobiotics, production of coagulation factors and other proteins. It further plays a role in the regulation of the endocrine system and is characterized by its high regenerative capacity[1].

In addition, the liver represents an important interface between the contents of the gut and the systemic circulation. As a result, the liver is exposed to a large quantity of gut-derived nutrients, potential antigens, toxins, and bacterial products such as the so-called pathogen-associated molecular patterns (PAMPS). The liver must tolerate this immunogenic load while still providing immunosurveillance for pathogenic infections and/or malignant cells[2].

This tolerance effect was first described in 1969 in porcine transplantation studies. It was shown that liver allografts with major histocompatibility complex mismatch were accepted without immunosuppression, whereas skin or kidney allografts were rejected[3]. In order to induce this ant-inflammatory state, a unique immunological milieu is required, which is created by mechanisms of the innate and adaptive immune system as well as by the anatomical structure of the liver[4]. The basis for understanding the immunologic role of the liver is knowledge of the characteristic liver architecture, the distinct hepatic circulation, and the specific cellular composition of the organ.

1.1.1. Liver architecture

The liver is the largest gland in the body, and accounts for approximately two percent of body weight. Macroscopically the liver is divided into four lobes by connective tissue. The four lobes are the quadrant lobe, the caudate lobe, the right, and left lobe[5]. In a more functional division, the liver can be divided into eight segments along the blood supply of the liver. Each of these segments has a vascular branch from the portal vein, hepatic artery,

and bile duct. There are various microscopic ways of dividing the liver into morphological or functional units [6]. The lobule and the acinus are the two most commonly described structures (Figure 1-1).

The lobule is the traditional concept, seen under the microscope as a hexagonal structure, and is composed of parenchymal cells (hepatocytes) lined by liver sinusoidal endothelial cells (LSECS), which together form the hepatic sinusoids. In each lobe, the hepatic sinusoids extend from the portal tract which consists of branches of the hepatic arteries, portal veins and bile ducts to the central veins in the center of the lobe.

Alternatively, the acinus describes a unit containing two portal tracts in the center and two central venules in the periphery. It is the smallest functional unit and is divided into zones 1, 2, and 3. Zone 1 surrounds the portal tract and has high levels of oxygen and nutrients, while zone 3, near the central vein, has low levels of both. Zone 2 is in between, with an intermediate concentration of oxygen and nutrients[7].

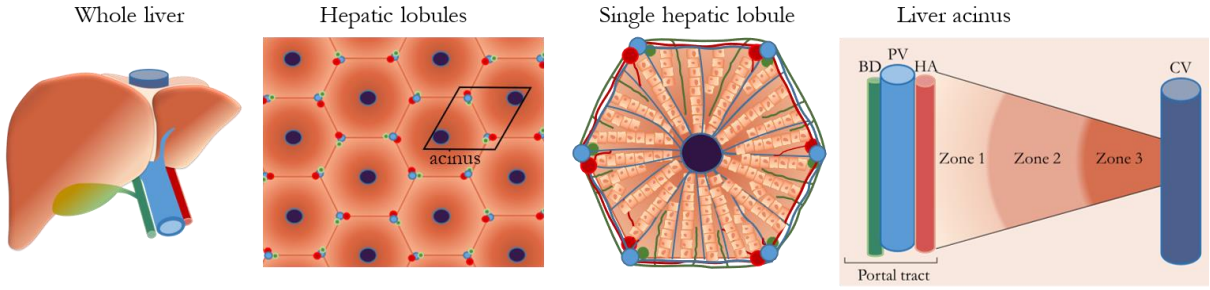


Figure 1-1. Liver structure and functional subunits
CV: central vein, HA: hepatic artery, PV: portal vein, BD: bile duct
(Adapted from Sanders *et al.* [8])

1.1.2. The hepatic circulation

The liver receives its blood supply from two sources, the hepatic portal vein and the hepatic artery. 20% of hepatic blood is oxygenated blood delivered by the hepatic artery, while 80% is delivered by the portal vein from the gastrointestinal tract[9]. The mixed blood from the liver artery and the portal vein flows in special capillaries, the so-called liver sinusoids, which lead to the central veins. Sinusoids have a discontinuous fenestrated endothelium without basement membrane. The fenestrations of LSECs and the absence of a basement membrane also allow direct cell contacts between circulating immune cells and liver resident cells such as hepatic stellate cells (HSCs) in the space of Dissé and, importantly, with hepatocytes, the parenchymal cells of the liver[10,11]. In other organs, transmigration of leukocytes across an endothelium with a continuous basement membrane

requires activation of endothelial cells with expression of selectins and integrins. This necessary activation ensures that only in the context of inflammation blood cells and especially immune cells migrate into the tissue[10,12].

In contrast, in the liver, the interaction of the different cell populations is facilitated further by another liver-specific peculiarity. Due to the small diameter of the sinusoids (5-7 µm), the blood flow rate in the sinusoids is reduced compared to peripheral blood vessels. This prolongs the contact of circulating lymphocytes with LSECs, Kupffer cells (KCs), and hepatocytes and allows selectin-independent adhesion to the endothelium[10].

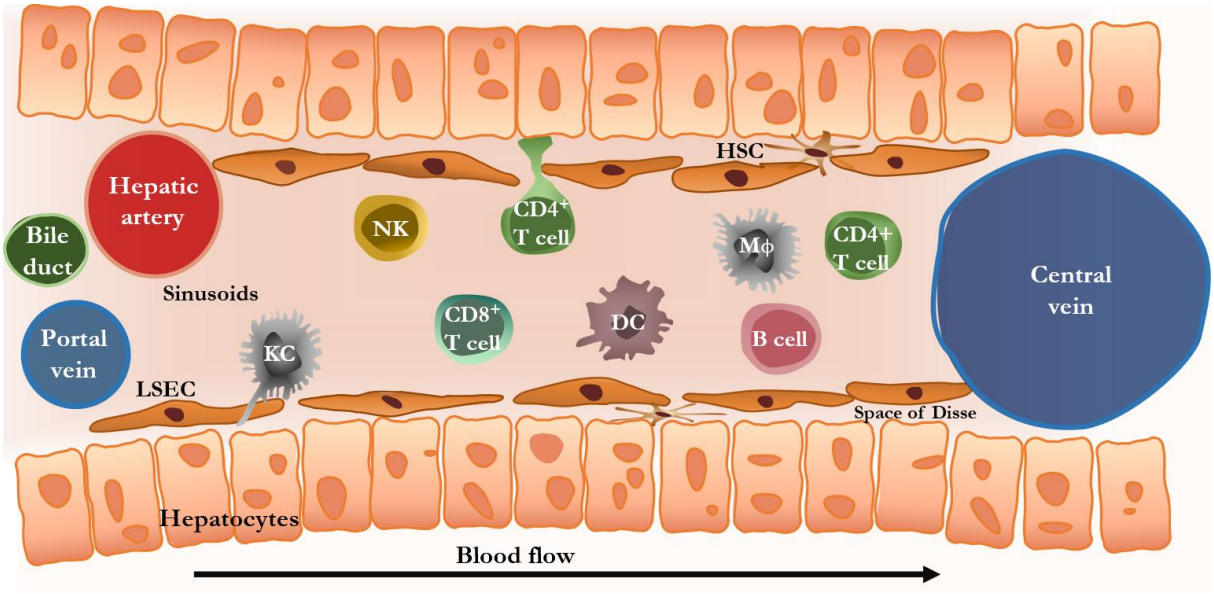


Figure 1-2. Immunological structure of the liver
Blood enters the liver through the portal vein and the hepatic artery and drains through the sinusoids to the central vein. The sinusoids are populated by diverse immune cells and are lined by a fenestrated monolayer of LSECs. Between the LSECs and the hepatocytes is the space of Dissé which is populated by HSCs.
LSEC: liver sinusoidal endothelial cell, HSC: hepatic stellate cell, DC: dendritic cells NK: natural killer cell, KC: Kupffer cell.

1.1.3. Cellular composition of the liver

The cellular composition of the liver can be divided into parenchymal cells (hepatocytes) and non-parenchymal cells. The latter include cholangiocytes, HSCs, endothelial cells and immune cells including phagocytes (macrophages, dendritic cells (DCs), and neutrophils) as well as lymphocytes (innate lymphoid cells (ILCs), NK cells, T cells, and B cells).

The immune cells maintain tolerance in the homeostatic state and are capable of mediating inflammation and liver injury during disease. Innate immune cells known to be

resident in the liver include DCs, KCs, mucosal-associated invariant T cells (MAIT cells) in man, invariant NKT (iNKT) cells predominantly in mice, γδ T cells, NK cells and ILCs. Despite being educated in a tolerogenic environment during homeostasis, hepatic innate cells participate in the clearance of common PAMPs, damage-associated molecular pattern (DAMPS) and other pro-inflammatory molecules[13].

The liver contains a large population of conventional αβ T cells for adaptive immune functions. These cells are either $CD8^+$ or $CD4^+$ T cells that recognize their antigen following presentation by antigen-presenting cells (APC) in a classical major histocompatibility complex (MHC) I- or MHC II-dependent manner, respectively. T cell activation depends not only on the interaction between the TCR and the peptide/MHC complex, but also on interaction with co-stimulatory receptors and the release of interleukin 2 (IL-2)[14]. $CD4^+$ and $CD8^+$ T cells are characterized by different effector functions.

$CD8^+$ T cells are cytotoxic effector cells that can be activated by any target cell after recognition of the MHCI/antigen complex without further costimulatory signals. Their cytotoxicity is exerted by the release of perforin and granzyme or through the Fas pathway[15,16].

1.2. $CD4^+$ T cells as a special cell subset in the liver

Naïve **$CD4^+$ T cells** differentiate after antigen-specific activation in the presence of inflammatory signals into specialized T helper cells (Th) with different phenotypes (Type 1, 2, 17 and 22 helper T cells (Th1, Th2, Th17, and Th22), and regulatory T cells). These can be subdivided based on effector functions such as secretion of defined cytokine profiles. The ability to differentiate into distinct subpopulations with appropriate effector functions depending on the nature of the threat, enables $CD4^+$ T cells to target specifically and direct the course of adaptive immune responses[17].

The composition of danger signals during T cell activation initiates the expression of characteristic transcription factors that regulate the differentiation into the different T helper cells. IL-2 plays a special role in this process. IL-2 does not specify the type of Th differentiation; instead, IL-2 modulates expression of receptors for other cytokines and transcription factors, thereby either promoting or inhibiting cytokine cascades that correlate with each Th differentiation state[18].

In the presence of IL-12 produced by APC, the transcription factors Signal Transducer and Activator of Transcription (STAT) 4 and T-bet are activated which regulate the differentiation (and maintenance) of $CD4^+$ T cells into **Th1 cells**. They are characterized by their cytokine production of interferon γ (IFNγ), IL-2, and tumor necrosis factor α (TNFα) and their ability to induce a cell-mediated immune response against intracellular pathogens[19].

Th2 cells differentiate under the influence of the transcription factors GATA binding protein (GATA) 3 and STAT6 in the presence of IL-4 and in turn produce IL-4, IL-5, IL-10, and IL-13, which promote the humoral immune response and defense against extracellular parasites[20].

Th17 cells are another $CD4^+$ effector population. They are generated in the presence of transforming growth factor β (TGF-β), IL-6, and IL-23[21,22] and are characterized by the production of IL-17, IL-21, and IL-22. They are involved in the clearance of extracellular bacteria and fungi but are also present in chronic inflammation and are considered to be mediators of autoimmune diseases[23].

In contrast, $CD4^+$ Th subsets with regulatory functions, so called T regulatory cells (**Tregs**), play an immunosuppressive role, and promote immune self-tolerance and homeostasis.

The prevailing view is, that there are two main subsets of Tregs, classified according to their cellular origin: natural Tregs (nTregs), which are differentiated in the thymus from bone marrow-derived precursors, and inducible Tregs (iTregs), which are generated in the periphery from populations of mature T cells under certain antigenic stimulation conditions[24].

Apart from nTregs and iTregs, several studies have identified other T cells with regulatory properties, including Th-like Treg subsets, $CD8^+$ Tregs, Th3 cells and Tr1 cells[25]. The best characterized Tregs are **nTregs** as well as **Tr1 cells**. nTregs, one of the largest subsets of Tregs, express the nuclear transcription factor forkhead box P3 (FoxP3), which is induced by TGFβ. They are characterized by their cell surface proteins CTLA-4 and CD25, and secret IL-10 and TGFβ[24]. While self-tolerance is mainly mediated by $Foxp3^+$ Tregs, Tr1 cells contribute strongly to peripheral tolerance and only to a limited extent to self-tolerance. Tr1 cells have been generally characterized by their ability to secrete IL-10 as well as IFNγ, and by the lack of constitutive Foxp3 expression[26].

The immune regulating effect of Tr1 cells and Tregs is mainly mediated by cytokines, but also other mechanisms critical for their immunosuppressive function have been described, which are introduced in the following section.

1.3. Mechanisms of immune regulation

1.3.1. IL-10 and IFNγ

$CD4^+$ T cells among other cells interact with and modulate their surroundings by releasing cytokines. There is a variety of different cytokines which cannot be solely attributed to one specific cell type. Cytokines have diverse and pleiotropic effects including immunologic, pro-, and anti-inflammatory activities. IL-10, for example, is considered to be a prototypical anti-inflammatory cytokine that plays an important role in the maintenance and re-establishment of immune homeostasis.

It was originally described as a unique product of Th2 cells that inhibits cytokine production by Th1 cells[27]. However, since then it has been shown that IL-10 can be produced and secreted by a variety of immune cells including DCs, B cells, macrophages, $CD8^+$ T cells and all $CD4^+$ T cell subsets[28]. In addition, IL-10 is a key cytokine produced by Treg subsets, such as $Foxp3^+$ Tregs, and Tr1 cells, in which the sensing of IL-10 is an important signal to maintain their phenotype and function. IL-10 has broad anti-inflammatory functions targeting a wide variety of cells. For example, IL-10 modulates the local cytokine microenvironment, and also limits antigen presentation, thereby preventing the efficient development of T cell responses[29]. Furthermore, IL-10 has been demonstrated to restrict T cell responses by acting directly on $CD4^+$ T cells, inducing unresponsiveness or anergy[30].

However, there is increasing evidence that the function of IL-10 is not limited to inducing immunomodulatory effects, but also promotes pro-inflammatory effects, in addition to its described anti-inflammatory effects[31].

In contrast to IL-10, IFNγ is widely recognized as a predominantly pro-inflammatory cytokine and is produced primarily by NK cells, NKT cells, cytotoxic $CD8^+$ T cells, DCs, macrophages, B cells, type I innate lymphoid cells, and $CD4^+$ Th1 cells[32,33].

Indeed, IFNγ plays an important role in several immune activation scenarios and its production can be induced by various antigens, mitogens, DAMPS or PAMPS. Besides its antiviral effects, it is crucial for immunity against intracellular bacteria and protozoa[34]. The

role of IFNγ in antitumor immunity is well established, and its anti-tumor effects are exerted both directly on cancer cells and by positively modulating cancer-directed immune effector cells[35].

More recently, an increasing number of observations, in which IFNγ played a role in achieving a tolerogenic immunologic outcome have challenged the view of IFNγ as a predominantly pro-inflammatory cytokine[36–39]. For example, IFNγ also been shown to exert protective and anti-inflammatory functions, including inhibition of T cell proliferation as well as recruitment and stimulation of Tregs[40].

1.3.2. Notch signaling

Notch signaling has been considered as a critical regulator for the differentiation and function of immune cells including T cells. The Notch signaling pathway consists of four transmembrane Notch receptors (Notch1, 2, 3, 4) and five corresponding ligands belonging to the Jagged (Jag-1, -2) and Delta-like ligand (Dll-1, -3, -4) family[41].

Binding of the extracellular domain of the Notch receptor to Delta and Jagged family ligands on an adjacent cell initiates the canonical Notch signaling cascade. This triggers a series of enzymatic reactions that result in the release of the intracellular domain of the Notch receptor, which translocates to the nucleus and forms an active transcription complex that regulates the expression of target genes[42].

In addition to influencing the differentiation of $CD4^+$ T cells into Th1 and Th2 cells, there is evidence that Notch has immunosuppressive functions.

Overexpressing the Notch ligand Jag-1 in an immortalized B-cell lineage has been shown to differentiate $CD4^+$ T-cells *in vitro*, resulting in suppressor activity[43,44]. Furthermore, DCs overexpressing Jag-1 induced T-cell tolerance *in vivo* that could be transferred to wild type mice by adoptive transfer[45].

In another study, the presence of soluble TGFβ promoted Notch-dependent expression of the Treg specific FoxP3, which is thought to result in the generation and maintenance of adaptive Tregs[46].

Moreover, Notch signaling, in synergy with IL-12 or IL-27, has been shown to stimulate Th1 cells to release large amounts of IL-10, which contributes to self-limitation of Th1 immunity by inhibiting the Th1 inflammatory potential. This effect was limited to the expression of Dll, but not Jagged ligands on DCs[47,48]. Exposure of $CD4^+$ T cells to Dll proteins under strong Th1 cell-polarizing conditions resulted in high levels of IL-10

production. It has been shown that hepatic inflammation is associated with the expression of Dll and Jag ligands in LSECs, which in turn, activates Notch in Th1 cells. When activated, Notch induces the production of IL-10 in Th1 cells, thereby switching them from a pro-inflammatory to an anti-inflammatory state. Consistent with this, Notch-deficient $CD4^+$ T cells express lower levels of IL-10 in the presence of LSECS, whereas the expression of Th1 cytokines such as IFNγ and TNFα remains unchanged[49].

1.3.3. Granzymes

Granzymes are a family of serine proteases released from cytoplasmic granules within cytotoxic cells of the immune system. Granzymes induce (apoptotic) cell death in the target cells. Currently, five human granzymes (granzyme A (GrA), GrB, GrH, GrK and GrM) have been identified, while ten mouse granzymes (GrA-G, GrK, GrM and GrN) are known. Granzymes A and B are the most abundant and best studied[50].

Granzyme B (GzmB) is a cytotoxic effector molecule that is stored in cytotoxic granules of NK cells and CTL and is released during cell lysis along with the pore-forming protein perforin. However, GzmB is also produced by various non-cytotoxic cell types with different functions[51].

Recent reports have shown that $CD4^+$ T cells are also able to synthesize GzmB and perforin[52,53]. In addition, GzmB is highly upregulated in activated $CD4^+$ T cells with a Tr1 phenotype, as shown in several studies. The GzmB expressing Tr1 cells are able to kill allogeneic target cells in a perforin-dependent manner[54,55]. Immunosuppressive roles in Tregs have been identified for both GzmA and GzmB expression. $CD4^+CD25^+$ Tregs have been shown to mediate suppression with GzmA requirement in the human system[56]. A GzmB-dependent suppressive mechanism of $CD4^+CD25^+$ Tregs was reported by the working group of Noelle *et. al*. They demonstrated an upregulation of GzmB by inducing regulatory activity on T cells. Furthermore, they showed a contact mediated, perforin independent suppressive mechanism of $CD4^+CD25^+$ Tregs with GzmB as one of the key components[57].

1.4. Antigen presentation and tolerance induction

Inducing and maintaining hepatic tolerance is mediated by the above-mentioned mechanisms, among others. Regulatory immune cells and APCs ensure that tolerance is

maintained under homeostatic conditions, but potent *ad hoc* immune responses to combat infections can still be initiated. The "classical" professional APCs found in the liver are DC and KC. However, in contrast to other solid organs, the non-hematopoietic cell populations of the liver (hepatocytes, LSEC, cholangiocytes, HSCs) can, under certain conditions, also present antigens to liver infiltrating NKT, $CD8^+$ and $CD4^+$ T cells due to their special properties, generally leading to an induction of cell anergy or apoptosis, and generation and expansion of regulatory T cells[58,59].

Many studies to date have focused on the role of LSECs in cross-presenting antigen to $CD8^+$ T cells, thereby regulating immunity[49,60–63]. In addition, hepatocytes also exert antigen presenting properties. They express pattern recognition receptors such as toll-like receptors and scavenger receptors. These receptors contribute to the clearance of pathogenic or other immunogenic foreign material delivered by portal blood, thereby reducing the immunogenic load. Another way in which hepatocytes regulate the inflammatory response is by influencing the activation and regulation of T cells by acting as APCs. Hepatocytes constitutively express MHC I, intercellular cell adhesion molecule 1 (ICAM-1)[64] and CD1d[65], but not costimulatory molecules. Under inflammatory conditions, MHC-II, ICAM-1, CD80, CD86, and CD40 can be upregulated[66].

This allows hepatocytes to interact with $CD8^+$ and $CD4^+$ T cells as well as NKT cells. T cells circulating in the sinusoids can interact directly with hepatocytes despite the barrier of LSEC and KC, due to the special architecture of the sinusoids. T cells form cytoplasmic projections (microvilli) that extend through the LSEC fenestrae into the space of Dissé and can contact the basolateral surface of hepatocytes. Hepatocytes also form microvilli that project into the sinusoidal lumen[10].

1.4.1. Antigen presentation and activation of $CD8^+$ T cells by hepatocytes

Hepatocytes act as APCs by expressing low levels of MHC-I under homeostatic condition, which may activate $CD8^+$ T cells. It was shown, that although hepatocytes promote rapid activation and proliferation of $CD8^+$ T cells, they do not express co-stimulatory molecules, including CD80 and CD86, and fail to induce functional CTL[67]. Furthermore, it has been shown that antigens expressed by hepatocytes can be cross-presented on LSECs *in vivo.* Cross-presentation of soluble antigens by LSECs results in defective activation of naïve $CD8^+$ T cells, as demonstrated in several studies[68]. $CD8^+$ T cells activated within the liver are rendered tolerogenic by a variety of mechanisms

that impair effector responses following intrahepatic activation. These mechanisms include non-apoptotic degradation of T cells in hepatocyte lysosomes within the first day of activation ("suicidal emperipolesis")[69] and Bim-dependent apoptosis of T cells that survive suicidal emperipolesis[70]. However, a recent study has shown that a threshold of antigen expression within the liver is the dominant factor determining the fate of $CD8^+$ T cells recognizing intrahepatic antigens, regardless of their affinity for the antigen or the site of initial antigen encounter, with high levels of antigen expression leading to exhaustion of T cell function[71]. Furthermore, the liver contains other liver-associated leukocytes, including NK cells, NKT cells, and regulatory T cells, whose immunoregulatory properties may also contribute to intrahepatic $CD8^+$ T cell tolerance[68].

1.4.2. Antigen presentation and activation of $CD4^+$ T cells and $CD4^+$ Tregs by hepatocytes

$CD4^+$ T cells may be activated by hepatocytes through MHC-II. MHC-II is upregulated in hepatocytes under inflammatory conditions, such as chronic liver disease and after stimulation with IFNγ[72–74].

In vitro, it has been shown that MHC-II^+ transgenic hepatocytes process antigen and present it to $CD4^+$ T cells, resulting in the polarization of naïve $CD4^+$ T cells to Th2 cells and their ability to suppress the secretion of IFNγ of activated Th1 cells[75].

Furthermore, MHC-II expression on hepatocytes during viral infection decreases IFNγ production by virus-specific $CD4^+$ and $CD8^+$ T cells and prolongs viral persistence. Therefore, in an inflammatory milieu, MHC-II^+ hepatocytes can induce differentiation of infiltrating $CD4^+$ T cells to a less pro-inflammatory phenotype, which does not contribute to increased inflammation but to an inability to clear viruses[76] . In addition, hepatocytes can also induce IL-10-producing $CD4^+$ T-cells in a Notch signaling dependent manner in a model of Th1- mediated liver injury (injection of the plant lectin Concanavalin A; ConA), suggesting that hepatocytes may help restore the immunological balance after liver injury[72,73]. In addition, Hall *et al.* showed that HCV-infected hepatocytes are able to directly induce the development of Tregs through the production of TGFβ and may contribute to impaired host T cell responses[77]. Burghardt *et al.* have demonstrated that hepatocytes induce IL-10^+IFNγ$^+CD4^+$ T cells, which are $Foxp3^-$ in a cell-cell contact-dependent manner. Moreover, the induction was shown to be strongly dependent

on Notch signaling. The cells were able to suppress responder T cells *in vitro* in an IL-10 independent manner. When TGFβ was added to the cultures of naïve $CD4^+$ T cells and hepatocytes, $CD25^+FoxP3^+CD4^+$ Tregs were induced instead of IL-10 producing $IFN\gamma^+CD4^+$ T cells. These cells had the same immunosuppressive capacity *in vitro* and their induction depended, similar to that of IL-10 producing Th 1 cells, on Notch signaling[72,73].

1.5. Aim of the study

The aim of this study was to investigate the characteristics and functional properties of hepatocyte-induced immunoregulatory $CD4^+$ T cells ($CD4^+$ T_{HC}). Based on previous studies demonstrating the immunosuppressive potential of hepatocyte-activated $CD4^+$ T cells[72,73], this study aimed to further characterize the heterogeneity of $CD4^+$ T_{HC} populations and their role in immunomodulation.

In particular, this study sought to elucidate the mechanisms underlying the dual production of the cytokines IFNγ and IL-10 and their potential synergy in the suppression of the proliferation and activation of naive $CD4^+$ T cells. In addition, the relationship between GzmB expression and the immunosuppressive and cytotoxic functions of $CD4^+$ T_{HC} was examined.

The study also aimed to investigate the involvement of the Notch signaling pathway in driving GzmB expression in $CD4^+$ T_{HC}. By addressing these research objectives, this study may contribute to a better understanding of the immunosuppressive mechanisms of hepatocyte-induced immunoregulatory $CD4^+$ T cells and their potential implications for recovery of immune homeostasis and control of immune-mediated liver disease.

2. Materials and Methods

2.1. Materials

2.1.1. Technical equipment

Table 2-1 Technical equipment

Equipment	Supplier
Centrifuge 5417R	Eppendorf, Hamburg
Centrifuge 5810R	Eppendorf, Hamburg
Clean Bench Hera cell 240	Thermo Fisher Scientific, Hamburg
Clean Bench, MSC advantag	Thermo Fisher Scientific, Hamburg
BD LSR Fortessa™	BD Biosciences, Heidelberg
BD FACSAriaTM III	BD Biosciences, Heidelberg
Freezer G3013 comfort	Liebherr, Biberach an der Riss
Freezer MDF U53V Ultra low	Sanyo, Munich
HandyStep® electronic repeating pipette	BRAND GmbH, Wertheim
Incubator Hercell 240	Thermo Fisher Scientific, Waltham, USA
Magnetic Stirrer IKAMAG® RCT	Janke und Kunkel, Staufen
Microscope CK40	Olympus, Hamburg
Mini Cell XCell Sure Lock	Invitrogen, Darmstadt
MyCyclerTM Thermal Cycler	Biorad, Hercules, USA
NanoDrop ND-100	Peqlab, Erlangen
Neubauer Improved	Chamber Roth, Karlsruhe
Pipetboy Integra	INTEGRA Biosciences, Fernwald
Pipettes Eppendorf Research® Plus	Eppendorf, Hamburg
PowerPac HC Power Supply	Biorad, Hercules, USA
QuadroMACS separation unit	Miltenyi Biotec, Bergisch Gladbach
Scale ATILON ATL-423-I milligram lab balance	Acculab Sartorius group, Göttingen
Scale TE124S analytical weight scale	Sartorius, Göttingen
Sonorex RK 102H	Bandelin electronics, Berlin
Tecan Infinite® M200	Tecan, Crailsheim
Tecan M8/2R ELISA washer	Tecan, Crailsheim
Thermal Cycler C1000	BioRad, Munich
CFX 96 ^{TM} Real-Time PCR Detection System	
Thermoleader Dry Block Heat Bath	Uniequip, Martinsried
Vortex Mixer	Heidolph, Schwabach

2.1.2. Consumables

Table 2-2 Consumables

Consumable	Supplier
Cell culture plates, flat or round bottom	Sarstedt, Nümbrecht
Flow cytometer tubes	Sarstedt, Nümbrecht
Hollow needles/canulaes	B. Braun, Melsungen AG, Melsungen,
MACS pre-seperation filters	Miltenyi Biotec, Bergisch Gladbach
MACS separation colums (LD, LS, MS)	Miltenyi Biotec, Bergisch Gladbach
Nunc 96 well microtiter plate,	Thermo Scientific, Hamburg
Protran WhatmanTM membran	GE Healthcare L. S., Little Chalfont, UK
Parafilm M ®	American National Can. USA
PCR tubes	Kisker Biotech, Steinfurt
Petridishes	Greiner Bio-One, Solingen
Pipette tips (10 µL, 200 µL, 1000 µL)	Sarstedt, Nümbrecht
Pipette tips, sterile and RNAse free (10 µL, 20 µL, 200 µL, 1000 µL)	Sarstedt, Nümbrecht
Pipettes (2 mL, 5 mL, 10 mL, 25 mL)	Sarstedt, Nümbrecht
Positive Displacement Tips (500 µL, 2.5 mL, 5 mL 12.5 mL)	BRAND GmbH, Wertheim
Reaction tubes (1.5 mL, 2 mL)	Sarstedt, Nümbrecht
Reaction tubes (15 mL, 50 mL)	Sarstedt, Nümbrecht
Reaction tubes, sterile and RNAse free (1.5 mL, 2 mL)	Sarstedt, Nümbrecht
Sealing Tape, optically clear	Sarstedt, Nümbrecht
Surgical blade	Feather, Osaka, Japan
Syringes	B. Braun, Melsungen AG, Melsungen
Syringe filter (0.22 µm)	TPP, Trasadingen, CH

2.1.3. Reagents and kits

Table 2-3 Reagents and kits

Reagents and kits	Supplier
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	Roth, Karlsruhe
Bovine serum albumin (BSA)	Serva, Heidelberg
Brefeldin A (BFA)	Sigma-Aldrich, Taufkirchen
CD11c MicroBeads, mouse	Miltenyi Biotec, Bergisch-Gladbach
$CD4^+$ T cell isolation kit	Miltenyi Biotec, Bergisch-Gladbach
Dimethylsulfoxid (DMSO; D2650)	Sigma-Aldrich, Taufkirchen
dNTPs (10mM)	Invitrogen GmbH; Darmstadt

Reagents and kits	Supplier
GSI N-[N-(3,5-difluorophenacetyl)-Lalanyl]-S-phenylglycine t-butyl ester (DAPT, 20 mM;)	Sigma-Aldrich, Taufkirchen
Cytofix/Cytoperm™ Solution Kit	BD Biosciences, Heidelberg
Ethylenediaminetetraacetic acid (EDTA)	Roth, Karlsruhe
Fetal calf serum (FCS)	Lonza, Cologne
Heparin-sodium-25000-ratiopharm ®	Ratiopharm, Ulm
Ionomycin	Sigma-Aldrich, Taufkirchen
Liberase	Roche, Basel, CH
Maxima™ SYBR Green/ROX qPCR Master Mix (2X)	Fermentas, Thermo Scientific, Hamburg
Mouse IFN-γDuo Set	R&D, Minneapolis, USA
Mouse IFNγ ELISA MAXTM Standard	BioLegend, San Diego, USA
nCounter® Custom CodeSets	NanoString Technologies, Inc, Seattle, USA
PCR Buffer (10x)	Invitrogen, Darmstadt
Penicillin/streptomycin (100 U/mL)	Gibco®, Invitrogen, Darmstadt
Percoll	GE Healthcare, Glattbrugg/Zürich, CH
Phorbol 12-myristate 13-acetate (PMA)	Sigma-Aldrich, Taufkirchen
rDNAse	Machery & Nagel, Düren
recombinant IL-10	BD Pharmingen, Heidelberg
recombinant IL-2	BD Pharmingen, Heidelberg
RPMI	Gibco®, Invitrogen, Darmstadt
Streptavidin horseradish peroxidase (HRP)	R&D, Minneapolis, USA
TMB Substrate Reagent Set	BD Opteia, Heidelberg
Tris-Base	Sigma-Aldrich, Taufkirchen
Tris-HCl	Roth, Karlsruhe
Trypan blue	Sigma-Aldrich, Taufkirchen
Tween 20	Roth, Karlsruhe
Verso cDNA Kit	Abgene, Thermo Scientific, Hamburg
William's E Medium	Thermo Scientific, Hamburg
Z-AAD-CMK	Enzo Life Sciences, New York, USA

2.1.4. Buffers and solutions

Table 2-4 Buffers and solutions

Buffer or solution	Recipe
Ammoniumchloride (NH_4Cl); pH 7.2 (erythrocyte-lysis solution)	19 mM Tris-HCl 140 mM NH_4Cl
Collagenase solution	0.05 % collagenase in GBSS

Buffer or solution	Recipe
ELISA blocking solution	1 % BSA 5 % Sucrose 0.8 mM NaN_3 (0,05 %) 1x PBS
ELISA coating buffer; pH 8.2	0.1 M Na_2HPO_4 0.1 M NaH_2PO_4
ELISA diluent buffer; pH 7.2 – 7.4	0.1 % BSA 0.05 % Tween 20 20 mM Tris 150 mM NaCl
ELISA washing buffer	1 x PBS 0.05 % Tween 20
Fluorescence activated cell sorting buffer	1 x PBS 1 % BSA 15.4 mM NaN_3 (0.1 %)
Hanks' Balanced Salt Solution (HBSS); pH 7.4	5.4 mM KCl 0.3 mM Na_2HPO_4 x 7 H_2O 4.2 mM $NaHCO_3$ 1.3 mM $CaCl_2$ 0.5 mM $MgCl_2$ x 6 H_2O 0.6 mM $MgSO_4$ x 7 H_2O 137 mM NaCl 5.6 mM D-glucose
Lysis buffer for protein isolation pH 8.0	137 mM NaCl 0.5 % NP 40 2 mM EDTA 50 mM Tris HCl 10 % glycerol
Magnetic Cell Separation (MACS) buffer	1x PBS 0.5 % BSA 2 mM EDTA
Phosphate Buffered Saline (PBS); pH 7.4	137.9 mM NaCl 6.5 mM Na_2HPO_4 x 2 H_2O 1.5 mM KH_2PO_4 2.7 mM KCl

Buffer or solution	Recipe
Perfusion medium (PM); pH 7,35	5.36 mM KCl 0.77 mM MgSO4 0.34 mM Na2HPO4 0.94 mM MgCl 138 mM NaCl 0.44 mM KH2PO4 10 mM glucose 2 mM CaCl 10 mM Hepes 100 U/L penicillin 100 U/L streptomycin 20% BSA 0.4 mg Liberase/ mL ad to 1 l ddH_2O;
Pre-Perfusion buffer (PPML) pH 7,4	5.36 mM KCl 0.44 mM KH_2PO4 4.17 mM $NaHCO_3$ 138 mM NaCl 0.38 Na_2HPO_4 5.00 mM Glucose 0.5mM EGTA 50.00 mM Hepes ad to 1 L ddH_2O

2.1.5. Software

Table 2-5 Software

Software	Company
Bio-Rad CFX Manager 2.0	Bio-Rad, Hercules, USA
BD FACS Diva	BD Biosciences, Heidelberg
FlowJo™10	BD Biosciences, Heidelberg
GraphPad Prism 6	GraphPad Software, San Diego, USA
MS Office 2013	Microsoft, Redmond, USA
nSolver™ Analysis Software 2.5	NanoString Technologies, Inc, Seattle, USA
Primer3	Whitehead Institute for Biomedical Research, Cambridge, USA
TBASE	Abase, 4D Deutschland GmbH, Eching
Tecan Magellan v6.5	Tecan, Crailsheim
Windows XP	Microsoft, Redmond, USA

2.2. Methods

2.2.1. Mice

For this study, 6-12 weeks old male C57BL/6J wild type (WT), CD45.1 expressing congenic (CD45.1), double-knockin Foxp3-IRES-mRFP (FIR) x IL-10-IRES-GFP enhanced reporter [tiger] (FIR x *tiger*), B6.129S2-Gzmbtm1Ley/J (*GzmB*$^{-/-}$), as well as C57BL/6.129S7-(Ifnγ)^{tm1Ts}/J (*Ifnγ*$^{-/-}$mice) mice were used. All transgenic mice were raised on a C57Bl/6J background.

WT and CD45.1 mice were obtained from the animal research facility of the University Medical Center Hamburg-Eppendorf (Hamburg, Germany). FIR x *tiger* reporter mice were a gift from Dr. Richard Flavell (Yale School of Medicine, USA) and Dr. Samuel Huber (UKE Hamburg, Germany). *GzmB*$^{-/-}$ mice were kindly provided by Xuefang Cao (Buffalo, USA). *Ifn-γ*$^{-/-}$ mice were kindly provided by Dr. Ulf Panzer (Hamburg, Germany).

Mice were kept at room temperature of 20 °C ± 2 °C and relative humidity of 50 % ± 5 % in individually ventilated microisolator cages. All mice were housed under specific pathogen-free conditions with autoclaved chow and water ad libitum exposed to a regular 12:12-h light-dark cycle. All mice received human care according to the guidelines of the National Institutes of Health as well as to the legal requirements in Germany.

2.2.2. Cell Isolation

Isolation of hepatocytes

Primary hepatocytes from WT mice were isolated by a modified two-step collagenase perfusion method first described by Seglen[78].

Briefly, mice were anesthetized by an intravenous injection of (100 µL/10 g mouse) a ketamine (120 mg/kg) xylazine (16 mg/kg) heparin (8333 I.E./kg) solution and were sacrificed by cervical dislocation. The abdomen was opened, the upper part of the vena cava superior was ligated, and the portal vein was cannulated. Subsequently, livers were perfused with 5 mL pre-warmed (37 °C) pre-perfusion medium (PPML) to wash out blood and circulating cells from the liver as well as to eliminate calcium. The livers were then perfused with 25 mL pre-warmed (37 °C) perfusion medium (PM) containing 0.4 mg/mL liberase to dissociate the extracellular matrix. Following removal of the liver, the liver capsules were gently disrupted in 25 mL pre-warmed (37 °C) PM Buffer. Single cell

suspensions were achieved by passing the liver tissue through a sterile 100 µm nylon mesh, and the hepatocytes were then allowed to settle by gravity for 20 min. To separate hepatocytes from non-parenchymal cells (NPC) the cell pellet was gently agitated and layered on a 90% Percoll density solution. After a centrifugation step at 60 x g at 4 °C for 10 min the pellet was redispersed in 15 mL Williams E Media and washed twice with Williams E Media at 60 x g at 4 °C for 3 min. Finally, hepatocytes were resuspended in Williams E Media for cultivation.

Isolation of dendritic cells

Dendritic cells (DCs) were isolated in a two-step isolation procedure. First non-parenchymal cells were isolated. For this purpose, splenic cell suspensions were prepared by pressing the organs through 100 µm nylon meshes in HBSS. After centrifugation at 500 x g at 4 °C for 5 min, the cell pellets were resuspended in HBSS. DCs were then isolated using magnetic activated cell sorting (MACS) with CD11c MicroBeads according to the manufactures' protocol. CD11c is expressed in mice on all defined DC subsets. $CD11c^+$ cells were enriched by a positive selection using LS columns.

Isolation of $CD4^+$ T cell subtypes

Magnetic activated cell sorting (MACS) was used to isolate $CD4^+$ T cells from the spleen of WT, FIR x *tiger*, CD45.1, *Ifnγ*$^{-/-}$ or *GzmB*$^{-/-}$ mice. Cells were isolated using the MACS $CD4^+$ T Cell Isolation Kit II according to the manufacturer's instructions. For suppression assays and messenger ribonucleic acid (mRNA) extraction $CD4^+$ T cells were sorted by fluorescence activated cell sorting (FACS) via BD FACSAriaTM III to achieve higher purity. Cells were collected in 3 mL medium containing 2 % penicillin/streptomycin during the FACS process due to the higher risk of contamination.

2.2.3. *In vitro* co-culture

$CD4^+$ T cells from WT or FIR x *tiger* mice were co-cultured with hepatocytes or DCs from WT mice in William's Medium in 24-well flat-bottom plates with plate-bound anti-CD3 antibody (5 µg/mL) in a CO_2 incubator at 37 °C, 5 % CO_2 and saturated humidity for 48 h. As a control, $CD4^+$ T cells were stimulated with anti-CD3 in the absence of APCs.

In order to analyze different polarization conditions, in one assay recombinant human transforming growth factor- β ((rhTGF- β; 4 ng/mL)) and interleukin-12 (IL-12; 10 ng/mL) were added to the co-culture.

In one assay, Notch signaling was blocked by adding GSI N-[N-(3,5-difluorophenacetyl)-lalanyl]-S-phenylglycine t-butyl ester (DAPT, 20 mM) dissolved in dimethylsulfoxid (DMSO) to the culture.

2.2.4. Suppression assay

For suppression assays, $CD4^+CD25^-$ responder T cells were isolated from spleens of CD45.1 mice via MACS using a $CD4^+$ T cell isolation Kit. The cells were then stained with an anti-CD25 antibody and sorted via FACS using a BD FACSAriaTM III.

Suppressor cells were either $CD4^+$ T cells from FIR x *tiger* -or *Ifn*$^{-/-}$ or *GzmB*$^{-/-}$ mice which were co-cultured with hepatocytes for 48 hours (h) ($CD4^+$ T_{HC}; see previous section) or IL-10^+ or IL-10^- $CD4^+$ T_{HC}, which were subsequently sorted by flow cytometry.

CD45.1 mice express the allelic variant CD45.1 and can therefore be distinguished in FACS analyses from the FIR x *tiger* -or *Ifn*$^{-/-}$ or *GzmB*$^{-/-}$ mice which express the allelic variant CD45.2.

$CD4^+CD25^-$ responder T cells were labeled with the cell proliferation dye eFlour 670 according to the manufacturer's instructions. Subsequently, $1x10^5$ responder T cells were co-cultured with FACS-sorted $CD4^+$ T_{HC} populations (1×10^5) or naïve $CD4^+$ T cells (1×10^5) in the presence of anti-CD3/anti-CD28-coated MACSiBeads (bead-to-cell ratio 1:1) in RPMI Medium (supplemented: FCS (10 %), penicillin/streptomycin (1 %), HEPES (10 mM), 2-ME (50 µM)) in 96-well U-bottom plates (ratio suppressor cells : responder cells= 1:1).

For some experiment different amounts of exogenous IL-2 (0.3 µg, 3.0 µg, 20 µg, 60 µg) were added to the culture.

After 72 h, cells were harvested and stained for surface markers and fixable viability dye eFluor 506. Proliferation of living responder $CD4^+$ T cells was assessed by flow cytometry. The function of GzmB was analyzed either by adding the GzmB inhibitor Z-Ala-Ala-Asp (OMe) Chlormethyl Ketone (Z-AAD-CMK, 100mM) dissolved in DMSO to the culture described above or by using $CD4^+$ T_{HC} derived from *GzmB*$^{-/-}$ mice.

The proliferation index was calculated using the algorithm PI = Log [FI_{nd}/MFI_{all}]/Log [2]. MFI_{all} is the median fluorescence intensity of all cells; FI_{nd} is the peak fluorescence of the cells that did not proliferate.

2.2.5. *In vitro* re-stimulation of $CD4^+$ T_{HCs}

$CD4^+$ T cells were restimulated *in vitro* in order to analyze cytokine production via FACS analysis. For that purpose, $CD4^+$ T_{HC} were incubated in RPMI medium containing FCS (10 %), penicillin/streptomycin (1 %), phorbol 12-myristate 13-acetate (PMA; 10 ng/mL), and ionomycin (250 ng/mL) for a total of 6 h at 37 °C. After 1 h of incubation Brefeldin A (1 µg/mL) and monensin (2 µM) were added to stop vesicular transport and secretion of cytokines. After stimulation, $CD4^+$ T_{HC} were washed and stained for surface antigens and intracellular cytokines as described in the chapter flow cytometry.

2.2.6. Cytokine determination by ELISA

To measure cytokine concentration of IFNγ, IL-2 and IL-10 in the cultures, supernatant was taken after 48h and stored at -20 °C until analysis via enzyme-linked immunosorbent assay (ELISA). The assay was performed according to manufacturer's instructions. The antibodies (Ab) used are listed in Table 2-6.

In brief, high-binding microtiter plates were coated with diluted capture/primary Ab and incubated over night at 4°C. Between the different steps the microtiter plate was washed three times with ELISA washing buffer. The plates were blocked for at least two hours with blocking solution to avoid unspecific binding. Afterwards, a standard series and samples were applied for at least two hours at 4°C. During that time, the primary monoclonal antibody (mAb) captured the antigen, which is then bound by the biotin-coupled secondary mAb, which was added at room temperature for additional two hours at 4°C. Finally, adding streptavidin-coupled horseradish peroxidase and TMB substrate resulted in a concentration-dependent color change. After the enzymatic reaction was stopped with sulphuric acid (1 M), the absorbance of the single wells was determined at a wavelength of 450 nm by a Tecan infinite, and the cytokine concentrations were calculated by Magellan V6.5 correlating he color changes of the samples with the standard curve.

Table 2-6 IL-2, IL-10, and IFNγ ELISA antibodies

name	host	clone	supplier	conjugate	conc.	final conc.
IL-2						
capture	rat	JES6-1A12	BD Pharm.	none	0.5 mg/mL	3 µg/mL
detection	rat	JES6-5H4	BD Pharm.	biotin	0.5 mg/mL	1 µg/mL
IL-10						
capture	rat	JES5-2A5	BioLegend	none	0.5 mg/mL	8 µg/mL
detection	rat	JES5-16E3	BioLegend	biotin	0.5 mg/mL	1 µg/mL
IFNγ						
capture	N/A	N/A	BioLegend	none	N/A	1:200 dil.
detection	N/A	N/A	BioLegend	biotin	N/A	1:200 dil.

2.2.7. Gene expression analysis

Gene expression analysis was performed using Nanostring Technologies' nCounter® system, a fully automated, next-generation digital gene expression analysis system. The nCounter assay is based on the direct digital detection of mRNA molecules of interest using target-specific, color-coded probe pairs. There is no need to reverse transcribe the mRNA into cDNA or amplify the resulting cDNA by PCR[79].

For this purpose, $CD4^+$ T_{HC} were stained with anti-CD3 and anti-CD4 antibodies and sorted by flow cytometry. After flow cytometry pellets of $1x10^5$ $CD4^+$ T_{HC} were collected and sent to Prof. Dr. Frank Tacke, Internal Medicine III, University Clinic Aachen. There, the analysis was performed using nCounter® Custom CodeSets according to the manufacturer's instructions with 562 selected target genes.

Results were analyzed using nSolver™ Analysis Software 2.5. Quantification is expressed as x-fold changes from corresponding housekeeping genes.

2.2.8. Flow cytometry

FACS a classical tool to measure and quantify intra- and extracellular molecules. Cells were stained using a standard staining protocol including pre-blocking of Fc receptors (anti-CD16/32) to prevent unspecific binding and the fixable viability dye eFlour506 to exclude dead cells. The BD Cytofix/Cytoperm™ Fixation/Permeabilization Solution Kit was used for cytokine and GzmB cell staining according to the manufacturer's instructions.

Details of the antibodies used for staining are summarized in Table 2-7. In general, staining was performed at 4 °C for 30 min.

Stained cells were analyzed using an LSR Fortessa™ system with FACS Diva™ software. Data analysis was performed using FlowJo™10 software. Forward sideward scatter and viability dye determined vital leucocytes. Lymphocytes were identified as $CD3^+$ and further divided into $CD4^+$ T cells. After this cytokine and GzmB expression was determined.

Table 2-7 Anti-mouse antibodies for flow cytometric analysis

name	host	clone	supplier	final conc.
anti-CD25	rat	BC96	BioLegend	1 µg/ml
anti-CD3	rat	17A2	BioLegend	1 µg/ml
anti-CD4	rat	RM4-5	BioLegend	1 µg/ml
anti-GzmB	mouse	GB11	BioLegend	1:50
anti-IFNγ	rat	XMG1.2	BioLegend	1:100
anti-IL-10	rat	JES5-16E3	BioLegend	1:100

2.2.9. Quantitative real-time reverse-transcriptase polymerase chain reaction

RNA was isolated from sorted liver NPCs using a RNeasy® Micro Kit according to the manufacturer's instructions. RNA concentration was determined photometrically using a NanoDrop ND-1000.

A total of one µg of RNA was then transcribed into complementary DNA (cDNA) using the Verso cDNA Synthesis Kit according to the manufacturer's instructions and a My Cycler™ thermal cycler. The Verso cDNA Synthesis Kit uses anchored oligo dT primers to specifically transcribe mRNA.

Real-time quantitative PCR was performed using the ABsolute™ QPCR SYBR Green Mix. 1 µl of cDNA was added to the master mix containing ABsolute™QPCR SYBR Green Mix and the primer pair specific for the target gene at a final concentration of 0.7 mM. Primers were designed using Primer3 software for detection of exon overlapping amplicons and were purchased from Metabion International AG (see Table 2-8).

Melting curves of the PCR products were analyzed to confirm the specificity of the amplification. mRNA expression was calculated by normalizing the relative threshold cycle (CT) to the mitochondrial adenosine triphosphate synthase subunit (mATPsy) for

each sample. The relative quantification of sample mRNA content (2ΔCT) was then calculated as the x-fold change from the corresponding control sample content.

Table 2-8 List of primers
(T = annealing temperature, time = elongation time)

Gene	Sequence	T [°C]	time [s]
mATPsyn 5’	ATT GCC ATC TTG GGT ATG GA	60	12
mATPsyn 3’	AAT GGG TCC CAC CAT GTA GA	60	12
GranzymeB 5‘	GCC CAC AAC ATC AAA GAA CAG	60	12
GranzymeB 3‘	AAC CAG CCA CAT AGC ACA CAT	60	12

2.2.10. Statistics

Data were analysed using the GraphPad Prism 7 software. All of the data is presented as mean ± SEM. Group comparisons were performed using one-way ANOVA (P = 0.05) with a Tukey’s post-hoc test. Comparison between 2 groups were performed using non-parametric Mann-Whitney test or student’s t test depending on whether the sample showed normal distribution. A p value of less than 0.05 was considered statistically significant with the following ranges *$p< 0.05$, ** $p< 0.01$, *** $p< 0.001$ and **** $p< 0.0001$.

3. Results

3.1. CD4$^+$ T_{HC} are a heterogeneous population

Previous work has shown that murine hepatocytes, specifically those of regenerating mouse livers from an experimental model of Th1-mediated liver injury, were capable to induce immunoregulatory CD4$^+$ T cells *in vitro*. These cells had immunosuppressive capabilities and were characterized by the production of IL-10[72]. The aim of this study was to further investigate the characteristics and suppressive properties of hepatocyte-induced immunoregulatory CD4$^+$ T cells (CD4$^+$ T_{HC}).

3.1.1. CD4$^+$ T_{HC} are a heterogeneous population consisting of single and co-producers of IL-10 and IFNγ

To investigate the cytokine production of CD4$^+$ T_{HC}, splenic MACS-sorted CD4$^+$ T cells from WT mice were co-cultured for 48 h with hepatocytes isolated from WT mice and stimulated with anti-CD3 mAb. Splenic CD4$^+$ T cells in monoculture were used as control. Concentrations of IL-10 and IFNγ were determined in the culture supernatants by ELISA. A significant increase of IL-10 (Figure 3-1A) as well as IFNγ (Figure 3-1B) was detected in the supernatant of the co-cultures with CD4$^+$ T_{HC} compared to the control group.

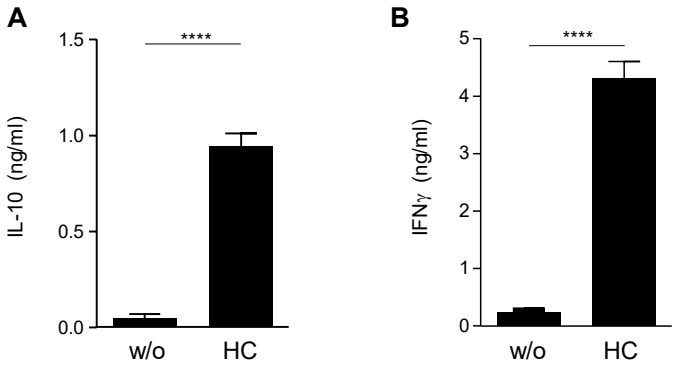


Figure 3-1. CD4$^+$T cells produce IL-10 and IFNγ when cultured with hepatocytes.
Splenic MACS-sorted CD4$^+$ T cells, from C57Bl/6 mice were cultured with hepatocytes (HC) in the presence of plate-bound anti-CD3 antibody for 48 h. After 48 h, the supernatant was collected, and IL-10 (A) and IFNγ (B) protein levels in the supernatant were determined via ELISA. **** $p \leq 0.0001$; w/o: without

The cells were then restimulated with ionomycin and PMA and analyzed by flow cytometry to determine the cytokine expression profile of the $CD4^+$ $T_{HC.}$

Figure 3-2 depicts a representative dot blot of the flow cytometry analysis showing an overall increase in the three subpopulations IL-$10^-IFN\gamma^+$, $IL10^+IFN\gamma^+$ and IL-$10^+IFN\gamma^-$ producing $CD4^+$ T_{HC}. Statistical analysis in Figure 3-2B reveals that each of the described subpopulations was significantly increased when $CD4^+$ T cells were co-cultured with hepatocytes.

Taken together, hepatocytes induced a significant increase in IL-$10^+IFN\gamma^-$, IL-$10^+IFN\gamma^+$, and IL-$10^-IFN\gamma^+$ producing $CD4^+$ T cells.

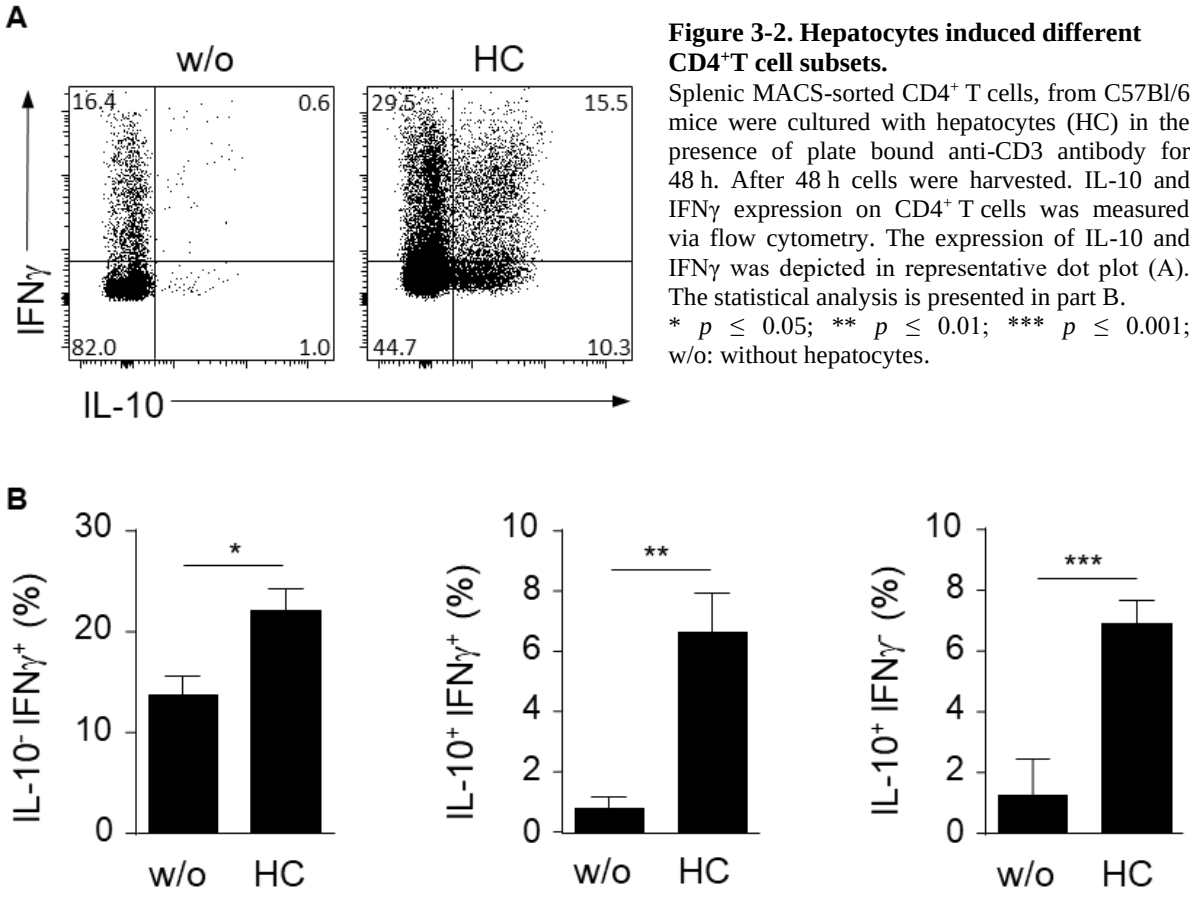


Figure 3-2. Hepatocytes induced different $CD4^+$T cell subsets.
Splenic MACS-sorted $CD4^+$ T cells, from C57Bl/6 mice were cultured with hepatocytes (HC) in the presence of plate bound anti-CD3 antibody for 48 h. After 48 h cells were harvested. IL-10 and IFNγ expression on $CD4^+$ T cells was measured via flow cytometry. The expression of IL-10 and IFNγ was depicted in representative dot plot (A). The statistical analysis is presented in part B.
* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; w/o: without hepatocytes.

3.1.2. $CD4^+$ T_{HC} suppressed activation and proliferation of naïve $CD4^+$ T cells independently of IL-10

Since hepatocytes induced both IL-$10^-$$IFN^+$ $CD4^+$ T cells and double positive IL-$10^+$$IFN^+$ $CD4^+$ T cells, the aim was to assess whether this is associated with any suppressive activity and to clarify whether IL-10 is the key mediator of immunosuppression in hepatocyte-induced $CD4^+$ T cells. A suppression assay comparing all different $CD4^+$ T cell subsets was not feasible due to the need for intracellular staining of IFNγ, respectively the lack of IL-10/IFNγ double reporter mice. Therefore, an *in vitro* suppression assay was first performed, comparing the IL-10^- and IL-10^+ hepatocyte-induced $CD4^+$ T cell populations.

Splenic MACS-sorted $CD4^+$ T cells from FIR x *tiger* mice were cultured with hepatocytes (48 h, with anti-CD3) and subsequently the $CD4^+$ T_{HC} were collected and sorted into IL-$10^-$$CD4^+$ T_{HC} and IL-10^+ $CD4^+$ T_{HC} by flow cytometry. These sorted T_{HCs} were cultured for an additional 72 h with CD45.1 expressing eFluor670 labeled $CD4^+CD25^-$ T cells (responder T cells, 1:1 ratio). As a control, splenic naïve T cells (not pre-cultured with hepatocytes) also derived from FIR x *tiger* mice were cultured with the responder T cells under the same conditions.

Responder cell proliferation was analyzed via flow cytometry. The results showed an immunosuppressive capacity of the IL-10^- $CD4^+$ and IL-10^+ $CD4^+$ T_{HC} as demonstrated by a decreased proliferation rate of the responder T cells. Representative histograms of the different responder T cell proliferation rates are shown in Figure 3-3A. In the control group, 11.9 % of the responder cells did not proliferate. In contrast, responder cells had a decreased proliferation rate when cultured with $CD4^+$ T_{HC}. Although the IL-10^+ $CD4^+$ T_{HC} subpopulation had a greater effect on the proliferation rate (80 % of the responder T cells did not proliferate), IL-10^- $CD4^+$ T_{HC} also caused a significant decrease in the proliferation rate (68% of the responder T cells did not proliferate).

Statistical evaluation of the immunosuppressive capacity of the $CD4^+$ T_{HC} subpopulations was performed by calculating the percentage of the inhibitory effect of the $CD4^+$ T_{HC} on responder T cells. Both, IL-10^+ $CD4^+$ T_{HC} and IL-10^- $CD4^+$ T_{HC} had high immunosuppressive properties, although it is it is clearly shown that hepatocyte-induced IL-10^+ $CD4^+$ T cells had a significantly higher capacity to suppress responder T cells *in vitro* (Figure 3-3B).

In addition to the suppressive properties of $CD4^+$ T_{HC} cells on T cell proliferation, their ability to inhibit the activation the responder T cells was analyzed. As a marker of T cell activation, the expression of CD25 on $CD4^+$ T cells was examined. Figure 3-3C shows representative histograms of the CD25 expression of the responder T cells.

The majority (94.7 %) of the responder T cells cultured with naïve $CD4^+$ T cells (control group) expressed CD25 on their surface. In contrast, CD25 expression of responder T cells were significantly reduced when cultured with IL-10^- $CD4^+$ T_{HC} or $IL10^+$ $CD4^+$ T_{HC} (31.7 % expressed CD25 in cells cultured with IL-10^- T_{HC} and 25.7 % when cultured with IL-10^+ T_{HC}). Similar to their inhibition of proliferation shown in Figure 3-3A,B, both $CD4^+$ T_{HC} subgroups significantly suppressed the CD25 expression of the responder T cells compared to the control group but in this case, there is no significant difference between the two subgroups (Figure 3-3D).

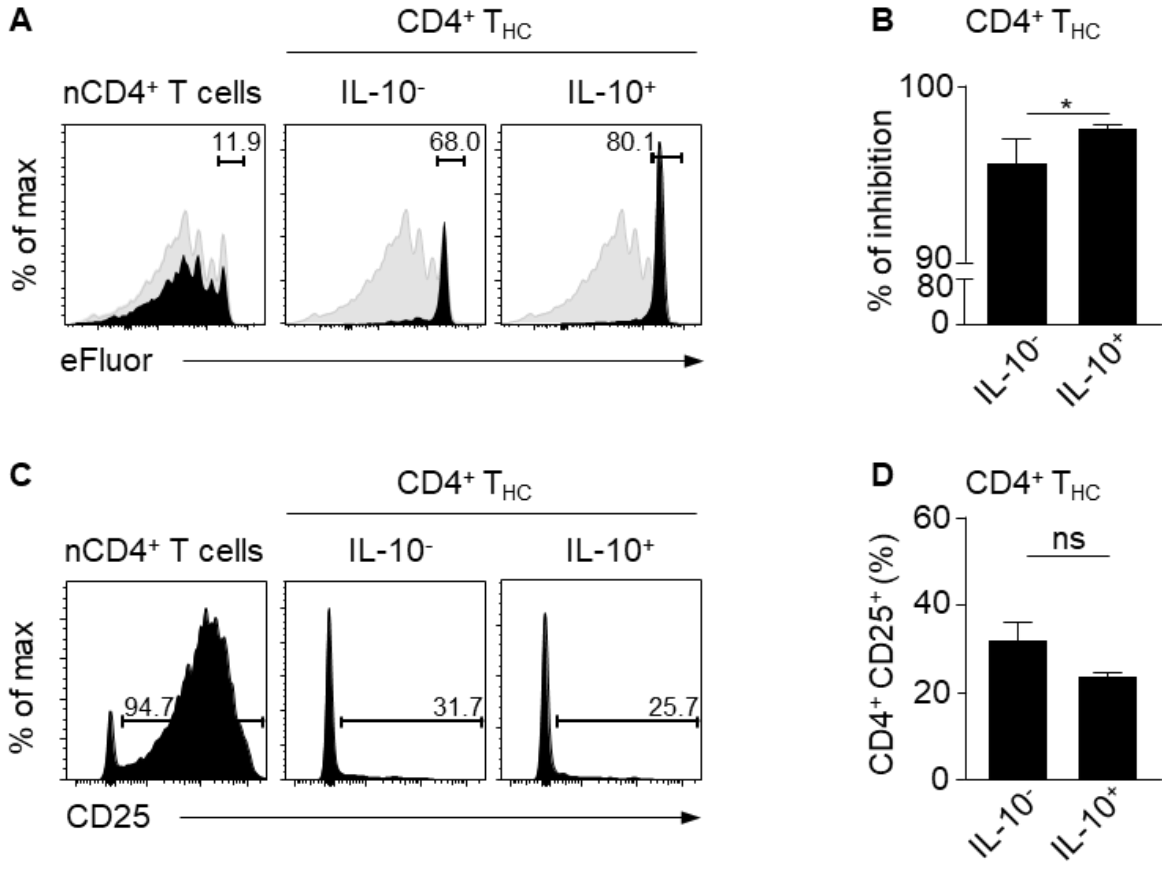


Figure 3-3. IL-10^+ and IL-10^- $CD4^+T_{HC}$ suppress activation and proliferation of responder T cells
Splenic MACS-sorted $CD4^+$ T cells isolated from FIR x *tiger* (foxp3-RFP and Il10-eGFP double reporter mice) mice were cultured with hepatocytes (HC) in the presence of plate bound anti-CD3 antibody for 48 h. After 48 h, cells were harvested and sorted into $IL10^+$ and $IL10^-$ cells via FACSAria. Cells were cultured with $CD4^+CD25^-$ eFluor670 labeled responder cells, isolated from CD45.1 transgenic mice, in the presence of anti-CD3/CD28 MicroBeads. After 72 h, the cells were harvested. Cells were analyzed by flow cytometry. Proliferation of the responder cells was analyzed by using the proliferation marker eFluor670 (A and B). The CD25 expression of the responder cells was measured via flow cytometry (C-D). * $p \leq 0.05$; ns: non-significant

Taken together, $CD4^+$ T_{HC} are immunosuppressive, and this effect is not exclusively mediated by IL-10 expression. Furthermore, the activation of the responder T cells is impaired when cultured with $CD4^+$ $T_{HC,}$ which was maintained even in the absence of IL-10 expression. The molecular mechanism responsible for this suppression is still elusive.

3.1.3. The suppressive capacity of $CD4^+$ T_{HC} *in vitro* is not dependent on IFNγ

Since it has been shown that the immunosuppressive capacity is independent of IL-10, the role of IFNγ was investigated using suppressor T cells from *IFNγ*$^{-/-}$ mice.

For this purpose, an *in vitro* suppression assay was performed with WT $CD4^+$ T_{HC} compared to *Ifnγ*$^{-/-}$ $CD4^+$ T_{HC}. Figure 3-4 shows the comparison between WT $CD4^+$ T_{HC} and *Ifnγ*$^{-/-}$ $CD4^+$ T_{HC} on the proliferation rate of responder T cells as well as their activation level, represented by CD25 expression. The representative data from the FACS staining and the corresponding statistical analysis show that $CD4^+$ T_{HC} from WT mice as well as $CD4^+$ T_{HC} from *Ifnγ*$^{-/-}$ mice had a high ability to inhibit the proliferation rate of responder T cells (Figure 3-4 C). Statistical analysis revealed a slight but significantly higher inhibition rate by *Ifnγ*$^{-/-}$ $CD4^+$ T_{HC} compared to WT $CD4^+$ T_{HC} (Figure 3-4 D).

Similar to the inhibition of proliferation shown in Figure 3-4 A, B, the expression of the activation marker CD25 is low in the suppression assay with both $CD4^+$ T_{HC} from WT- and *Ifnγ*$^{-/-}$ mice (Figure 3-4 C). However, WT $CD4^+$ T_{HC} showed a higher suppression of CD25 expression than *Ifnγ*$^{-/-}$ $CD4^+$ T_{HC} (Figure 3-4 D). The results implicate that IFNγ affects (directly or indirectly) the ability of $CD4^+$ T cells to inhibit proliferation and the expression of CD25 in responder T cells. However, both effects are not very pronounced, although they are significant.

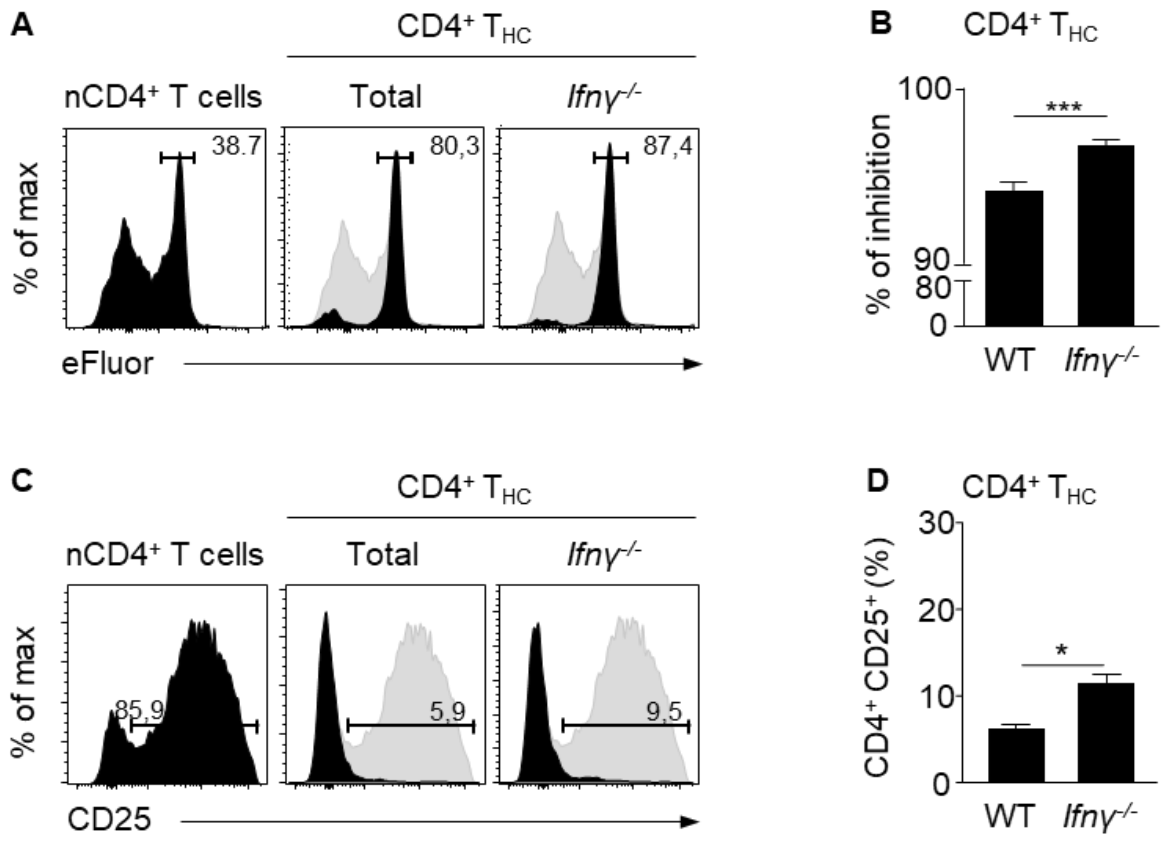


Figure 3-4. The suppressive capacity of CD4$^+$T$_{HC}$ *in vitro* is not dependent of IFNγ.
Splenic MACS-sorted CD4$^+$ T cells isolated from WT and *Ifnγ*$^{-/-}$ mice were cultured with hepatocytes (HC) in the presence of plate bound anti-CD3 antibody for 48 hours. After 48 hours, cells were harvested. Cells were cultured with CD4$^+$CD25$^-$ eFluor670 labeled responder cells, isolated from CD45.1 transgenic mice, in presence of anti-CD3/CD28 Microbeads. After 72 hours, the cells were harvested. The cells were analyzed by flow cytometry. Proliferation of the responder cells was analyzed by using the proliferation marker eFluor670 (A and B). The CD25 expression of the responder cells was measured via flow cytometry (C-D). * $p \leq 0.05$; *** $p \leq 0.001$

3.2. CD4$^+$ T$_{HC}$ showed a high expression of GzmB

3.2.1. HCs specifically induced GzmB expression in CD4$^+$ T cells

In order to analyze which molecular mechanisms are affected in CD4$^+$ T$_{HCs}$, an nCounter® gene expression assay with 562 target genes, was performed. Therefore, MACS-sorted WT splenic CD4$^+$ T cells were cultured with WT hepatocytes (48 h, with anti-CD3). To achieve higher purity, the cells were then sorted for CD4 by flow cytometry. CD4$^+$ T cells monocultured and sorted under the same conditions were used as a control. The miRNA expression was measured in three independent samples from CD4$^+$ T$_{HC}$ and the control group using the nCounter® Gene expression assay.

The heat map in Figure 3-5A shows the correlation of miRNA levels of $CD4^+$ T_{HC} compared to the control group. The most striking up-regulation was seen in the miRNA level of *GzmB* with a 47-fold higher expression level than in the control group of $CD4^+$ T cells cultured without hepatocytes.

In order to confirm the results of the nCounter® gene expression assay, the mRNA expression of *GzmB* in $CD4^+$ T with and without hepatocyte co-culture was measured by RT-PCR analysis. The result of the RT-PCR analysis showed a significant increase of *GzmB* mRNA in $CD4^+$ T_{HC} (24.31-fold) compared to the control group (Figure 3-5C). Although the increase is lower than that seen in the nCounter® gene expression assay, the results show the same strong upward trend consistent with the result of the nCounter® gene expression assay.

To determine whether the induction of GzmB production of $CD4^+$ T_{HC} was specific to hepatocytes and not a general response of $CD4^+$ T cells cultured with APCs, the influence of DCs on GzmB production by $CD4^+$ T cells was examined.

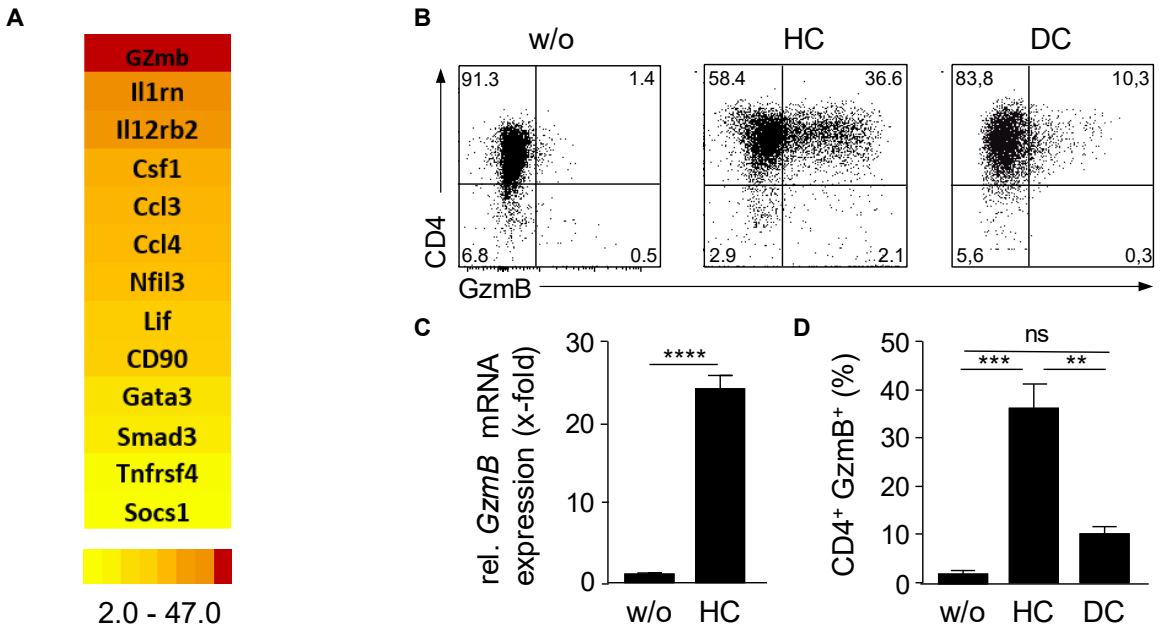


Figure 3-5. $CD4^+$T cells produce high levels of GzmB when cultured with hepatocytes.
Splenic WT MACS-sorted $CD4^+$ T cells were cultured with hepatocytes (HC) in the presence of plate-bound anti-CD3 antibody for 48 h and compared with $CD4^+$ T cells cultured in the absence of HCs. The cells from culture were resorted for $CD4^+$T cells via FACSAria and the mRNA expression profile was determined by the NanoString nCounter gene expression system (A). Furthermore rel. *GzmB* expression of $CD4^+$ T_{HC} in comparison to the control was measured via RT-PCR (B) and flow cytometry, shown by a representative dot blot (D). As a further control group $CD4^+$ T cells were cultured with DC under the same conditions and measured via flow cytometry (C).
** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$; ns: non-significant; w/o: without hepatocytes

For this purpose, $CD4^+$ T cells were cultured in the presence of hepatocytes, dendritic cells or in the absence of any other cell type as a negative control. After 48 h the GzmB expression was measured by flow cytometry.

$CD4^+$ T_{HC} were additionally analyzed for GzmB protein expression by flow cytometry. A distinct population of cells expressing GzmB (36.2 %) was detected in the $CD4^+$ $T_{HC,}$ which was largely absent in the cells cultured without hepatocytes (3.4 %) (Figure 3-5B). In Figure 3-5D, a representative dot plot of GzmB $CD4^+$ T cells and $CD4^+$ T_{HC} is depicted.

$CD4^+$ T cells cultured with DCs showed a non-significant increase of GzmB expression compared to the control group. However, the increase of GzmB expression was significantly more pronounced in $CD4^+$ T_{HC} (Figure 3-5D).

Taken together, $CD4^+$ T_{HC} expressed high levels of GzmB. In comparison to this finding, $CD4^+$ T cells expressed significantly less GzmB when they are cultured with DCs.

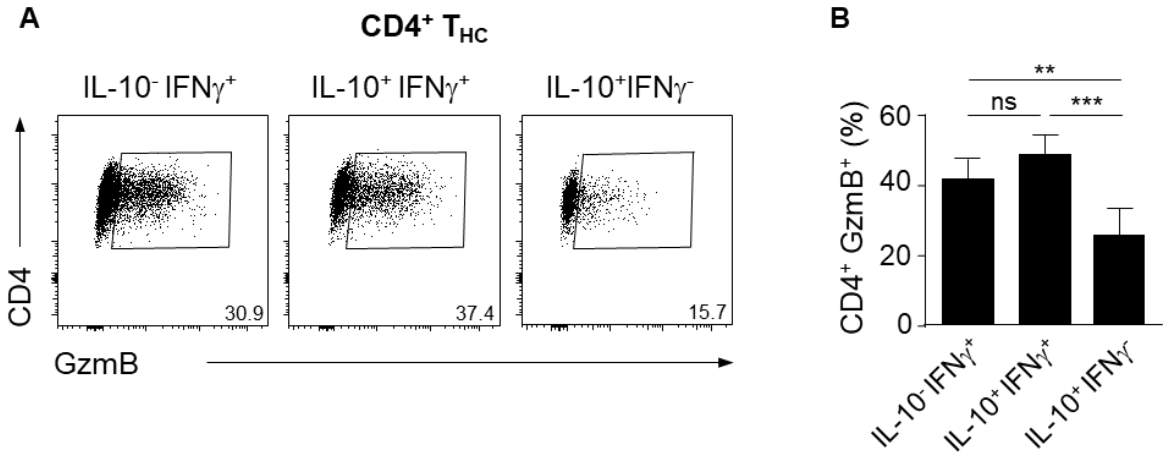
3.2.2. GzmB was predominantly expressed by IFNγ$^+$ CD4$^+$ T_{HC}

As shown in chapter 3.1, hepatocytes induce different subpopulations of $CD4^+$ T cells capable of immunosuppression. The subpopulations are characterized by the production of either IL-10 or IFNγ or both IL-10 and IFNγ.

Since $CD4^+$ T_{HC} were also shown to produce a high level of GzmB, the aim was to assign GzmB expression to the individual $CD4^+$ T_{HC} subpopulation. Therefore, the expression of GzmB by IL-10$^-$IFNγ$^+$ $CD4^+$ $T_{HC,}$ IL-10$^+$IFNγ$^+$ $CD4^+$ T_{HC} and IL-10$^+$ IFNγ$^-$ $CD4^+$ T_{HC} was analyzed by flow cytometry.

Figure 3-6A-B shows significantly increased amount of GzmB expressing cells in $CD4^+$ T_{HC} that also expressed IFNγ, either alone (41.2 %) or in combination with IL-10 (44.9 %), compared to IL-10 single positive $CD4^+$ T_{HC} (22.4 %).

Figure 3-6. IFNγ$^+$ CD4$^+$ T$_{HC}$ produce a significant higher amount of GzmB.
Splenic MACS-sorted CD4$^+$ T cells, from C57Bl/6 mice were cultured with hepatocytes (HC) in the presence of plate bound anti-CD3 antibody for 48 h. After 48 h, the cells were harvested. GzmB expression on the different subsets of CD4$^+$ T$_{HC}$ cells was measured via flow cytometry (A and B). ** $p \leq 0.01$; *** $p \leq 0.001$; ns: non- significant



3.2.3. GzmB induction in CD4$^+$ T$_{HC}$ occured independently of IFNγ

Having shown that the IFNγ-producing CD4$^+$ T$_{HC}$ express more GzmB, it was investigated whether the production of GzmB is dependent on the co-expression of IFNγ. Therefore, splenic CD4$^+$ T cells from both *IFN-γ*$^{-/-}$ and WT mice were cultured with WT hepatocytes (48 h, with anti-CD3).

Flow cytometry analysis confirmed the absence of IFNγ expression in the *IFNγ*$^{-/-}$ CD4$^+$ T$_{HC}$. GzmB production of IFNγ$^{-/-}$ CD4$^+$ T$_{HC}$ was only slightly reduced compared to that of WT CD4$^+$ T$_{HC}$ (Figure 3-7A). Statistical analysis showed no significant difference in GzmB production between *IFN-γ*$^{-/-}$ CD4$^+$ T$_{HC}$ and WT CD4$^+$ T$_{HC}$ (Figure 3-7).

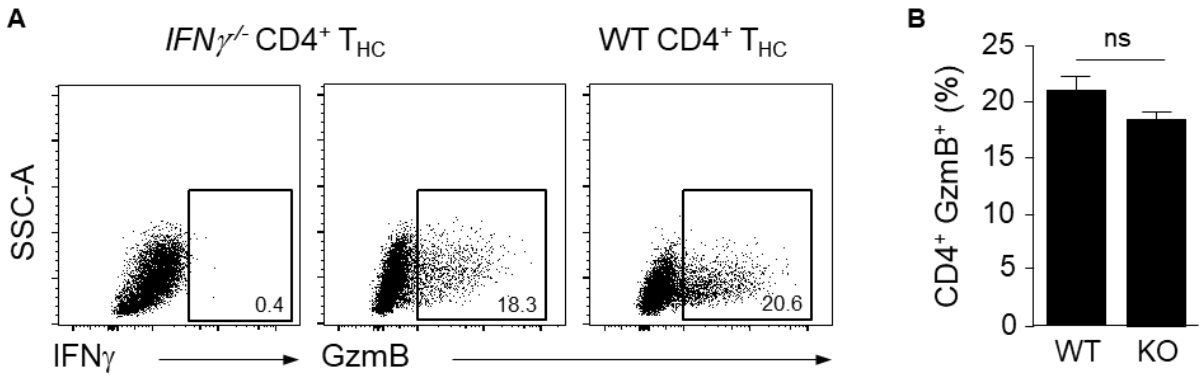


Figure 3-7. GzmB production in CD4$^+$ T_{HC} is not dependent on T cell derived IFNγ.
Splenic MACS-sorted CD4$^+$ T cells, from C57Bl/6 and *IFNγ*$^{-/-}$ mice were cultured with hepatocytes (HC) in the presence of plate bound anti-CD3 antibody for 48 h. After 48 h, the cells were harvested. The GzmB expression on CD4$^+$ T cells from C57Bl/6 and *IFNγ*$^{-/-}$ was measured via flow cytometry (A and B). ns: non-significant

In conclusion, the IFNγ$^+$ CD4$^+$ T_{HC} expressed higher levels of GzmB than the IFN-γ$^-$ CD4$^+$ T_{HC} in WT T_{HC}. Even though IFNγ and GzmB are co-expressed, the GzmB expression is not dependent on T cell derived IFNγ.

3.2.4. TGFβ abrogated whereas IL-12 further increased expression of GzmB in CD4$^+$ T_{HC}

Since GzmB production in CD4$^+$ T_{HC} is not dependent of T cell derived IFNγ, the effect of different polarization conditions on GzmB production in CD4$^+$ T_{HC} was investigated. For this purpose, either IL-12 or TGFβ was added to *in vitro* cultures of MACS-sorted splenic CD4$^+$ T cells with or without hepatocytes. Cultures without exogenous IL-12 or TGFβ were used as controls.

The potential role of TGFβ on GzmB production in CD4$^+$ T_{HC} was investigated, as it is known to promote the polarization of CD4$^+$ T cells into Foxp3$^+$ Tregs[73]. The results of the *in vitro* cultures with and without TGFβ are shown in Figure 3-8A-B. The representative flow cytometry blots (Figure 3-8A) and the corresponding statistical analysis (Figure 3-8B) show that the addition of TGF-β almost completely abolished the GzmB expression. This was a significant effect in both the monocultured CD4$^+$ T cells and the CD4$^+$ T_{HC}.

In contrast, the addition of the Th1-polarizing cytokine IL-12 had the opposite effect (Figure 3-8C-D). The GzmB production of the monocultured CD4$^+$ T cells was significantly increased in the presence of IL-12. Similarly, the significant increase of GzmB production of CD4$^+$ T_{HC} further increased in the presence of IL-12 (Figure 3-8).

Taken together, the data show that the production of GzmB by $CD4^+$ T cells is severely impaired in the presence of TGFβ and increased in the presence of IL-12.

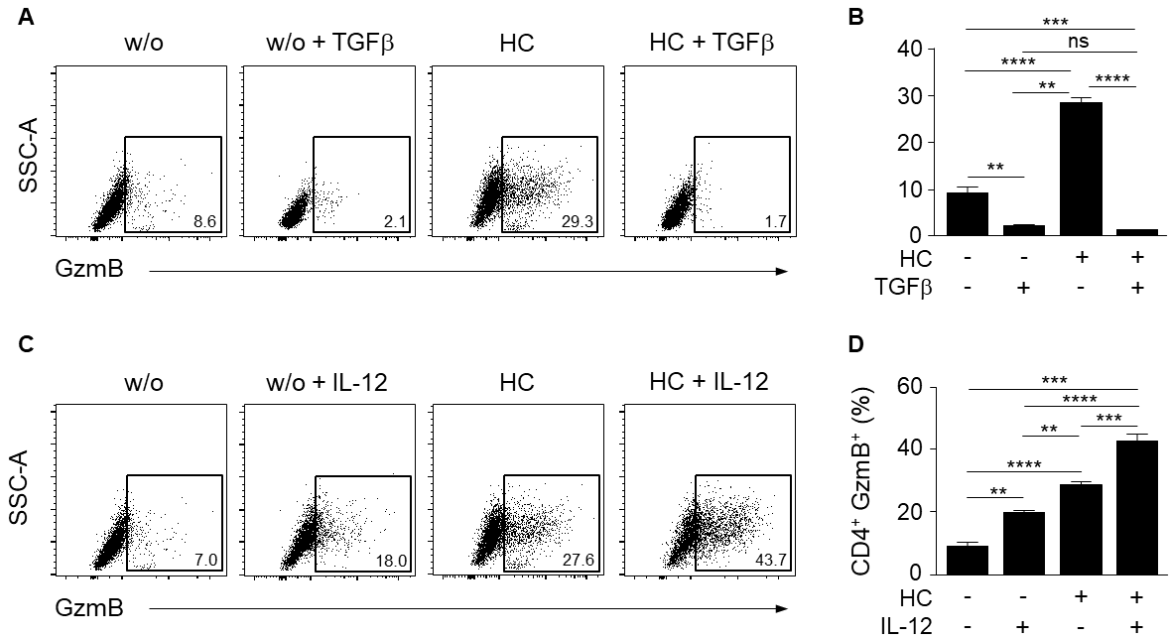


Figure 3-8. TGFβ abrogates, and IL-12 further increases GzmB production in CD4$^+$ T$_{HC}$.
Splenic MACS-sorted CD4$^+$ T cells, from C57Bl/6 mice were cultured with hepatocytes (HC) in the presence of plate bound anti-CD3 antibody for 48 h. In some cultures, TGFβ or IL-12 was added. After 48 h, the cells were harvested. The GzmB expression on CD4$^+$ T cells from the different cultures was measured via flow cytometry. The representative flow cytometry blots and the corresponding statistical analysis if adding TGFβ (A and B) or IL-12 (C and D) are presented. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$; ns: non- significant; w/o: without hepatocytes

3.2.5. Hepatocytes induced GzmB expression in CD4$^+$ T cells via the Notch pathway

GzmB expression in CD4$^+$ T$_{HC}$ was not dependent on T cell-derived IFNγ. Published data have shown that the expression of IL-10 in CD4$^+$ T$_{HC}$ is highly regulated by the Notch signaling pathway[72]. Therefore, it was investigated whether Notch signaling plays a similar role in the expression of GzmB in CD4$^+$ T$_{HC}$.

For this purpose, the Notch signaling inhibitor DAPT was added to the co-culture of CD4$^+$ T cells with hepatocytes (48 h, with anti-CD3). GzmB expression of the CD4$^+$ T$_{HC}$ cells with or without DAPT was measured by flow cytometry.

Representative flow cytometry blots and statistical analysis of GzmB expression in CD4$^+$ T$_{HC}$ are shown in Figure 3-9A-B. Only 6.9% of the CD4$^+$ T$_{HC}$ express GzmB when Notch signaling was abrogated, compared to 25.1% in the CD4$^+$ T$_{HC}$ with a functional Notch pathway.

This significant difference indicated a critical role for Notch signaling in the induction of GzmB expression in CD4$^+$ T$_{HC}$.

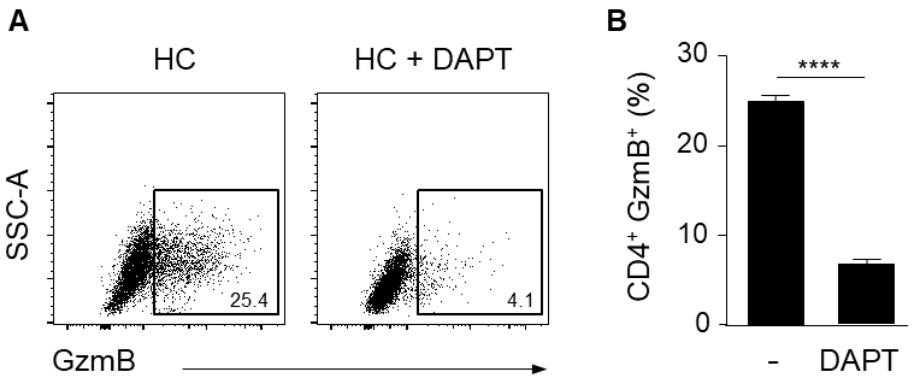


Figure 3-9. GzmB production in CD4$^+$T$_{HC}$ is dependent on Notch signaling.
Splenic MACS-sorted CD4+ T cells, from C57Bl/6 mice were cultured with hepatocytes (HC) in the presence of plate bound anti-CD3 antibody for 48 h. DAPT, a Notch signaling inhibitor, was added to half of the cultures. After 48 h, the cells were harvested. GzmB expression on CD4+ T cells was compared between the cultures with DAPT and without DAPT via flow cytometry (A and B). **** $p \leq 0.0001$

3.2.6. CD4$^+$ T$_{HC}$ did not suppress CD4$^+$ T cell activation and proliferation by expression of GzmB

Since the immunosuppressive mechanism of CD4$^+$ T$_{HC}$ cells remained elusive and GzmB was highly expressed by CD4$^+$ T$_{HC}$, the aim was to investigate whether GzmB played a critical role for their immunosuppressive capacity.

For this purpose, *in vitro* suppression assays were performed with either WT CD4$^+$ T$_{HC}$ treated with the GzmB inhibitor Z-AAD-CMK or *GzmB*$^{-/-}$ CD4$^+$ T$_{HC}$. In both cases, the suppressive capacity was compared to untreated WT CD4$^+$ T$_{HC}$.

Figure 3-10 shows the influence of the GzmB inhibitor Z-AAD-CMK on the proliferation rate of the responder T cells as well as their activity level, represented by CD25 expression. The representative data from the FACS staining and the corresponding statistical analysis showed that blocking GzmB expression did not affect the ability of CD4$^+$ T$_{HC}$ to inhibit the proliferation rate of responder T cells. Both cultures, with and without the GzmB inhibitor Z-AAD-CMK, strongly inhibited responder T cell proliferation (Figure 3-10A). CD25 expression of the responder T cells was also unaffected by the GzmB inhibitor (Figure 3-10B).

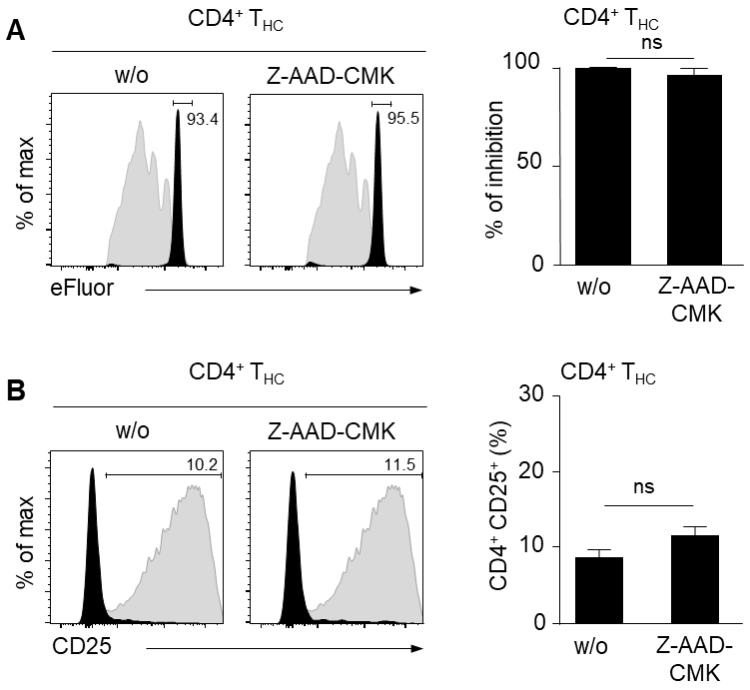


Figure 3-10. The suppressive capacity of CD4$^+$ T$_{HC}$ *in vitro* is not dependent on GzmB. Splenic MACS-sorted CD4$^+$ T cells isolated from C57Bl/6 mice were cultured with hepatocytes (HC) in the presence of plate bound anti-CD3 antibody for 48 h. After 48 h, cells were harvested and cultured with CD4$^+$CD25$^-$ eFluor670 labeled responder cells, isolated from CD45.1 transgenic mice, in the presence of anti-CD3/CD28 MicroBeads. In one half of the cultures Z-AAD-CMK was added to the cultures. After 72 h the proliferation and CD25 expression of the responder cells was analyzed via flow cytometry. The proliferation (A and D) and CD25 expression (C and D) of the responder cells cultured with CD4$^+$ T$_{HC}$ with or without Z-AAD-CMK was compared. ns: non-significant; w/o: without hepatocytes

Figure 3-11A shows the results of the comparison of suppression assays with WT CD4$^+$ T$_{HC}$ and *GzmB*$^{-/-}$ CD4$^+$ T$_{HC}$. The results were similar to the cultures with or without GzmB inhibitor. The proliferation rate of responder T cells and their activity level (CD25 expression) were not affected by the genetic ablation of *GzmB* (Figure 3-11B).

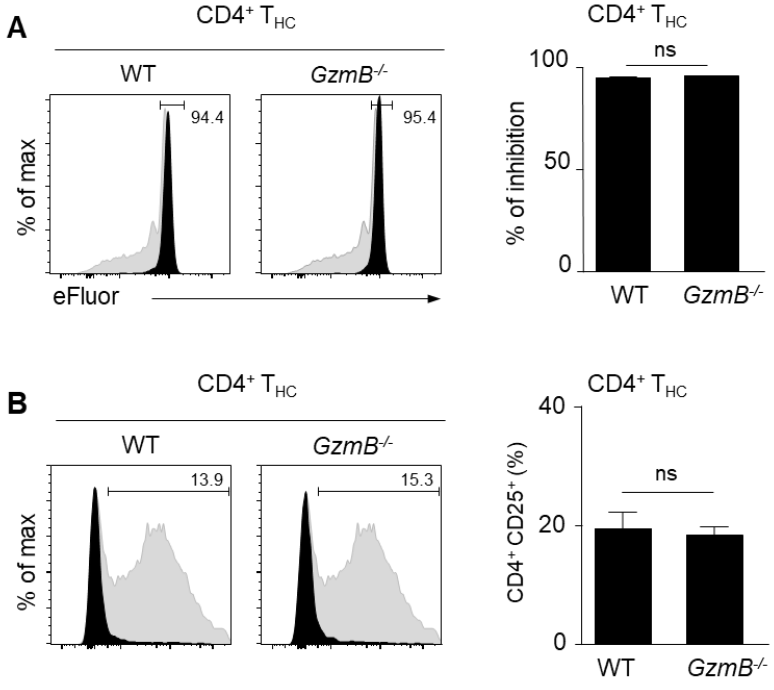


Figure 3-11. The suppressive capacity of CD4$^+$ T$_{HC}$ *in vitro* is not dependent on GzmB. Splenic MACS-sorted CD4$^+$ T cells isolated from C57Bl/6 mice and *GzmB*$^{-/-}$ mice were cultured with hepatocytes (HC) in the presence of plate bound anti-CD3 antibody for 48 h. After 48 h, CD4$^+$ T$_{HC}$ were cultured with CD4$^+$CD25$^-$ eFluor670 labeled responder cells, isolated from CD45.1 transgenic mice, in the presence of anti-CD3/CD28 MicroBeads. After 72 h the proliferation and CD25 expression of the responder cells was analyzed via flow cytometry. The proliferation (A and D) and CD25 expression (C and D) of the responder cells cultured with CD4$^+$ T$_{HC}$ from WT mice or *GzmB*$^{-/-}$ mice was compared. ns: non-significant; w/o: without hepatocytes

In conclusion, neither the GzmB inhibitor Z-AAD-CMK nor the use of $CD4^+$ T cells from *GzmB*$^{-/-}$ mice could abolish the immunosuppressive capacity of $CD4^+$ T_{HC}.

3.2.7. The suppressive effect of $CD4^+$ T_{HC} could not be overcome by exogenous IL-2

Having shown that the immunosuppressive mechanism of $CD4^+$ T_{HC} was independed of GzmB, and neither IL-10 nor IFNγ were key mediators, the exact mechanism was still elusive.

Since T cell activation is correlated with the presence of IL-2[18], another explanation for the limited activation of responder T cells would be a reduced supply of IL-2, possibly due to increased IL-2 consumption by $CD4^+$ T_{HC}. To analyze if the IL-2 concentration is reduced in co-cultures of responder T cells if cultured with $CD4^+$ T_{HC}, suppression assays were performed, and the supernatant was collected after 72 hours. IL-2 concentration was measured by ELISA. The results show a significant reduction of IL-2 concentrations in the supernatant in the presence of $CD4^+$ T_{HC} (Figure 3-12A).

If the lack of IL-2 would be responsible for the shown suppression of the responder cells, it should be overcome by the addition of exogenous IL-2 to the cultures.

In line with this hypothesis, suppression assays were performed with the addition of different amounts of exogenous IL-2 (0.3 µg/ml, 3.0 µg/ml, 30 µg/ml, 60 µg/ml). To verify that the added IL-2 was still in excess after 72 h of culture, the supernatant was collected, and the IL-2 concentration was measured by ELISA. The results showed that after 72 h (60 µg not shown), IL-2 was detectable in each supernatant collected. The concentrations of IL-2 in the supernatant correlated directly with the amount of IL-2 added, with the cultures treated with 0.3 µg IL-2 having the lowest and those treated with 30 µg IL-2 having the highest (above the measurable maximum) levels of IL-2 after 72 h. In all cases, a surplus was retained (Figure 3-12C). The effect of the exogenous IL-2 on the proliferation rate of the responder T cells was analyzed by flow cytometry (Figure 3-12B). In addition, the activity level of the responder T cells was again represented by the CD25 expression and measured by flow cytometry (Figure 3-12D).

It was shown that the inhibition of the responder T cells was slightly reduced in cultures supplemented with at least 3.0 µg/ml of exogenous IL-2. However, the percentage of inhibition remained above 90% in these cultures. Similar results were observed for CD25 expression. The CD25 expression of the responder T cells was increased with the

addition of at least 3.0 µg/ml of exogenous IL-2 but was still greatly reduced compared to the control group. Furthermore, CD25 expression was not further increased when 60 µg/ml

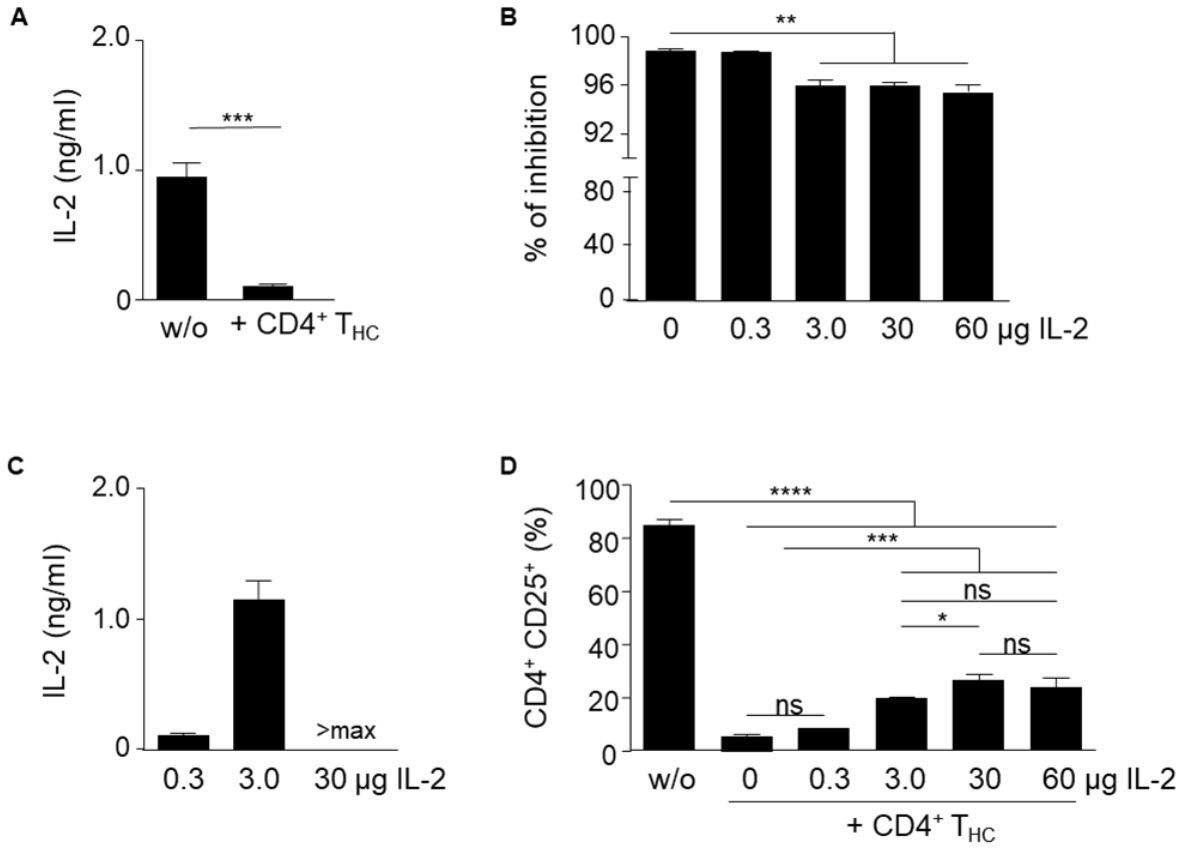


Figure 3-12. The suppressive capacity of $CD4^+$ T_{HC} cannot be overcome by the addition of IL-2.
Splenic MACS-sorted WT CD4+ T cells were cultured with hepatocytes (HC) in the presence of plate bound anti-CD3 antibody for 48 h. After 48 h, the cells were harvested. The $CD4^+$ T_{HC} were cultured with $CD4^+CD25^-$ eFluor670 labeled responder cells, isolated from CD45.1 transgenic mice, in presence of anti-CD3/CD28 MicroBeads. In addition, different amounts of IL-2 (none, 0.3 µg; 3 µg; 30 µg; 60 µg) were added to the cultures. After 72 h, the cells were harvested, and the supernatant was collected. The IL-2 level was determined in the different cultures by sandwich ELISA (A +C). Cells were analyzed by flow cytometry. Proliferation of the responder cells was analyzed by using the proliferation marker eFluor670 (B). Activation of the responder cells was analyzed by measuring the CD25 expression (D). * $p \leq 0.01$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.001$; ns: non-significant; w/o: without hepatocytes

IL-2 was added instead of 30 µg/ml.

Taken together, the results showed a reduced but still effective inhibition of responder T cell proliferation and activation by $CD4^+$ T_{HC}, leading to the conclusion that the suppressive effect of $CD4^+$ T_{HC} cannot be reversed by the addition of exogenous IL-2.

4. Discussion

The liver is endowed with distinct tolerogenic capabilities. However, the precise cellular and molecular mechanisms responsible for the liver's ability to induce tolerance are not fully understood. Functional studies have revealed remarkable properties of both non-professional and professional liver APCs. It's noteworthy that hepatocytes can also act as non-professional APCs, especially under inflammatory conditions, by engaging in direct cell-to-cell interactions with T lymphocytes, thereby contributing to T cell activation and immune regulation in the liver[80].

Hepatocytes have been shown to be capable of promoting $CD4^+$ T cell tolerogenicity, but the underlying molecular interactions remain to be elucidated[72].

The results presented in this study revealed the heterogeneity and functional characteristics of hepatocyte-induced immunoregulatory $CD4^+$ T cells ($CD4^+$ T_{HC}). The study further investigated the phenotype of $CD4^+$ T_{HC} and their potential immunosuppressive activities, focusing on the roles of IFNγ, IL-10, and GzmB. The following discussion provides a comprehensive interpretation of the results and their implications in the broader context of immunology.

4.1. Heterogeneous $CD4^+$ T cell population induced by hepatocytes

The results of the present study showed that hepatocytes induce the differentiation of $CD4^+$ T cells into immune suppressor cells under artificial conditions *in vitro*, emphasizing their immunosuppressive capabilities. In particular, $CD4^+$ T_{HC} were shown to produce large amounts of IL-10 and IFNγ, which was confirmed by ELISA. Detailed flow cytometry analysis revealed distinct $CD4^+$ T_{HC} subsets consisting of single and co-producers of IL-10 and IFNγ.

A suppression assay with the single and co-producers of IL-10 and IFNγ could not be performed since IFNγ must be stained intracellularly. Consequently, IFNγ cannot be used for the isolation of viable cells for a suppression assay. In addition, double reporter mice for IL-10 and IFNγ were not available. Despite these constraints, the particular effect of either cytokine on the immunosuppressive function of CD4 T_{HC} were analyzed in

suppression assays with either WT $CD4^+$ T_{HC} sorted for IL-10 expression or with *Ifnγ*$^{-/-}$ CD4 T_{HC}.

Inhibition of proliferation was consistently high in all cell populations analyzed, indicating that hepatocyte-induced $CD4^+$ T cells are highly suppressive (> 90 %). However, $CD4^+$ T_{HC} from *Ifnγ*$^{-/-}$ mice showed a slightly but significantly higher suppressive effect compared to WT $CD4^+$ T_{HC}. In contrast to the greater suppressive effect of *Ifnγ*$^{-/-}$ $CD4^+$ T_{HC}, the activation of responder $CD4^+$ T cells, as represented by the CD25 expression, is more impaired in the presence of IFNγ. This suggests a possible involvement of IFNγ in inhibiting $CD4^+$ T cell activation and thus may mediate a functional immunosuppressive effect.

Whether there was also a reduction in the total number of responder $CD4^+CD25^-$ T cells was not analyzed here. Therefore, it is possible that the $CD4^+$ T_{HC} induce cell death of naïve or activated responder T cells, a mechanism that needs to be further investigated.

While IL-10-producing cells are strongly associated with immunosuppressive activity, this cannot be applied to IFNγ-producing cells, which are a relevant part of $CD4^+$ T_{HC} subsets. However, there is evidence that $CD4^+$ T cells co-producing IL-10 and IFNγ do have a regulatory function. In a murine *T. gondii* infection model, $IL\text{-}10^+$ $IFN\gamma^+$ $CD4^+$ T cells are required to prevent early mortality from excessive inflammation[81]. Similar results have been reported in cutaneous models of *L. major* infection where it was shown that these cells are responsible for the maintenance of chronic, non-resolving infection. $IL\text{-}10^+$ and $IFN\gamma^+$ $Foxp3^-$ $CD4^+$ T cells (but not $IL\text{-}10^+Foxp3^+CD4^+$ T cells) suppressed the healing process in *L. major*-infected mice in an adoptive transfer experiment[82]. Induction of IL-10 in Th1 cells has been shown to contribute to self-limitation of the immune response and protection of the host from Th-1-associated pathology in models of infectious disease[81,82]. Furthermore, Neumann *et al*. showed in an *in vitro* culture system that LSECs induce high IL-10 expression in Th1 cells under inflammatory conditions without reducing IFNγ production. These IL-10-expressing Th1 cells suppress inflammatory T cell-induced immune responses in an IL-10-dependent manner[49]. In addition, previous research has shown that hepatocytes from regenerating livers of an experimental model of Th1-mediated liver injury (ConA) can induce $CD4^+$ T cells with immunosuppressive capabilities *in vitro*. These $CD4^+$ T cells, which are similar to the recently described IL-10-producing Th1 cells, were characterized by the co-expression of IL-10 and IFNγ, and the lack of Foxp3 expression[72].

In addition, there is another immunosuppressive $CD4^+$ T cell, the Tr1 cell, which has been described as a dual producer of IL-10 and IFNγ. However, the expression of the latter cytokine is not obligatory in Tr1 cells, which can also secrete other cytokines such as IL-5, IL-4, or TGF-β[83]. The induction of these cells by hepatocytes has not been studied so far, and to date, there is only one study showing that LSECs can induce autoreactive $CD4^+$ recent thymic emigrant lymphocytes into Tr1 cells[84].

Several suppressive mechanisms of Tr1 cells to inhibit effector cells in immune responses have been described. The main mechanism is cytokine-mediated, through the secretion of IL-10 and TGFβ[26]. In addition, it has been shown that mechanisms implying direct cell-cell communication are also involved[85,86]. These mechanisms represent further possibilities for immune regulation of these cells and should be further investigated. The exact phenotype of the different $CD4^+$ T_{HC} was not defined in this study and should be further investigated.

However, it was clearly shown that hepatocytes induce a highly suppressive phenotype especially $IL\text{-}10^+$ $CD4^+$ T_{HC} suggesting IL-10 is an immunosuppressive driver in this system. One possible mechanism for the induction of IL-10 in $CD4^+$ T cells was identified as the activation of the Notch signaling pathway in response to pro-inflammatory cytokines such as IL-12 or IL-27[87]. This pathway was also identified as the mechanism by which LSECs induce IL-10 expression in Th1 cells, since LSECs expressed high levels of the Notch ligands and induced the expression of the Notch target gene expression in Th1 cells. Furthermore, IL-10 induction in Th1 cells by LSECs was selectively inhibited by the blockade of Notch signaling. [49]. Burghardt *et al.* also demonstrated that hepatocytes-induced $IL\text{-}10^+$ $IFN\gamma^+$ $CD4^+$ T cells are generated upon activation of Notch signaling. They observed a significant increase of Notch1 receptor density in $CD4^+$ T cells co-cultured with hepatocytes from regenerating livers of a Th1 cell-mediated liver injury model (ConA mouse model). In addition, the secretion of IL-10 could be prevented by inhibiting the cleavage and thus the activation of Notch[72].

Besides the described suppressive phenotype of the $IL\text{-}10^+$ $CD4^+$ T_{HC}, this study also showed that $IL10^-$ $CD4^+$ T_{HC} were able to suppress proliferation and activation of responder $CD4^+CD25^-$ T cells. This implies that much of the immunosuppressive activity of $CD4^+$ T_{HC} *in vitro* is independent of IL-10 and requires alternative mechanisms of immunomodulation, possibly driven by other cytokines or regulatory pathways.

However, it is important to note, that IL-10 per se is known to play a critical role in immune tolerance and is the most widely studied anti-inflammatory cytokine. Numerous *in vivo* data also support this fact[82,88].

At present, it has to be acknowledged that the applied experimental design limited the insights gained into the diverse subsets of immunosuppressive cells. This should be addressed in future research.

Nevertheless, taken together, all investigated subsets of $CD4^+$ T_{HC} strongly inhibited the proliferation and activation of naive $CD4^+$ T cells. Both cytokines, IFNγ and IL-10 are involved, but none of them is indispensable for the suppressive mechanism. Other mediators that might play a role in the immunosuppressive effect of $CD4^+$ T_{HC} will be discussed in the following section and in the outlook.

4.2. $CD4^+$ T_{HC} expressed high amounts of GzmB

Subsequent analysis focused on the potential mechanisms underlying the suppressive effects of $CD4^+$ T_{HC}. The nCounter® gene expression assay, which analyzed 562 target genes, identified *GzmB*, as a gene of interest. *GzmB* expression was upregulated 47-fold in $CD4^+$ T_{HC} compared to $CD4^+$ T cells. The expression of no other gene in the panel was affected as strong as that of *GzmB* by the co-culture with hepatocytes. mRNA and flow cytometry analysis confirmed a significantly higher expression of *GzmB* in $CD4^+$ T cells induced by hepatocytes.

Several studies have shown that activated $CD4^+$ T cells express GzmB and a subset of these cells exhibit cytotoxic effects[89,90]. This GzmB-mediated cytolytic function of antigen specific $CD4^+$ T cells can be initiated in response to bacterial and viral infections[91,92]. Similarly, certain tumor infiltrating $CD4^+$ T cells exhibit GzmB-mediated cytotoxicity against tumor cells[93]. In addition, one strategy used by regulatory $CD4^+$ T cells in both humans and mice to modulate immune responses is to induce cell death via GzmB[55,57,94,95].

In this study, it was shown that GzmB expression varies between the $CD4^+$ T_{HC} subpopulations. The $IL10^+IFN\gamma^+$ $CD4^+$ T_{HC} as well as the $IL10^-$ $IFN\gamma^+$ $CD4^+$ T_{HC} expressed significantly more GzmB than the $IL10^+IFN\gamma^-$ $CD4^+$ T_{HC}. Higher expression in $IFN\gamma^+$ $CD4^+$ T cells is not unexpected, as GzmB levels have been shown to correlate positively with IFNγ levels[96].

Having shown that the IFNγ-producing $CD4^+$ T_{HC} had higher GzmB expression, it was investigated whether the production of GzmB is dependent on the co-expression of IFNγ.

The results of this work show that neither GzmB expression nor the immunosuppressive potential of $CD4^+$ T_{HC} is dependent on IFNγ.

Therefore, this apparent correlation between IFNγ and GzmB, does not imply a causal relationship. Whether the co-expression is the result of so far unknown upstream signaling events causing a simultaneous up-regulation of IFNγ and GzmB or whether IFNγ is up-regulated in the presence of higher GzmB levels in $CD4^+$ T_{HC} remains to be elucidated.

Thus far, this study has shown that GzmB is highly expressed in hepatocyte-induced $CD4^+$ T cells that have a suppressive effect on other $CD4^+$ T cells, but not in a T cell-derived IFNγ-dependent manner. Although IFNγ may be dispensable for the immunosuppressive potential of $CD4^+$ T_{HC}, the surprisingly high expression of GzmB in $CD4^+$ T_{HC} remains of particular interest in this study.

4.3. Different polarization conditions affected GzmB production in $CD4^+$ T_{HC}

The expression of granzymes may be one of the mechanisms by which Tregs are able to suppress the function of other immune cells, i.e. by killing them. Human Tregs have been shown to express granzyme A and/or B to kill various autologous immune cells in a perforin-dependent but FasL-independent manner [56]. In addition, Zhao *et al.* showed that activated Tregs inhibit the proliferation of B cells in a perforin and GzmB-dependent manner[97]. It has also been shown that TGFβ potentiates the induction of $Foxp3^+$ $CD4^+$ Tregs when co-cultured with hepatocytes[73].

The results of this study clearly showed that GzmB is less expressed on $CD4^+$ T cells in the presence of TGFβ, and even co-culture with hepatocytes could not counteract this effect. This is consistent with gene expression profiles of T cell activation *in vitro* which have shown that *GzmB* is downregulated in the presence of TGFβ[98]. Therefore, TGFβ negatively regulates the expressing of GzmB, implying that Tr1 cells which are known to induce their immunosuppressive activity via IL-10 and TGFβ (see 4.1), are not the responsible cell type.

IL-12 is involved in the differentiation of naïve T cells into Th1 cells[99]. Furthermore, it has been demonstrated that IL-12 stimulates the production of IFNγ and TNFα by T cells

and NK cells[100]. By adding IL-12 to $CD4^+$ T cell cultures with or without hepatocytes, we detected an increase in the expression of GzmB in these cells. This IL-12-induced GzmB expression was also detected in several cell types, including cytotoxic T cells and NK cells[101]. While in these cells GzmB induction was associated with an increase in cytotoxic and inflammatory potential, these results show a similar association in CD4 cells with an immunosuppressive phenotype. This result highlights a Th1-like phenotype that induces immunosuppressive effects.

Overall, the complex regulatory network influencing $CD4^+$ T_{HC} functions and their plasticity under different cytokine environments is still the focus of several research and will further shed light on these results.

4.4. Notch signaling might play a role in the induction of GzmB expression in $CD4^+$ T_{HC}

Together, this study identified high GzmB expression in $CD4^+$ T_{HC} that could be enhanced by the addition of IL-12. Notch signaling might be a mechanism in the induction of GzmB expression in $CD4^+$ T_{HC}.

The importance of Notch signaling was discussed in section 4.1. In addition, it has also been shown that Notch signaling regulates IL-12 expression via interferon regulatory factor 8[102]. Furthermore, a role of Notch pathway in inducing IL-10, IFNγ, GzmB, as well as IL-22 which lead to increased proliferation was shown in T cells expressing chimeric antigen receptors[103]. Therefore, given that GzmB expression in $CD4^+$ T cells is upregulated by Notch signaling[104] and that $CD4^+$ T_{HC} are dependent on Notch signaling[72], the study investigated whether Notch signaling also plays a role in GzmB expression in $CD4^+$ T_{HC}. The use of a Notch signaling inhibitor in the co-culture of hepatocytes and $CD4^+$ T cells resulted in a substantial decrease in GzmB expression in $CD4^+$ T_{HC}, implying its involvement in the molecular mechanism governing GzmB production in $CD4^+$ T_{HC}. In accordance with Burghardt *et al.*, this leads to the conclusion that $CD4^+T_{HC}$ have a similar phenotype to previously described IL-10 producing Th1 cells[87]. In line with this, our data have shown that GzmB production can be enhanced by creating a Th1 polarizing environment.

4.5. Role of GzmB in immunosuppressive capacity of $CD4^+$ T_{HC}

Since a suppressive phenotype was observed for both $IL10^+$ $CD4^+$ T_{HC} as well as $IL10^-$ $CD4^+$ T_{HC} and GzmB was also highly expressed in these cells, a possible suppressive mechanism of GzmB should be considered.

In an infectious disease model, IFN-γ/IL-10-producing $CD4^+$ T cells induced by herpes simplex virus plasmacytoid DCs (pDCs) acquire anergic and regulatory properties through the action of pDC-derived type I IFNs and T cell-derived IL-10[95]. Furthermore, it was demonstrated that a major bacterial virulence factor directly induces IL-10 secreting/GzmB-expressing T cells with a Tr1-like phenotype and immunosuppressive properties[94]. In addition, the study by Gondek *et. al.* identified GzmB as one of the key components of Treg-mediated suppression. Their study demonstrated that the induction of regulatory activity coincides with the upregulation of GzmB expression. The functional relevance of GzmB in contact-mediated suppression by Tregs was substantiated by the observed reduction in the suppressive capacity of Tregs from *GzmB*$^{-/-}$ mice compared to those from WT mice. This finding leads to the hypothesis that GzmB plays a critical role in the suppression of responder CD4 T cells by $CD4^+T_{HC}$.

In contrast, in this study, despite the high expression of GzmB in $CD4^+$ T_{HC}, inhibition of GzmB or the absence of GzmB did not abrogate the immunosuppressive effect of $CD4^+$ T_{HC} on responder T cells. This suggests that other mechanisms beyond GzmB contribute to the suppressive capacity of $CD4^+$ T_{HC}.

4.6. Role of IL-2 in co-culture with $CD4^+T_{HC}$

IL-2 is a pivotal cytokine for T cell activation and proliferation and is produced by naïve T cells in a primary response[105] . Our data revealed lower IL-2 levels in co-cultures of activated responder T cells indicating that the differentiated $CD4^+$ T_{HC} consummated the IL-2 in the cultures[106].

Since the results showed a consumption of IL-2, this leads to the hypothesis that this might be a potential way to inhibit the activation and suppression of responder T cells.

However, the addition of exogenous IL-2 did not completely reverse the suppressive effect of $CD4^+$ T_{HC}.

Taken together, the results suggest that while IL-2 availability may partially contribute to the suppressive mechanism of $CD4^+ T_{HC}$, the observed immunosuppression is likely to be multifactorial and involves mechanisms beyond IL-2 consumption. This highlights the complexity of the interactions between $CD4^+ T_{HC}$ and responder T cells, potentially involving various regulatory molecules and pathways.

In conclusion, this study improved our understanding of hepatocyte-induced immunoregulatory $CD4^+$ T cells and their immunosuppressive capabilities. The possibly multifactorial nature of $CD4^+ T_{HC}$ immunomodulation suggests that their impact on immune responses goes beyond individual cytokines or cytotoxic molecules. This research sets the stage for future investigations into the detailed mechanisms governing the suppressive effects of $CD4^+ T_{HC}$, contributing to the broader understanding of immune regulation and paving the way for potential therapeutic applications.

5. Outlook

This study sheds light on the heterogeneity and functional characteristics of hepatocyte-induced immunoregulatory $CD4^+$ T cells ($CD4^+$ T_{HC}) and their potential mode of action.

Based on the findings and insights presented in this study, several avenues for further research could expand our understanding of the cellular and molecular mechanisms underlying the immunosuppressive capabilities of $CD4^+$ T_{HC} and their role in immune regulation and tolerance induction. The study indicates the presence of heterogeneous $CD4^+$ T_{HC} subsets, including those that co-produce IL-10 and IFNγ, and express GzmB.

Further investigation is needed to elucidate the precise phenotypic markers and functional characteristics of these subpopulations. This could include advanced flow cytometry techniques, single cell RNA sequencing, and proteomic analyses to unravel the distinct roles and regulatory functions of these subpopulations. Defining the molecular signatures and regulatory pathways associated with these subsets could provide a deeper understanding of their immunoregulatory mechanism.

For example, $CD4^+$ T cells that secrete both IL-10 and IFNγ might be both Th1 and Tr1. This study favors a Th1 – like phenotype.

However, for a clearer picture, Th1 can be distinguished from other $CD3^+CD4^+CD8^-$ T cells based on the cell surface expression of IL-12 R beta 2, IL-27 R alpha/WSX-1, IFNγ R2, IL-18 R, CCR5, and CXCR3. In addition, the expression of the transcriptional regulators STAT4 and T-bet, the latter of which is considered to be the master transcriptional regulator required for Th1 cell development, can be analyzed[27].

For Tr1 cells, many candidate surface molecules have been identified, in particular the co-expression of CD49b and LAG-3[107]. However, no lineage-specific biomarkers have been identified. In addition, Tr1 cells have a unique cytokine expression profile, $IL\text{-}10^{++}TGF\beta^+IFN\text{-}\gamma^+IL\text{-}5^+IL\text{-}4^-IL\text{-}2^{low/neg}$[108] . Mechanisms by which Tr1 cells inhibit effector cells include the secretion of the anti-inflammatory mediators IL-10 and TGFβ[26], and Tr1 cells have been shown to secrete cytolytic vesicles containing GzmB and perforin to inhibit effector T cells. Suppressive activity and GzmB expression have already been demonstrated, but perforin expression should be analyzed in further experiments, as well as other mechanism by which Tr1 may suppress other effector cells. These include receptor analysis of CTLA-4 and PD-1[85], indicating contact-dependent inhibition, as well as CD39

and CD73, which have been shown to produce adenosine, thereby increasing intracellular cAMP concentrations and disrupting effector T cell metabolism[86].

Irrespective of the precise phenotype of $CD4^+$ T_{HC}, it should be investigated whether $CD4^+$ T_{HC} exert their suppressive effects via direct cell-cell interactions, secretion of soluble factors, or a combination of both.

GzmB expression was found to be high in $CD4^+$ T_{HC}, but it did not directly contribute to the immunosuppressive function of these cells. Hence, elucidating the function of GzmB in $CD4^+$ T_{HC} could be a focus of future research. This could include to investigate whether GzmB-mediated cell death plays a role in dampening responder T cell activation and how it synergizes with other immunoregulatory factors. Therefore, *in vitro* cytotoxicity assays should be performed, to analyze a possible cytotoxic activity of $CD4^+$ T_{HC}. Furthermore, phenotypic analysis of T cells for eomesodermin, perforin, and CD107 could further clarify whether the effect is mediated by the potential cytotoxic properties of $CD4^+$ T_{HC}.

The induction of apoptosis by $CD4^+$ T_{HC} on target cells should be considered. $CD4^+$ T cells exert their cytotoxic effect through the expression of GzmB and/or Fas ligand (FasL)[109]. Like GzmB, FasL, after binding to its receptor Fas on target cells, activates caspase-3 and induces apoptosis in these cells. Since the number of responder $CD4^+$ T cells was not examined in this study, it is possible that the $CD4^+$ T_{HC} induced apoptosis in the co-cultures. To verify this, apoptosis could be measured in the responder $CD4^+$ T cells using annexin V/propidium iodide staining.

The interaction between $CD4^+$ T_{HC} and responder T cells was the main subject of this study. However, the liver is a complex immunological organ with multiple immune cell types interacting with each other. A more comprehensive view of the immunoregulatory role of $CD4^+$ T_{HC} could be obtained by studying the interaction between $CD4^+$ T_{HC} and other immune cells, such as $CD8^+$ T cells, B cells, NK cells and myeloid cells, by using different co-culture setups.

While this study focuses on *in vitro* findings, translating these observations to *in vivo* models is crucial for validating the relevance of $CD4^+$ T_{HC} in real-world immune responses. Animal models and translational studies could help to determine the significance of $CD4^+$ T_{HC} in various physiological and pathological contexts, shedding light on their potential contributions to immune homeostasis and disease progression. A possible first approach would be to adoptively transfer different subpopulations of $CD4^+$ T_{HC} into WT mice. The mice will then be injected with ConA, a model for a Th1-mediated liver injury. In the ConA model, IL-10 was shown to play a key anti-

inflammatory role by reducing ConA-induced liver injury, most likely through downregulation of IFNγ and TNFα. Using *Il-10* knockout mice and anti-IL10R-treated mice, a more pronounced hepatitis was observed upon ConA challenge[110,111]. Whether and which of the adoptively transferred subpopulations have a protective effect in this model would be a first *in vivo* experiment.

New integrative multiple omics datasets and the use of systems biology approaches may help to construct comprehensive regulatory networks involving cytokines, signaling pathways, and cellular interactions. This holistic perspective may reveal the interconnected mechanisms that orchestrate $CD4^+$ T_{HC} mediated immune regulation.

In summary, by addressing the outlined research direction, future investigations could unravel the molecular features of these $CD4^+$ T_{HC} cells that mediate immunosuppression and may help to advance our understanding of liver immunology.

6. Summary

The liver is the largest metabolic organ in the body and performs numerous metabolic functions. Among other things, nutrients absorbed from the intestine are transported via the portal vein to the liver for further processing. In addition to nutrients, potential antigens and toxins continuously enter the liver via the portal vein. The liver needs a tolerogenic environment to avoid a permanent inflammatory response. To create this milieu, numerous professional and non-professional immune cells interact in the liver.

Burghardt *et al.* were able to show in *in vitro* experiments with murine cells that hepatocytes, the parenchymal cells of the liver, interact with $CD4^+$ T cells and generate an immunosuppressive IL-10 -and IFNγ-producing phenotype.

This thesis focuses on the further characterization and functional properties of hepatocyte induced $CD4^+$ T cells ($CD4^+$ T_{HC}). For this purpose, co-cultures of hepatocytes and naïve $CD4^+$ T cells from different mouse models as well as suppression assays with $CD4^+$ T_{HC} and responder T cells were analyzed.

Hepatocytes were shown to induce different $CD4^+$ T_{HC} phenotypes consisting of single and co-producers of IL-10 and IFNγ. Both populations, $IL\text{-}10^+$ $CD4^+$ T_{HC} and $IL\text{-}10^-$ $CD4^+$ T_{HC}, were shown to inhibit the proliferation and activation of naïve $CD4^+$T cells. The ability to inhibit proliferation and activation of T cells was also shown for $CD4^+$ T_{HC} from *Ifnγ*$^{-/-}$ mice. This led to the conclusion that neither IL-10 nor IFNγ play a key role in the immunosuppressive abilities of $CD4^+$ T_{HC}.

To further analyze the gene expression of $CD4^+$ T_{HC}, the cells were analyzed using Nanostring Technologies' nCounter® system. In particular, GzmB was significantly higher expressed in the $CD4^+$ T_{HC} than in the control group. This led to the hypothesis that GzmB may play an important role in the immunosuppressive capabilities of $CD4^+$ T_{HC} and was therefore further analyzed.

GzmB production by $CD4^+$ T_{HC} is highest in IFNγ-expressing cells. However, its production was not dependent on IFNγ as its expression was unchanged in *Ifnγ*$^{-/-}$ mice. In addition, GzmB expression was enhanced under Th1 polarizing conditions and almost eliminated under Th2 polarizing conditions. It was shown by Burghardt *et al.* that the Notch signaling pathway is critical for the induction of $CD4^+$ T_{HC}, and this could also be demonstrated for GzmB expression in this study.

In order to test whether GzmB plays a key role in the immunosuppressive effect of $CD4^+$ T_{HC}, suppression assays were performed with *Gzmb*$^{-/-}$ mice and a GzmB inhibitor. Contrary to the hypothesis, inhibition of GzmB or the use of knockout mice failed to abrogate the inhibitory effect of $CD4_+$ T_{HC}.

Furthermore, the data showed that $CD4^+$ T_{HC} in co-culture with naïve T cells consumed IL-2 and thus IL-2 was not available to the naïve T cells. Externally added IL-2 increased proliferation and activation of $CD4^+$ T cells in suppression assays but could not abolish the suppressive effect of $CD4^+$ T_{HC}.

In summary, this thesis impressively demonstrated that the different subpopulations of $CD4^+$ T_{HC} were highly suppressive. As possible immunosuppressive mechanisms, the involvement of the cytokines IL-10 and IFNγ as well as the relationship with the expression of GzmB were investigated.

However, neither IL-10, IFNγ nor GzmB play a key role in the immunosuppressive effect of $CD4^+$ T_{HC} in vitro.

Further studies are needed to determine the differentiation and function of the $CD4^+$ T_{HC} in more detail. This includes the analysis of additional surface markers to differentiate the cell type more precisely. In addition, the immunosuppressive mechanism remains to be elucidated. Because of the high expression of GzmB, testing whether $CD4^+$ T_{HC} can induce cell death should be part of further investigations. Another important step would be to transfer the $CD4^+$ T_{HC} populations into an in vivo model, e.g. by transferring them into mice that subsequently receive concanavalin A, which leads to Th1-induced liver injury.

Although further studies are needed, this work demonstrates the influence of liver parenchymal cells on the generation of the specific immune milieu. These results may contribute to a better understanding of the restoration of immune homeostasis and the control of immune-mediated liver diseases.

7. Deutschsprachige Zusammenfassung

Die Leber ist das größte Stoffwechselorgan des Körpers mit zahlreichen metabolischen Funktionen. Unter anderem werden die aufgenommenen Nährstoffe des Darmes über die Portalvene in die Leber transportiert und dort weiterverarbeitet. Mit den Nährstoffen über die Portalvene kommen auch kontinuierlich potentielle Antigene und Toxine in die Leber. Hierfür benötigt die Leber ein tolerogenes Milieu zur Vermeidung einer permanenten Entzündungsreaktion. Um dieses Milieu zu erzeugen, sind in der Leber zahlreiche professionelle und nicht professionelle Immunzellen lokalisiert, welche miteinander interagieren. Burghardt *et al.* konnten in *in vitro* Experimenten mit murinen Zellen zeigen, dass Hepatozyten, die Parenchymzellen der Leber, mit $CD4^+$ T Zellen interagieren und ein immunosuppressiver IL-10- und IFNγ produzierender Phänotyp entsteht.

In dieser Dissertationsschrift geht es um die weitere Charakterisierung und funktionelle Eigenschaften der durch Hepatozyten induzierten $CD4^+$ T Zellen ($CD4^+$ T_{HC}). Hierfür wurden Ko-Kulturen aus Hepatozyten und $CD4^+$ T Zellen aus verschiedenen Maus Modellen sowie Suppressionsassays mit $CD4^+$ T_{HC} und Responder-T Zellen analysiert.

Es konnte gezeigt werden, dass durch Hepatozyten verschiedene $CD4^+$ T_{HC} Phänotypen, bestehend aus Einzel- und Koproduzenten von IL-10 und IFNγ induziert werden. Mittels Auftrennung nach IL-10^+ und IL-10^- $CD4^+$ T_{HC} wurde dargestellt, dass beide Populationen die Proliferation und Aktivierung von naiven $CD4^+$ T-Zellen inhibieren. Die Fähigkeit Proliferation und Aktivierung von T-Zellen inhibieren traf ebenfalls auf $CD4^+$ T_{HC} aus *Ifnγ*$^{-/-}$-Mäusen zu. Dies führte zur Schlussfolgerung, dass weder IL-10 noch IFNγ eine Schlüsselrolle für die immunsuppressiven Fähigkeiten der $CD4^+$ T_{HC} spielen.

Um die $CD4^+$ T_{HC} weiter zu analysieren wurde die Gen Expression der Zellen mittels Nanostring Technologies' nCounter® system analysiert. Hierin konnte gezeigt werden, dass insbesondere *GzmB* in den $CD4^+$ T_{HC} deutlich höher exprimiert wurde als in der Kontrollgruppe. Dies führte zur Hypothese, dass GzmB möglicherweis eine wichtige Rolle für die immunsuppressiven Fähigkeiten der $CD4^+$ T_{HC} einnimmt.

Die GzmB Produktion der $CD4^+$ T_{HC} ist am höchsten in den IFNγ exprimierenden Zellen, jedoch ist die Produktion nicht abhängig von IFNγ da die Expression von GzmB in *Ifnγ*$^{-/-}$-Mäusen unverändert blieb. Zusätzlich wurde die Expression von GzmB unter Th1

polarisierenden Bedingungen verstärkt und unter Th2 polarisierenden Bedingungen nahezu eliminiert. Bereits Burghardt *et al.* zeigte, dass der Notch Signalweg entscheidend für die Induktion von $CD4^+$ T_{HC} war. Dies konnte in dieser Arbeit für die GzmB Expression ebenfalls gezeigt werden.

Zur Überprüfung ob GzmB eine Schlüsselrolle für die immunsupressive Wirkung von $CD4^+$ T_{HC} spielt, wurden Suppressionsassays mit *Gzmb*$^{-/-}$ Mäusen und mit einem GzmB Inhibitor durchgeführt. Entgegen der Hypothese zeigten jedoch die Inhibition oder die Abwesenheit von GzmB keinen Unterschied auf den inhibitorischen Effekt der $CD4^+$ T_{HC}.

Darüber hinaus zeigten die Daten, dass $CD4^+$ T_{HC} in Ko-Kultur mit naiven T-Zellen IL-2 verbrauchen und es somit den naiven T-Zellen nicht zur Verfügung steht. Extern hinzugefügtes IL-2 führte zwar zu einer verstärkten Proliferation und Aktivierung von $CD4^+$ T-Zellen im Suppressionsassay, konnte aber den suppressiven Effekt der $CD4^+$ T_{HC} nicht aufheben.

Zusammenfassend stellte diese Arbeit eindrücklich dar, dass die durch Hepatoyzten induzierten IL-10 und/ oder IFNγ produzieren Subpopulationen suppressiv wirken. Als mögliche immunsuppressive Mechanismen wurde die Beteiligung der Zytokine IL-10 und IFNγ als auch der Zusammenhang mit der Expression von GzmB untersucht.

Die Ergebnisse zeigten, dass weder IL-10, IFNγ noch GzmB *in vitro* eine Schlüsselrolle bei der immunsuppressiven Wirkung der $CD4^+$ T_{HC} einnehmen.

Weitere Studien sind erforderlich, um die Differenzierung und Funktion der $CD4^+$ T_{HC} genauer zu bestimmen. Dazu gehört die Analyse weiterer Oberflächenmarker zur genaueren Differenzierung des Zelltyps. Darüber hinaus ist der immunsuppressive Mechanismus noch nicht geklärt.

Aufgrund der hohen Expression von GzmB sollte im Rahmen weiterer Untersuchungen geprüft werden, ob $CD4^+$ T_{HC} den Zelltod induzieren können. Ein weiterer wichtiger Schritt wäre die Übertragung der $CD4^+$ T_{HC} Populationen in ein *in vivo* Modell, z.B. durch adoptiven Transfer in Mäuse, die anschließend Concanavalin A erhalten, welches zu einer Th1 induzierten Leberschädigung führt.

Auch wenn weitere Studien erforderlich sind konnte der Einfluss von Leberparenchymzellen auf die Entwicklung des spezifischen Immunmilieus der Leber gezeigt werden. Diese Ergebnisse können zu einem besseren Verständnis der Wiederherstellung der Immunhomöostase und der Kontrolle immunvermittelter Lebererkrankungen beitragen.

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List of Publications

Abstracts of congress presentations

M. Pfaff, K. Neumann, K. Karimi, F. Tacke, G. Tiegs. Hepatocyte-dependent induction of regulatory $CD4^+$ T cells. *Zeitschrift für Gastroenterologie* 2015; 53 DOI:10.1055/s-0035-1567953

Further congress presentations

M. Pfaff, K. Karimi, L. Diehl, G. Tiegs. Hepatocyte dependent induction of regulatory T-cell subsets. 4th European Congress of Immunology - ECI 2015 - Vienna, Austria

List of Abbreviations

Ab	antibody
ANOVA	analysis of variance
APC	antigen-presenting cell
BSA	bovine serum albumin
cDNA	copy DNA
ConA	Concanavalin A
DAMP	damage-associated molecular pattern
DC	dendritic cell
DMSO	Dimethylsulfoxid
dNTP	deoxynucleosidtriphosphate
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked-immunosorbent assay
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
Foxp3	forkhead box p3
h	hours
HBSS	Hank's Balanced Salt Solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IFN	interferon
IL	interleukin
i.p.	intraperitoneal
LSEC	liver sinusoidal endothelium cell
mAb	monocolonal antibody
MACS	magnetic activated cell sorter
mg	milligram
MHC	major histocompatibility complex
min	minute
ml	millilitres
mRNA	messanger ribonucleic acid
NK cell	natural killer cell
NKT cell	natural killer T cell
NPC	non-parenchymal cell
PCR	polymerase chain reaction
qRT-PCR	quantitative real-time PCR
RT	room temperature
TCR	T cell receptor
TGFβ	tumor growth factor β
Th cell	T helper cell
TNF	tumor necrosis factor
Tregs	regulatory T cells
WT	wild type

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Der Lebenslauf wurde aus datenschutzrechtlichen Gründen entfernt

Eidesstattliche Versicherung

Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe.

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Ich erkläre mich einverstanden, dass meine Dissertation vom Dekanat der Medizinischen Fakultät mit einer gängigen Software zur Erkennung von Plagiaten überprüft werden kann.

Hamburg, den