Chemokine receptor CCR6-dependent accumulation of IL-17 producing γδ T cells in injured liver restricts hepatic inflammation and fibrosis

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List of Abbreviations

actT	Activated T cell
ALT	Alanine aminotransferase
APC	Antigen presenting cell
APC	Allophycocyanine
AST	Aspartate aminotransferase
BC	B cell
BSA	Bovine serum albumine
CC	Cysteine-cysteine
CCL	CC chemokine ligand
CCl_4	Carbon tetrachloride
CCR	CC chemokine receptor
CD	Cluster of differentiation
cDNA	Complementary DNA
ConA	Concanavalin A
CTL	Cytotoxic T lymphocyte
CXC	Cysteine-other amino acid-cysteine
CXCL	CXC chemokine ligand
CXCR	CXC chemokine receptor
Cy7	Cyanine dye 7
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic cell
DEPC	Diethyl Pyrocarbonate
DMEM	Dulbecco´s modified eagle medium
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
EAE	Experimental autoimmune encephalomyelitis
EBSS	Earle´s balanced salt solution
ECM	Extracellular matrix
EDTA	Ethylene Diamine Tetra-Acetic Acid
EGTA	Ethylene Glycol Tetra-Acetic Acid
ELISA	Enzyme linked immunosorbent assay
EtOH	Ethanol

FACS	Fluorescence associated cell sorting
FasL	Fas ligand
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FoxP3	Forkhead-box protein P3
Gata3	Trans-acting T-cell-specific transcription factor 3
GBSS	Gey´s balanced salt solution
GFP	Green fluorescent protein
GM-CSF	Granulocyte macrophage colony stimulating factor
Granulo	Granulocyte
H+E	Hematoxylin/eosin
HBSS	Hank´s balanced salt solution
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HSC	Hepatic stellate cell
iDC	Immature dendritic cell
IL	Interleukin
IFN	Interferon
ip	Intraperitoneally
iv	Intravenously
KC	Kupffer cell
LSEC	Liver sinusoidal endothelial cell
MCD	Methionine- choline- deficient
mDC	Mature dendritic cell
MIP3α	Macrophage inflammatory protein 3α
MMP	Matrixmetalloproteinase
Mono	Monocyte
mRNA	Messenger RNA
NaCl	Sodium chloride
NAD	Nicotinamide adenine dinucleotide
NASH	Non-alcoholic steatohepatitis
nd	Not detectable
ns	Not significant

NK	Natural killer cell
NKT	Natural killer T cell
Neutro	Neutrophil
OD	Optical density
OHP	Hydroxyproline
PCR	Polymerase chain reaction
PBC	Primary biliary cirrhosis
PBS	Phosphate buffered salt solution
PE	Phycoerythrin
PerCP	Peridinin-chlorophyll-protein complex
PI	Propidium iodide
PMA	phorbol 12-myristate 13-acetate
PMSF	Phenylmethylsulfonylfluorid
PP	Peyer´s patches
qPCR	Quantitative PCR
RBC	Red blood cell
RNA	Ribonucleic acid
RIPA	Radio Immuno Precipitation assay
RORγt	Retinoid-related orphan receptor-gamma t
RPMI	Roswell Park Memorial Institute
RT	Reverse transcriptase
SEM	Standard error of the mean
STAT	Signal transducer and activator of transcription
T	T cell
T-bet	T-box expressed in T cells
TCR	T cell receptor
TGF	Transforming growth factor
Th	T helper cell
TIMP	Tissue inhibitor of metalloproteinase
TLR	Toll-like receptor
TMB	Tetramethylbenzidine
TNF	Tumor necrosis factor
Treg	Regulatory T cell
wt	wildtype

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1 Zusammenfassung

Als Folge verschiedener Lebererkrankungen können chronische Entzündungsprozesse in der Leber entstehen, die typischerweise zur Entwicklung einer Leberfibrose führen. In der vorliegenden Arbeit wurde die Rolle des Chemokinrezeptors CCR6 und seines Liganden CCL20 in chronischer Leberschädigung und Entstehung von Leberfibrose untersucht. CCR6 wird von T-Helfer Zellen, gamma/delta (γδ) T-Zellen, dendritischen Zellen und einigen B-Zellen exprimiert. CCR6 spielt mutmaßlich eine wichtige Rolle für die Migration von Th17 Zellen, regulatorischen T-Zellen und γδ T-Zellen, aber die Bedeutung von CCR6/CCL20 für Lebererkrankungen ist noch weitgehend unklar.

Um deren Rolle genauer zu untersuchen, wurde die Expression von CCR6 und CCL20 in Lebergewebeproben von Patienten mit verschiedenen chronischen Lebererkrankungen untersucht. Zusätzlich wurden wildtyp (wt) und CCR6 knock-out (*Ccr6*$^{-/-}$) Mäuse für 4 Wochen mit dem Hepatotoxin Tetrachlorkohlenstoff (CCl_4) behandelt oder für 8 Wochen mit einer Methionin-Cholin-defizienten Diät gefüttert, um eine Leberfibrose zu induzieren. Leberschädigung, -entzündung und –fibrose wurden mittels biochemischer Methoden, Histologie, Durchflusszytometrie und Genexpressionsanalysen untersucht.

Die Expression von *CCR6* und *CCL20* war in Leberbiopsien von Patienten mit chronischen Leberkrankungen (n=50) sowie fibrotischen murinen Lebern signifikant erhöht. Die *Ccl20*-Expression war besonders auf primären murinen Hepatozyten aus geschädigter Leber stark erhöht. Beide experimentellen Modelle zeigten eine deutlich gesteigerte Fibrose in *Ccr6*$^{-/-}$ Mäusen. Darüberhinaus zeigten *Ccr6*$^{-/-}$ Mäuse nach chronischer CCl_4-Behandlung eine deutlich verstärkte Leberentzündung mit einer stärkeren Einwanderung von Immunuzellen in die Leber im Vergleich zu wt Mäusen. Die Differenzierung von T-Helfer Zellen war in *Ccr6*$^{-/-}$ Mäusen unverändert, während die Expression von Interleukin-17 (IL-17) in der Leber deutlich verringert war. Bemerkenswerterweise zeigten wt Mäuse eine deutliche Einwanderung von IL-17 produzierenden γδ T-Zellen in die geschädigte Leber, wohingegen dieser Effekt in *Ccr6*$^{-/-}$ Mäusen nicht beobachtet werden konnte. Entsprechend führte der adoptive Transfer von wt γδ T-Zellen, jedoch nicht von wt T-Helfer Zellen, in *Ccr6*$^{-/-}$ Mäuse zu einer verringerten Leberentzündung und –fibrose nach CCl_4-Behandlung, unabhängig von der IL-17 Produktion dieser Zellen. Außerdem konnte eine Kolokalisation von γδ T-Zellen mit den Kollagen-produzierenden hepatischen Sternzellen (HSC) *in vivo* sowie eine Inhibition der HSC-Funktion durch γδ T-Zellen *in vitro* nachgewiesen werden. Hepatische γδ T-Zellen inhibierten die Kollagen-Synthese von HSC und lösten Fas-abhängig Apoptose in HSC aus.

Zusammenfassend konnte in dieser Arbeit gezeigt werden, dass IL-17 produzierende γδ T-Zellen CCR6 benötigen, um in die geschädigte Leber einzuwandern, und dass diese Zellen die Leber vor Inflammation und Fibrogenese schützen können.

2 Abstract

Chronic liver injury promotes hepatic inflammation, representing a prerequisite for organ fibrosis, a common consequence of various liver diseases. In this study we investigated the contribution of the chemokine receptor CCR6 and its ligand CCL20 to chronic liver injury and hepatic fibrosis. CCR6 is known to be expressed on some T helper cells and gamma/delta ($\gamma\delta$) T cells, monocyte derived dendritic cells and B cell subsets. It plays an important role in mucosal immunity and has been implicated in the migration of Th17, regulatory (Treg) and $\gamma\delta$ T cells, but its role in liver disease is largely unknown.

Therefore we analyzed human liver samples of patients with chronic liver disease of various etiologies for expression of CCR6 and CCL20. Additionally, wildtype (wt) and CCR6 knock-out (*Ccr6*$^{-/-}$) mice were treated with the hepatotoxic agent carbon tetrachloride (CCl_4) thrice weekly over a period of 4 weeks or subjected to methionine-choline-deficient (MCD) diet for 8 weeks to induce liver fibrosis. Liver damage, inflammation, and fibrosis development were then assessed by biochemical methods, histology, immunostaining, flow cytometry, and quantitative realtime PCR.

CCR6 and *CCL20* expression was significantly up-regulated in the livers of patients with chronic liver diseases (n=50) and fibrotic murine livers. *Ccl20* was strongly induced in primary murine hepatocytes upon injury. In both models of chronic liver injury, *Ccr6*$^{-/-}$ mice developed more severe fibrosis than wildtype mice. Furthermore, in CCl_4-induced liver injury *Ccr6*$^{-/-}$ mice showed strongly enhanced liver inflammation compared to wt mice, as indicated by increased overall hepatic immune cell infiltration. While CCR6-deficiency did not affect the composition of hepatic T helper cell subtypes, expression of interleukin-17 (IL-17) was significantly reduced in livers of *Ccr6*$^{-/-}$ mice. Wt mice showed an accumulation of IL-17-producing $\gamma\delta$ T cells in the liver upon CCl_4 treatment, which was markedly reduced in livers of *Ccr6*$^{-/-}$ mice.

Concordantly, the adoptive transfer of wt $\gamma\delta$ T cells, but not of $CD4^+$ T cells, into *Ccr6*$^{-/-}$ mice reduced hepatic inflammation and fibrosis in chronic injury, but this function was independent of their IL-17 production. Moreover, hepatic $\gamma\delta$ T cells colocalized with hepatic stellate cells (HSC) *in vivo* and directly inhibited HSC function by limiting their collagen synthesis and facilitating apoptosis in a Fas-dependent manner *in vitro*.

Thus, IL-17 producing $\gamma\delta$ T cells specifically require CCR6 to migrate to the liver upon chronic injury and are able to protect the liver from excessive inflammation and fibrosis by inhibiting HSC.

3 Introduction

Inflammation is the body´s reaction to infection or injury. It is carried out by cells of the immune system and supposed to protect the body from disease. Acute inflammation is characterized by an influx of leukocytes to the site of injury to clear infectious organisms or debris. Once the harmful stimulus is removed, healing can take place and the body goes back to homeostasis. However, inflammation can also become chronic if the immune system is dysregulated or impaired. Nowadays, chronic inflammation is believed to be the underlying mechanisms causing plenty of diseases including (among many others) arthritis, diabetes, Alzheimer's, cancer and organ fibrosis. Therefore it is absolutely necessary that inflammation is tightly regulated by the body.[1]

3.1 Cytokines and Chemokines

3.1.1 Cytokines

Cytokines are small cell signaling proteins that facilitate intercellular communication between various cells. They can act as growth factors, differentiation factors, or immunomodulatory agents. Cytokines function through cell surface receptors and downstream signaling induces an alteration of cell function and phenotype. By means of their receptors cytokines can influence transcription of genes, expression of proteins and induce feedback loops leading to downregulation of their own expression or that of their receptors. How a specific cytokine acts on its target cells depends on the cytokine, cell type, expression of its receptor, and abundance of the cytokine itself. Cytokines can be classified into families according to their function, cellular origin, or structure of the corresponding receptor. Cytokines can be assigned to functional groups such as interferons, interleukins, colony-stimulating factors, tumor necrosis factor family members, and chemokines.[2] Interferons mainly stimulate anti-viral and anti-tumoral immune responses and are mostly produced by monocytes and macrophages, but also by lymphocytes and fibrocytes.[3] Interleukins are mediators for the communication between leukocytes or leukocytes and their target cells. This term is used rather independent of the cytokine function for molecules that either act on leukocytes or are produced by leukocytes, mainly lymphocytes.[4] Colony-stimulating factors are growth factors for hematopoietic cells such as erythrocyte and leukocyte precursors.[5] Members of the tumor necrosis factor superfamily are mainly produced by macrophages

during immune reactions and can elicit a variety of effects on target cells, including apoptosis, proliferation, differentiation, and others.[6] Chemokines are chemotactic cytokines that primarily induce migration of immune cells, but can also have additional other effects, for instance, on cell differentiation or survival.

3.1.2 Chemokines and chemokine receptors

Chemokines are chemotactic cytokines specialized in regulating the migration of immune cells. Their main function is to promote the extravasation of immune cells from the blood into tissue and to regulate the migration of immune cells through the tissue, and additionally, chemokines are involved in angiogenesis and development.[7-8] The so-called inflammatory chemokines are produced upon proinflammatory stimuli to attract leukocytes to the site of injury or infection, whereas a variety of other chemokines is constitutively expressed under homeostatic conditions and participates in the formation and correct organization of lymphoid tissue. Chemokines released upon inflammation form a concentration gradient and attract leukocytes expressing the corresponding chemokine receptor(s), which then migrate towards the highest concentration of the chemokine. They can be released by a lot of different cell types including immune cells, endothelial cells, fibroblasts, and parenchymal cells and act through G protein-linked transmembrane receptors on the cell surface of their target cells.

Chemokines are classified into four groups according to the spacing of their first two cysteine residues near their amino-termini: CC chemokines that have two adjacent cysteine residues, CXC chemokines that have two cysteines separated by one amino acid, CX_3C chemokines that have 3 amino acids separating their cysteine residues, and C chemokines that only have one cysteine residue near the amino terminus. So far, 48 different chemokines and 19 different chemokine receptors have been identified, which can act on a variety of cell types (Table 1).[9-10]

Chemokine	alternative name(s)	chemokine receptor	responding cell type
CC Chemokines			
CCL1	I-309, TCA-3	CCR8	*i*DC, *act*Th2
CCL2	MCP-1	CCR2	Granulo, Mono, *act*T, NK, *i*DC
CCL3	MIP-1α	CCR1, CCR5	Granulo, Mono, *act*T, NK, *i*DC
CCL4	MIP-1β	CCR5	Mono, *act*T, NK, *i*DC
CCL5	RANTES	CCR1, CCR3, CCR5	Granulo, Mono, *act*T, NK, *i*DC
CCL7	MCP-3	CCR1, CCR2, CCR3, CCR5	Granulo, Mono, *act*T, NK, *i*DC
CCL8	MCP-2	CCR1, CCR2, CCR5	Granulo, Mono, *act*T, NK, *i*DC
CCL9/10	MIP-1γ, MRP-2	CCR1	Granulo, *act*T
CCL11	Eotaxin	CCR3, CCR5	Granulo, Mono, *act*T, *i*DC
CCL12	MCP-5	unknown	Granulo, Mono, *act*T, NK, *i*DC
CCL13	MCP-4	CCR2, CCR3	Granulo, Mono, *act*T, NK, *i*DC
CCL14	HCC-1	CCR1, CCR3, CCR5	Granulo, Mono
CCL15	MIP-5, LKN-1, HCC-2	CCR1, CCR3	Granulo, Mono
CCL16	LEC, HCC-4	CCR1, CCR2, CCR5, CCR8	Mono, *act*T
CCL17	TARC, ABCD-2	CCR4, CCR8	*act*T
CCL18	MIP-4, PARC, DC-CK1	unknown	T, *i*DC
CCL19	ELC, Exodus-3	CCR7	T, *act*T, *m*DC
CCL20	MIP-3α, LARC, Exodus-1	CCR6	T, B, *i*DC
CCL21	SLC, 6Ckine, Exodus-2	CCR7	T, *act*T, *m*DC
CCL22	MDC, STCP-1	CCR4	*act*T, *i*DC
CCL23	MIP-3, MPIF-1	CCR1	Granulo, Mono, T
CCL24	Eotaxin-2, MPIF-2	CCR3	Granulo, *act*T, *i*DC
CCL25	TECK	CCR9	T, thymocytes
CCL26	MIP-4α, Eotaxin-3	CCR3	Granulo, *act*T, *i*DC
CCL27	CTACK, ILC, Eskine	CCR10	*act*T
CCL28	MEC	CCR3, CCR10	*act*T
CXC Chemokines			
CXCL1	Gro-α, KC	CXCR2	Granulo, Mono, Mast cells
CXCL2	Gro-β, MIP-2α	CXCR2	Granulo, Mono, Mast cells
CXCL3	Gro-γ, MIP-2β	CXCR2	Granulo, Mono, Mast cells
CXCL4	PF-4	CXCR3B	Fibroblasts
CXCL5	ENA-78	CXCR2	Granulo, Mono, Mast cells
CXCL6	GCP-2	CXCR1, CXCR2	Granulo, Mono, Mast cells
CXCL7	NAP-2, CTAPIII	CXCR1, CXCR2	Granulo, Mono, Mast cells
CXCL8	IL-8	CXCR1, CXCR2	Granulo, Mono, Mast cells
CXCL9	MIG	CXCR3	*act*T
CXCL10	IP-10	CXCR3	*act*T
CXCL11	I-TAC	CXCR3, CXCR7	*act*T
CXCL12	SDF-1α/β	CXCR4, CXCR7	Lymphocytes
CXCL13	BCA-1, BLC	CXCR3, CXCR5	BC
CXCL14	BRAK, bolekine	unknown	Mono
CXCL15	Lungkine	unknown	Granulo
CXCL16	SCYB16, SRPSOX	CXCR6	*act*T, NKT
CXCL17	DMC, VCC-1	unknown	Mono, *i*DC
C Chemokines			
XCL1	Lymphotactin α, SCM-1α	XCR1	T
XCL2	Lymphotactin β, SCM-1β	XCR1	T
CX_3C Chemokine			
CX_3CL1	Fractalkine, Neurotactin	CX_3CR1	Mono, *act*T, NK

Table 1 *Chemokines and chemokine receptors.* Granulo= Granulocytes; Mono= Monocytes; iDC= immature dendritic cells; mDC= mature dendritic cells; T= T cells; actT= activated T cells; BC= B cells.

3.1.3 CCR6 and its ligand CCL20

The chemokine receptor CCR6 is a lymphocyte associated receptor with important functions in mucosal immunity. It has been found to be expressed on specific subsets of T[11-12] and B lymphocytes[13-14], monocyte derived dendritic cells[15-16] and some neutrophils[17], especially under inflammatory conditions.[18] In the $CD4^+$ T cell compartment CCR6 is only expressed on Th17 and Treg, but not Th1 or Th2 cells,[12, 19-21] while CCR6 expression appears to be even more specific for Th17 cells than Treg, as all Th17 cells are $CCR6^+$ whereas CCR6 expression on Treg is organ specific.[22]

The only known CC-type ligand for CCR6 is CCL20, also termed Macrophage Inflammatory Protein-3alpha (MIP-3α), but beta-defensins can also bind to CCR6.[23-24] The importance of the CCR6-CCL20 axis has been unravelled best in the gut. In the intestine CCL20 is constitutively expressed by epithelial cells facilitating homing of the residing leukocytes during steady state conditions.[25-27] During inflammatory reactions this constitutive expression can become pathogenic by enhancing the recruitment of $CCR6^+$ proinflammatory cells.[28-29] CCR6 is also a homing receptor for Th17 cells to the Peyer's patches (PP) and required for their normal migration behavior, since mice lacking CCR6 develop excessive colonic inflammation.[22] Dendritic cells are recruited to sites of potential antigen entry via CCR6.[30]

CCR6-mediated chemotaxis of T cells has been described to be important in several immune-mediated diseases. CCR6-deficiency aggravated experimental glomerular nephritis and experimental autoimmune encephalomyelitis (EAE) in the murine system. In both cases infiltration of Th17 and Treg was markedly reduced, and enhanced disease severity was due to the fact that the inflammatory reaction could not be controlled by regulatory T cells anymore.[31-33] Adoptive transfer of $CCR6^+$ T cells into CCR6-deficient mice ameliorated both diseases.[32-33] Descriptive analyses from patients with hepatocellular carcinoma indicated that the CCR6-CCL20 axis might be involved in the recruitment of Treg in liver cancer as well.[34] In contrast, a different role for CCR6 has been described in psoriasis, as *Ccr6*$^{-/-}$ mice do not develop psoriasiform inflammation upon IL-23 treatment of the ears.[35] This is due to reduced recruitment of $CCR6^+$ leukocytes to the ears and subsequently lower production of IL-22, which is necessary for IL-23 induced changes.

More recently CCR6 has also been identified on some subsets of $\gamma\delta$ T cells, and CCR6 expression is clearly associated with IL-17 expression of these cells.[36-38] Similar to

CD4 T cells also IL-17 producing γδ T cells have been described to play a role in immune mediated diseases like EAE[39-41], lung fibrosis[36] or collagen-induced arthritis[37]. In corneal epithelial healing, CCR6 and CCL20 play crucial roles in recruiting γδ T cells producing both IL-17 and IL-22.[42] IL-22 contributes to the healing reaction and without these cells corneal healing is impaired.

In human liver the CCR6-CCL20 axis has been suggested to be involved in positioning of Th17 cells.[43] CCL20 is expressed by human biliary epithelial cells in inflamed liver, which recruits infiltrating Th17 cells to bile ducts. Nevertheless, the functional role of CCR6 in liver diseases is currently unclear. A preliminary study investigating 34 patients with various liver diseases revealed elevated levels of CCR6-expressing hepatic T cells and enhanced intrahepatic levels of CCL20 in fibrotic livers,[44] suggesting that the CCR6-CCL20 axis might indeed be involved in modulating the inflammatory and fibrotic response in liver injury.

3.2 T cells in homeostasis and immunity

3.2.1 T-helper cell subsets

$CD4^+$ T-helper cells (Th cells) are important components in adaptive immunity and one of the two major populations of conventional T cells. Conventional T cells are also called αβ T cells because they express highly antigen specific T cell receptors (TCR) consisting of one alpha and one beta chain. Th cells provide help for antigen presenting cells and $CD8^+$ cytotoxic T lymphocytes to initiate and promote adaptive immune responses. Activation of $CD4^+$ T cells is critical for the elimination of many invading pathogens, but inadvertently they can also become responsive to self antigens, thus leading to autoimmune diseases. In order to prevent this, the differentiation and activation of $CD4^+$ T-helper cells is tightly regulated.

Nowadays $CD4^+$ T-helper cells are divided into four major subsets, based on their expression profile of transcription factors and secreted cytokines: Th1, Th2, Th17 and regulatory T cells (Treg) (Fig.1).[45] The first two subsets, Th1 and Th2, were identified already in the 1980s.[46] Th1 cells are characterized by the secretion of IFNγ, a proinflammatory cytokine which is necessary for the activation of macrophages and involved in immunity against intracellular pathogens.[47-48] They have also been linked to cell-mediated autoimmune

diseases.[49] Th2 cells produce mainly IL-4, IL-5 and IL-13 and play an important role in allergy as well as for the clearance of various extracellular pathogens and parasites.[47-48]

Figure 1 summarizes key cytokines that drive the differentiation of T-helper cell populations, their main effector cytokines, and the characteristic transcription factors for the different subsets. The differentiation of Th1 cells is mainly induced by IL-12 and can be further enhanced by IFNγ.[48, 50] Th2 cells develop in the presence of IL-4.[50] Th1 and Th2 negatively regulate each other through actions of their specific cytokines: IL-12 represses induction of Th2 cells, whereas IL-4 inhibits Th1 cell development. On the transcriptional level Th1 polarizing cytokines induce the transcription factors T-bet and STAT4, whereas Th2 cells require action of GATA3 and STAT6.[51]

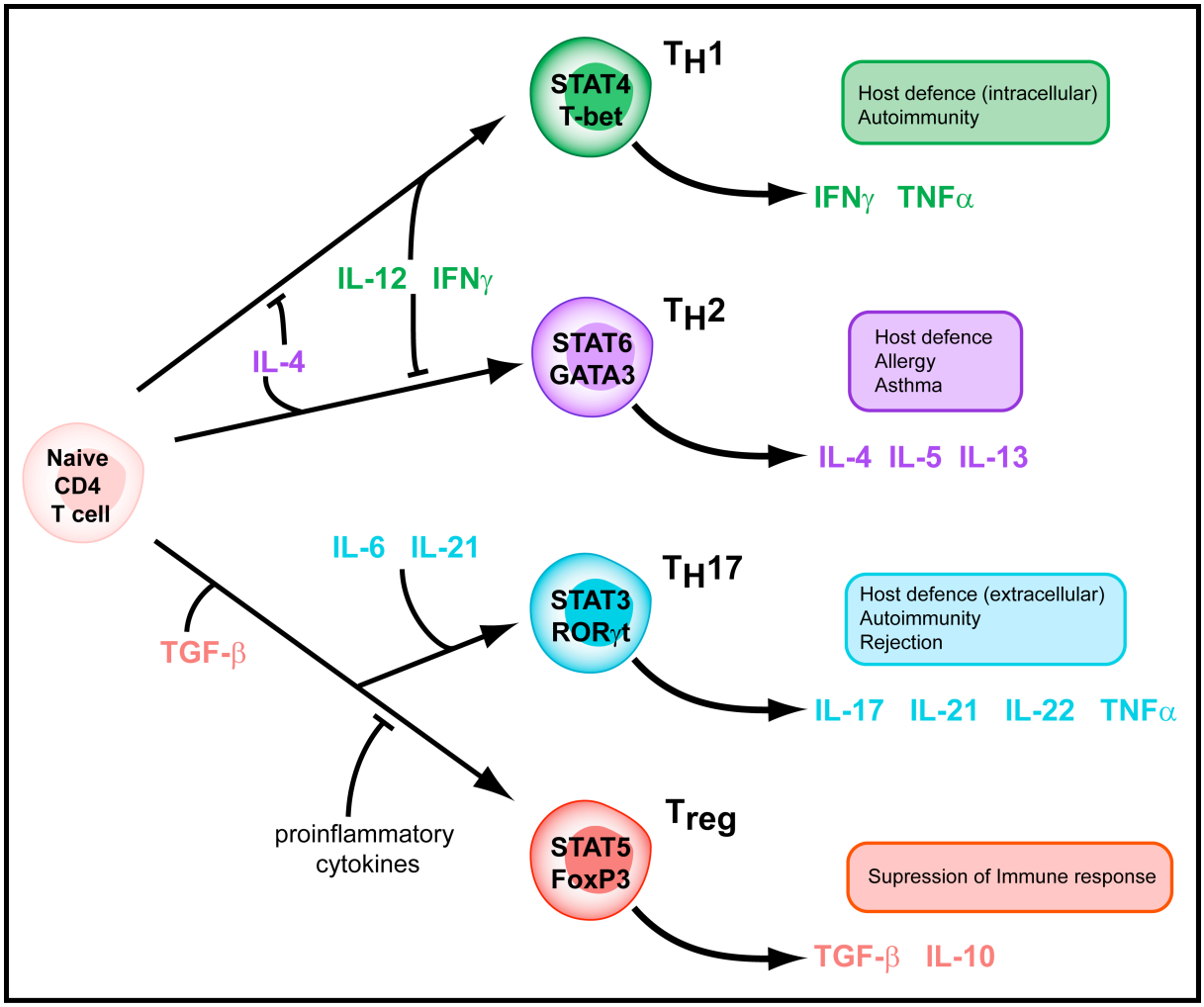


Figure 1. *Differentiation of CD4⁺ T cell subsets.*[45]

Upon activation, naïve CD4⁺ T cells can differentiate into different subsets depending on the surrounding cytokine milieu. The different subpopulations show distinct expression patterns of transcriptions factors and can be characterized by secretion of signature cytokines that are unique for each subset. Each subset takes part in different kinds of immune responses against various pathogens or in mediating autoimmunity.[45]

Regulatory T cells (Treg) are a unique subset of $CD4^+$ T helper cells that control effector T cell responses to prevent (auto-) immune reactions. Activated Treg produce the anti-inflammatory cytokines IL-10 and TGFβ, thus suppressing the development of functional immune reactions.[52] The differentiation of Treg is induced by TGFβ[53-54], but inhibited in the presence of proinflammatory cytokines. Treg are characterized by the expression of the transcription factors Foxp3 and STAT5 and expression of CD25 on their surface.[55]

Th17 cells are a more recently discovered subset of $CD4^+$ T-helper cells characterized by the production of their signature cytokine IL-17. They represent another subtype of proinflammatory T-helper cells that differs from Th1 and Th2 cells in development and function. Differentiation of Th17 cells needs the combined action of TGFβ, IL-6, and IL-21 in mice,[56-58] whereas IL-6 and IL-21 can be replaced by IL-23 or IL-1β in humans.[59] These cytokines induce expression of the transcription factors orphan nuclear receptor RORγt and STAT3.[60-61] Development of Th17 cells is suppressed by IFNγ and IL-4 that promote Th1 or Th2 cells, respectively.[62] In the absence of other proinflammatory cytokines like IL-6, TGFβ induces $FoxP3^+$ regulatory T cells instead of Th17 cells, which shows the close relationship between Th17 and Treg. Activated Th17 cells secrete IL-17, IL-21, IL-22, and TNFα, which then promote tissue inflammation by induction of other proinflammatory mediators and recruitment of leukocytes, mainly neutrophils, to the site of inflammation.[63-64] Th17 cells are involved in the clearance of extracellular bacteria and fungi,[65-67] but they have also been linked to several autoimmune diseases.[68-69]

3.2.2 Gamma/delta T cells

Gamma/delta T cells (γδ T cells) are a small subpopulation of non-conventional T cells that are negative for classical differentiation markers like CD4 or CD8 and express the γδ T cell receptor (TCR) instead of the αβ TCR.[70] In secondary lymphoid organs they account for 2-3% of all $CD3^+$ cells and the highest abundance of γδ T cells is seen in the gut mucosa. γδ T cells are often described to link innate and adaptive immunity as they share features with innate immune cells as well as with conventional T cells that belong to the adaptive immune system.[71] In contrast to αβ T cells, γδ T cells leave the thymus after their maturation as mature T cells with a defined functional potential in a so-called pre-activated status.[72] Although γδ T cells are able to recognize antigens presented on MHC molecules, they can also be sufficiently activated through cytokines without TCR engagement.[73-74] Therefore, activation in the periphery can occur very rapidly allowing γδ T cells to respond much faster

than αβ T cells. Similar to conventional T cells, γδ T cells can kill target cells via Fas or TNF-induced apoptosis or by releasing cytotoxic granules and are able to produce large amounts of immunomodulatory cytokines.[75-76] So far they have been shown to secrete IFNγ, IL-17, IL-4, IL-5, IL-13 and GM-CSF as well as immunosuppressive cytokines like IL-10 or TGFβ.[77] According to their cytokine production they can be divided into two major subpopulations, one that mainly secretes IFNγ and one that mainly secretes IL-17. The IFNγ secreting subpopulation expresses NK1.1 and CD27 on the cell surface, expands upon stimulation through IL-12 and is associated with T-bet expression - features similar to $CD4^+$ Th1 cells.[72, 78] The IL-17^+ subpopulation expresses CCR6 and CD25 on the cell surface, expands on IL-6 and/or IL-23 signaling and is associated with expression of RORγt and STAT3 in the nucleus - features similar to those of $CD4^+$ Th17 cells.[79-80] Recently, NK1.1 and CCR6 have been established as specific markers to distinguish between IFNγ-producing and IL-17-producing γδ T cells, respectively.[38] Interestingly, γδ T cells have been shown to be the major source of IL-17 in different immune-mediated diseases often producing much higher amounts than Th17, even if responding in similar numbers as Th17 cells.[81-82]

3.2.3 Other T cell subtypes

$CD8^+$ T cells are the second population of conventional T cells besides T helper cells that are called cytotoxic or killer T lymphocytes (CTL) and are very important for the clearance of infected cells. Like T helper cells they express the α/β T cell receptor, but instead of CD4 they express CD8 as a co-receptor on their surface. CTL recognize antigens presented on MHC class I-molecules[83] and are able to directly kill infected or damaged target cells. However, engagement of the TCR with its specific antigen-MHC complex is not sufficient to fully activate cytotoxic T cells, a mechanism that prevents autoimmune reactions. CTL need at least one costimulatory signal, which is usually provided by the antigen presenting cell (APC). APC that process foreign antigens and have been licensed and activated by T helper cells upregulate costimulatory molecules on their surface, which will then bind to receptors on the CTL and activate it.[84] This effect can be further enhanced through proinflammatory cytokines produced by other activated leukocytes, like IL-12.[85] If costimulation is absent antigen recognition will lead to a tolerant stage of the CTL.[86] As MHC I-molecules are expressed by all host cells, this prevents self-reactive T cells from killing intact cells that present self-antigens on their surface. Once a CTL is fully activated it can kill every infected target cell wearing the same antigen-MHC I complex on the surface by inducing programmed cell death without the need of further costimulation. CTL can release

cytolytic granules containing perforins, that form pores in the target cell's membrane, and granzymes that induce apoptosis by activating the caspase cascade inside the cell.[87] Apoptosis can also be induced by binding of so-called death receptors like Fas, which is expressed by most somatic cell types.[87] An activated CTL will upregulate Fas ligand on its cell surface and binding to Fas on the target cell will lead to apoptosis of this cell. Activated CTL also release proinflammatory cytokines like IFNγ and TNFα, which will in turn activate other immune cells such as T helper cells and enhance expression of MHC class I molecules.[88]

A very small but nonetheless important subset of unconventional T cells are natural killer T cells (NKT cells) that share features with conventional T cells and natural killer cells. They account for less than 1% of all peripheral T cells[89] and express a αβ TCR with a very limited diversity as the alpha chain of the receptor is invariant. In contrast to conventional T cells NKT cells do not recognize MHC molecules and peptide antigens, but glycolipid antigens presented on CD1d.[90] NKT cells were originally defined as T cells expressing the natural killer (NK) cell marker NK1.1 on their surface, but nowadays the term NKT cell refers to all CD1d restricted T cells, which include $NK1.1^+$ and $NK1.1^-$ cells. Many but not all NKT cells also express CD4, CD8 or both. NKT cells can kill their target cells similar to cytotoxic T cells. Upon engagement of the TCR they can release cytolytic granules containing perforins and granzymes and also induce apoptosis via Fas-Fas ligand interactions.[91] NKT cells are often considered as innate lymphocytes because they respond much faster than conventional $CD4^+$ or $CD8^+$ T cells. Within minutes to hours of antigen recognition they can release a variety of cytokines including IFNγ, IL-4, IL-13, IL-17, IL-21, IL-10, TNFα and GM-CSF.[92-93] As many of these cytokines are T helper cell related NKT cells can modulate immune responses by driving them in the direction of Th1, Th2 or Th17 responses. Furthermore, IFNγ can also activate NK cells and macrophages.

3.2.4 Natural killer cells

Natural killer cells (NK cells) are non-T cytotoxic lymphocytes that belong to the innate immune system as they do not have antigen-specific receptors. In mice they are characterized by expression of NK1.1 on their surface.[94] Because they are not antigen specific, NK cells can rapidly respond to infections or tissue damage, long before antigen-specific T cell responses are generated. NK cells are activated in response to proinflammatory cytokines like IL-12 produced by macrophages or IFNγ released by T lymphocytes. Once activated NK cells kill target cells by releasing cytolytic granules or

binding of death receptors, as already described for NKT cells and CTL, and release cytokines like IFNγ[95], TNFα or IL-10 that will activate other leukocytes. In order to distinguish between infected and uninfected cells they express a variety of activating and inhibiting receptors on their surface that determine whether the NK cell is activated or not.[96] Activating receptors recognize MHC-peptide complexes or non-self MHC molecules on the surface of target cells and transmit signals that will activate the NK cell. Inhibitory receptors bind to normal MHC molecules as expressed on healthy cells and inhibit NK cell function. By this mechanism NK cells are not only able to spare normal host cells, but also to kill virus infected cells that downregulate MHC molecules on their surface after infection and therefore do not provide sufficient inhibitory signals.[97] NK cells are very important for containing infections until adaptive immune responses can be mounted to clear the infection.

3.3 Liver biology

The liver is the major organ in terms of metabolism in the body of vertebrates. It has important functions in metabolism and storage of glucose, fat and proteins and degradation of toxic and harmful substances. Therefore proper liver function is absolutely necessary to keep up the body's homeostasis. Furthermore, the liver also plays an important role during inflammation as it produces acute-phase proteins and can provide the body with energy sources like glucose.[98]

The liver has a unique blood supply as it is not only provided with oxygen-rich arterial blood via hepatic arteries but also with venous blood coming from the gastrointestinal tract via the portal vein. About 30% of the total blood volume passes the liver per minute.[99] The importance of the liver is further underlined by its unique potential to regenerate after organ damage,[100] a fact that is very useful for liver transplantation as it allows splitting one donor organ between several recipients or living donor transplantations.

The parenchymal cells of the liver are called hepatocytes and account for about two third of all the cells in the liver. They build up the functional subunits of the liver, the lobules, small hexagonal areas. The corner areas between adjacent lobules are called portal tracts and each portal tract contains a terminal hepatic artery, vein, and bile duct (portal triad). Blood enters through these vessels and then passes through the sinusoids and leaves the liver via the central hepatic vein that is located in the center of each lobule. The sinusoids are the capillaries of the liver, which are located between the hepatocytes and lined by a single layer of epithelial cells, the liver sinusoidal endothelial cells (LSEC).[101] The LSEC build up a fenestrated endothelium which facilitates the extravasation of leukocytes from the blood. The

space between sinusoids and hepatocytes is called the space of Dissé;[102] it contains the fat-storing cells of the liver, the hepatic stellate cells (HSC), which are also the main producers of hepatic extracellular matrix.

3.3.1 The liver as an immunological organ

Despite its vital metabolic functions the liver is also an important immunological organ. The blood coming from the gastrointestinal tract carries manifold potential antigens, hormones and nutritional components derived from food. The liver is not only able to mount immune responses against pathogenic antigens, e.g. from invading microorganisms, but also to induce tolerance against harmless substances, e.g. food antigens. These functions are facilitated by the vast number of immune cells that reside in the liver even under homeostatic conditions.[103]

3.3.2 Intrahepatic immune cells

Even during homeostasis the liver tissue is scattered with lymphocytes. They are evenly distributed throughout the parenchyma and the portal tracts. Liver lymphocytes include conventional $CD4^+$ and $CD8^+$ positive T cells, unconventional gamma/delta T cells and NKT cells as well as NK cells and B cells. Compared to other organs the liver is selectively enriched in NK and NKT cells, with about 10% NK and 30% NKT cells among the hepatic lymphocytes in mice and about 50% NK and 10% NKT cells among human liver lymphocytes.[104] It is also one of the richest sources for $\gamma\delta$ T cells in the body as 15-25% of the hepatic T cells express the gamma/delta TCR.[105]

The liver also contains several populations of resident antigen presenting cells, which are involved in the upkeep of tolerance under non-inflammatory conditions. The most extensively studied population so far are the liver resident macrophages, called Kupffer cells. Around 20% of all non-parenchymal cells in the liver are Kupffer cells, which are derived from bone marrow precursors and reside in the periportal areas. Here, they clear endotoxins and phagocytose debris and microorganisms from the blood that enters the sinusoids. They maintain tolerance against harmless antigens by suppressing T cell activation and reducing antigen uptake by dendritic cells. If Kupffer cells encounter pathogenic antigens they can

initiate host defense by releasing cytokines that will then activate and attract other immune cells, mainly lymphocytes and neutrophils.[106]

Dendritic cells (DC) derived from the bone marrow are located around the central veins and portal tracts. They constantly capture and process antigen, but under homeostatic conditions they remain immature. Once they get activated upon proinflammatory stimuli they migrate towards lymphatic vessels via the space of Dissé and travel to the draining lymph node to initiate immune responses.[103]

Moreover, it has been shown that also LSEC are able to take up and present antigens in a way similar to professional DC. As the blood flow in the liver sinusoids is very slow and sometimes discontinuous LSEC are in constant interaction with leukocytes in the blood stream. This allows them to prime naive $CD4^+$ T cells to differentiate into regulatory T cells and therefore induce hepatic immune tolerance.[107]

3.3.3 Chemokines and chemokine receptors in liver disease

Chemokines can be produced by almost all liver cell types upon inflammation and are very important for recruitment and positioning of leukocytes during liver disease. CCL2 seems to be the major chemokine recruiting proinflammatory monocytes to the injured liver. CCL2 is produced by hepatocytes, hepatic stellate cells, Kupffer cells, and epithelial cells during liver fibrogenesis and numbers of infiltrating $CCR2^+$ monocytes correlate with expression levels of CCL2. Important insights on the functional role of chemokines and chemokine receptors for regulating immune cell infiltration upon liver injury has been gained from knock-out mice subjected to experimental models of liver damage, for instance, chronic toxic injury by carbon tetrachloride (CCl_4) or metabolic induction of steatohepatitis via a methionine-choline deficient (MCD) diet.[108-110] In such models, *Ccr2*$^{-/-}$ mice are protected from acute and chronic liver damage.[108-109] In contrast, the only CX_3C chemokine Fractalkine has been shown to control differentiation and survival rather than recruitment of intrahepatic monocytes in mice.[111]

Neutrophils express CXCR2 and are mainly recruited to the liver via CXCR2 ligands. This has been demonstrated in *Cxcr2*$^{-/-}$ mice that show reduced liver damage and less neutrophil infiltration after ischemia/reperfusion or acetaminophen treatment.[112-113]

In case of T cells the picture is much more complex. The chemokine receptors CCR5 and CXCR3 have been described to be involved in $CD4^+$ T cells recruitment to the

human liver.[114-115] However, they show differential function in positioning of Th cells within the organ; $CCR5^+$ T cells end up in the portal tracts, whereas $CXCR3^+$ T cells are found throughout the parenchyma.[115] Interestingly, CXCR3 is mainly expressed by Th1 cells, Th17 cells and regulatory T cells, but not Th2 cells.[114, 116-117] Therefore, *Cxcr3*$^{-/-}$ mice display enhanced liver fibrosis after CCl_4 treatment as infiltration of Th1 cells is significantly reduced whereas recruitment of profibrotic Th2 cells is not impaired.[118] Likewise, *Cxcr3*$^{-/-}$ mice also develop more severe acute hepatitis after Concanavalin A treatment, which is due to reduced infiltration of Treg to the liver.[119] It has been shown that after entering the liver using CXCR3, different chemokine receptors are responsible for positioning of T helper cells. Th17 cells and Treg localize to inflamed bile ducts via engagement of CCR6 and CCR10, respectively.[120-121]

CCR7 has been associated with infiltration of $CD8^+$ T cells to the liver as sinusoidal endothelial cells express CCR7 ligands in human liver.[122] However, it is still unclear whether this mechanism is supposed to recruit circulating T cells from the blood stream or to recruit intrahepatic T cells to the lymphatics allowing them to travel to the draining lymph node. CXCL4 is also involved in recruitment of $CD8^+$ T cells as *Cxcl4*$^{-/-}$ mice show reduced T cell numbers in the liver along with decreased fibrogenesis.[123]

CXCR6 has been implicated in homing of NKT cells to the liver during homeostasis. CXCL16, the ligand for CXCR6, is expressed on sinusoidal endothelial cells, and *Cxcr6*$^{-/-}$ mice show severely reduced numbers of NKT cells in the liver, which makes them much less susceptible to T-cell dependant hepatitis.[124]

Almost nothing is known about mechanisms recruiting other innate T cell subsets like gamma delta T ($\gamma\delta$ T cells) cells to the liver, although the liver is one of the richest sources of $\gamma\delta$ T cells in the body.[103]

3.4 Liver fibrosis

Hepatic fibrosis is a result of chronic inflammation and a common consequence of various liver diseases, including viral, toxic, metabolic and autoimmune diseases. In humans, it occurs after years or decades of constant or repetitive organ damage leading to a persisting immune response. It is characterized by an increase in and changed composition of extracellular matrix (ECM) with deposition of collagen and other fiber proteins such as elastin in the space of Dissé.[125] If too much ECM is produced the liver architecture gets

disrupted, which impairs correct organ function, alters intrahepatic blood flow and may progress to liver cirrhosis.

Upon the first mediators released during acute inflammation are anti-fibrinolytic factors that induce blot-clotting and matrix metalloproteinases (MMP) that degrade extracellular matrix to alleviate access of immune cells.[126] When inflammation subsides debris is cleaned up by macrophages and neutrophils, which also release anti-inflammatory chemokines. This leads to attraction of endothelial cells and blood vessel growth and activation of tissue macrophages and myofibroblasts to rebuild and reform the ECM, which will eventually close the wound. If inflammation cannot be resolved and therefore persists it comes to an excessive deposition of ECM, which replaces normal tissue. This is called the fibrotic scar.[127]

In the liver, damaged cells rapidly produce inflammatory mediators, among them chemokines and cytokines that will recruit leukocytes to the site of injury. The first cells to arrive in damaged areas are monocytes/macrophages and neutrophils, which clear up dead cells and debris, and lymphocytes, which are activated to produce fibrotic mediators that will in turn activate macrophages and fibroblasts. Hepatic stellate cells (HSC) are activated to transdifferentiate to myofibroblasts;[128] they lose their Vitamin A and start to produce alpha smooth muscle actin (αSMA) and collagen I. They also express inhibitors of matrix degradation, so called tissue inhibitors of metalloproteinases (TIMPs), and proinflammatory cytokines. This production of ECM components along with reduced degradation of ECM is part of the normal organ repair process that will close the wound. During this process lost cells will be replaced by cells of the same kind and normal parenchyma is replaced by connective tissue to keep up organ integrity and function. But if this regeneration occurs uncontrolled like in chronic inflammation it leads to an excessive formation of ECM and normal tissue is replaced with scar tissue. This scar tissue is non-functional and disturbs organ architecture and therefore ongoing fibrogenesis can lead to organ failure and eventually death.

Hepatic stellate cells have been shown to be the main producers of ECM during hepatic fibrogenesis.[129] In the resting liver they metabolize and store retinoids and are responsible for the turnover of the extracellular matrix. Once they get activated by profibrotic stimuli they start to produce ECM components (αSMA, collagen I), and TIMPs. This increases production of ECM and reduces its degradation leading to an excessive formation of scar tissue. Myofibroblasts show a contractile phenotype and can migrate to the areas of active fibrogenesis where they are needed for wound contraction.[128] Furthermore activated HSC also produce chemokines, e.g. CCL2 that will recruit immune cells to the site of

inflammation.[130] HSC can be activated by a variety of signals including cytokines derived from activated lymphocytes or Kupffer cells, pathogen associated molecular patterns and toll-like receptor ligands. The best studied profibrotic factor so far is TGF-beta (TGFβ). TGFβ is produced by infiltrating lymphocytes and monocytes, but also tissue macrophages and damaged hepatocytes. It potently stimulates HSC to transdifferentiate and TGFβ signaling induces production of collagens.[131]

Although TGFβ might be the most important profibrogenic mediator, a lot of other cytokines and chemokines are also produced during very early stages of liver fibrogenesis. Among the first chemokines released are CCL2/3/4 and CXCL9/10, which will recruit monocytes via CCR2 and Th1 cells via CXCR3 and CCR5. The profibrogenic role of the CCL2-CCR2 pathway is emphasized by the fact that *Ccr2*$^{-/-}$ mice develop significantly less fibrosis compared to wildtype mice.

In the context of T-helper cell derived cytokines liver fibrosis is strongly linked to Th2 responses. The Th2 cytokines IL-4 and IL-13 have been shown to act profibrotic as they can activate fibroblasts, [132-133] whereas the Th1 cytokines IL-12 and IFNγ have a rather antifibrotic effect.[134] More recently it has been shown that IL-17 derived from Th17 cells is also a potent profibrotic mediator as mice deficient for the IL-17 receptor were resistant to fibrosis development and IL-17 itself induced production of TGFβ in the liver and production of collagen by HSC.[135] However, IL-10, an anti-inflammatory cytokine produced by regulatory T cells, has been shown to inhibit hepatic fibrogenesis. IL-10 signaling suppressed synthesis of collagen I,[136] and IL-10 knockout mice have been proven to be more susceptible to develop liver fibrosis.[137]

NKT cells seem to play a dual role in liver fibrosis. For many years they have been thought to be rather profibrogenic as they can promote liver fibrosis by the production of profibrotic cytokines like IL-4 and IL-13.[138] But in the recent years it has been shown that they can also inhibit fibrosis by producing IFNγ and killing HSC under specific conditions.[139] NKT cells do not only influence fibrogenesis directly but by releasing cytokines like IFNγ or IL-4 they can also polarize towards antifibrotic Th1 or profibrotic Th2 responses, respectively.

In contrast, NK cells appear to have a protective function during fibrosis development. They can kill activated HSC through cell lysis and induction of apoptosis in mice[140-141] and men[142] and they also produce the antifibrotic cytokine IFNγ. These effects promote resolution of extracellular matrix and balance towards Th1 responses. However, recent studies have shown that this protective functions might critically depend on the subtype of NK cells or disease progression. In patients with chronic HCV infection CXCR3^{+} NK cells seem to be functionally impaired and therefore promoting liver fibrosis,[143] and in

mice subjected to CCl_4 treatment the anti-fibrotic activity of NK cells is lost during advanced stages of the disease, possibly due to increased TGFβ production by HSC.[144]

3.5 Experimental models of liver fibrosis

In order to study the mechanisms underlying liver inflammation and fibrogenesis it is very important to use in vivo models of fibrosis. Mouse models provide a good tool as they are easy to handle und develop liver fibrosis within a few weeks after experimental fibrosis induction. As liver fibrosis can be the result of many different diseases various models of liver fibrosis have been established, which can be subdivided into immunologically mediated, cholestatic, chemically induced, and dietary models.

Immunologically mediated models include infection with parasites[145] and repetitive injection of the T cell activating agent Concanavalin A.[146] In these models, various immune cells get activated, secrete cytokines and activate hepatic stellate cells (HSC). Both models depend on T cells and show an increase in TGFβ expression.

Bile duct ligation is used as a cholestatic model that mimics primary sclerosing cholangitis in humans.[147] Surgical ligation of the bile duct leads to accumulation of bile acids and oxidative stress that will activate the immune system. Mice develop strong liver fibrosis after two to three weeks, and hepatic stellate cells get activated without carcinogenic effects. TGFβ-1 and Kupffer cells seem to be the major players in this model of liver fibrosis.[148]

Models of chemically induced fibrosis are used to study steatohepatits and fibrosis and the role of different parts of the immune system. They are highly reproducible and can be induced through different chemical agents. The most widely used model of toxic liver injury is application of carbon tetrachloride (CCl_4). It mimics human liver fibrosis induced by alcohol or other toxins. Usually, CCl_4 is applied intraperitoneally at a dose of 0.5-2 ml/kg body weight 2-3 times weekly over a period of 4-8 weeks. CCl_4 is transformed into CCl_3 radicals in the liver, which damage hepatocytes and activate HSC.[149] This leads to the production of ECM and upregulation of TGFβ, a hallmark of many fibrosis models. Kupffer cells also play an important role in this model and the robustness of this model led to the identification of several other important factors in this model, like growth factor receptors and angiotensins.[150-151]

Other chemically induced models of fibrosis include treatment with Thioacetamide[152] or Dimethylnitrosamine.[153] Both substances will cause liver fibrosis after several weeks and subsequently lead to tumor formation.

Dietary models like methionine choline-deficient (MCD) diets or high-fat diets are more commonly used to mimic human non-alcoholic steatohepatitis (NASH), but both diets will also cause liver fibrosis. Mice fed a MCD diet will develop hepatic steatosis and inflammation after 1-2 weeks, which progresses to liver fibrosis after 8 weeks of feeding.[154]

3.6 Aims of this study

The role of CCR6 in liver diseases is largely unknown up to now. A preliminary study investigating 34 patients with various liver diseases found elevated levels of CCR6-expressing hepatic T cells and enhanced intrahepatic levels of CCL20 in fibrotic livers [44], and very recently CCR6-CCL20 has been described to be important in positioning of Th17 cells around inflamed portal tracts in human liver.[120] This suggests that CCR6-CCL20 might indeed be involved in modulating the inflammatory and fibrotic response in liver injury.

In this study the functional relevance of CCR6 in hepatic inflammation and fibrosis was investigated. Based on the findings about the involvement of CCR6 in other immune mediated diseases, we hypothesized that CCR6 is functionally important for limiting liver inflammation and fibrogenesis. To test this hypothesis the following questions were addressed in detail:

- Are CCR6 and CCL20 upregulated in liver tissue of patients with chronic liver disease and fibrotic murine livers?
- Which liver cells populations produce CCL20 upon organ damage?
- Do *Ccr6*$^{-/-}$ mice develop accelerated hepatic fibrosis in experimental models of chronic liver injury?
- Is the inflammatory response enhanced in *Ccr6*$^{-/-}$ mice upon chronic liver injury?
- Is differentiation of CD4^{+} T cells altered upon CCR6 deficiency?
- Which cell types in the liver express CCR6 and/or IL-17?
- Do γδ T cells accumulate in the liver upon injury?
- Is CCR6 needed by γδ T cells to migrate to the liver?
- Can hepatic fibrogenesis be limited by adoptive transfer of wt γδ or CD4^{+} T cells into *Ccr6*$^{-/-}$ mice?
- Is restriction of fibrosis through CCR6^{+} γδ T cells dependent on IL-17?
- Do γδ T cells interact with hepatic stellate cells (HSC)?
- Are γδ T cells able to induce HSC apoptosis in vitro?
- By which mechanisms do γδ T cells induce apoptosis in HSC?

4 Materials and Methods

4.1 Materials

4.1.1 Reagents

Accutase - Enzyme cell detachment medium	eBioscience (Frankfurt, Germany)
Acetic Acid (glacial)	Merck (Darmstadt, Germany)
Aluminium Potassium Sulfate Dodecahydrate	Merck (Darmstadt, Germany)
APC Calibrite beads	BD (Heidelberg, Germany)
Bovine Serum Albumin (BSA)	Sigma-Aldrich (St. Louis, USA)
Brefeldin A	BDBioscences/eBioscience
Calcium chloride dihydrate	Sigma-Aldrich (St. Louis, USA)
Carbon Tetrachloride (CCl_4)	Sigma-Aldrich (St. Louis, USA)
Chloral Hydrate	Merck (Darmstadt, Germany)
Chloramine T Hydrate	Sigma-Aldrich (St. Louis, USA)
Chloroform	Mallinckrodt Baker (Center Valley, USA)
Citric Acid Monohydrate	Sigma-Aldrich (St. Louis, USA)
Collagenase P	Roche (Mannheim, Germany)
Collagenase Type II	Worthington (Lakewood, USA)
Collagenase Type IV (280 U/mg)	Worthington (Lakewood, USA)
Concanavalin A (Con A)	Sigma-Aldrich (St. Louis, USA)
Diethyl Pyrocarbonate (DEPC) 97% (v/v)	Sigma-Aldrich (St. Louis, USA)
4-Dimethylaminobenzaldehyde	Sigma-Aldrich (St. Louis, USA)
Dithiothreitol	AppliChem (Darmstadt, Germany)
DnaseI	Roche (Mannheim, Germany)
Dulbecco´s Modified Eagle Medium (DMEM)	PAA (Pasching, Austria)
Dulbecco´s Phosphate Buffered Saline (PBS)	PAA (Pasching, Austria)
Earle's Balanced Salt Solution (EBSS-)	Gibco (Paisley, UK)
Earle's Balanced Salt Solution with Ca/Mg (EBSS+)	Gibco (Paisley, UK)
Eosin	Merck (Darmstadt, Germany)
Ethanol (absolute)	AppliChem (Darmstadt, Germany)
Ethylene Diamine Tetra-Acetic Acid (EDTA)	AppliChem (Darmstadt, Germany)
Ethylene Glycol Tetra-Acetic Acid (EGTA)	AppliChem (Darmstadt, Germany)
Fetal Calf Serum (FCS)	PAA (Pasching, Austria)
Fixation/Permeabilization Concentrate	eBioscience (Frankfurt, Germany)
Fixation/Permeabilization Diluent	eBioscience (Frankfurt, Germany)
Formaldehyde 4% (w/v)	Fischar (Saarbrücken, Germany)
Formaldehyde 37% (w/v)	Carl Roth (Karlsruhe, Germany)
Glucose	Sigma-Aldrich (St. Louis, USA)
Haematoxilin	Merck (Darmstadt, Germany)
Hank´s Balanced Salt Solution (HBSS)	PAA (Pasching, Austria)
Hoechst 33258	Invitrogen (Paisley, UK)
Hydrochloric Acid 37% (v/v)	Carl Roth (Karlsruhe, Germany)
cis-4-Hydroxy-D-Proline	Sigma-Aldrich (St. Louis, USA)
Ionomycin Calcium Salt	Sigma-Aldrich (St. Louis, USA)
Isopropanol	Carl Roth (Karlsruhe, Germany)
Ketamin 10% (v/v)	CEVA (Düsseldorf, Germany)

Lymphocyte seperation medium LSM 1077	PAA (Pasching, Austria)
Magnesium Chloride Hexahydrate	AppliChem (Darmstadt, Germany)
Magnesium Sulfate Heptahydrate	AppliChem (Darmstadt, Germany)
Methionine-choline-deficient diet	MP Biomedicals (Solon, USA)
Methanol	VWR (Radnor, USA)
Nonidet P40 Substitute (NP40)	AppliChem (Darmstadt, Germany)
Nycodenz	Axis-Shield PoC AS (Oslo, Norway)
Penicillin/Streptomycin (100x premix)	PAA (Pasching, Austria)
Perchloric Acid 70% (v/v)	Sigma-Aldrich (St. Louis, USA)
Permeabilization Buffer (10x)	eBioscience (Frankfurt, Germany)
Pharmlyse 10x	BD Biosciences (Heidelberg, Germany)
Phenylmethanesulfonylfluoride (PMSF)	AppliChem (Darmstadt, Germany)
Phorbol-12-myristate-13-acetate (PMA)	AppliChem (Darmstadt, Germany)
Picric Acid	Chroma Waldeck (Münster, Germany)
Potassium chloride	Sigma-Aldrich (St. Louis, USA)
Potassium Hydrogen Phosphate	Sigma-Aldrich (St. Louis, USA)
Pronase E	Merck (Darmstadt, Germany)
Protease Inhibitor Cocktail Tablets	Roche (Mannheim, Germany)
R1/R2 ALT Detection Reagents	Roche (Mannheim, Germany)
Roswell Park Memorial Institute (RPMI) 1640	PAA (Pasching, Austria)
Roti®-Histokitt (mounting medium for histology)	Carl Roth (Karlsruhe, Germany)
Saponin	Sigma-Aldrich (St. Louis, USA)
Serum, Human	Sigma-Aldrich (St. Louis, USA)
Serum, Mouse	Sigma-Aldrich (St. Louis, USA)
Serum, Rabbit	Sigma-Aldrich (St. Louis, USA)
Sirius Red F3B	Chroma Waldeck (Münster, Germany)
Sodium Acetate Trihydrate	Sigma-Aldrich (St. Louis, USA)
Sodium Azide	AppliChem (Darmstadt, Germany)
Sodium Chloride	Sigma-Aldrich (St. Louis, USA)
Sodium Citrate	Sigma-Aldrich (St. Louis, USA)
Sodium Deoxycholate	AppliChem (Darmstadt, Germany)
Sodium Hydrogen Carbonate	Sigma-Aldrich (St. Louis, USA)
Sodium Hydrogen Phosphate (Mono/Dihydrate)	AppliChem (Darmstadt, Germany)
Sodium Hydroxide Pellets	Merck (Darmstadt, Germany)
Sodium Iodate	Merck (Darmstadt, Germany)
Sodium Orthovanadate	AppliChem (Darmstadt, Germany)
Sodium Phosphate	Sigma-Aldrich (St. Louis, USA)
Tissue-Tek O.C.T Compound	Sakura (Heppenheim, Germany)
Tris Hydrochloride (Tris-HCl)	Carl Roth (Karlsruhe, Germany)
Triton X-100	AppliChem (Darmstadt, Germany)
Trypan blue	Sigma-Aldrich (St. Louis, USA)
Trypsin Inhibitor	Sigma-Aldrich (St. Louis, USA)
Tween 20 SigmaUltra	Sigma-Aldrich (St. Louis, USA)
Vecta shield Mounting Medium with DAPI	Vector (Burlingame, USA)
Xylazin 2% (v/v)	Medistar (Hannover, Germany)
Xylol	Fischar (Saarbrücken, Germany)

Table 2. *Reagents and chemicals.*

4.1.2 Kits

ArrayPure Nano-scale RNA Purification Kit	Epicentre Biotechnologies (Madison, USA)
Dako ChemMate™ detection kit peroxidase/DAB	DAKO (Hamburg, Germany)
DC Protein Assay	Bio-Rad (Hercules, USA)
peqGOLD TriFast	peqLab (Erlangen, Germany)
Ready SET-Go® ELISA Kit IFNγ	eBioscience (Frankfurt, Germany)
Ready SET-Go® ELISA Kit IL-4	eBioscience (Frankfurt, Germany)
Ready SET-Go® ELISA Kit IL-10	eBioscience (Frankfurt, Germany)
Ready SET-Go® ELISA Kit IL-17	eBioscience (Frankfurt, Germany)
SYBR GreenqPCR Supermix	Invitrogen (Paisley, UK)
Transcriptor First Strand cDNA Synthesis Kit	Roche (Mannheim, Germany)

Table 3. *Kits*

4.1.3 Equipment

7300 Real Time PCR System	Applied Biosystems (Darmstadt,Germany)
accu-jet® pro	Brand (Wertheim, Germany)
Autoklav varioklav	Thermo Scientific (Waltham, USA)
AxioCam Icc3 Camera	Zeiss (Göttingen, Germany)
AxioCam MRm Camera	Zeiss (Göttingen, Germany)
Axio Imager.Z1 Fluorescence Microscope	Zeiss (Göttingen, Germany)
BD FACS Aria II-SORP	BD (Franklin Lakes, USA)
BD FACSCanto II Flow Cytometer	BD (Franklin Lakes, USA)
BD LSR Fortessa Flow Cytometer	BD (Franklin Lakes, USA)
Biophotometer	Eppendorf (Hamburg, Germany)
Calibra 852 8-channel pipet	Socorex (Ecublens, Switzerland)
Concentrator Plus Speedvac	Eppendorf (Hamburg, Germany)
Dako TechMate™ immunostainer	DAKO (Hamburg, Germany)
Dispenser HandySTep	Brand (Wertheim, Germany)
DM1000 Bright Field Microscope	Leica (Wetzlar, Germany)
EC3 Camera	Leica (Wetzlar, Germany)
Eppendorf Research Pipets 10/20/100/200/1000µl	Eppendorf (Hamburg, Germany)
HM 550 Cryostat	Microm (Walldorf, Germany)
Heracell 150i Incubator	Thermo Scientific (Waltham, USA)
Megafuge 1.0R	Thermo Scientific (Waltham, USA)
MilliQ water purification system	Millipore (Schwalbach, Germany)
Onyx 2000 incubator	Hermle (Wehingen, Germany)
potter homogenizer with teflon pestle	VWR (Radnor, USA)
PB11 ph-Meter	Sartorius (Göttingen, Germany)
Reax Top vortex	Heidolph (Schwabach, Germany)
µQuant Microplate Spectrophotometer	Bio-Tek (Winooski, USA)
Sartorius pro 32/34 F balcance	Sartorius (Göttingen, Germany)
Table top centrifuge 1457R	Eppendorf (Hamburg, Germany)
T3000 Thermocycler	Biometra (Göttingen, Germany)

Thermomixer compact	Eppendorf (Hamburg, Germany)
Ultra Turrax (for RNA)	IKA (Staufen, Germany)
VMS-A Magnetic stirrer	VWR (Radnor, USA)

Table 4. *Equipment*

4.1.4 Consumables

cell culture plates (6-well/96-well)	BD Falcon (Franklin Lakes, USA)
Cell Strainer (70 µm)	BD Falcon (Franklin Lakes, USA)
Cryotube vials 1,0ml	Nunc (Penfield, USA)
Costar® 96-well plates for ELISA	Corning (Tewksbury, USA)
Cover slips 24x48mm	Carl Roth (Karlsruhe, Germany)
Disposable serological pipette 5/25/50ml	Corning (Tewksbury, USA)
Disposable serological pipette 10ml	Corning (Tewksbury, USA)
Falcon tubes for FACS (5ml)	BD Falcon (Franklin Lakes, USA)
False bottom tubes	Roche (Mannheim, Germany)
Filter Papers MN 640 w Ø 90 mm	Macherey-Nagel (Düren, Germany)
Flip Top conical centrifuge tubes 15ml	Nunc (Penfield, USA)
Flip Top conical centrifuge tubes 50ml	Nunc (Penfield, USA)
Kapillary 30mm 0,6mm diameter	Hilgenberg (Malsfeld, Germany)
Microlance 3 20G Needle	BD (Franklin Lakes, USA)
Microlance 3 25G Needle	BD (Franklin Lakes, USA)
Microlance 3 27G Needle	BD (Franklin Lakes, USA)
Microtube 1,3ml K3E	Sarstedt (Nümbrecht, Germany)
Microtube 1,1ml Z-Gel	Sarstedt (Nümbrecht, Germany)
Microscope Slides Super Frost	Carl Roth (Karlsruhe, Germany)
Millex GP Filter Unit (0.22 µm)	Millipore (Schwalbach, Germany)
Nylon mesh 100µm	VWR (Radnor, USA)
Omnican F 0.3x12 mm 1ml Syringe	Braun (Melsungen, Germany)
Parafilm	Pechiney packaging
Pipet tips TipOne (1-0/2-200/100-1000µl)	StarLab (Hamburg, Germany)
Safe-lock tubes (0,5ml/1,5ml/2ml)	Eppendorf (Hamburg, Germany)
Semper Care nitrile examination gloves	SemperMed (Vienna, Austria)
Stericup (0.22 µm)	Millipore (Schwalbach, Germany
Syringe without needle 2/5/10/20ml (disposable)	Terumo (Eschborn, Germany)
Syringe without needle 1ml (disposable)	BD (Franklin Lakes, USA)
Tissue-Tek Cryomold® standard	Sakura (Staufen, Germany)

Table 5. *Consumables*

4.1.5 Buffers and solutions

BSA Solution (30% (w/v))	4.5 mM BSA in PBS
Buffer A for hydroxyproline assay	850mM NaOH 162 mM Citric Acid Monohydrate 881 mM Sodium Acetate Trihydrate in Aqua dest Adjust pH to 6.0 with Acetic Acid
Buffer B for hydroxyproline assay	60 ml Isopropanol 100% (v/v) 33 ml Aqua dest 39 ml Buffer A
Chloramine T Solution	26.4 mM Chloramine T in Buffer B
Citrate Buffer 10mM pH6,0	9 mM citric acid monohydrate in Aqua dest
Collagenase Stock Solution	2% (w/v) Collagenase in RPMI
Collagenase Working Solution	0.2% (w/v) Collagenase in HBSS
DEPC water	6.2 mM DEPC in Aqua dest (autoclaved)
EBSS- for hepatocyte isolation	0.5 mM EGTA in EBSS-
EBSS++ for hepatocyte isolation	0.04% (w/v) Collagenase Type II 0.004% (w/v) Trypsin Inhibitor in EBSS+
EDTA stock solution	100 mM EDTA (pH 8.0) in Aqua dest
Ehrlich´s Reagent	634 mM 4-Dimethylaminobenzaldehyde 26 ml Isopropanol 100% (v/v) 8 ml 50% (v/v) Perchloric Acid

ELISA wash buffer	0,1% (v/v) Tween20 in PBS
Eosin Solution	1% (w/v) Eosin in 100 ml Aqua dest 1 drop of Acetic Acid (glacial)
FACS Blocking Buffer (2x)	2% (v/v) Mouse Serum 2% (v/v) Rabbit Serum 2% (v/v) Human Serum 2% (w/v) BSA in sterile PBS
FACS Staining Buffer	FACS Blocking Buffer 1x in FACS Buffer
FACS Buffer	0,1% (w/v) BSA 2mM EDTA in PBS
Formalin Solution	4% (w/v) Formaldehyde in PBS
GBSS-Buffer	5 mM KCl 1 mM $MgCl_2$ x 6 H_2O 284 µM $MgSO_4$ x 7 H_20 481 µM Na_2HPO_4 x 2 H_2O 220 µM KH_2PO_4 5.5 mM Glucose 2.7 mM $NaHCO_3$ 1.5 mM $CaCl_2$ x 2 H_2O 137 mM NaCl 17 µM Phenol-Red in Aqua dest pH 7,3-7,4
HBSS++	15 µM BSA 2 mM EDTA in HBSS
Haemalum Solution	3.3 mM Haematoxylin 1 mM Sodium Iodate 194 mM Aluminum Potassium Sulfate 303 mM Chloral Hydrate 5.2 mM Citric Acid in Aqua dest

Hydroxyproline stock solution for standard (1mg/ml)	7.6 mM cis-4-Hyroxy-D-Proline in 50% (v/v) Isopropanol
Lysis Buffer (for RBC)	1x Pharmlyse (BD) in Aqua dest
Nicoletti buffer	0.1% (v/v) Triton-X100 3.9 mM Sodium Citrate in Aqua dest
Real Time Amplification PCR Mix	50% (v/v) SYBR Green Mix 200 nM Forward Primer 200 nM Reverse Primer in DEPC water containing cDNA
Reverse Transcription PCR Mix	RNAse Inhibitor (2.86 U/µl) Reverse Transcriptase (1.43 U/µl) dNTP Mix (2.86 mM) in Reverse Transcriptase Buffer
RIPA (Radio Immuno Precipitation Assay) Buffer	50 mM Tris-HCl pH 7.4 150 mM NaCl 1% (v/v) NP40 12.7 mM Deoxycholic Acid 1 mM PMSF
RPMI++	10% (v/v) FCS Penicillin/Streptomycin (1x) in RPMI 1640
Saponin buffer	0,5% (w/v) Saponin in aqua dest
SC1-Medium	137 mM NaCl 5.4 mM KCl 565 µM NaH_2PO_4 x H_2O 848 µM Na_2PO_4 x 2 H_2O 10 mM HEPES 4.2 mM $NaHCO_3$ 500 µM EGTA 5 mM Glucose 17 µM Phenol-Red in Aqua dest pH 7,3-7,4
SC2-Medium	3.8 mM $CaCl_2$ x 2 H_2O in SC1 without EGTA and Glucose

Sirius red staining solution	0.1% (w/v) sirius red 1,2% (v/v) picric acid pH 2,0
Tissue Lysis Buffer	1 mM Sodium Orthovanadate 1 mM DTT 1 Protease Inhibitor Cocktail Tablet 1 mM PMSF in 10ml RIPA Buffer
Tris	pH 6.8/pH 8.8 1 M Tris in Aqua dest

Table 6. *Buffers and Solutions*

4.1.6 Primers for genotyping

All primers were synthesized by Eurofins MWG Operon (Ebersberg, Germany)

Target	**Sequence**
CCR6	fw-GCGATGCACTGTGTAAACTGAT rev-TGTTGACCGCAGTCACGAGGA
IL-17A/F (knockout allele)	fw-ATCCAATCCCCCATCACCTT rev-GTTGGGACTTGCCATTCTGA
IL-17 A/F (Exon 3 of IL-17 gene)	fw-CTTTCAGGGTCGAGAAGATGCTG rev-AAGCAGTTTGGGACCCCTTTACA

Table 7. *Primers for gernotyping*

4.1.7 Primers for qPCR human

All primers were synthesized by Eurofins MWG Operon (Ebersberg, Germany)

Target	**Sequence**
β-actin	fw-TCACCCACACTGTGCCCATCTACGA rev-CAGCGGAACCGCTCATTGCCAATGG
CCL20 (MIP3α)	fw-TGCTGTACCAAGAGTTTGCTC rev-CGCACACAGACAACTTTTTCTTT
CCR6	fw-GCCCACAATGAGCGGGGAATC rev-AGCCTGGAGAACTGCCTGAC

Table 8. *Primers for qPCR human*

4.1.8 Primers for qPCR murine

All primers were synthesized by Eurofins MWG Operon (Ebersberg, Germany)

Target	Sequence
β-actin	fw-CTCTAGACTTCGAGCAGGAGATGG rev-ATGCCACAGGATTCCATACCCAAGA
Bax	fw-CGAGCTGATCAGAACCATCA rev-GGTCCCGAAGTAGGAGAGGA
Bcl2	fw-GCTGAGCAGGGTCTTCAGAG rev-AGTACCTGAACCGGCATCTG
CCL20 (MIP3α)	fw-ATCAACTCCTGGAGCTGAGAATGG rev-ATGTACGAGAGGCAACAGTCGTAG
CCR6	fw-GGTACGCTCCAGAACACTGACG rev-ACCTGTACCGTGGCTCACAGAC
FoxP3	fw-CAGTCTCTGGAGCAGCAGCTGGA rev-CCTGAGTACTGGTGGCTACGATG
GATA-3	fw-CCCGAGTACAGCTCTGGACTCTTC rev-TCACACACTCCCTGCCTTCTG
IFNg	fw-GGAGGAACTGGCAAAAGGATGG rev-TGTTGCTGATGGCCTGATTGTC
IL-4	fw-CGTCCTCACAGCAACGAAGAACAC rev-AAGAGTCTCTGCAGCTCCATGA
IL-10	fw-GGCTGAGGCGCTGTCATCG rev-TCATTCATGGCCTTGTAGACACC
IL-17A	fw-GCGTGTCCAAACACTGAGGC rev-GGGTCTTCATTGCGGTGGAG
RORγt	fw-GGCATGTCCCGAGATGCTGTC rev-TGTGTCTGCTCCGCGGCTCC
T-bet	fw-AGAAGGCAGCATGCCAGGGAAC rev-GCACTGCAGGCAGCCTCTGG

Table 9. *Primers for qPCR murine*

4.1.9 Antibodies

Antigen	Clone	Conjugate	Company
B220	RA3-6B2	PerCP-Cy5.5	BD Pharmingen
CD3a	145-2C11	PE-Cy7	eBioscience
CD4	RM4-5	APC	BD Pharmingen
	RM4-5	FITC	BD Pharmingen
	RM4-5	PerCP-Cy5.5	BD Pharmingen
	RM4-5	PE	BD Pharmingen
CD8a	53-6.7	APC	BD Pharmingen
	53-6.7	FITC	BD Pharmingen
CD11b	M1/70	PE	eBioscience
	M1/70	APC	eBioscience
CD45	30-F11	APC-Cy7	eBioscience
	30-F11	Purified	BD Pharmingen
CD45.1	A20	FITC	eBioscience
CD45.2	104	PE	eBioscience
CD115	AFS98	PE	eBioscience
Desmin	polyclonal	Purified	Thermo Scientific
F4/80	BM8	PE-Cy7	eBioscience
	CI:A3-1	Biotin	AbD Serotec
FoxP3	FJK-16s	PE-Cy7	eBioscience
gamma/delta TCR	eBioGL3	PE	eBioscience
	eBioGL3	APC	eBioscience
	eBioGL3	Purified	eBioscience
Gr1 (Ly6C+Ly6G)	RB6-8C5	PerCP-Cy5.5	BD Pharmingen
IFNγ	XMG1.2	Alexa647	eBioscience
IL-4	11B11	PE	BD Pharmingen
IL-17	TC11-18H10	PE	BD Pharmingen
Ly6G	1A8	FITC	BD Pharmingen

NK1.1	PK136 PK136	PE Biotin	eBioscience eBioscience
RORγt	AFKJS-9	PE	eBioscience
T-bet	eBio4B10	PerCP-Cy5.5	eBioscience
donkey anti-rabbit IgG1	polyclonal	Alexa594	Invitrogen
goat anti-hamster IgG	polyclonal	DyLight488	AbD Serotec
rat IgG1 isotype control	R3-34	PE	BD Pharmingen
hamster IgG1 isotype control	G235-2356	Biotin	BD Pharmingen
Streptavidin-conjugates			
Biotin		Streptavidin PE-Cy7 Streptavidin PerCP-Cy5.5	eBioscience BD Pharmingen

Table 10. *Antibodies and Streptavidin-conjugates*

4.2 Methods

4.2.1 Human samples

Human liver tissue was acquired either from biopsies for routine clinical purposes or explants of cirrhotic livers obtained during liver transplantation. An independent pathologist (Prof. Nikolaus Gassler, Institute of Pathology, University Hospital Aachen) blinded to experimental data performed grading (grade of inflammation) and staging (stage of fibrosis) according to the Desmet-Scheuer score.[155] *CCR6* and *CCL20* mRNA expression was analysed in a total of 50 liver specimens from varying etiologies encompassing n=15 hepatitis C (HCV), n=10 hepatitis B (HBV), n=11 alcoholic liver disease, n=11 primary biliary cirrhosis (PBC), n=3 miscellaneous. Tissue from tumour-free margins of resected hepatic metastasis within normal liver parenchyma (n=5) served as controls.

4.2.2 Animals

All mice were housed under standardized and specific pathogen-free conditions according to german legal requirements (Deutsches Tierschutzgesetz, FELASA, GV-SOLAS) in the Institute of laboratory animal science at the University Hospital Aachen. Central air conditioning ensured for a constant temperature of 20-24°C and humidity of 50-60% and a 12 hours light-dark rhythm was maintained. Mice were kept in Type 2 long cages in groups of 6 to 8 animals per cage. They were provided with food and water ad libitum. Experiments were performed with age and sex-matched animals at 6 to 12 weeks of age under ethical conditions approved by the appropriate authorities according to German legal requirements (Tierversuchsantrag 50.203.2 AC 5a, 30/06 and 87-51.04.2010.A199).
The following mouse strains were used:

C57Bl/6	Wild type mice (CD45.2 allele)	Jackson Laboratory, Bar Harbor, Maine USA
Ccr6$^{-/-}$	C57Bl/6 mice deficient for CCR6 gene	Kindly provided by Sergio A. Lira, New York, USA[16]
C57Bl/6 *Ptprc*	C57Bl/6 mice transgenic for CD45.1 gene	Jackson Laboratory, Bar Harbor, Maine USA
Il-17$^{-/-}$	C57Bl/6 mice deficient for IL-17 A/F gene	Kindly provided by Immo Prinz, Hannover, Germany[156]

Table 11. *Mouse strains*

4.2.3 Induction of liver damage and fibrosis

To induce liver damage mice were injected intraperitoneally (ip) with 0.6 ml/kg body weight of carbon tetrachloride (CCl_4, Merck) mixed with corn oil. Per injection every mouse received 50µl of the CCl_4/oil mix depending on the body weight according to the table below. For induction of liver fibrosis, CCl_4 was injected three times a week over a period of 4 weeks. Mice were sacrificed 48 hours after the last injection.

Mouse weight range (g)	CCl_4 volume (µl)	Oil volume (µl)
17-20	222	778
20-23	258	742
23-26	294	706
26-29	336	664

Alternatively, mice were fed a methionine-choline-deficient (MCD) diet (MP Biomedicals) over a period of 8 weeks to induce hepatic steatosis and, subsequently, fibrosis.

4.2.4 Measurement of liver enzymes in serum

Serum levels of alanine-aminotransferase (ALT) and aspartate-aminotransferase (AST) are important parameters when assessing liver damage. ALT is found predominantly in the liver while AST is also found in other organs like heart, brain, skeletal muscle, and kidney making ALT the more specific marker for liver damage. Both enzymes are released to serum from the cytoplasm of damaged hepatocytes, therefore serum activity of ALT and AST closely correlates with liver damage.[157] Measurement of this activity was performed by the clinical routine laboratory of the University hospital Aachen. For this purpose blood was drawn from the heart of the mouse and centrifuged at 10000x g for 10 minutes at 4°C in a microcentrifuge. Plasma was diluted with NaCl and subjected to an enzyme activity assay according to the IFCC method. This method is a UV based assay where the oxidation of NADH to NAD^+ is photometrically measured. In principal, samples are mixed with all the substances necessary for the transfer reaction: enzymes (lactatedehydrogenase/malatdehydrogenase), coenzyme (pyridoxalphosphate), substrates (L-aspartate/L-alanine), a-ketoglutarat and NADH. The expected products for the transaminase reactions are glutamate and pyruvate for ALT and glutamate and oxalacetate for AST. Pyruvate and oxalacetate are then reduced by lactatedyhdrogenase or malatdehydrogenase, respectively, leading to oxidation of NADH to NAD^+. Accumulation of

NAD^+ correlates with transaminase activity and causes a change in absorbance of UV light that can be measured on a photometer.

4.2.5 Hydroxyproline assay

Hydroxyproline is an amino acid abundantly present in collagen but almost no other proteins. Therefore it is commonly used as a measure for total liver collagen. Liver samples were hydrolyzed with 6N HCl at a concentration of 1mg liver per 10µl of HCL for 16 hours at 110°C. Samples were then mixed with methanol in a 1:1 ratio and all liquid removed by vacuum centrifugation. Crystallized samples were then resuspended in 50% (v/v) isopropanol and mixed with 0,6% (w/v) Chloramine-T solution. As a standard purified hydroxyproline was dissolved in 50% (v/v) isopropanol followed by serial dilutions to allow measurement of a standard curve. Standards were also mixed with 0,6% (w/v) Chloramine-T solution. Samples and standards were then incubated for 10 minutes at room temperature. During incubation Chloramine-T would react with the hydroxyproline molecules by oxidizing them resulting in the formation of pyrrole and pyrrole-derivatives. Subsequently Erlich´s reagent was added to samples and standards followed by incubation at 50°C for 45 minutes in a shaker to allow the Ehrlich´s reagent to react with the pyrrole-derivatives to form a chromophore. Absorbance could then be measured at 570nm, all samples and standards were measured in duplicates. 50% (v/v) isopropanol served as a blank. The standard curve allowed to determine absolute hydroxyproline concentrations in liver samples.

4.2.6 Isolation of RNA and qPCR

4.2.6.1 Isolation of RNA from liver samples

Liver samples were homogenized in 1.5ml PeqGold Trifast for 15 seconds using an Ultra-Turrax and incubated at room temperature for 15 minutes. The homogenate was transferred to a microcentrifuge tube and vortexed thoroughly after addition of 300µl of cold chloroform. Samples were incubated on ice for 5 minutes under occasional shaking and subsequently centrifuged for 10 minutes at 4°C in a microcentrifuge at 15000x g. 750µl of the supernatant were transferred to a new microcentrifuge tube without touching the interphase. The same volume of isopropanol was added and samples were inverted for a few times before being incubated on ice for 10 minutes. RNA was pelleted by centrifugation for 10 minutes at 4°C and 15000x g and the supernatant was discarded. Pellets were washed twice by adding 750µl of 70% (v/v) ethanol and centrifugation for 3 minutes at 4°C and 15000x g. Ethanol was removed and pellets left to air-dry for 10-15 minutes. Dry pellets were resuspended in 80µl of DEPC-water and RNA concentration was determined using a

spectrophotometer. Samples were diluted 1:50 in aqua dest and optical densitiy (OD) was measured at 260 and 280 nm, water served as blank. Samples were stored at -80°C until needed.

4.2.6.2 Isolation of RNA from sorted cells

Purification of RNA from cells isolated by FACS sorting was performed using the ArrayPure Nano-scale RNA purification kit (Epicentre Biotechnologies) according to manufacturer´s protocol. In short, cells were washed in PBS and pelleted by centrifugation. Cell pellets were then resuspended in Nano-scale lysis solution containing proteinase K, mixed thoroughly by vortexing and incubated at 65°C for 15 minutes. The samples were cooled on ice for 5 minutes before MCP protein precipitation reagent was added. Samples were vortexed for 10 seconds and debris was pelleted by centrifugation at 10000xg at 4°C for 7 minutes. Supernatants were transferred to new microcentrifuge tubes and mixed with the same volume of isopropanol. RNA was pelleted by centrifugation at 10000xg at 4°C for 5 minutes and residual isopropanol was discarded. After air-drying pellets were incubated with DNase I for 30 minutes at 37°C. Samples were again mixed with lysis solution and MCP reagent and after chilling on ice for 5 minutes pelleted by centrifugation as before. Supernatants were recovered and RNA precipitated with isopropanol. Pelleted RNA was washed once with ethanol and resuspended in DEPC water after air-drying.

4.2.6.3 cDNA synthesis

Reverse transcription of RNA to receive cDNA was carried out using the Transcriptor First Strand cDNA Synthesis Kit (Roche) and all steps were performed on ice if not stated otherwise. 2µg of RNA were mixed with 2µl of a random hexamer primer mix and the reaction volume was adjusted to 13µl with water. Samples were incubated at 65°C for 10 minutes to remove any secondary RNA structures. 7µl of Reverse Transcription PCR Mix containing nucleotides and reverse transcriptase were added and reverse transcription was performed according to the following protocol:

Step 1	Start	25°C	10 min
Step 2	RT reaction	50°C	60 min
Step 3	Inactivation	85°C	5 min
Step 4	Stop	4°C	∞

cDNA solution was diluted 3 times with water and stored at -20°C until further usage.

4.2.6.4 Real time gene expression analysis

2µl of cDNA and specific primers (200nM) were added to 23µl of SYBRGreen qPCR master mix containing, SYBR Green I Dye, dNTPs and DNA polymerase. Realtime PCR was performed on the 7300 realtime PCR System (Applied Biosystems) and amplification of genes was measured in duplicates using β-actin as internal reference (house-keeping gene). Realtime PCR protocol:

Step		Temperature	Time	Cycles
1	Warm up	50°C	2 min	1
2	Hot start	95°C	15 sec	40
	Amplification	60°C	1 min	
3	Melting Curve	95°C	15 sec	1
		60°C	30 sec	
		90°C	15 sec	

At the beginning of each cycle the samples were heated to 95°C to separate the two DNA strands from each other and then cooled down to 60°C to allow annealing of the primers and amplification of the target gene. SYBR Green is a dye emitting fluorescence when bound to double-stranded DNA and being non-fluorescent when unbound. Therefore fluorescence intensity correlates with the amount of DNA molecules and was measured at the end of each amplification step. SYBR Green fluorescence is detectable as soon as it rises above background fluorescence level. The cycle when this occurs is called the threshold cycle and the number of this cycle is the Ct value. All samples were normalized against β-actin as house-keeping gene before samples of interests were compared to control samples. The delta-delta Ct method was used to relatively quantify amplification of target genes:

ΔCt= Ct (target gene) – Ct (reference gene)

$\Delta\Delta Ct$= ΔCt (control sample) – ΔCt (treated sample)

fold induction= $2^{\Delta\Delta Ct}$

4.2.7 Isolation and quantification of total protein from liver samples

Frozen liver samples were thawn in 500µl of tissue lysis buffer and manually broken up using a potter homogenizer with a Teflon pestle. Samples were then centrifuged at

10.000rpm in a microcentrifuge at 4°C for 15 minutes. Supernatants containing the proteins were collected and transferred into clean tubes and pellets containing debris were discarded. Protein concentration in the supernatant was determined using DC protein Assay (Biorad). The DC protein assay is a colorimetric assay similar to the Lowry assay optimized for protein solutions containing detergents. Samples are mixed with an alkaline copper tartrate solution and incubated shortly to allow proteins to build a complex with the copper. Then a solution containing Folin´s reagent is added and samples incubated for 15 minutes at room temperature. The protein-copper-complex causes a reduction of the Folin´s reagent and reduced species have a characteristic blue color. The absorbance increases with protein concentration and can be measured on a spectrophotometer at 750nm. To assess absolute protein concentrations a standard curve was determined every time using different dilutions of bovine serum albumin in the same buffer as the samples.

4.2.8 Enzyme-linked immuno sorbent assay (ELISA)

All ELISAs were performed using Ready-SET-Go!® ELISA-kits from eBioscience. 96-well plates were coated over night at 4°C with the capture antibody. Plates were then washed 5 times with wash buffer. To block unspecific binding ELISA plates were incubated with assay diluent for 1 h at room temperature before being washed as before. After washing 100µl of each sample were added to the appropriate wells at a concentration of 1µg protein/1µl in Assay Diluent. The recombinant cytokine standard provided with the kit was diluted in Assay Diluent according to the manufacturer´s protocol and a series of 2-fold dilutions was prepared to determine the standard curve. Samples and standards were all measured in duplicates. Subsequently plates were incubated at room temperature for 2 hours. After washing biotinylated detection antibody was added to each well and plates were incubated for 1 hour at room temperature. Plates were washed for 5 times before Avidin-HRP in Assay Diluent was added and incubated for another 30 minutes at room temperature to allow binding of avidin and biotin. This time plates were washed for 7 times and substrate solution containing Tetramethylbenzidine (TMB) was added to each well. The avidin-bound HRP converts the TMB substrate into a TMB-diimine which has a blue color. After 10-15 minutes at room temperature the reaction was stopped by adding 2N sulfuric acid (H_2SO_4), which changes the color of the solution from blue to yellow. Absorption of the plate could then be read at 450nm. The standard curve was used to extrapolate absolute cytokine concentrations in the liver extracts, results were normalized to total protein concentration of the extracts.

4.2.9 Isolation of hepatic cell types

4.2.9.1 Isolation of primary hepatocytes

Mice were anaesthetized using a mixture of ketamine and xylazine and heparine was injected intraperitoneally to avoid coagulation. Livers were then perfused via the vena cava inferior with 50ml of EBSS- containing EGTA. Subsequently livers were perfused with 30ml of EBSS+ followed by another 50ml of EBSS++ (EBSS+ containing collagenase and trypsin inhibitor). All solutions were warmed to 37°C before perfusion. Livers were then transferred to a cell culture dish containing medium and hepatocytes were removed by gentle strokes with a cell scraper over each liver lobe. Cell suspension was filtered to remove debris and then centrifuged at 300x g at 4°C to collect the cells. Pellets were either stored at -80°C for later RNA extraction or resuspended in medium for plating on cell culture dishes.

4.2.9.2 Isolation of hepatic stellate cells

Mice were anaesthetized using a mixture of ketamine and xylazine and livers were perfused via the portal vein. All solutions were warmed to 37°C before perfusion. First, livers were perfused with 30ml of SC1 medium to remove all the blood from the liver. Then, 30ml SC2-medium containing Pronase E were used, followed by 30ml of SC2-medium containing collagenase P. Livers were taken out and manually broken up in a cell culture dish with SC2. They were further digested with pronase E, collagenase P and DNase I in SC2 at 37°C for 20 minutes. Cell suspensions were filtered through a nylon mesh, pelleted and treated twice with Dnase. Cells were washed with GBSS buffer, mixed with Nycodenz, covered with GBSS and subjected to density gradient centrifugation at 1500x g for 22 minutes at 4°C without break. The interphase was collected and washed with GBSS. The cell pellet was resuspended in FACS blocking buffer and stained for CD45, F4/80, CD11b and Ly6G. Hepatic stellate cells were then isolated by FACS-sorting as identified by negative staining for CD45, F4/80, CD11b and Ly6G and positive signal in the UV-channel. HSC store vitamin A, which can be excited with a UV-Laser causing the positive signal. Cell sorting was performed on a FACS Aria-II-SORP (BD).

4.2.9.3 Isolation of blood leukocytes

For flow cytometry of blood leukocytes 100µl of blood were drawn from the heart and coagulation was inhibited by EDTA. Red blood cells were removed by using BD Pharm

Lyse lysing buffer (BD) and incubating for 8-10 minutes at room temperature. Cells were washed with PBS to stop lysis and resuspended in PBS to be stored at 4°C until further usage.

4.2.9.4 Isolation of splenic leukocytes

Spleens were homogenized by forcing the tissue through a 70µm nylon mesh using the plunger of a 2ml syringe. Cell suspensions were then filtered again to remove debris. Cells were pelleted at 450xg for 5 minutes at 4°C and resuspended in Pharm Lyse lysing buffer (BD) to remove red blood cells. After 8 minutes of incubation at room temperature lysis was stopped by addition of PBS, cells pelleted as before and resuspended in appropriate volumes of PBS. Cells were stored at 4°C until further usage.

4.2.9.5 Isolation of hepatic leukocytes

Mice were perfused post mortem via the heart with 10ml of PBS to remove all blood from the system. Subsequently livers were taken out and the gall bladder was removed. Livers were then put into cell culture plates with HBSS and cut into small pieces with a scissor. Then they were subjected to digestion using Collagenase Type IV (Worthington) at a concentration of 0,2% (w/v). Samples were incubated at 37°C for 30 minutes. Afterwards digestion was stopped by adding HBSS++ and livers were further homogenized mechanically to get cell suspensions. Cells were filtered through a 70µm nylon mesh and either directly stained to identify neutrophils or exposed to density gradient centrifugation using the lymphocyte separation medium LSM-1077 (PAA). The LSM was carefully pipetted underneath the cell suspensions to build the bottom layer and samples were centrifuged for 20 minutes at 2000 rpm at room temperature without brake. The leukocytes were then collected from the interphase and washed with PBS to remove all residual LSM.

4.2.10 Flow cytometric analysis

4.2.10.1 Surface staining of leukocytes

Staining of leukocytes was performed in FACS blocking buffer using monoclonal antibodies against F4/80 (Serotec), Ly6G (BD Pharmingen), B220, CD3, CD8, CD4, CD11b, CD45, FoxP3, NK1.1, RORγt, T-bet, γδTCR, rat IgG or hamster IgG isotype controls (all eBioscience). All antibodies were used at a dilution of 1:400 and samples were incubated

with antibodies for 15 minutes at 4°C in the dark. Cells were washed with PBS to remove unbound antibodies and resuspended in 100-300µl of FACS buffer for measurement. Hoechst 33258 was added at a final concentration of 0.5µg/ml to exclude dead cells that would be Hoechst positive. APC Calibrate beads (BD) were used as internal standard to determine absolute cell numbers. 20000 beads were added to each sample right before measurement to allow calculation of cell numbers as the proportion of measured beads correlates with the proportion of cells counted. Samples were analyzed using a FACS Canto-II (BD). Data were analyzed with FlowJo (TreeStar).

4.2.10.2 Intracellular staining of cytokine expression

For intracellular staining, cells were cultured in RPMI 1640 containing 10% FCS (v/v), 40 ng/mL PMA (Applichem) and 500 ng/mL ionomycin (Sigma) for 4h at 37°C in a humidified incubator with 5% CO_2. Cytokine secretion was blocked by adding GolgiPlug (BD) according to manufacturer´s protocol for the last 3 hours. Surface staining was performed as described before and cells were fixed in 2% (w/v) Formalin for 15 minutes at 4°C. After washing cells were resuspended in saponin buffer and incubated for 15 minutes at room temperature for permeabilization. Intracellular staining was performed in Saponin solution using antibodies against IFN-γ (eBioscience) or IL-17 (BD Pharmingen) (both 1:400) for 15 minutes at 4°C. Cells were washed as before and resuspended in 200µl of FACS buffer for measurement.

4.2.10.3 Intracellular staining of transcription factors

Staining of transcription factors was performed using the Transcription factor staining Buffer set from eBioscience. Cells were stained for surface markers as described and subsequently fixed and permeabilized with eBioscience Fixation/Permeabilization working solution. Samples were incubated for 30 minutes at 4°C in the dark and washed with 1x eBioscience Permeabilization Buffer. Cells were then stained with antibodies against T-bet, RORγt or FoxP3 1:400 in 1x Permeabilization Buffer for 30 minutes at room temperature. Cells were washed as before and resuspended in 200µl of FACS buffer for measurement.

4.2.10.4 Detection of apoptotic cells using propidium iodide

For detection of apoptotic cells, cells were fixed with 4% (w/v) PFA for 15 minutes at room temperature. After washing with PBS cells were resuspended in Nicoletti buffer and

incubated for 15 minutes at room temperature for permeabilization. Cells were washed again and resuspended in PI/RNAse staining solution (BD) according to manufacturer's protocol. Cells were analyzed immediately after staining.

4.2.11 Sorting of different leukocyte populations

Hepatic or splenic leukocytes were isolated and surface-stained as described for flow cytometric analysis. Cell sorting was then performed on a FACS Aria-II-SORP (BD). Macrophages were identified as $Ly6G^-$ $F4/80^+$ $CD11b^+$ cells, NK cells were identified as $CD3^-$ $NK1.1^+$ cells, and T cell populations were identified as $CD3^+$ $CD4^+$ or $CD3^+$ $CD4^-$ $CD8^-$ $\gamma\delta TCR^+$ cells for $CD4^+$ T cells and $\gamma\delta$ T cells, respectively. Cell sorting was performed on a FACS Aria-II-SORP (BD). Cells were either stored at -80°C for later RNA extraction or resuspended in sterile PBS for adoptive transfer experiments.

4.2.12 Adoptive transfer of T cells

Splenic $CD4^+$ T cells of wildtype or splenic $\gamma\delta$ T cells of wildtype, Actin-eGFP, *Il-17*$^{-/-}$ or *Ccr6*$^{-/-}$ mice were isolated using FACS-Sorting as described before. The cells were identified as $CD3^+$ $CD4^+$ or $CD3^+$ $CD4^-$ $CD8^-$ $\gamma\delta TCR^+$ cells for $CD4^+$ T cells and $\gamma\delta$ T cells, respectively. Cells were then injected once a week into the lateral tail vein of wt and *Ccr6* knockout mice that were treated with CCl_4 thrice a week over a period of four weeks. Between $1x10^6$ and $1{,}5x10^6$ $CD4^+$ T cells or between $1x10^5$ and $1{,}5x10^5$ $\gamma\delta$ T cells were used for every injection.

4.2.13 Coculture experiments

The murine HSC-like cell line GRX or primary HSC were cultured in DMEM in 6-well plates at 50,000 cells per well (GRX) [108] or 150,000 cells per well (HSC). Hepatic $\gamma\delta$ T cells, $CD4^+$ T cells or NK cells were isolated from livers of wt mice 48h after a single CCl_4 injection. Isolated cells were either added directly to the GRX/HSC culture or incubated overnight for production of cytokines, followed by transfer of the supernatant to GRX/HSC after 24h. After

48h of coculture, lymphocytes were washed off thoroughly and GRX cells/HSC were harvested for isolation of RNA or staining with propidium iodide (PI) for FACS analysis.

4.2.14 Histology

All histology samples were fixed in 4% (w/v) formalin to ensure tissue integrity; if not stated otherwise. Embedding and cutting of the samples was performed by the Institute of Pathology of the University Hospital Aachen.

4.2.14.1 Haematoxylin and Eosin (H+E) staining

H+E staining is one of the most widely used staining procedures in histology, because it gives a good overview of tissue structure and characteristics, therefore also indicating tissue integrity and damage. Haematoxylin or haemalum is used to stain basophilic structures like nuclei in blue while eosin stains eosinophilic structures like cytoplasm in different shades of red. For H+E staining formalin-fixed paraffin-embedded liver samples were cut into 1-2µm thick slices and stored at room temperature until usage. For staining slides were heated to 60°C for 30 minutes to melt the paraffin. The samples were then dipped in Xylol 3 times for several minutes to remove all paraffin from the slides. The sections were rehydrated by immersing in descending concentrations of Ehanol (100% (v/v), 96% (v/v) and 70% (v/v), each used twice) for 5 minutes each before being incubated for 5 minutes in aqua dest. The sections were then stained with Mayer´s haemalum solution for 7 minutes at room temperature and afterwards rinsed in running tap water for several minutes. Subsequently slides were counterstained with Eosin solution (1% (w/v)) for 5 minutes and dipped shortly in tap water to remove excess eosin. To dehydrate the stained sections again, slides were immersed in ascending concentrations of ethanol (10 seconds in 70% (v/v) EtOH, 1-3 minutes in 96% (v/v) EtOH and 2x 5 minutes in 100% (v/v) EtOH) and finally dipped three times in xylol. A mounting medium consisting of synthetic resins was used to seal the sections between slide and coverslip. Samples were then examined on a bright field microscope (Leica). H+E staining was performed by the Institute of Pathology of the University Hospital Aachen.

4.2.14.2 Sirius red staining

Sirius red is a dye commonly used to stain collagen fibers in tissue, which serves as a semi-quantitative assessment of tissue fibrosis. Collagen fibers appear red on a yellow

background in bright light, but when examined with polarized light fibers are bright orange (collagen I) or green (collagen III) on black background.

For Sirius red staining paraffin slides were prepared in the same way as for H+E staining (deparaffinization and rehydration). After being incubated 5 minutes in aqua dest slides were immersed in sirius red staining solution for 60 minutes. Excessive staining solution was removed by letting the slides drain on a piece of paper tissue. Slides were then dipped twice in aqua dest containing 0,5% (v/v) acetic acid for 15 seconds each time. Subsequently sections were dehydrated again by immersing in ascending concentrations of ethanol (10 seconds in 70% (v/v) EtOH, 2 minutes in 96% (v/v) EtOH and 2x 5 minutes in 100% (v/v) EtOH) and finally three times for 5 minutes in Xylol. Coverslips were placed on the slides and sealed with synthetic resins containing mounting medium. Samples were examined on a bright-field microscope (Leica) using polarized light to allow quantification of the staining. Morphometric analysis of Sirius Red staining was then performed using ImageJ 1.42q (NIH). Sirius red staining was performed by the Institute of Pathology of the University Hospital Aachen.

4.2.14.3 Immunohistochemistry

For immunostaining of CD45 paraffin slides were pretreated as for H+E staining (deparaffinization and rehydration). Before staining antigen retrieval was conducted by boiling the slides in citrate buffer (pH6.0) in a pressurized heating chamber. Staining and detection of the antibody was performed fully automated using a DAKO TechMate™ immunostainer. The slides were incubated with the primary antibody against CD45 at 4°C over night at a dilution of 1:500. The antibody was detected using Dako ChemMate™ Peroxidase/DAB detection kit and slides were counterstained with heamalum solution to stain nuclei. CD45 staining was performed by the Institute of Pathology of the University Hospital Bonn and positively stained cells were counted by a blinded pathologist.

4.2.14.4 Immunofluorescent staining of cryosections

Unfixed liver samples were embedded in Tissue Tek® O.C.T.™ compound and snap frozen in liquid nitrogen. Samples were then cut into 5µm thick sections on a cryostat. Sections were left to air-dry and fixed in ice-cold acetone for 5 minutes. Acetone-fixed sections were left to air-dry and rehydration was performed in PBS containing 0.02% sodiumazide for 3x 3 minutes followed by blocking with 1% (w/v) BSA in PBS for 5-30 minutes at room temperature. Slides were then incubated with the first antibody (monoclonal

hamster-anti-mouse γδTCR 1:100, polyclonal rabbit-anti-mouse desmin 1:200 or monoclonal rabbit-anti-mouse F4/80 1:200) in PBS containing 1% (w/v) BSA at 4°C over night. Samples were washed 3x 3minutes with PBS and incubated with the secondary antibody (anti-rabbit Alexa594 1:200 for desmin and F4/80 or anti-hamster DyLight488 1:100 for γδTCR) in PBS containing 1% (w/v) BSA for 1 hour at room temperature. Slides were washed as before and mounted using Vectashield® Mounting Medium with DAPI to counterstain cell nuclei. Cover slips were sealed with nail polish before microscopy.

4.2.15 Statistical analysis

Data were analyzed using PRISM 5 software (GraphPad) and are expressed as mean ±standard error of the mean (SEM). Differences between groups were assessed by means of a two-tailed unpaired Student t-test. Differences were considered significant when p-values were below 0.05.

5 Results

5.1 The chemokine receptor CCR6 and its ligand CCL20 are significantly up-regulated in chronic liver injury in mice and men

5.1.1 Expression of *CCR6* and *CCL20* in human tissue

To investigate the potential role of the chemokine receptor CCR6 in chronic liver disease, liver samples of patients with different stages of chronic hepatic injury were assessed for mRNA expression of *CCR6* and its cognate ligand *CCL20*. In comparison to healthy controls both markers were significantly up-regulated in liver tissue of patients with chronic liver diseases (Fig.2A+D). Upon subgroup analysis of patients with either fibrosis or cirrhosis, a clear upregulation of *CCR6* expression compared to controls could be observed in both groups, with even higher expression in cirrhosis than fibrosis (Fig.2B). Also for *CCL20* both groups showed significantly increased expression compared to healthy controls, but *CCL20* expression was even higher in fibrotic compared to cirrhotic livers (Fig.2E). We also analyzed the liver samples of cirrhotic patients according to their underlying disease etiology. Of note, highest expression of *CCR6* was seen in patients with viral hepatitis compared to other disease etiologies (Fig.2C), while highest *CCL20* expression was observed in patients with cholestasis-related primary biliary cirrhosis (Fig.2F).

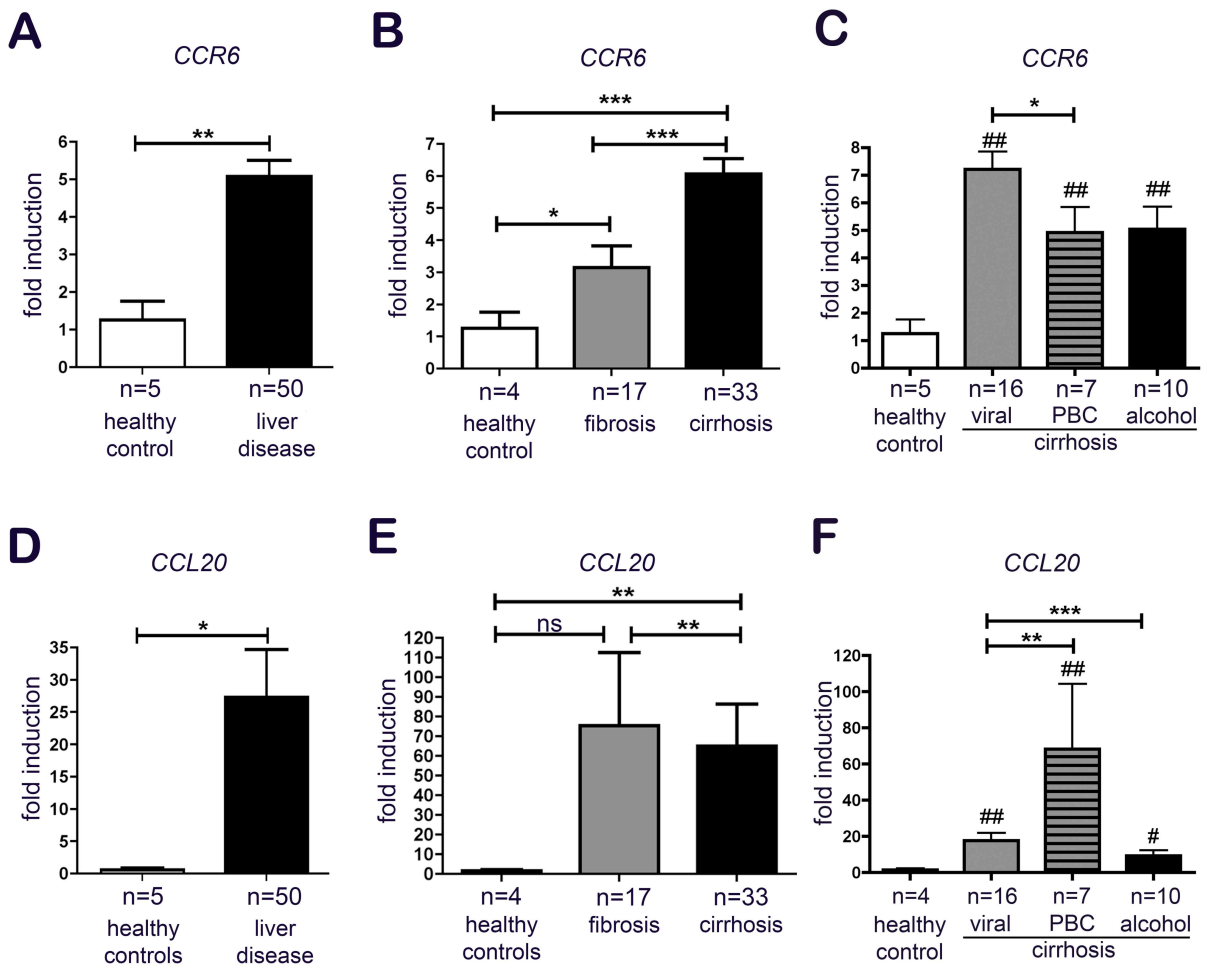


Figure 2. *CCR6 and CCL20 are up-regulated in human chronic liver disease.*

RNA was extracted from liver samples (see Methods section 4.2.6.1) of patients with chronic liver disease and control tissue. *CCR6* (**A-C**) and *CCL20* (**D-F**) expression levels were measured by quantitative real-time PCR (qPCR) (see Methods section 4.2.6.4), normalized to the housekeeping gene β-actin. **C+F** Only patients with cirrhosis were included in the analysis. Viral: viral hepatitis, PBC: primary biliary cirrhosis, alcohol: alcoholic liver disease. */# P<0.05 **/## P<0.01 ***/### P<0.001. #=compared to healthy controls. Data are shown as mean ± SEM.

5.1.2 Expression of *Ccr6* and *Ccl20* in experimental murine chronic liver injury

To assess whether CCR6 and CCL20 are also up-regulated in a murine model of chronic liver disease, c57bl/6 mice were intraperitoneally (ip) injected with the hepatotoxic agent carbon tetrachloride (CCl_4) three times a week over a period of 4 weeks. Mice that had been treated with CCl_4 showed significantly higher hepatic gene expression levels of *Ccr6* and *Ccl20* compared to controls (Fig.3A+B), resembling the results obtained in human samples. Interestingly, when mice deficient for *Ccr6* were treated with CCl_4, the hepatic *Ccl20* expression was even higher compared to CCl_4 treated wildtype mice (Fig.3B), indicating a direct feedback loop between CCL20 and CCR6 in chronic liver injury.

In order to dissect which cell compartment(s) in the inflamed liver induce CCL20 upon chronic injury, different cell types from murine livers were isolated and checked for their *Ccl20* expression. As expected, freshly isolated hepatocytes from wt livers treated with CCl_4 appeared severely affected and were lower in numbers compared to hepatocytes from untreated livers (Fig.3C). However, hepatocytes clearly up-regulated *Ccl20* upon CCl_4 treatment compared to cells from untreated livers, whereas in freshly isolated primary hepatic stellate cells (HSC) and Kupffer cells/macrophages no expression of *Ccl20* could be detected (Fig.3D). Together, these results demonstrate that the CCR6/CCL20 pathway is activated in chronic liver disease in men and mice, and the experimental murine data indicate that injured hepatocytes strongly induce *Ccl20* expression, thus likely regulating CCL20-mediated chemotaxis on CCR6-expressing leukocytes upon liver injury.

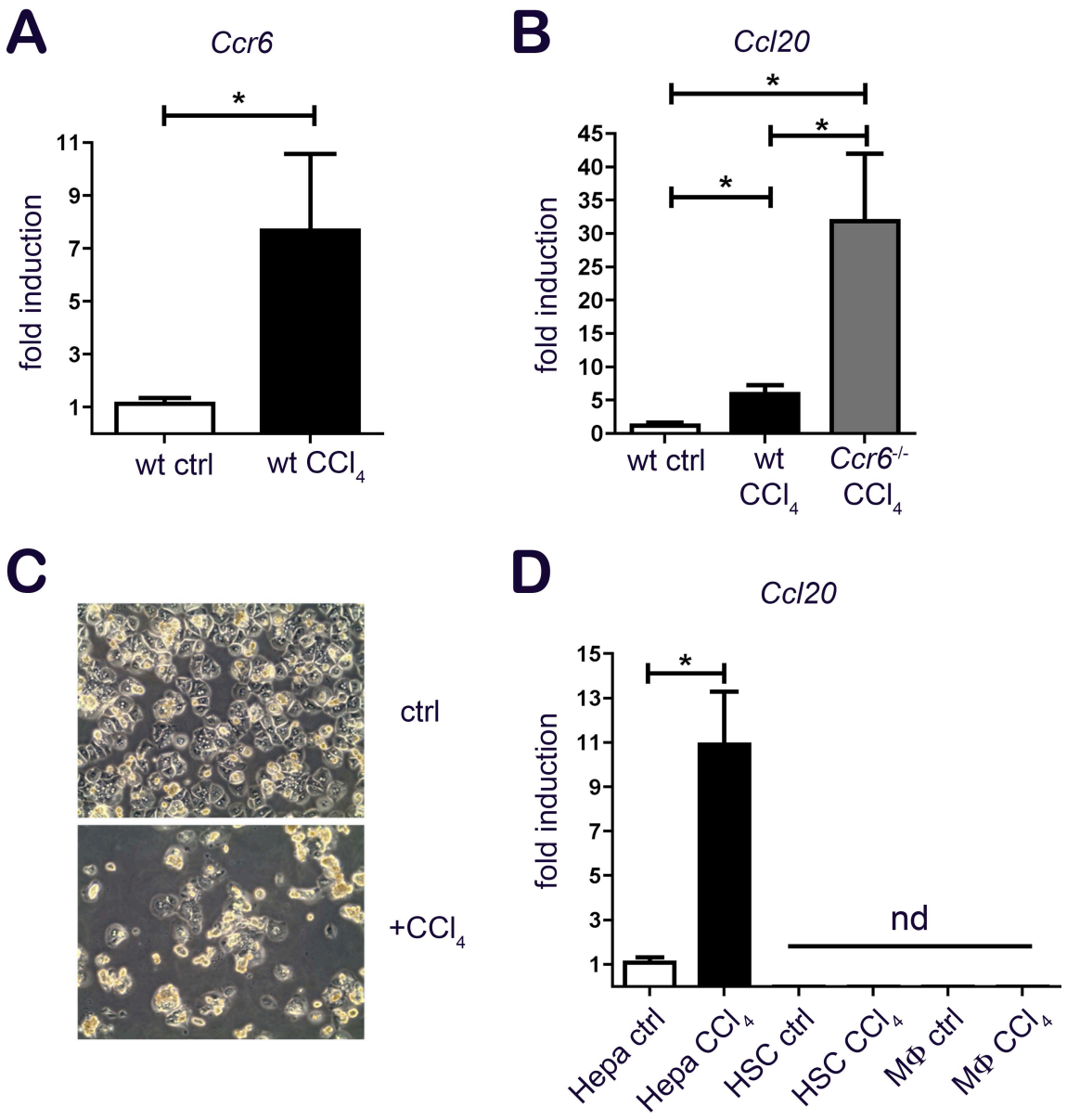


Figure 3. *Ccr6 and Ccl20 are upregulated in murine chronic liver injury.*

A-B C57bl/6 wt mice were treated with CCl_4 thrice weekly over 4 weeks, and RNA was extracted from liver (see Methods section 4.2.6.1). *Ccr6* and *Ccl20* expression were measured by qPCR (see Methods section 4.2.6.4). **C** Primary hepatocytes were isolated from control or CCl_4-treated wt mice and plated on cell culture dishes (see Methods section 4.2.9.1). Representative pictures, taken immediately after isolation and seeding. **D** Hepatocytes (Hepa), hepatic stellate cells (HSC) and macrophages (MΦ) were isolated from livers of control or CCl_4-treated wt mice (see Methods section 4.2.9.1ff.), and *Ccl20* expression was determined by qPCR. *P <0.05. Data are shown as mean ± SEM of 4-7 mice per group from two independent experiments.

5.2 *Ccr6*$^{-/-}$ mice develop more severe hepatic fibrosis upon chronic liver injury

In order to elucidate the functional relevance of CCR6/CCL20 for liver disease progression, liver fibrosis was induced by thrice weekly ip administrations of CCl_4 over a period of 4 weeks in wt and *Ccr6*$^{-/-}$ mice. Conventional (hematoxylin/eosin) histology stainings showed no difference between untreated wt and *Ccr6*$^{-/-}$ mice and severely damaged liver architecture in both wt and *Ccr6*$^{-/-}$ mice after CCl_4 treatment. Injured livers displayed large areas of necrotic hepatocytes and dense periportal inflammatory infiltrates (Fig.4A). We also determined the serum activity of liver enzymes in these mice. Damaged or dying hepatocytes release aminotransferases into the circulation, which can then be measured in the serum and are used as a clinical marker for liver damage. Accordingly, serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were increased in wt and *Ccr6*$^{-/-}$ mice after CCl_4 treatment, with a tendency towards higher aminotransferases in CCl_4-treated *Ccr6*$^{-/-}$ animals (Fig.4B).

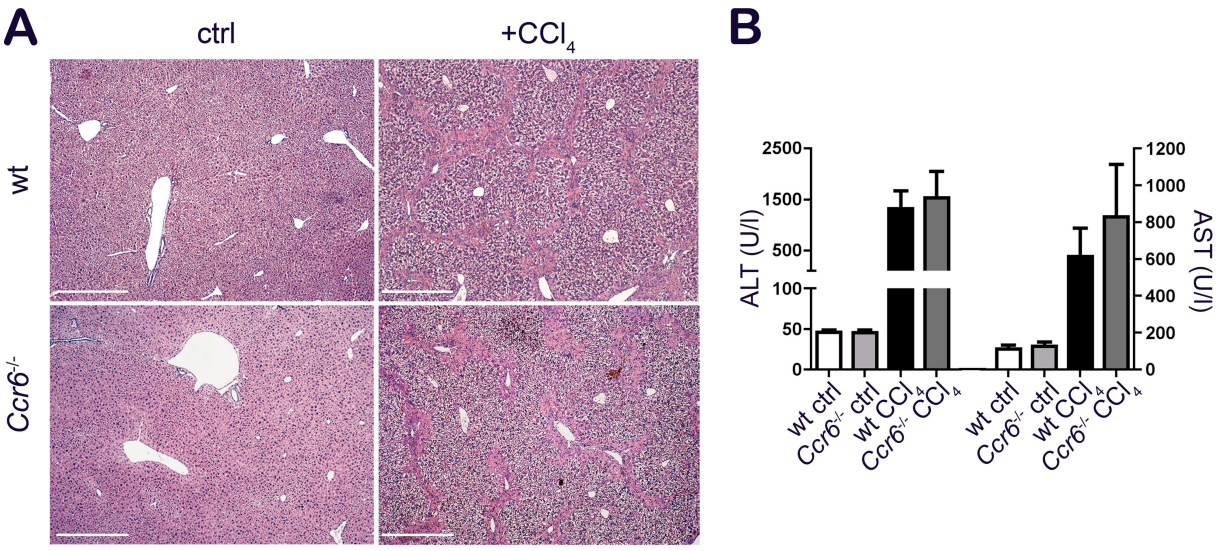


Figure 4. *Severe liver damage after CCl_4 treatment.*

A Hematoxylin-eosin stainings from livers of wt and Ccr6$^{-/-}$ mice challenged with CCl_4 three times per week for 4 weeks or controls (see Methods section 4.2.14.1). Scale bar: 500µm. **B** Alanine (ALT) and aspartate (AST) aminotransferase serum activities of wt and Ccr6$^{-/-}$ mice (see Methods section 4.2.4). Data are shown as mean ± SEM of n=10-13 mice per group from 4 independent experiments.

As a consequence of chronic CCl_4 administration, severe hepatic fibrosis develops in this model. Chronic inflammation leads to formation of scar tissue characterized by excessive production of extracellular matrix and deposition of collagen. Quantification of extracellular collagen deposition by Sirius Red stainings revealed significantly enhanced liver fibrosis in *Ccr6*$^{-/-}$ mice compared to wt mice (Fig.5A+B). This was confirmed by increased hepatic concentrations of hydroxyproline (Fig.5B), an amino acid abundantly present in collagen matrices but almost no other proteins. Therefore, hydroxyproline is used as a measure of total liver collagen and fibrotic tissue.

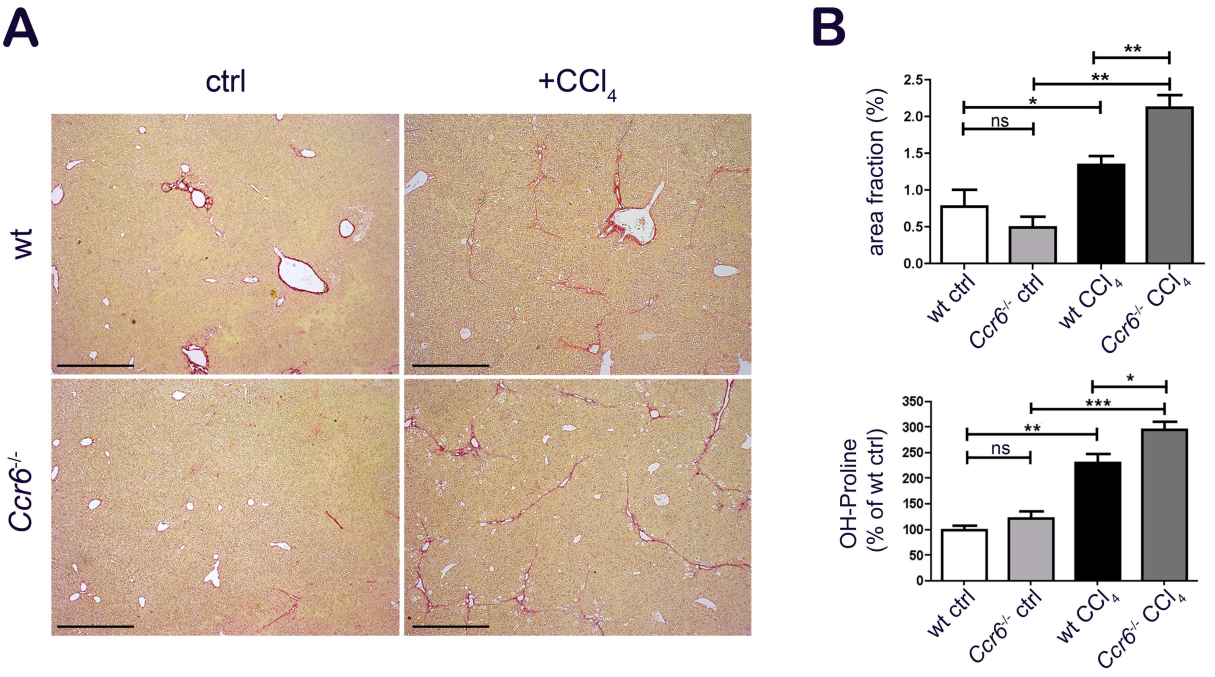


Figure 5. *CCR6 deficiency promotes hepatic fibrosis in mice.*

A Sirius Red stainings (see Methods section 4.2.14.2). Scale bar: 500µm. **B** Quantification of matrix deposition from Sirius Red stainings and hydroxyproline (OH-Proline) measurement (see Methods section 4.2.5 and 4.2.14.2) from livers of wt and *Ccr6*$^{-/-}$ mice. ns: not significant, *P <0.05 **P <0.01 ***P <0.001. Data are shown as mean ± SEM of n=5 (ctrl) or n=15 (treated) mice per group from 4 independent experiments.

In order to exclude model-specific confounding effects, mice were also subjected to a methionine-choline-deficient (MCD) diet as an independent model of chronic metabolic liver injury, in which mice develop severe steatohepatitis.[110] Remarkably, liver injury was enhanced in *Ccr6*$^{-/-}$ compared with wt mice after 8 weeks of MCD diet, as evidenced by histology and liver enzymes (Fig.6A+B). Furthermore, *Ccr6*$^{-/-}$ mice showed significantly enhanced liver fibrosis compared to wt mice, as evidenced by quantification of extracellular collagen deposition by Sirius Red stainings (Fig.6C+D). Collectively, these results show that

CCR6/CCL20 represents a yet unrecognized protective chemokine pathway during chronic liver injury and hepatofibrogenesis.

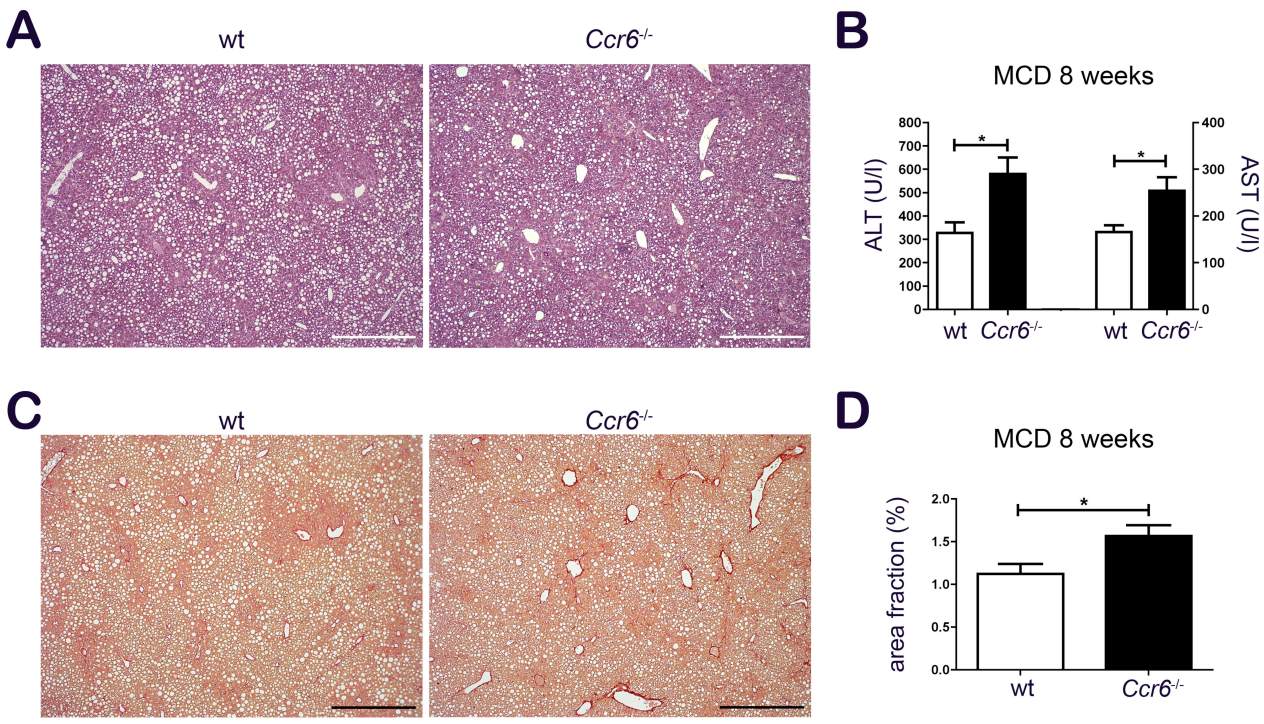


***Figure 6**. CCR6 deficiency enhances liver damage and fibrosis after MCD diet in Ccr6$^{-/-}$ mice.*
Wt and *Ccr6*$^{-/-}$ mice were subjected to a MCD diet for 8 weeks. **A** Hematoxylin-eosin stainings from livers of wt and *Ccr6*$^{-/-}$ mice (see Methods section 4.2.14.1). Scale Bar: 500µm. **B** Alanine (ALT) and aspartate (AST) aminotransferase serum activities of wt and *Ccr6*$^{-/-}$ mice (see Methods section 4.2.4). **C** Sirius Red stainings (see Methods section 4.2.14.2). Scale bar: 500µm. **D** Quantification of matrix deposition from Sirius Red stainings shown in C. *P <0.05. Data are shown as mean ± SEM of n=5 mice per group from 1 experiment.

5.3 Hepatic inflammation and infiltration of lymphocytes into damaged liver is enhanced in *Ccr6*$^{-/-}$ mice

After having observed accelerated fibrosis development in *Ccr6*$^{-/-}$ mice we assessed whether *Ccr6* deficiency also altered hepatic inflammation. Therefore, liver sections of wt and *Ccr6*$^{-/-}$ mice were stained for the pan-leukocyte marker CD45 to visualize infiltrating immune cells and evaluated by a blinded pathologist. In untreated animals, we detected similar numbers of immune cells in the livers of wt and *Ccr6*$^{-/-}$ mice. However, livers of *Ccr6*$^{-/-}$ mice showed significantly higher intrahepatic leukocyte accumulation upon chronic injury

compared to livers of wt mice (Fig.7A+B), suggesting enhanced liver inflammation in these animals.

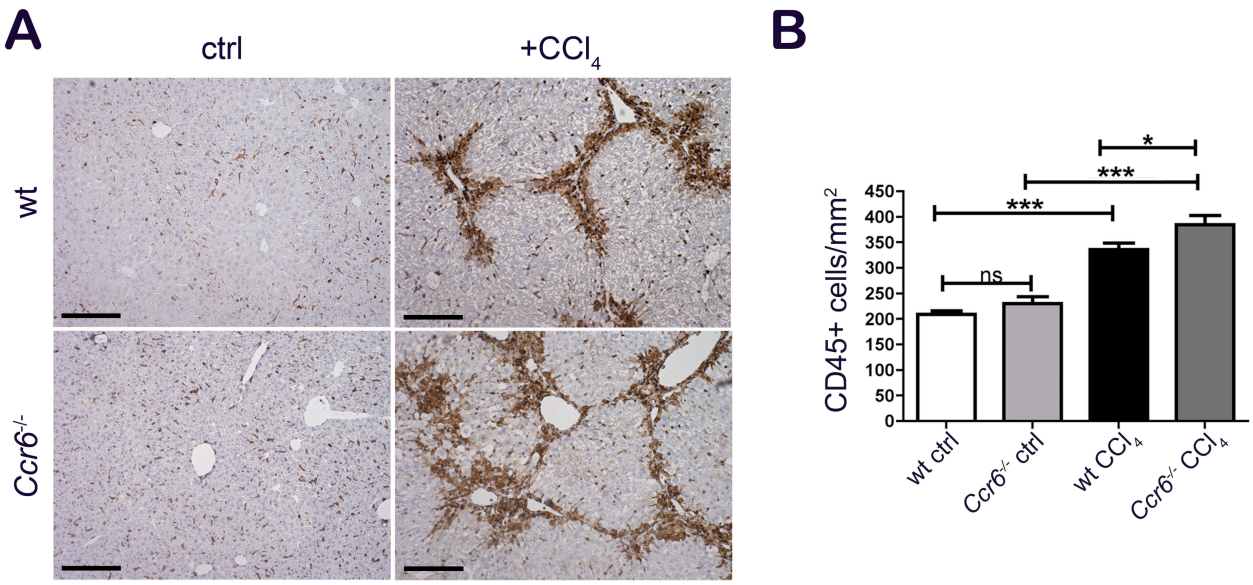


Figure 7. *Enhanced leukocyte infiltration in CCR6 deficient mice.*

A Immunohistochemistry for the pan-leukocyte marker CD45 in liver paraffin sections (see Methods section 4.2.14.3) of wt and *Ccr6*$^{-/-}$ mice treated with CCl_4 over 4 weeks or control mice. Scale bar: 200µm. **B** $CD45^+$ cells were counted manually by a blinded pathologist. ns: not significant, *P <0.05 **P <0.01 ***P <0.001. Data are shown as mean ± SEM of n=6-10 mice per group from 3 independent experiments.

We next aimed at addressing whether CCR6 deficiency altered the infiltration of immune cell subtypes into the injured liver. Therefore we again treated wt and *Ccr6*$^{-/-}$ mice with CCl_4 for 4 weeks and analyzed the composition and absolute numbers of the intrahepatic immune cell subsets using flow cytometry. We could not observe differences in the numbers of neutrophils or macrophages, whereas all major lymphocyte subsets (T-, B- and NK cells) were significantly increased in livers of CCl_4-treated *Ccr6*$^{-/-}$ mice compared to wt animals (Fig.8A+B). Importantly, the relative composition of the different major immune cell populations in injured livers did not significantly differ between wt and *Ccr6*$^{-/-}$ mice (Fig.8A+C) suggesting that *Ccr6*$^{-/-}$ mice develop an overall enhanced inflammatory response upon chronic liver injury.

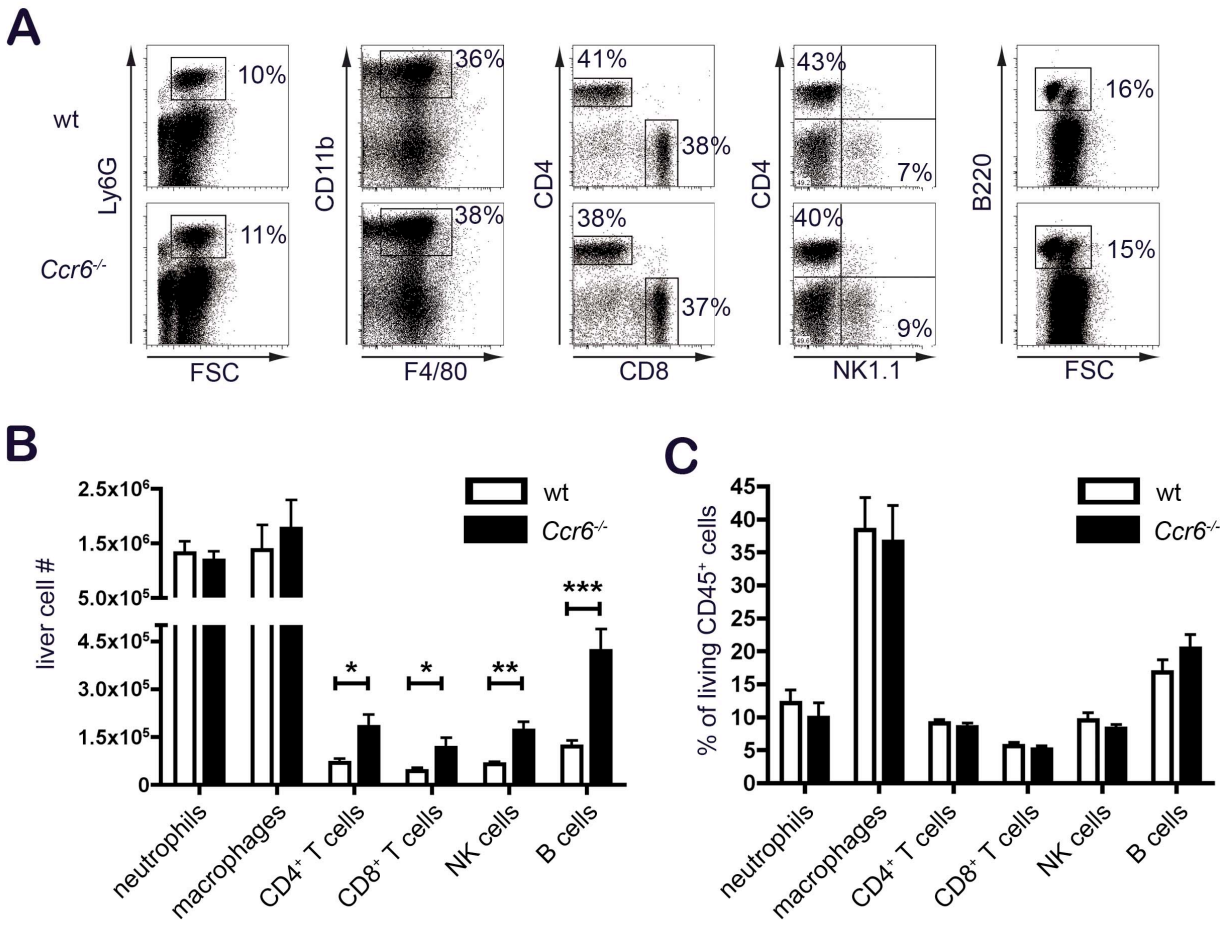


Figure 8. *Leukocyte infiltration upon chronic injury in livers of WT and CCR6 deficient mice.*
Wt and *Ccr6*$^{-/-}$ mice were treated thrice weekly with CCl_4 for 4 weeks. **A** Cells were isolated from liver (see Methods section 4.2.9.5) and stained for CD45 to identify leukocytes, dead cells were excluded by Hoechst 33258. Leukocytes were stained with Ly6G for neutrophils, CD11b and F4/80 for macrophages, CD3 and CD4 or CD3 and CD8 for T cells, CD3 and NK1.1 to identify NK-cells (as $CD3^-NK1.1^+$), and B220 for B cells (see Methods section 4.2.10.1). **B** Absolute numbers of intrahepatic leukocyte subpopulations. **C** Relative amounts of the individual immune cell subpopulations as fractions of living $CD45^+$ cells are shown. *P<0.05 **P<0.01 ***P<0.001. Data are shown as mean ± SEM of n=6-10 mice per group from three independent experiments.

5.4 Differentiation of T helper cells is not altered in *Ccr6*$^{-/-}$ mice

CCR6 expression had been reported on distinct $CD4^+$ T cell subsets, in particular Th17 cells and regulatory T cells (Treg).[11, 158] Thus we investigated whether a shift in inflammation-regulating T helper cell populations might be responsible for the enhanced overall hepatic inflammation in CCl_4-treated *Ccr6*$^{-/-}$ mice. In CCl_4-treated animals we analyzed the expression of the signature cytokines for Th1, Th2, Th17 and Treg cells in the liver, which are IFNγ, IL-4, IL-17 and IL-10, respectively. There was no difference in IFNγ or

IL-10 gene expression and protein concentration between wt and *Ccr6*$^{-/-}$ mice (Fig.9A+B). *Il-4* gene expression was significantly reduced in the livers of CCl_4 treated *Ccr6*$^{-/-}$ mice compared to wt mice (Fig.9A), but lower IL-4 levels could not be confirmed on protein level (Fig.9B).

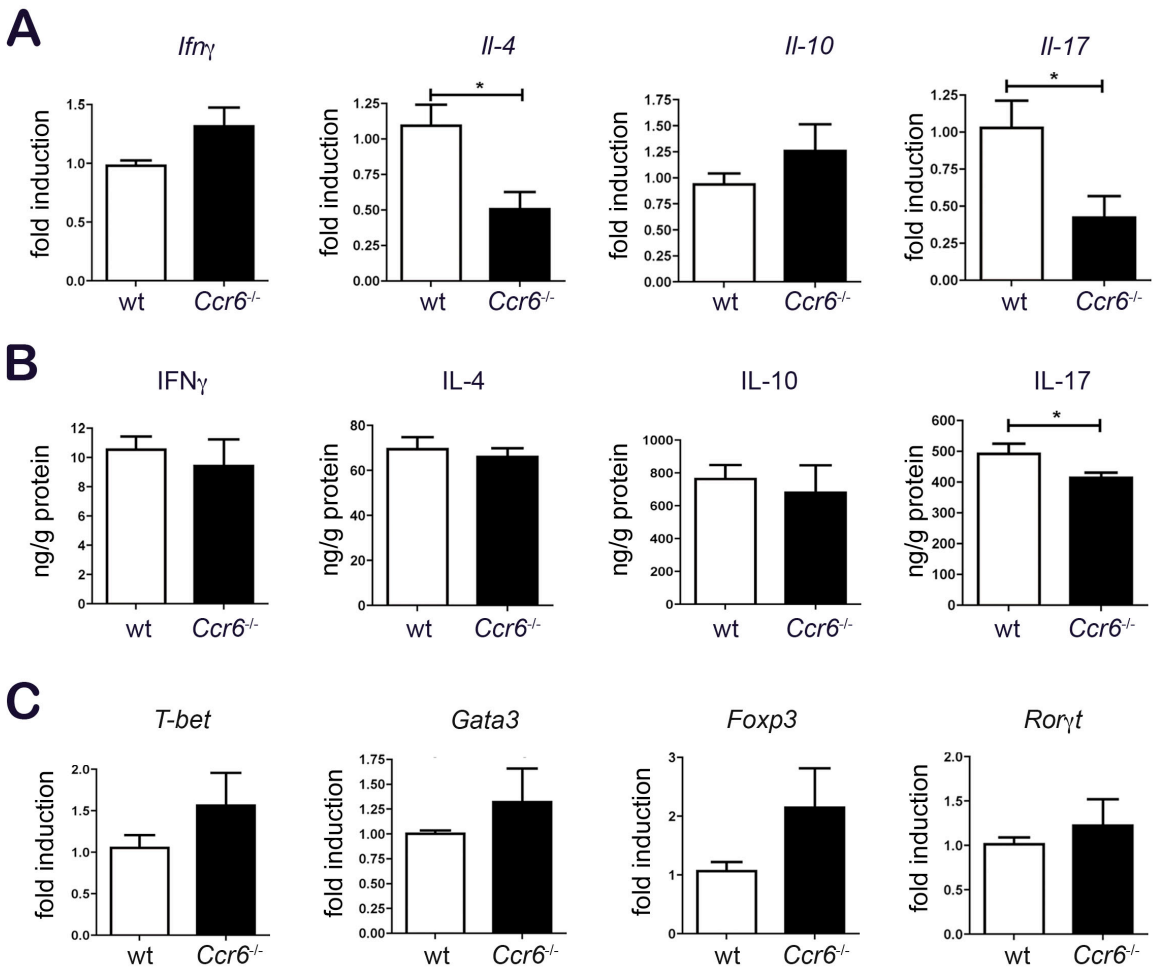


Figure 9. *T helper cell subset composition in injured livers of WT and CCR6 deficient mice.*
Wt and *Ccr6*$^{-/-}$ mice were treated thrice weekly with CCl_4 for 4 weeks. **A** Expression of signature cytokines for CD4 T helper cell subtypes was measured by qPCR from total liver (see Methods section 4.2.6.1 and 4.2.6.4), namely *Ifnγ* for Th1 cells, *Il-4* for Th2 cells, *Il-10* for Treg, and *Il-17* for Th17 cells. **B** Concentrations of cytokines were measured by ELISA from liver and normalized to total protein content in liver extracts (see Methods section 4.2.7f.). **C** Expression of signature transcription factors for $CD4^+$ T helper cell subtypes was measured by qPCR (see Methods section 4.2.6.1 and 4.2.6.4), namely *T-bet* for Th1 cells, *Gata*3 for Th2 cells, *Foxp3* for Treg, and *Rorγt* for Th17 cells. *P<0.05. Data are shown as mean ± SEM of n=6 mice per group from two independent experiments.

More strikingly, IL-17 expression was significantly reduced in livers of *Ccr6*$^{-/-}$ compared to wt mice, both on mRNA as well as on protein level (Fig.9A+B). Nonetheless, we also checked for expression of Th-lineage specific transcription factors *T-bet* (Th1), *Gata3*

(Th2), *FoxP3* (Treg) and *Rorγt* (Th17) (Fig.9C). We could not detect significant differences in expression of any of these transcription factors between wt and *Ccr6*$^{-/-}$ mice, indicating that differentiation of intrahepatic T helper cells did not change upon absence of CCR6. These results could be further confirmed on protein level by flow cytometric analysis of hepatic T helper cells. Here we could observe similar numbers of Th1, Th17 and Treg cells between wt and *Ccr6*$^{-/-}$ mice, in untreated as well as CCl_4 treated mice (Fig.10A+B).

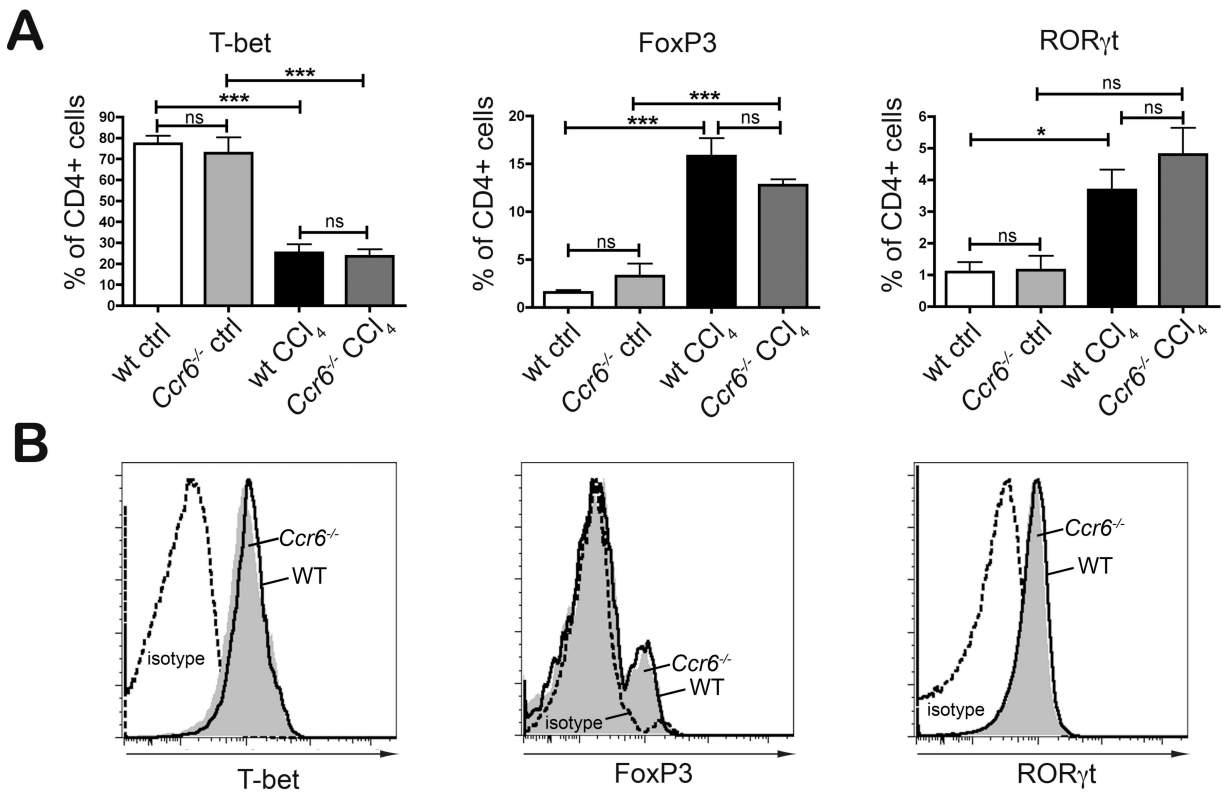


Figure 10. *Flow cytometric analysis of hepatic T helper cells.*
Wt and *Ccr6*$^{-/-}$ mice were treated thrice weekly with CCl_4 for 4 weeks or left untreated. **A** Leukocytes were isolated from livers of wt and *Ccr6*$^{-/-}$ mice and stained for CD3, CD4 and subsequently T-bet, FoxP3 and RORγt to identify T helper cell subsets (see Methods section 4.2.9.5 and 4.2.10.3). Relative amounts of $CD4^+$ T cells are shown. **B** Representative FACS plots of same $CD4^+$ T cell subsets as in A. Dashed histogram: isotype control, grey histogram: *Ccr6*$^{-/-}$, black histogram: wt. ns: not significant, *P<0.05 **P<0.01 ***P<0.001. Data are shown as mean ± SEM of n=5 (ctrl) or n=12 (treated) mice per group from two independent experiments.

Collectively, while our results demonstrate that *Ccr6*$^{-/-}$ mice develop more severe hepatic inflammation after CCl_4 treatment with higher immune cell infiltration compared to wt mice, this effect appears not related to alterations in the $CD4^+$ T-helper cell compartment. Thus, the results strongly suggest the critical involvement of another CCR6-dependent, IL-17-producing cell type that restricts the inflammatory response in the injured liver.

5.5 Infiltration of IL-17 producing γδ T cells is reduced in the absence of CCR6

5.5.1 γδ T cells express high amounts of *Ccr6* and *Il-17* in chronic liver injury

To identify the CCR6-dependent cellular compartment responsible for limiting the inflammatory response in the liver upon chronic injury, different cell types were isolated from livers of wt mice and checked for *Ccr6* expression. We could not detect *Ccr6* expression in liver resident cell populations like hepatocytes and HSC, but we noted a clear induction of *Ccr6* in macrophages and $CD4^+$ T cells upon injury (Fig.11A). The most remarkable *Ccr6* upregulation, however, was detected on γδ T cells after CCl_4 treatment (Fig.11A), a small subpopulation of unconventional T cells negative for classical differentiation markers like CD4 or CD8 and expressing the γδ T cell receptor. We next isolated hepatic $CD4^+$ T cells and γδ T cells from wt mice after CCl_4 treatment and untreated mice and checked for *Il-17* expression. Both cell types significantly upregulated *Il-17* after CCl_4 treatment, but again expression in γδ T cells was much higher than in $CD4^+$ T cells (Fig.11B), suggesting that γδ T cells might be the main IL-17 producing cells in CCl_4-treated liver.

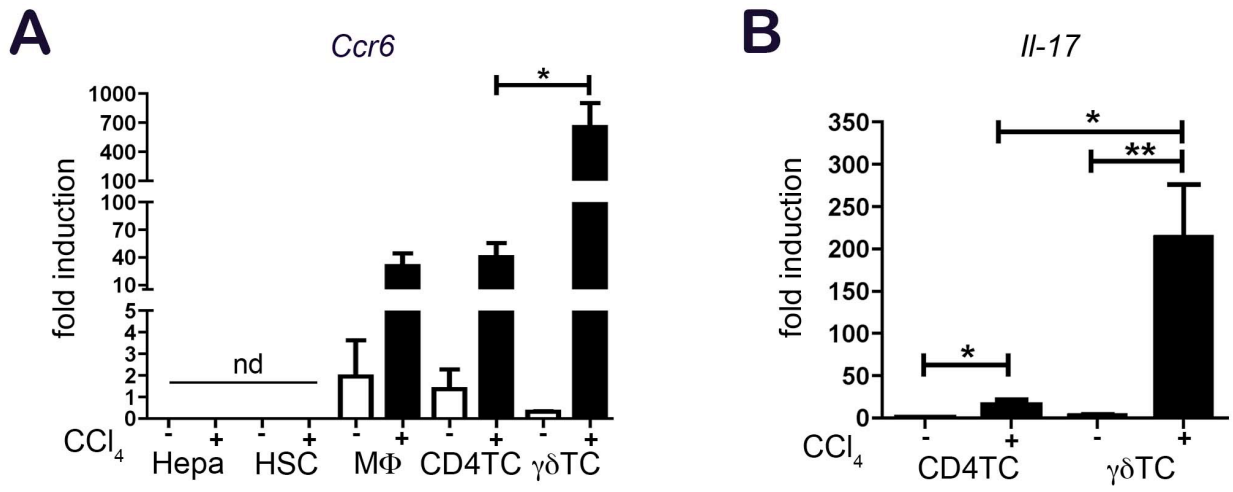


Figure 11. *Ccr6 and IL-17 expression of γδ T cells in chronic liver injury.*

A Hepatocytes (Hepa), hepatic stellate cells (HSC), macrophages (MΦ) , $CD4^+$ T cells (CD4TC) and γδ T cells (γδTC) were isolated from livers of wt mice treated with CCl_4 or control mice (see Methods section 4.2.9 and 4.2.11), and *Ccr6* expression was determined by qPCR (see Methods section 4.2.6.1 and 4.2.6.4). **B** $CD4^+$ T cells and γδ T cells were isolated from livers of wt mice and checked for *Il-17* expression by qPCR. nd: not detectable. *P <0.05 **P <0.01 ***P <0.001. Data are shown as mean ± SEM of n=4-6 mice per group from two independent experiments.

5.5.2 Differential infiltration of IFNγ and IL-17 producing γδ T cells

These analyses hinted at a CCR6-dependent hepatic recruitment of γδ T cells in chronic liver injury. Therefore we analyzed livers of CCl_4 treated wt and *Ccr6*$^{-/-}$ mice and untreated animals for intrahepatic γδ T cells using flow cytometry. In fact, we could detect a clear γδ TCR$^+$ T cell population in livers of both wt and *Ccr6*$^{-/-}$ mice, which increased upon CCl_4-induced hepatic fibrosis (Fig.12A+B). While absolute numbers of these cells did not vary at baseline conditions between wt and *Ccr6*-deficient mice, after CCl_4 treatment *Ccr6*$^{-/-}$ mice showed significantly reduced intrahepatic γδ T cells compared to wt mice (Fig.12B).

γδ T cells can be classified into two major subsets according to their surface expression of NK1.1 or CCR6. NK1.1$^+$ γδ T cells produce mainly IFNγ, while CCR6$^+$ γδ T cells primarily produce IL-17 and share characteristic features with Th17 cells.[38, 79] In contrast to Th17 cells, which belong to the adaptive immune system, γδ T cells can produce IL-17 immediately after recruitment to sites of inflammation, therefore representing one of the earliest sources of IL-17 after injury.[82, 159] We thus further analyzed liver leukocytes for γδ TCR, NK1.1 and IL-17 by flow cytometry. In healthy livers around half of the γδ T cells were NK1.1 positive in wt, but only 25% in *Ccr6*$^{-/-}$ mice (Fig.12D). After CCl_4 treatment the relative amount of NK1.1$^+$ γδ T cells decreased significantly in wt mice, due to an influx of CCR6$^+$ γδ T cells (Fig.12C+D). In contrast, the NK1.1$^+$ subset did not change considerably in *Ccr6*$^{-/-}$ after CCl_4 treatment, shifting the balance of both subtypes towards NK1.1$^+$ γδ T cells in the injured liver of *Ccr6*$^{-/-}$ mice (Fig.12C+D). Importantly, in *Ccr6*$^{-/-}$ mice the numbers of IL-17$^+$ γδ T cells was significantly lower than in wt mice upon CCl_4 treatment (Fig.12E+F). Taken together, these results strongly indicate that the infiltration of IL-17 producing γδ T cells into injured liver depends on the chemokine receptor CCR6.

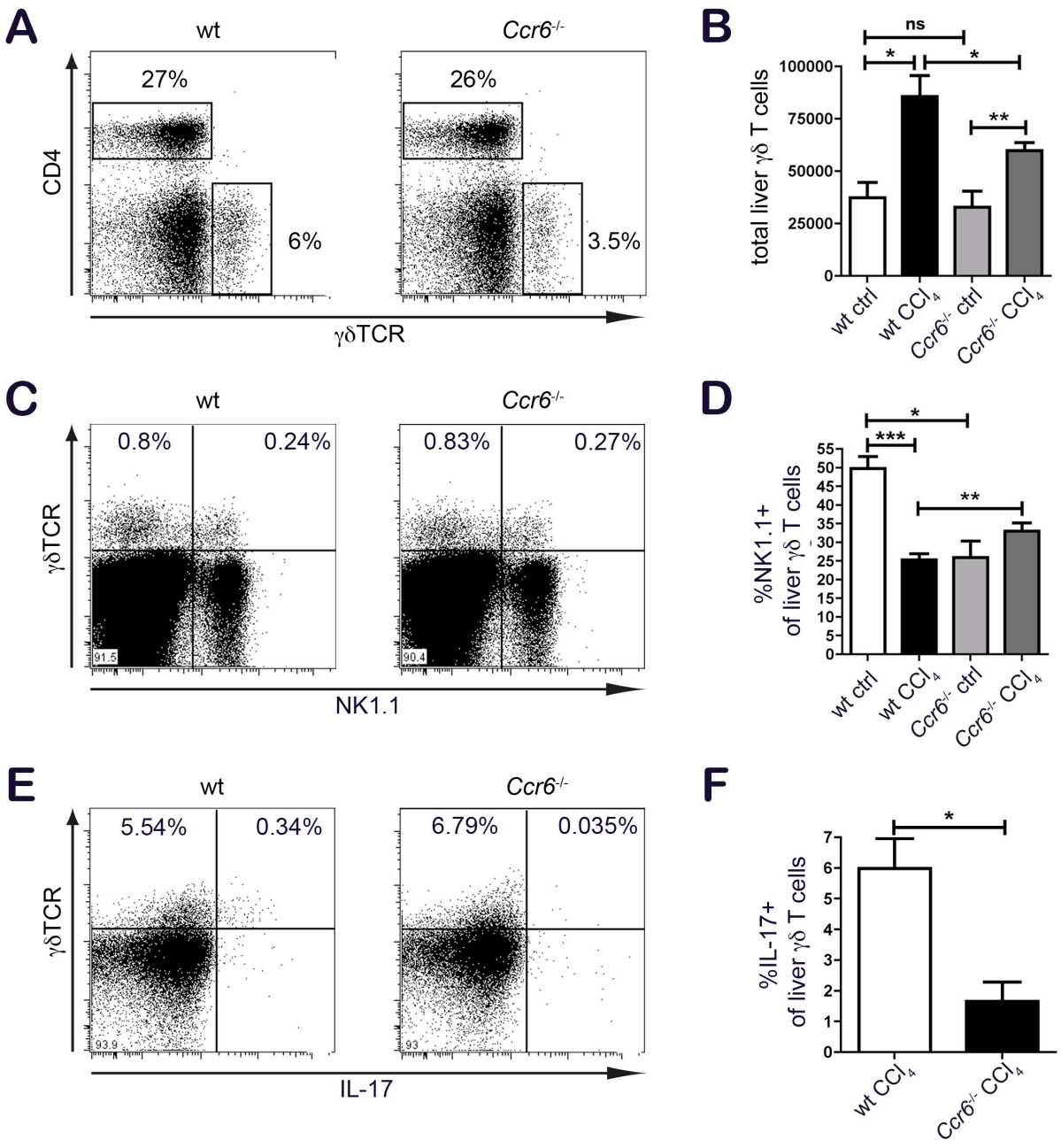


Figure 12. *Intrahepatic accumulation of IL-17 producing γδ T cells is impaired in Ccr6$^{-/-}$ mice upon chronic injury.*
A Leukocytes were isolated from livers (see Methods section 4.2.9.5) of wt and *Ccr6*$^{-/-}$ mice after CCl_4 treatment and stained for CD3, CD4, CD8 and γδTCR to identify γδ T cells (see Methods section 4.2.10.1). Representative FACS plots are shown. **B** Absolute numbers of γδ T cells in livers of wt or *Ccr6*$^{-/-}$ mice treated with CCl_4 or control mice. γδ T cells were identified as CD3$^+$ CD4$^-$ CD8$^-$ γδTCR$^+$ cells. **C-F** Same as A with additional staining for NK1.1 and IL-17 after restimulation with PMA/ionomycin to discriminate γδ T cell subtypes (see Methods section 4.2.10.2). **C** Representative dot plots of NK1.1 γδTCR staining in the liver (CCl_4 treated mice). **D** Quantification of NK1.1$^+$ γδ T cells. **E** Representative FACS plots of IL-17$^+$ γδ T cells in the liver (CCl_4 treated mice). **F** Quantification of E. ns: not significant. *P <0.05 **P <0.01 ***P <0.001. Data are shown as mean ± SEM of n=7-10 mice per group from two or three independent experiments.

5.6 IL-17 producing γδ T cells require CCR6 to traffic to the liver

We thus tested the hypothesis that the IL-17-producing subset of γδ T cells employs CCR6-CCL20 mediated chemotaxis to enter the injured liver. Therefore, splenic γδ T cells were isolated from eGFP-transgenic wt mice and GFP-negative *Ccr6*$^{-/-}$ mice, which are both from the CD45.2 background, and mixed in a ratio of 1:1. The cells were then injected iv into congenic wt mice from the CD45.1 background that had been treated once with CCl_4 24h prior to γδ T cell transfer (Fig.13A). 24 hours after transfer liver and spleen were analyzed for CD45.2$^+$ (=transferred) γδ T cells. In the livers we found overwhelming numbers of GFP$^+$ (=wt) γδ T cells, but only very few GFP$^-$ (=*Ccr6*$^{-/-}$) γδ T cells (Fig.13B). Only few transferred cells could be found in the spleen with no difference in numbers of wt and *Ccr6*$^{-/-}$ T cells, corroborating that CCl_4 administration induced a strong recruitment signal for transferred γδ T cells to the liver (Fig.13B). These results indicate that γδ T cells indeed employ CCR6 to enter the liver upon toxic injury.

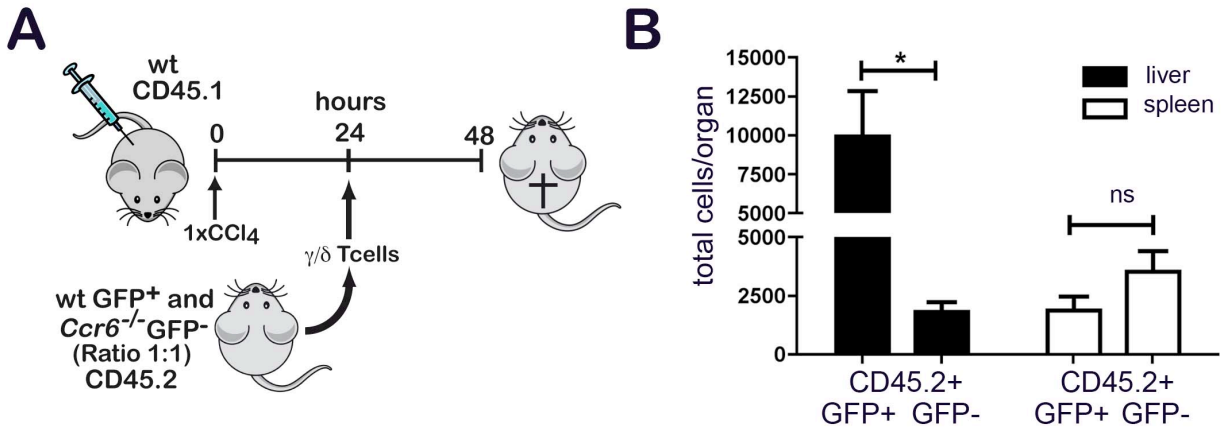


Figure 13. *γδ T cells require CCR6 to travel to the liver.*

A Splenic γδ T cells were isolated from Actin-eGFP or *Ccr6*$^{-/-}$ mice (see Methods section 4.2.11), mixed in a 1:1 ratio and injected into CD45.1$^+$ B6-mice that had been treated with CCl_4 once 24h prior to T cell injection. **B** 24h after injection of γδ T cells mice were sacrificed and absolute numbers of CD45.2$^+$ GFP$^+$ WT and CD45.2$^+$ GFP$^-$ *Ccr6*$^{-/-}$ γδ T cells in liver and spleen were determined (see Methods section 4.2.9.4f.). Ns: not significant, *P<0.05. Data are shown as mean ± SEM of n=5 mice per group from 2 independent experiments.

5.7 Adoptive transfer of WT γδ T cells, but not of CD4 T cells, limits hepatic fibrogenesis in *Ccr6*$^{-/-}$ mice independent of IL-17

5.7.1 Adoptive transfer of WT γδ T cells limits hepatic fibrogenesis in *Ccr6*$^{-/-}$ mice

In order to provide functional evidence that the recruitment of CCR6-expressing γδ T cells is responsible for limiting CCl_4-mediated hepatic injury and fibrogenesis, we reconstituted *Ccr6*$^{-/-}$ mice with wt γδ T cells. Therefore, wt and *Ccr6*$^{-/-}$ mice (both from the CD45.2 background) were treated with CCl_4 thrice weekly for 4 weeks, and the mice additionally received wt γδ T cells (100,000 to 150,000 cells per injection) isolated from spleens of congenic mice (CD45.1) via iv injection once per week (Fig.14A). After four weeks of CCl_4 treatment a clear population of CD45.1$^+$ (=transferred) cells could be found in the liver that expressed the γδ TCR but not CD4 (Fig.14B). *Ccr6*$^{-/-}$ mice that received wt γδ T cells showed significantly less hepatic fibrosis compared to *Ccr6*$^{-/-}$ mice that received no γδ T cells, as visualized by Sirius Red staining, quantification of fibrotic areas and measurement of hepatic hydroxyproline content (Fig.14C+D). Moreover, the adoptive transfer of wt γδ T cells to *Ccr6*$^{-/-}$ mice was capable of fully restoring the fibrosis phenotype to the level of wt mice (Fig.14C+D). Notably, the adoptive transfer of γδ T cells into wt mice did not significantly affect fibrosis progression, which might be related to the overall low numbers of transferred γδ T cells compared to the endogenous hepatic γδ T cell pool already present in fibrotic wt mice.

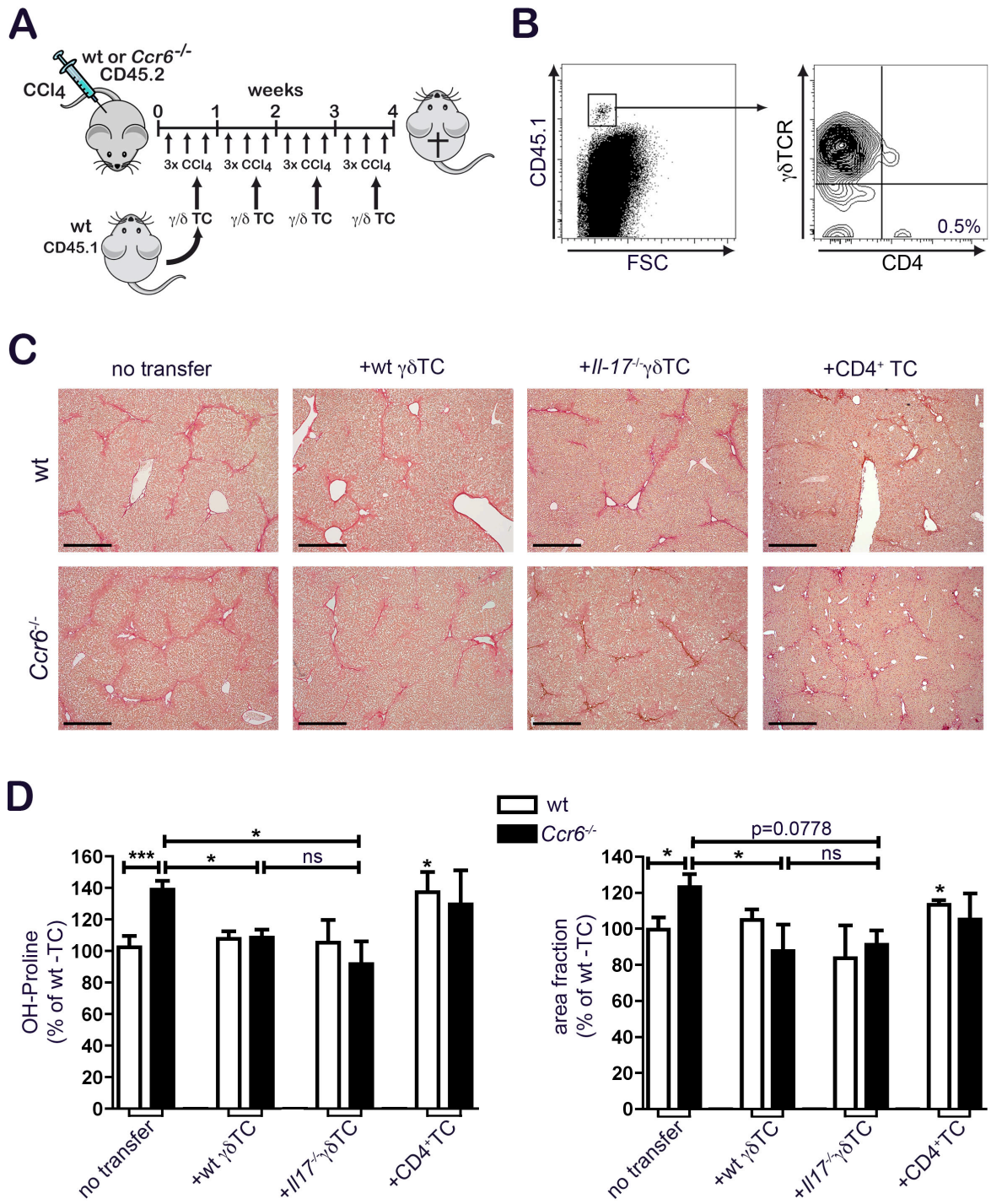


Figure 14. *Reconstitution of Ccr6$^{-/-}$ mice with γδ T cells but not with CD4^{+} T cells reduces hepatic fibrosis.*

A-D Wt and *Ccr6*$^{-/-}$ mice were treated with CCl_4 three times a week over a period of 4 weeks. Simultaneously, splenic CD4^{+} or γδ T cells were isolated from CD45.1^{+} mice and injected intravenously into CCl_4-treated mice once per week (see Methods section 4.2.12). Mice received either wt γδ T cells (100,000-150,000 cells), *Il-17*$^{-/-}$ γδ T cells (100,000-150,000 cells), wt CD4^{+} T cells (1,000,000-1,500,000 cells) or no adoptively transferred T cells (-TC) during CCl_4 treatment. **B** Leukocytes were isolated from liver and stained for CD45.1 to identify transferred cells that were found to express the γδ TCR but not CD4 (representative plot shown) (see Methods section 4.2.9.5ff.). **C** Sirius Red stainings of liver paraffin sections (see Methods section 4.2.14.2). Scale bar: 500µm. **D** Measurement of hepatic hydroxyproline (see Methods section 4.2.5) and quantification of extracellular matrix deposition. Ns: not significant, *P<0.05. Data are shown as mean ± SEM of n= mice per group from 4 independent experiments.

5.7.2 γδ T cells limit hepatofibrogenesis independent of IL-17

We next investigated the mechanisms by which CCR6-expressing γδ T cells restrict hepatic inflammation and fibrosis. IL-17 was the primary cytokine produced by hepatic $CCR6^+$ γδ T cells in injured liver, but a recent study had described that IL-17 signaling in macrophages and HSC exacerbated liver fibrosis in mice.[160] To test whether CCR6-expressing γδ T cells limit hepatofibrogenesis via IL-17 we reconstituted *Ccr6*$^{-/-}$ mice with IL-17-deficient but CCR6-expressing γδ T cells. Wt and *Ccr6*$^{-/-}$ mice were treated with CCl_4 thrice weekly for 4 weeks, and the mice additionally received *Il-17*$^{-/-}$ γδ T cells (100,000 to 150,000 cells per injection) isolated from spleens of *Il-17*$^{-/-}$ mice via iv injection once per week. After four weeks of CCl_4 treatment *Ccr6*$^{-/-}$ mice that received *Il-17*$^{-/-}$ γδ T cells showed significantly less hepatic fibrosis compared to *Ccr6*$^{-/-}$ mice that did not receive γδ T cells, as visualized by Sirius Red staining, quantification of fibrotic areas and measurement of hepatic hydroxyproline content (Fig.14C+D). The fibrosis phenotype of these mice was fully restored to wt level, similar to *Ccr6*$^{-/-}$ mice that were reconstituted with *Il-17*$^{+}$ γδ T cells (Fig.14C+D).

5.7.3 Adoptive transfer of CD4 T cells does not limit hepatic fibrogenesis

To ensure that accelerated fibrosis in *Ccr6*$^{-/-}$ mice was a consequence of reduced γδ T cells, but not of alterations in the $CD4^+$ T cell compartment, we also transferred wt $CD4^+$ T cells into wt and *Ccr6*$^{-/-}$ mice that were treated as before. As anticipated,[161] the adoptive transfer of $CD4^+$ T cells enhanced collagen deposition in wt mice, but did not further increase the strong fibrosis in *Ccr6*$^{-/-}$ mice (Fig.14C+D), corroborating that CCR6 was specifically important for γδ T cells *in vivo*. Taken together, our results provide experimental proof that γδ T cells modulate hepatic inflammation and restrict fibrosis progression, but these effects are unrelated to IL-17 secretion by these cells.

5.8 Hepatic γδ T cells directly impact functionality of hepatic stellate cells

5.8.1 Hepatic γδ T cells induce apoptosis in GRX cells

We then tested the hypothesis that γδ T cells interact with hepatic stellate cells (HSC), a key resident cell population in the liver that perpetuates hepatic inflammation by releasing pro-inflammatory chemokines and promotes fibrosis by transdifferentiation into collagen-producing myofibroblasts.[162] We isolated γδ T cells, $CD4^+$ T cells and natural killer (NK) cells from livers of wt mice that had received a single CCl_4 injection and cocultured these cells as well as their supernatants with GRX cells, a murine HSC cell line, for 48 hours. As a positive control, GRX cells were stimulated with TGFβ1 that activates HSC, while untreated cells served as negative controls. Hepatic NK cells were included in this experiment, because they have been shown to exert anti-fibrotic functions through killing of activated HSC.[140-141] After 48h of co-culture, lymphocytes were washed off thoroughly, and GRX cells were harvested for RNA isolation or FACS analysis. GRX cells that were left untreated, incubated with TGFβ or co-cultured with $CD4^+$ T cells showed a typical appearance of proliferating, myofibroblast-like cells after 48h (Fig.15A). In contrast, upon co-culture with hepatic γδ T cells, GRX cells grew less dense and had more round-shaped apoptotic myofibroblasts in culture (Fig.15A). As expected,[162] stimulation of GRX cells with TGFβ1 resulted in up-regulated expression of *Collagen1*, the HSC activation marker *PDGF-receptor-β* (Fig.15B), the pro-inflammatory chemokine *Ccl2* (Fig.15C) and did not affect expression of apoptosis-related genes (Fig.15D). In contrast, co-culture with NK cells reduced *Collagen1* and *PDGF-receptor-β* expression (Fig.15B) and increased expression of the proapoptotic mediator *Bax* (Fig.15D). Co-culture of GRX cells with γδ T cells, but not with its supernatant, also significantly reduced *Collagen1* expression, suggesting that γδ T cells impair HSC-mediated extracellular matrix production (Fig.15B). Co-culture of γδ T cells did not affect HSC activation marker or chemokine synthesis, but significantly induced expression of the proapoptotic mediator *Bax* in GRX cells (Fig.15D). Co-culture with $CD4^+$ T cells did not alter expression of any of these markers (Fig.15B-D).

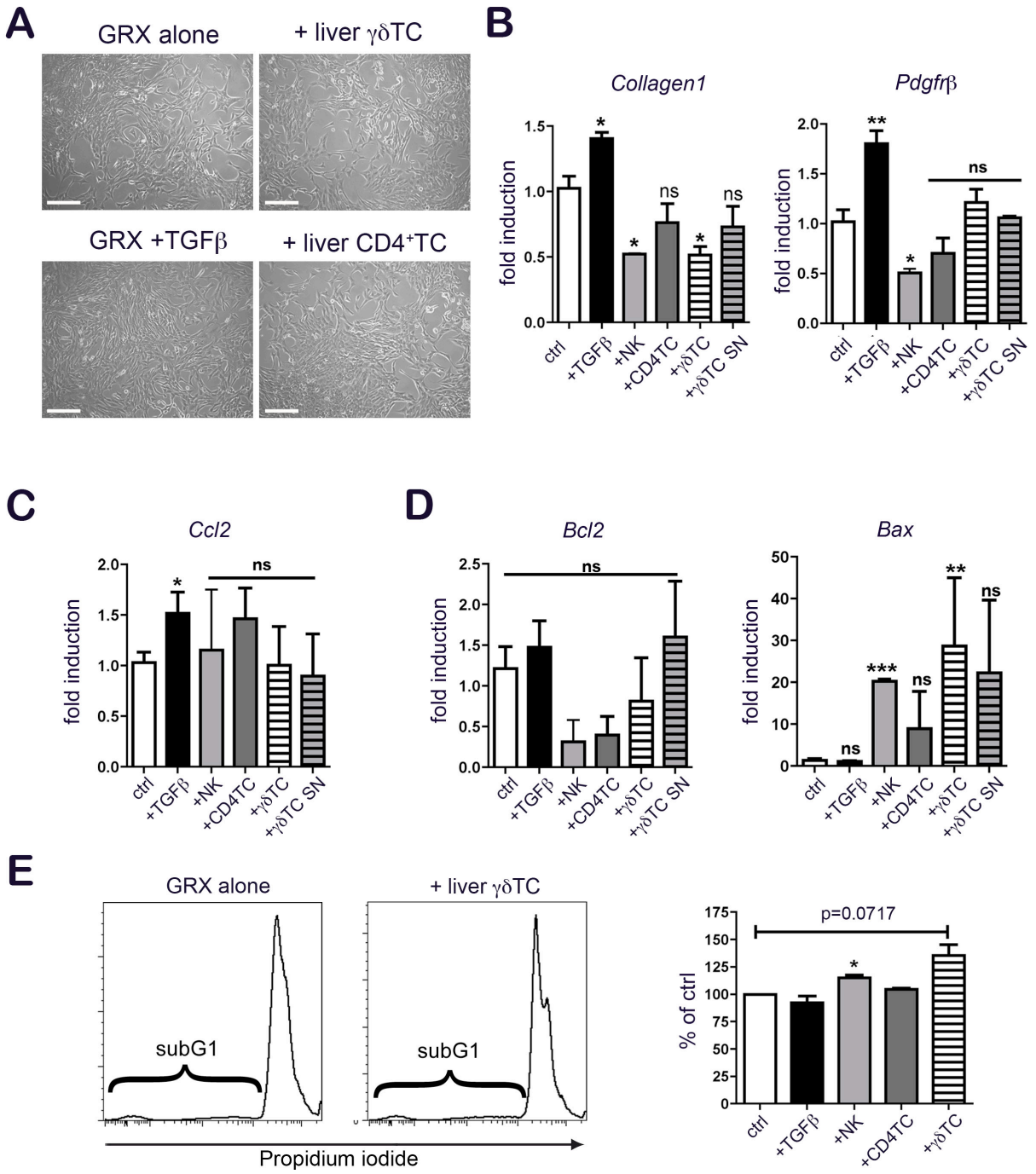


Figure 15. *γδ T cells directly inhibit stellate-cell like GRX cells.*

A-E Hepatic $CD4^+$ T cells, γδ T cells and NK cells were isolated from wt mice (see Methods section 4.2.11) 48h after single CCl_4 injection. Either isolated cells or supernatant from overnight cultures were incubated with the HSC cell line GRX (see Methods section 4.2.13). GRX cells cultured in the presence of 5ng/ml TGF-β1 served as positive control; as a negative control cells were left untreated. After 48h lymphocytes were washed off, and GRX cells were harvested for RNA isolation or FACS analysis. **A** Representative pictures of co-cultures. Scale bar: 200µm. **B-D** Gene expression of Collagen1 and Pdgfrβ (B), the chemokine Ccl2 (C) and the apoptosis-related genes Bcl2 and Bax (D) in GRX cells. **E** GRX cells were fixed, permeabilized and stained with propidium iodide (PI) for FACS analysis (see Methods section 4.2.10.4). Representative histogram with sub G1 cells is shown. Amounts of apoptotic cells are shown as percent of control. Ctrl: control, SN: supernatant, Ns: not significant, *P<0.05 **P <0.01 ***P<0.001. Data are shown as mean±SEM of n=4-6 samples per condition from 2 independent experiments.

Furthermore, co-culture of GRX cells with hepatic γδ T or NK cells, but not with hepatic T cells, significantly increased the fraction of apoptotic GRX cells in culture (Fig.15E), which was evidenced by propidium iodide (PI) staining of the cells. PI is a DNA intercalating dye that is commonly used for cell cycle analysis and detection of apoptosis.[163] Healthy cells in G1 phase give a clear peak in the PI signal and every cell giving a signal below this peak (and therefore containing less DNA) is considered as apoptotic (subG1). Our data strongly support direct inhibitory effects of hepatic γδ T cells on GRX cells by favoring apoptosis and limiting collagen synthesis.

5.8.2 Hepatic γδ T cells colocalize with hepatic stellate cells in vivo and induce apoptosis in primary HSC

To investigate whether γδ T cells might also directly interact with HSC *in vivo*, we stained liver sections of CCl_4-treated wt mice for γδ TCR and desmin to identify γδ T cells and HSC, respectively. We could detect clusters of γδ T cells in periportal infiltrates and HSC throughout the liver tissue with increased numbers in areas of tissue damage (Fig.16A). In periportal areas γδ T cells and HSC were usually found adjacent to each other (Fig.16A, white arrows). Interestingly, when we stained liver tissue for γδ TCR and F4/80 to identify macrophages we did not typically see direct colocalization of γδ T cells and macrophages (Fig.16B).

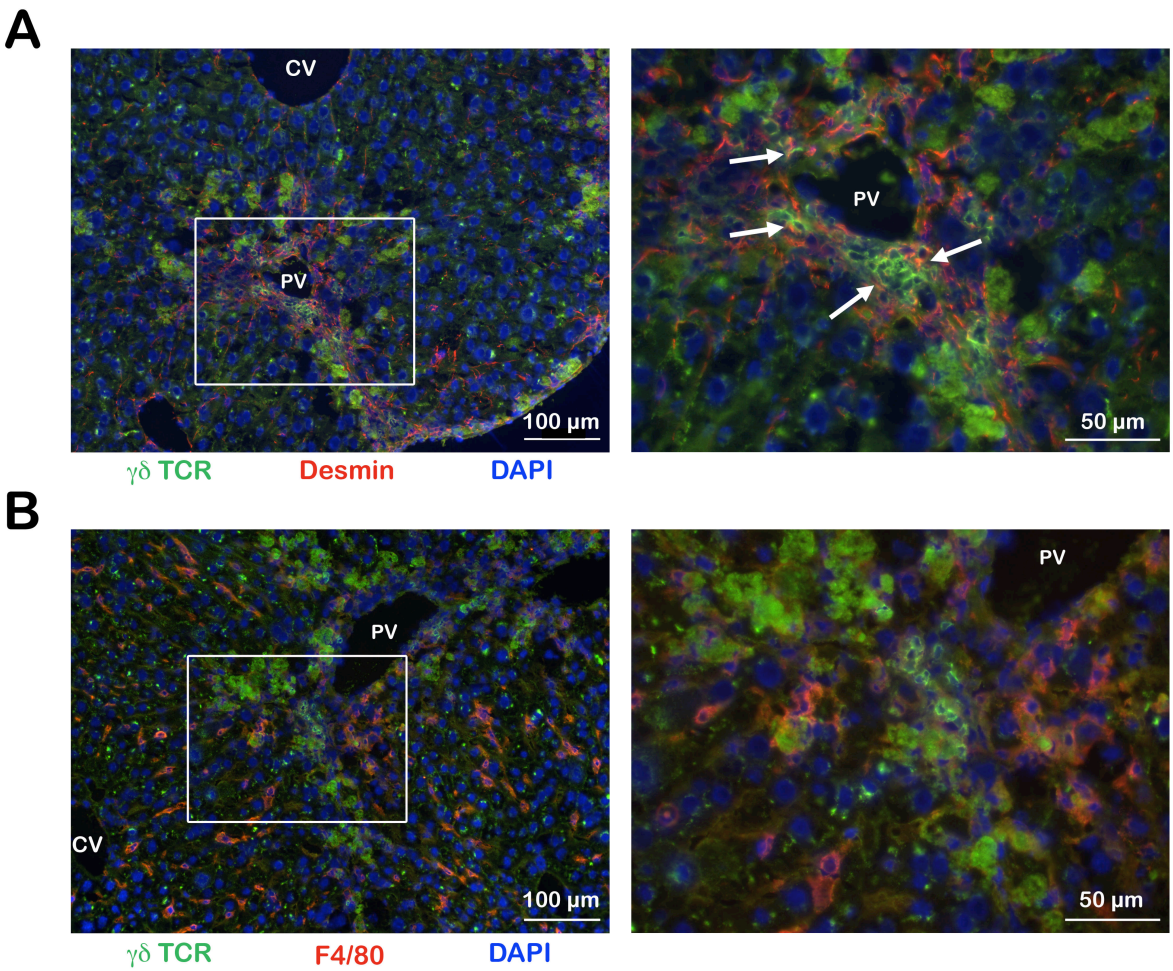


Figure 16. *Hepatic γδ T cells colocalize with HSC but not macrophages in vivo.*
Wt mice were treated with CCl_4 thrice weekly over 4 weeks. Livers were snap frozen in liquid nitrogen and cut into 5µm sections. **A** Liver sections were stained for γδ TCR (green) and desmin (red) (see Methods section 4.2.14.4). Nuclei were stained with DAPI (blue). White arrows show colocalization of γδ T cells and HSC. **B** Liver sections were stained for γδ TCR (green) and F4/80 (red) (see Methods section 4.2.14.4). Nuclei were stained with DAPI (blue). PV: portal vein. CV: central vein.

Based on these results we examined whether γδ T cells are able to induce apoptosis in primary HSC *in vitro*. Therefore we again isolated γδ T cells, $CD4^+$ T cells and natural killer (NK) cells from livers of wt mice that had received a single CCl_4 injection and cocultured these cells as well as their supernatants with primary HSC isolated from wt mice. After 48h of co-culture, lymphocytes were washed off thoroughly, and HSC cells were harvested for flow cytometric analysis. Untreated HSC, which will usually get activated when plated on plastic dishes,[162] showed the typical phenotype of activated and differentiating HSC, while HSC cocultured with γδ T cells or NK cells grew less dense and appeared smaller and more

rounded with less protrusions (Fig.17A). When analyzed for apoptotic cells by propidium iodide (PI) staining, HSC cocultured with γδ T cells or NK cells showed significantly higher numbers of apoptotic cells in culture compared to untreated HSC (Fig.17B). Coculture with $CD4^+$ T cells or the supernatant of γδ T cells did not lead to increased apoptosis.

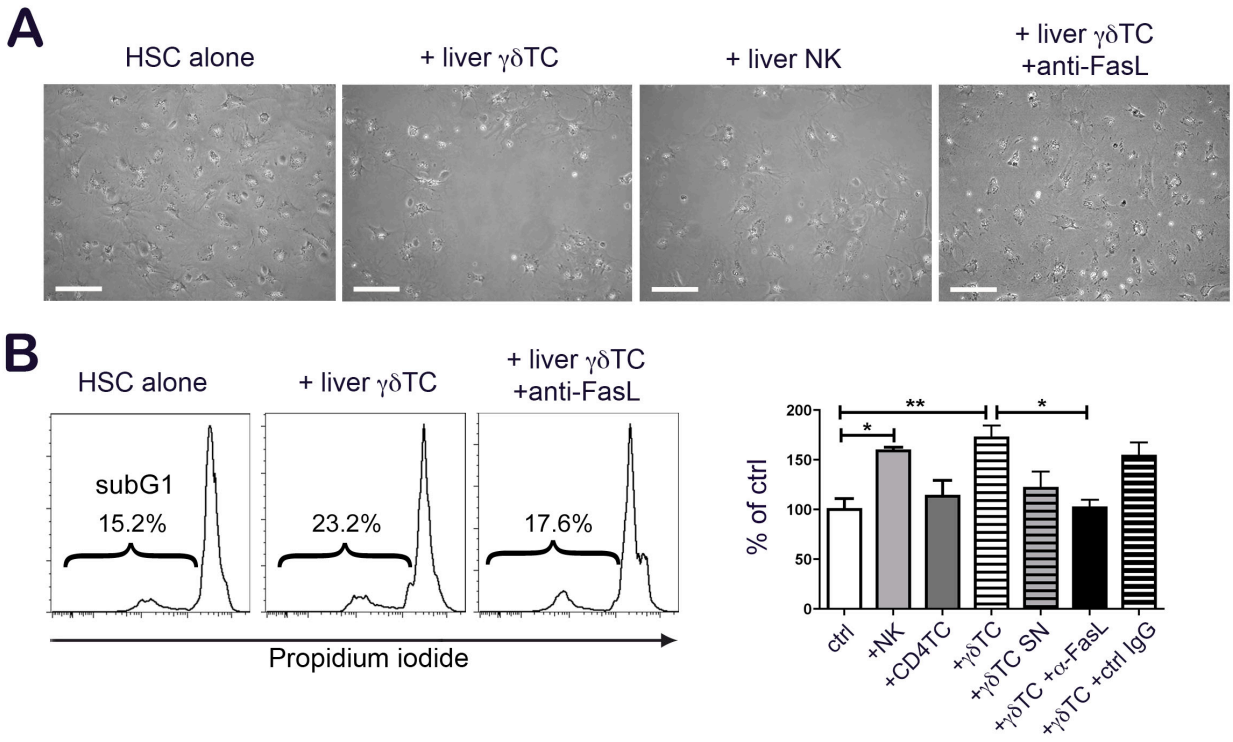


Figure 17. *γδ T cells directly inhibit primary hepatic stellate cells.*

A+B Hepatic $CD4^+$ T cells, γδ T cells and NK cells were isolated from wt mice (see Methods section 4.2.11) 48h after single CCl_4 injection. Either isolated cells or supernatant from overnight cultures were incubated with primary HSC in absence or presence of a Fas-ligand blocking antibody (see Methods section 4.2.13). After 48h lymphocytes were washed off, and HSC were harvested for RNA isolation or FACS analysis. **A** Representative pictures of co-cultures. Scale bar: 200µm. **B** HSC were fixed, permeabilized and stained with propidium iodide (PI) for FACS analysis (see Methods section 4.2.10.4). Representative histogram with sub G1 cells is shown. Amounts of apoptotic cells are shown as percent of control. HSC: hepatic stellate cell, ctrl: control, SN: supernatant, FasL: Fas-ligand. *P<0.05 **P <0.01 ***P<0.001. Data are shown as mean ± SEM of n=4-6 samples per group from 2-3 independent experiments per condition.

Taken together, our results indicate that γδ T cells indeed interact with HSC upon liver damage *in vivo*, and that they induce apoptosis in activated HSC through direct cell-cell interactions.

5.8.3 Apoptosis induction in primary HSC is Fas-dependent

To further elucidate how γδ T cells might induce HSC apoptosis, we analyzed expression of Fas ligand (CD95L or FasL) on hepatic γδ T cells. FasL expression was significantly increased on γδ T cells isolated from fibrotic compared to control livers (Fig.18).

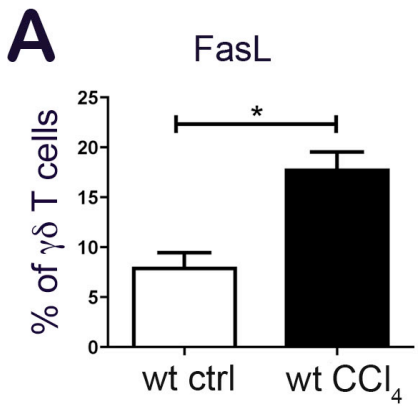


Figure 18. *Hepatic γδ T cells upregulate Fas ligand upon chronic liver injury.*
γδ T cells were isolated from livers of wt mice (see Methods section 4.2.9.5) that were treated with CCl_4 thrice weekly over 4 weeks or control mice and stained for CD3, CD4, CD8 and γδTCR to identify γδ T cells and Fas ligand (see Methods section 4.2.10.1). *P <0.05. Data are shown as mean ± SEM of n=3 mice per group.

Thus we cocultured primary HSC with hepatic γδ T cells as described before and added a FasL-blocking antibody or control antibody to the culture. HSC cocultured with γδ T cells in the presence of the FasL-blocking antibody showed the same phenotype as untreated HSC (Fig.17A), whereas HSC cocultured with γδ T cells in the presence of the control antibody looked more like HSC cocultured with γδ T cells alone. By PI staining and flow cytometric analysis we could show that addition of FasL-blocking antibody to the coculture reduced the amount of apoptotic HSC to the amount observed in untreated HSC cultures (Fig.17B). Addition of the control antibody did not influence the amount of apoptotic HSC. These results suggest that inhibition of HSC through γδ T cells is Fas-dependent.

6 Discussion

Chronic organ injury, such as in conditions of liver diseases, characteristically provokes a complex inflammatory reaction, in which the interplay between damaged parenchymal cells, resident and infiltrating immune cells is tightly regulated. The nature and the extent of the inflammation then determine the pathological consequences, e.g. full recovery or progression to organ fibrosis. Fibrotic remodelling of the liver is a major health problem worldwide with mortality attributable to cirrhosis and primary liver cancer of around 1.5 million deaths per year.[164] During recent years, many investigations had revealed an increased expression of CC and CXC chemokines and their receptors in hepatic fibrosis.[161] Functional studies in mice using experimental models of liver fibrosis identified various pro-inflammatory and pro-fibrogenic chemokine pathways, including CCR2-CCL2 and CCR8-CCL1 for monocytes/macrophages,[108, 165] CXCR1/CXCR2-CXCL1 (CXCL8 in humans) for neutrophils,[166] or CCR5/CCR1-CCL5 for T cells.[167] In contrast, very little is known about chemokine-mediated pathways that down-regulate inflammation or exert antifibrotic actions. As such, the CX_3CR1-CX_3CL1 axis represents an anti-inflammatory and pro-survival signal for intrahepatic monocyte-derived macrophages in liver fibrosis,[168] or CXCR3 and its ligand CXCL9 modulate intrahepatic T cell responses towards a net antifibrotic effect.[118] In our current study, we provide compelling evidence that the CCR6-CCL20 axis is functionally involved in chronic liver injury and fibrogenesis. Our data revealed that the CCR6-dependent recruitment of IL-17 producing $\gamma\delta$ T cells into the injured liver critically limits hepatic inflammation and fibrosis *in vivo*.

6.1 The CCR6-CCL20 pathway is activated in chronic liver injury and represents an antifibrogenic mechanism in vivo

There have been several descriptive studies in human inflammatory diseases as well as functional analyses from murine models (comprehensively summarized in table 12) indicating that the CCR6-CCL20 pathway might be of outstanding relevance for the regulation of innate and adaptive immune responses *in vivo*. However, only scarce data existed on this chemokine pathway in the liver and in conditions of liver disease. Before conducting functional studies in murine liver fibrosis, we thus analyzed expression of CCR6 and CCL20 in human liver tissue samples derived from patients with different stages of hepatic fibrosis. Both markers were significantly upregulated in fibrotic as well as cirrhotic

liver tissue, independent of the underlying disease etiolgy. Our findings were well in agreement with a prior smaller study investigating 34 patients with various liver diseases that had reported elevated levels of CCR6-expressing hepatic T cells and enhanced intrahepatic levels of CCL20 in fibrotic livers,[44] and similar observations had been also described for patients with hepatocellular carcinoma.[34] A very recent study about CCR6-CCL20 in the human liver had shown that CCR6 is especially important for positioning of Th17 cells within the inflamed liver.[120] The authors concluded that CCL20 was secreted by biliary epithelial cells in injured liver and subsequently led to recruitment of $CCR6^+$ T cells to inflamed bile ducts. This production of CCL20 could be further enhanced by IL-17 signaling, indicating a positive feedback loop by which Th17 cells amplify secretion of CCL20 and recruitment of more $IL\text{-}17^+$ effector cells to bile ducts. Moreover, the authors provided experimental indications that CCR6 was not involved in recruitment of Th17 cells via sinusoids into the liver itself, this function was carried out by CXCR3.[43]

Concordant with data from patients with liver diseases, our experiments confirmed that the activation of the CCR6-CCL20 pathway was resembled in the most widely used murine model of liver fibrosis, namely chronic injury induced by the hepatotoxic agent carbon tetrachloride (CCl_4). As in the human situation both markers were significantly higher expressed in liver tissue of mice treated with CCl_4 compared to control mice. Additionally, we also treated CCR6-deficient mice with CCl_4 and observed even higher expression of *Ccl20* compared to CCl_4 treated wt mice. This strongly suggests a direct feedback loop between CCR6 and CCL20, which is abrogated in $Ccr6^{-/-}$ mice and therefore leading to an overshooting CCL20 response.

Upon injury, CCL20 expression was strongly induced by murine hepatocytes but none of the other liver resident cell compartments investigated, corroborating that this chemokine pathway is directly activated by damaged parenchymal cells. This stands in contrast to the results observed in inflamed human liver, where CCL20 is upregulated only by biliary epithelial cells,[120] indicating differences between the human and murine system. However, we cannot exclude that our isolated hepatocytes also include a fraction of cholangiocytes. Further studies using more sophisticated genetic targeting (with cholangiocyte specific promoter) or cell isolation strategies are thus warranted to elucidate the contribution of CCL20 from hepatocytes or cholangiocytes, respectively. These studies are largely hampered by the fact that both cell types have likely a common stem cell precursor in the liver and share many molecular markers.[169-170]

Experimental mouse model	overall phenotype	proposed mechanism	ref.
Experimental colitis	*Ccr6*$^{-/-}$ Th17 cells aggravate colitis in SCID mice	T cell balance is changed towards more Th1 and less Th17/Treg cells	22
Experimental autoimmune encephalomyelitis (EAE)	*Ccr6*$^{-/-}$ mice have delayed disease onset	Impaired recruitment of pathogenic CD4^{+} T cells into CNS	32, 171
	Ccr6$^{-/-}$ mice have aggravated chronic EAE	decreased presence of PD-L1^{+} (regulatory) mDC in the spleen	31
		Impaired recruitment of Treg to CNS	32
Glomerulonephritis	*Ccr6*$^{-/-}$ mice show aggravated glomerulonephritis	Impaired recruitment of Th17 and Treg to inflamed kidney	33
Psoriasis-like inflammation	*Ccr6*$^{-/-}$ prevents from psoriasiform dermatitis	Lower levels of IL-22 in ears of *Ccr6*$^{-/-}$ mice due to Impaired γδ T cell recruitment into epidermis	35, 172-173
Corneal epithelial abrasion	Anti-CCL20 treatment reduces inflammation and epithelial healing	Reduced accumulation of IL-17^{+} IL-22^{+} γδTC	42
Respiratory syncytial virus infection	*Ccr6*$^{-/-}$ mice show reduced airway inflammation	Reduced accumulation of dendritic cells	174
Leishmania infection	*Ccr6*$^{-/-}$ mice have enhanced local inflammation	Reduced recruitment of Treg	175

Inflammatory human disease	main observation	proposed mechanism	ref
inflammatory liver diseases	Increased frequency of CCR6^{+} T helper cells in inflamed liver tissue	CCL20 production by cells in periportal areas	44
	Positioning of CCR6^{+} Th17 cells around inflamed bile ducts	Secretion of CCL20 by cholangiocytes	43
Hepatocellular carcinoma (HCC)	Increased tumor infiltrating Treg are associated with poor prognosis	Selective recruitment of Treg to tumors via CCR6-CCL20	34
Inflammatory bowel diesease (IBD)	Patients show elevated levels of CCL20 in colonic tissue	CCL20 is produced by colonic epithelial cells in IBD patients	28
Renal inflammation	Increased CCR6^{+} cells in nodular infiltrates		176
Multiple sclerosis	Increased frequency of CCR6^{+} γδ T cells in patients		177
glioma	Enhanced expression of CCR6 and CCL20 in tumor tissue predicts poor prognosis		178

Table 12. *Role of CCR6 in experimental inflammatory mouse models or human inflammatory diseases.*

Based on our findings about enhanced CCR6/CCL20 expression in inflamed human and murine livers, we at first assumed that the CCR6-CCL20 pathway is functionally involved in the pathogenesis of liver fibrosis, possibly through recruitment of proinflammatory leukocytes to the liver, and that CCR6-deficiency would ameliorate chronic liver disease. But unexpectedly, mice deficient for CCR6 showed stronger hepatic inflammation and developed more severe hepatic fibrosis compared to wt mice, thus indicating that CCR6-dependent immune responses functionally restrict inflammation and fibrogenesis *in vivo*. We did not detect any differences in liver architecture or serum activity of aminotransferases between untreated wt and *Ccr6*$^{-/-}$ mice, indicating that this chemokine pathway is not involved in liver homeostasis or development. After induction of chronic liver injury by CCl_4-treatment, wt and *Ccr6*$^{-/-}$ mice showed a similar extent of liver damage, but measurement of aminotransferase activity in serum revealed a tendency towards higher transferase activity in *Ccr6*$^{-/-}$ mice compared to wt mice, suggesting that liver damage is increased in these mice. Moreover, we could also show that *Ccr6*$^{-/-}$ mice display enhanced liver fibrogenesis compared to wt mice upon chronic CCl_4 treatment, which was secured by two independent methods revealing higher collagen levels in the livers of *Ccr6*$^{-/-}$ mice, namely quantification of Sirius red stainings and measurement of hydroxyproline. These results could be confirmed in a second liver injury model of diet-induce steatoheopatitis. In this case aminotransferase activities in serum were significantly enhanced in *Ccr6*$^{-/-}$ mice accompanied by higher fibrosis development compared to wildtype mice. Collectively, these results confirm the general relevance of the CCR6-CCL20 pathway and indicate that CCR6/CCL20 represents a yet unrecognized protective chemokine pathway during chronic liver injury and hepatofibrogenesis.

6.2 Role of CCR6 in liver inflammation and fibrosis: Focus on T helper cells

Consistent with the previous results we could also show that infiltration of $CD45^+$ immune cells into the livers of CCl_4 treated *Ccr6*$^{-/-}$ mice was elevated in comparison to wt mice, while intrahepatic leukocyte numbers did not differ between both strains in untreated mice. When we analyzed intrahepatic leukocyte subsets by flow cytometry, we detected significantly increased lymphocyte numbers in the livers of injured *Ccr6*$^{-/-}$ mice, but relative composition of immune cells was similar in wt and *Ccr6*$^{-/-}$ mice. This suggests that *Ccr6*$^{-/-}$ mice develop an overall enhanced inflammatory response upon chronic injury, which is not shifted towards a specific major leukocyte subset. As chronic inflammation is the key factor promoting hepatic fibrogenesis, these findings nicely correspond to the more severe hepatofibrogenesis observed in *Ccr6*$^{-/-}$ mice.

The functional relevance of CCR6 in inflammatory models appears to be very much restricted to the type of inflammatory stimulus (sterile vs. infectious, acute vs. chronic, Th1 vs. Th2 type immune reaction), as revealed by studies of *Ccr6*$^{-/-}$ mice from injury models (summarized in table 12). However, most of the chronic non-infectious injury models revealed overall anti-inflammatory net effects of CCR6 activation, specifically experimental colitis[22], chronic autoimmune encephalomyelitis[31-32] or glomerulonephritis[33]. In contrast, some distinct allergic or infectious inflammation models, specifically psoriasis[35], *Leishmania*[175] or respiratory viral infections[174], appeared to be reduced in *Ccr6*$^{-/-}$ mice indicating proinflammatory actions mediated via CCR6 as well.

We therefore hypothesized that the immune cells responsible for mediating CCR6-dependent functions might differ between organs and inflammation models. In most experimental studies CCR6 deficiency reduced the recruitment of $CCR6^+$ T cells or dendritic cells to the site of inflammation (see table 12). In fibrotic liver we detected CCR6 expression on macrophages, $CD4^+$ T cells and $\gamma\delta$ T cells. Interestingly, we did not observe a migratory defect in $CD4^+$ T cells or macrophages in *Ccr6*-deficient mice in our model. While numbers of macrophages did not differ between wt and *Ccr6*$^{-/-}$ mice, $CD4^+$ T cells were significantly elevated in the absence of CCR6. This contrasts studies with *Ccr6*$^{-/-}$ mice in autoimmune-mediated inflammatory diseases like EAE or glomerulonephritis,[31, 33] in which lack of CCR6 significantly impaired recruitment of distinct CD4 T cell subsets, namely regulatory T cells and Th17 cells, to the inflamed organ. In both cases, *Ccr6*$^{-/-}$ mice developed aggravated disease, most probably due to reduced numbers of regulatory T cells. As also summarized in Table 12, inflammatory human diseases are commonly associated with enhanced expression

of CCR6 and/or CCL20 in the respective organ and accompanied by an increased presence of $CCR6^+$ leukocytes.

It is important to emphasize that our model of CCl_4-induced liver fibrosis by itself already markedly differs from the inflammatory diseases mentioned above and in table 12, because liver injury by CCl_4 is not directly immune-mediated, but inflammation evolves as a consequence of tissue damage. Moreover, the liver is anatomically distinct from other organs like kidney or brain due to its vast blood supply from portal veins and hepatic arteries and its rather leaky endothelium as well as due to its abundance of resident innate immune cells including macrophages ('Kupffer cells'), NKT or other unconventional T cells.[103] Our results clearly showed that neither migration of hepatic $CD4^+$ T cells was impaired in *Ccr6*$^{-/-}$ compared to wt mice in CCl_4-induced fibrogenesis, nor was the differentiation of the main T helper cell subsets affected in fibrotic *Ccr6*$^{-/-}$ animals. We could not detect differences in the numbers of Th1, Th17 and Treg cells or expression of the Th-lineage specific transcription factors in fibrotic livers of wt and *Ccr6*$^{-/-}$ mice. Also, expression of the signature cytokines for T helper cells was unaltered, with the exception of IL-17, which was reduced in the livers of *Ccr6*$^{-/-}$ mice compared to wt mice. But as numbers of Th17 cells did not vary between both strains, this suggests the critical involvement of another CCR6-expressing and IL-17-producing cell type in liver inflammation.

In addition, we could show that reconstitution of *Ccr6*$^{-/-}$ mice with wt $CD4^+$ T cells did not affect fibrosis progression in these mice, which further supports our hypothesis that increased fibrogenesis is not the result of impaired $CD4^+$ T cell recruitment. However, wt mice that received $CD4^+$ T cells showed enhanced fibrogenesis compared to wt mice that did not receive T cells. This is most likely due to the fact that all T helper cell subsets, including those that are profibrogenic (e.g. Th17 cells),[160] were enhanced through the transfer and therefore leading to increased fibrogenesis similar to that observed in *Ccr6*$^{-/-}$ mice, which already exhibit elevated levels of T helper cells.

6.3 Role of CCR6 in liver inflammation and fibrosis: Focus on γδ T cells and interleukin-17

In fact, our experiments revealed that the main CCR6-expressing and –responsive cell type in the injured liver are γδ T cells. By isolating primary immune cells from fibrotic murine livers, γδ T cells were found to express CCR6 in much higher amounts than $CD4^+$ T cells or macrophages, alongside a massively increased IL-17 expression compared to $CD4^+$ T cells. This is consistent with recent work describing CCR6 and NK1.1 as surface markers of γδ T cells that allow distinguishing between IL-17 and IFNγ producing γδ T cells, respectively.[38] Although it has been noted before that the liver is highly enriched for γδ T cells in general,[179] their functional role in homeostasis or hepatic injury had remained elusive. In experimental fibrosis, we found significantly less hepatic γδ T cells in *Ccr6*$^{-/-}$ compared to wt mice, while numbers of intrahepatic γδ T cells did not differ between untreated mice. When we analyzed hepatic γδ T cells for their NK1.1 expression, we could show that in untreated wt livers around half of the γδ T cells were $NK1.1^+$, whereas the amount of $NK1.1^+$ γδ T cells decreased upon chronic injury, which is most probably due to an influx of $CCR6^+$ γδ T cells. In contrast, in the livers of *Ccr6*$^{-/-}$ mice the subset of $NK1.1^+$ γδ T cells did not change considerably upon organ damage. These results indicate that the reduction of hepatic γδ T cells in fibrotic *Ccr6*$^{-/-}$ mice was specifically caused by a lack of recruitment of CCR6-expressing IL-17-producing γδ T cells.

Furthermore, adoptive transfer experiments revealed that $CCR6^+$ IL-17 producing γδ T cells utilize the CCR6-CCL20 axis to migrate to the injured liver. Upon transfer of wt and *Ccr6*$^{-/-}$ γδ T cells into CCl_4 treated congenic mice, we could only find considerable numbers of wt γδ T cells but not of *Ccr6*$^{-/-}$ γδ T cells in the livers of these mice.

Although these results nicely explain the reduced expression of IL-17 observed in injured livers of *Ccr6*$^{-/-}$ mice, they were at first very surprising to us. IL-17 is usually considered as a proinflammatory cytokine in the context of liver diseases.[45] In many experimental liver injury models (summarized in table 13) IL-17 has been shown to enhance immune responses and promote disease development, e.g. in immune-mediated hepatitis or liver fibrogenesis. Only in *Listeria monocytogenes* infection of the liver $IL\text{-}17^+$ T cells were assigned a protective function, but this seems to be also due to an enhancement of the immune response, which helps to contain the infection in the liver.[82] Therefore we would have expected that CCR6-deficiency reduces the accumulation of IL-17 producing leukocytes in the liver, leading to decreased liver injury and fibrogenesis. Nonetheless, our

results still do not contradict a proinflammatory and profibrotic function of IL-17 as we could show in later experiments that reduced hepatic IL-17 alone was not the cause of accelerated fibrosis development in *Ccr6*$^{-/-}$ mice.

Experimental liver injury	overall phenotype	proposed mechanism	ref.
ConA-induced hepatits	Deletion or blockade of IL-17 or IL-17RA ameliorates disease	IL-17 is functionally involved in promoting the inflammatory reaction	180 181
	STAT3-deletion in T cells reduces IL-17 production and hepatitis		182
Liver fibrosis	IL-17RA$^{-/-}$ protects against hepatofibrogenesis	IL-17 signaling promotes hepatic fibrosis by activating inflammatory, Kupffer and hepatic stellate cells	135
Non-alcoholic fatty liver disease	Neutralization of IL-17 ameliorates LPS-induced liver injury after high fat diet	IL-17 facilitates transition from steatosis to steatohepatitis	183
Ischemia reperfusion injury	Depletion of IL-17 ameliorates liver injury	IL-17 produced by NK cells promotes liver injury through neutrophil recruitment	184 185
Schistosoma mansoni infection	IL-23-deficient mice fail to develop immunopathology and secrete IL-17	IL-17 produced by Th17 cells contributes to severe immunopathology	186
	Tbet-deficiency exacerbates disease and enhances IL-17 levels	Tbet-dependent signaling negatively regulates Th17-mediated immunopathology	187
Primary biliary cirrhosis (induced by IL-2Rα deletion)	*Il-2R$\alpha^{-/-}$* mice show elevated levels of IL-17 and Th17 cells in the liver	IL-2 signaling represses induction of Th17 cells	188 189
Halothane induced liver injury	Neutralization of IL-17 supresses hepatotoxic effects of halothane	IL-17 contributes to pathology by induction of chemokines and neutrophil recruitment	190
Listeria monocytogenes infection	Mice deficient for TCRδ or IL-17A fail to contain infection in liver	IL-17 produced by $\gamma\delta$ T cells protects against early *L. monocytogenes* infection	82

Table 13. *Role of interleukin-17 (IL-17) in experimental models of liver injury.*

6.4 Regulation of liver inflammation and fibrosis by CCR6$^+$ γδ T cells

The previous results suggested that CCR6$^+$ γδ T cells play an important role in regulating hepatic inflammation upon chronic liver injury and protect the liver from excessive inflammation and fibrogenesis. Very little has been known about the functional role of CCR6$^+$ γδ T cells in inflammatory conditions *in vivo* (table 14 summarizes current concepts and key findings on γδ T cells in inflammatory mouse models). CCR6$^+$ γδ T cells were described to express toll-like receptors TLR1 and TLR2, possibly enabling them to directly interact with certain pathogens. It had been concluded that γδ T cells may act as an efficient first line of defense that could orchestrate an inflammatory response to pathogen-derived as well as environmental signals, long before adaptive immunity, e.g. Th17 cells, has sensed bacterial invasion.[79] Similar observations were reported in a model of crescentic glomerulonephritis, in which renal γδ T cells produced IL-17 and IL-23, thus providing pro-inflammatory signals very early in the course of disease.[191] Moreover, IL-17-producing γδ T cells were also found at high frequency in the brain of mice with EAE. In this model, γδ T cells could be activated by IL-1beta or IL-23 and promoted IL-17 production by CD4$^+$ T cells, suggesting that γδ T cells act in an amplification loop for IL-17 production by Th17 cells.[41] Overall, γδ T cells increased autoimmune-mediated inflammation in this setting.[41]

Nevertheless, γδ T cells have also been shown to exert protective functions in several experimental models of inflammatory disease, like Concanavalin A (ConA)-induced hepatitis,[192] adriamycin nephropathy[193] or corneal epithelial abrasion,[42] where depletion of γδ T cells aggravated inflammation and disease severity. In these models γδ T cells function as regulators of inflammation by inhibition of other proinflammatory leukocytes (ConA-hepatitis, adriamycin nephropathy) or secretion of anti-inflammatory cytokines like TGFβ (nephropathy) or IL-22 (corneal epithelial abrasion). As illustrated in Table 14 summarizing findings about γδ T cells in experimental models of immune-mediated diseases, pathogenic functions of γδ T cells are often associated with the production of IL-17 by these cells or promoting IL-17 production of other cells, whereas protective functions shown for γδ T cells seem to be rather unrelated to IL-17 in most cases.

Experimental mouse model	main phenotype	proposed mechanism	ref.
Protective function of γδ T cells			
ConA-induced hepatitis	γδ T cell deficiency exacerbates hepatitis	γδ T cells inhibit NKT cell function	192
Inflammatory bowel disease (IBD)	γδ T cell depletion/deficiency aggravates intestinal inflammation in different IBD models	γδ T cells control early inflammation possibly through control of IFNγ-production and epithelial regeneration	194
Adriamycin nephropathy	Depletion of γδ T cells exacerbates disease	γδ t cell regulate kidney inflammation through production of TGFβ and inhibition of $CD4^+$ T cell proliferation	193
Listeria monocytogenes infection	Mice deficient for TCRδ or IL-17A fail to contain infection in liver	γδ T cells protect against early *L. monocytogenes* infection by production of IL-17	82
Pulmonary fibrosis	Deficiency in Vγ4/6 T cells increases pulmonary fibrosis	Impaired recruitment of immunorgulatory γδ T cells	36
Corneal epithelial abrasion	γδ T-cell-deficiency reduces inflammation and epithelial healing	IL-22 produced by γδ T cells promotes epithelial healing	42
Pathogenic function of γδ T cells			
Experimental glomerulonephritis	γδ T cell deficiency ameliorates renal injury	γδ T cells promote kidney injury and neutrophil recruitment through production of IL-17	191
Experimental autoimmune encephalomyelitis (EAE)	γδ T cell-deficiency decreases EAE severity	Decreased immune response with less cytokine production (IFNγ, TNFα)	39 40
		γδ T cells promote IL-17 production by $CD4^+$ T cells	41
Mouse hepatitis virus (MHV) infection	Increased numbers of γδ T cells accelerate disease and decrease survival	Hyperactivated γδ T cells are cytotoxic to infected hepatocytes	195
Interstitial lung disease	*Tcrδ*$^{-/-}$ reduces disease severity	Reduced accumulation and proliferation of NK cells	196
Experimental silicosis	γδ T-cell-deficiency abrogates pulmonary IL-17 expression	γδ T cells are the major cellular source of IL-17 in silicosis	197
Collagen-induced arthritis (CIA)	Depletion of $Vγ4^+$ cells attenuated disease	$Vγ4^+$ γδ T cells exacerbate CIA through production of IL-17	37

Table 14. *Role of γδ T cells in experimental mouse models of inflammatory diseases.*

In CCl_4-induced chronic liver damage, we could demonstrate that CCR6-expressing γδ T cells accumulated in the injured liver, a process that is critically dependent on CCR6 as accumulation of these cells was abrogated in *Ccr6*$^{-/-}$ mice. Furthermore, *Ccr6*$^{-/-}$ mice that were reconstituted with wt γδ T cells while treated with CCl_4 developed significantly less

fibrosis compared to *Ccr6*$^{-/-}$ mice that did not receive T cells. The severity of fibrosis in those mice was even restored to wt level. In contrast, reconstitution of *Ccr6*$^{-/-}$ mice with wt CD4^{+} T cells did not affect fibrosis progression in these mice. Hence we could prove that the subset of CCR6-expressing γδ T cells was functionally responsible for restricting inflammation and fibrosis in the chronic liver injury model. This was unexpected as this subset of γδ T cells is considered to be the IL-17 producing subtype, and our results contrast findings from autoimmune inflammatory models in other organs, where IL-17 production by γδ T cells was associated with accelerated disease (Table 14). However, in our model, the anti-inflammatory and anti-fibrotic effects of hepatic γδ T cells were not mediated by its signature cytokine IL-17, as reconstitution of *Ccr6*$^{-/-}$ mice with *Il-17*$^{-/-}$ γδ T cells during CCl_4 treatment reduced fibrosis severity to the same degree as reconstitution with wt (*Il-17*$^{+}$) γδ T cells. This is in agreement with prior studies which identified pro-fibrogenic functions for IL-17, derived by Th17 and myeloid cells, in the inflamed liver (Table 13).[45, 160]

After having observed that the limiting effect of γδ T cells on liver fibrosis was independent of their IL-17 production we also investigated by which other mechanisms γδ T cells might influence hepatic fibrosis. Our experiments unraveled direct interactions of hepatic γδ T cells with stellate cells in the liver. By immunoflouorescent stainings of liver sections we could show that in the injured liver γδ T cells colocalize with HSC but not with macrophages *in vivo*. Moreover, hepatic γδ T cells were found to reduce collagen production and facilitate HSC apoptosis *in vitro*. Hepatic γδ T cells were able to carry out this function on an immortalized HSC-like cell line (GRX) as well as on primary HSC. As primary HSC get activated and start to transdifferentiate as soon as they are plated on plastic, induction of apoptosis by γδ T cells was even able to overcome this activation stimulus. Furthermore, triggering of apoptosis occurred to the same extent as in coculture with NK cells, which have already been described to kill activated HSC.[140-141] Our results also show that apoptosis induction happens through direct cell-cell interactions between γδ T cells and HSC. Supernatants from cultured hepatic γδ T cells were not able to induce apoptosis when added to HSC cultures, which makes it unlikely that apoptosis is triggered through a soluble factor released by γδ T cells. This hypothesis is further supported by the observation that this process is Fas-dependent. When we added a Fas ligand (FasL) blocking antibody to the cocultures, HSC apoptosis could be reduced to the same level seen in untreated HSC cultures. This effect was not observed when a control antibody was added to the culture. Taken together, we could show that γδ T cells directly interact with HSC.

6.5 Final conclusions and outlook

Taken together, our study not only unraveled a yet unrecognized anti-inflammatory and anti-fibrotic function for CCR6/CCL20 in liver fibrosis *in vivo*, but also illuminates for the first time functional aspects of the pathogenic involvement of hepatic γδ T cells in chronic liver injury. The subset of IL-17-producing γδ T cells is recruited via CCR6 to injured liver and restricts inflammation and fibrogenesis, likely by inhibiting HSC functionality through induction of apoptosis (Fig.19).

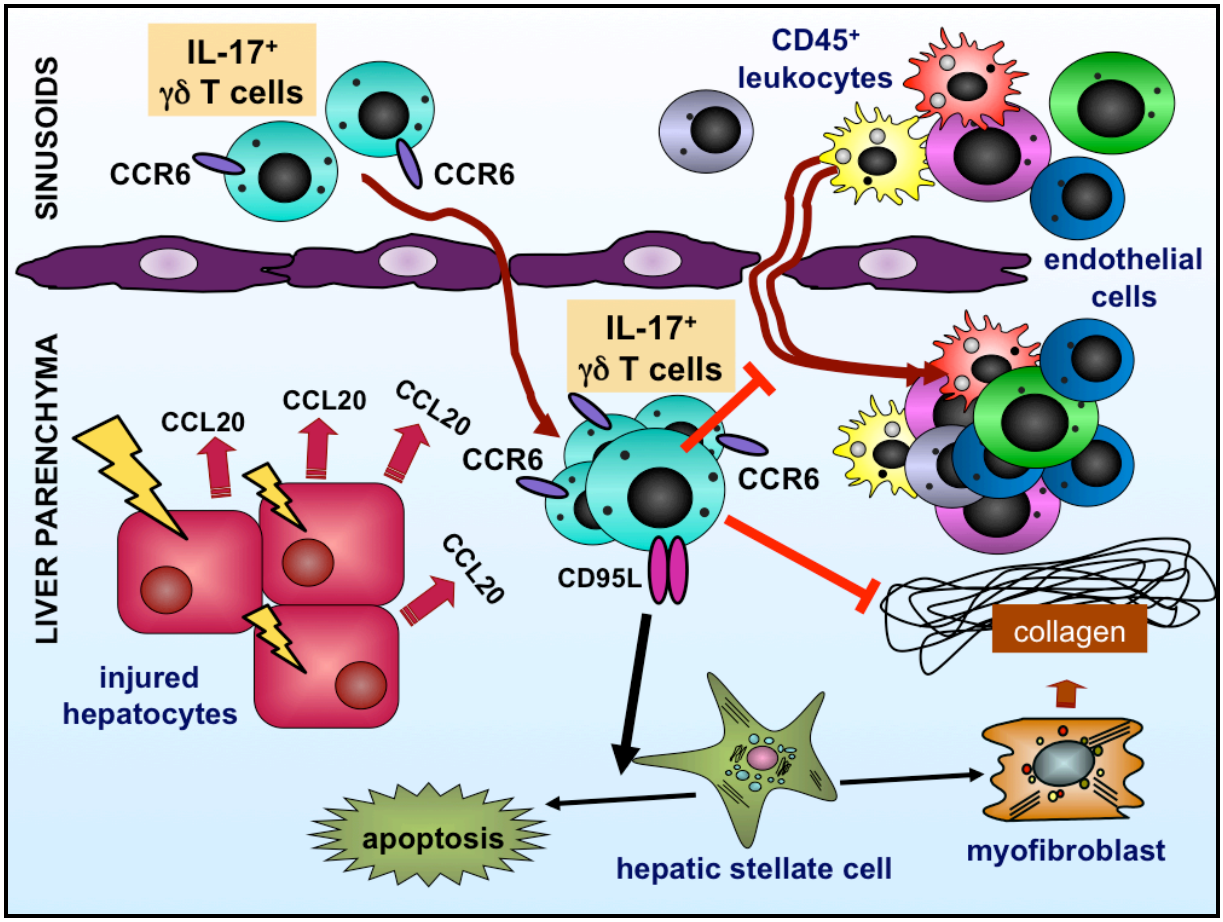


Figure 19. *CCR6+ γδ T cells limit hepatic fibrogenesis by directly inhibiting hepatic stellate cells.*
Upon chronic liver damage CCR6+ γδ T cells are recruited to the liver through CCL20, which is released by parenchymal cells. In the liver γδ T cells directly interact with hepatic stellate cells (HSC) and induce apoptosis in these cells via Fas-Fas ligand (CD95L) interactions. Furthermore, they reduced collagen production by HSC and limit infiltration of additional leukocytes into the liver.

However, future studies are required to address the therapeutic potential of augmenting the CCR6/CCL20 pathway and/or γδ T cell functionality in liver fibrosis. Enhancing CCR6-signaling, e.g. through induction of CCL20-production in the liver, harbours

the risk that not only antifibrotic γδ T cells but also profibrotic leukocytes like $CD4^+$ T cells will be recruited to the liver. This could possibly increase fibrosis development instead of reducing it. Therefore, it might be more reasonable to target $CCR6^+$ γδ T cells more specifically to enhance this cell type in the liver without influencing other lymphocyte subsets. Still, the full potential of γδ T cells to prevent liver fibrogenesis should be determined. We could clearly show that γδ T cells can protect from excessive inflammation and fibrogenesis in the liver, but our transfer experiments did not show a further reduction of fibrosis in wt mice when the pool of γδ T cells was augmented. This might be due to the fact that the number of transferred cells was relatively low compared to the endogenous γδ T cell population. Thus, it should be more precisely investigated whether γδ T cells can also reduce fibrogenesis in wt animals when given (transplanted) in higher numbers. Also, our results do not exclude that $CCR6^+$ γδ T cells might reduce hepatic inflammation and/or fibrogenesis by other mechanisms besides the induction of apoptosis in hepatic stellate cells. Future experiments are needed to determine which cytokines, apart from IL-17, these cells produce in the inflamed liver as γδ T cells have been shown to produce anti-inflammatory cytokines like IL-10 or TGFβ.[77] Another anti-inflammatory cytokine that should be considered in this setting is IL-22, which is often produced by the same cells as IL-17. In ConA-induced hepatitis IL-22 plays a protective role as a survival factor for hepatocytes[198-199] and in corneal epithelial abrasion IL-22 produced by $IL\text{-}17^+$ γδ T cells promotes wound healing.[42]

In addition, further translational studies should address the degree of comparability of CCR6-CCL20 functions between the human and murine system. The expression data of CCR6 and CCL20 in inflamed liver are very similar between human and murine samples, but the cellular source of CCL20 in the liver might be different (hepatocytes vs. cholangiocytes). This could indicate differences in how this pathway is regulated depending on the species. Therefore, the mechanism discovered in murine hepatofibrogenesis needs to be confirmed in human liver disease before therapeutic options can be considered.

7 References

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8 Publications

Hammerich L, Bangen JM, Govaere O, Zimmermann HW, Gassler N, Huss S, Liedtke C, Prinz I, Lira SA, Luedde T, Roskams T, Trautwein C, Heymann F, Tacke F
Chemokine receptor CCR6-dependent accumulation of γδ T cells in injured liver restricts hepatic inflammation and fibrosis
J Clin Invest. (submitted)

Heymann F, **Hammerich L**, Storch D, Huss S, Rüsseler V, Gassler N, Lira SA, Luedde T, Trautwein C, Tacke F
Hepatic macrophage migration and differentiation critical for liver fibrosis is mediated by the chemokine receptor CCR8.
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Hammerich L, Heymann F, Tacke F
Role of IL-17 and Th17 cells in liver diseases.
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Hochheiser K, Engel D, **Hammerich L**, Heymann F, Knolle PA, Panzer U, Kurts C
Kidney Dendritic Cells Become Pathogenic during Crescentic Glomerulonephritis with Proteinuria.
J Am Soc Nephrol. 2011 Feb;22(2):306-16

Heymann F, Meyer-Schwesinger C, Hamilton-Williams EE, **Hammerich L**, Panzer U, Kaden S, Quaggin SE, Floege J, Gröne HJ, Kurts C
Kidney dendritic cell activation is required for progression of renal disease in a mouse model of glomerular injury.
J Clin Invest. 2009 May;119(5):1286-97

Poster presentations

The 63rd Annual Meeting of the American Association for the Study of Liver Diseases 2012 in Boston, USA
L. Hammerich, F. Heymann, H.W. Zimmermann, S. Huss, S.A. Lira, C. Trautwein, F. Tacke
Loss of chemokine receptor CCR6 diminishes recruitment of IL-17-producing γδ T cells to the liver leading to increased liver inflammation and fibrosis.

European congress of Immunology 2012 in Glasgow, Scotland
L. Hammerich, F. Heymann, H.W. Zimmermann, S. Huss, S.A. Lira, C. Trautwein, F. Tacke
Loss of chemokine receptor CCR6 diminishes recruitment of IL-17-producing γδ T cells to the liver leading to increased liver inflammation and fibrosis.

2011 Joint annual meeting of the Italian Society for Immunology (SIICA) and the German Society for Immunology (DGfI) in Riccione, Italy
Linda Hammerich, Felix Heymann, Dunja Storch, Sebastian Huss, Vanessa Rüsseler, Tom Luedde, Christian Trautwein, Frank Tacke
Hepatic macrophage migration and differentiation critical for liver fibrosis is mediated by the chemokine receptor CCR8.

Oral presentations at national and international meetings

The 47th annual meeting of the European Association for the Study of the Liver (EASL) 2012 in Barcelona, Spain (20.04.2012)
The chemokine receptor CCR6 reduces liver inflammation and fibrosis by mediating recruitment of IL-17 producing γδ T cells to the liver.

29. Jahrestagung der Deutschen Arbeitsgemeinschaft zum Studium der Leber (GASL) 2013 in Hannover, Germany (25.01.2013)
Der Chemokinrezeptor CCR6 limitiert Entzündung und Fibrogenese der Leber durch Rekrutierung von IL-17 produzierenden gamma/delta T-Zellen.

9 Eidesstattliche Erklärung

Hiermit erkläre ich, Linda Hammerich, dass ich die Dissertation mit dem Titel:

Chemokine receptor CCR6-dependent accumulation of IL-17 producing γδ T cells in injured liver restricts hepatic inflammation and fibrosis

selbständig verfasst habe.

Bei der Anfertigung wurden folgende Hilfen Dritter in Anspruch genommen:

1. In der Abteilung für Gastroenterologie, Stoffwechselerkrankungen und internistische Intensivmedizin (Medizinische Klinik III) am Universitätsklinikum Aachen habe ich die Versuche mit den im Kapitel *„Materials and Methods“* aufgeführten Hilfen und Hilfsmitteln durchgeführt.

2. Die Anfertigung erfolgte unter Leitung von Prof. Dr. Frank Tacke, Abteilung für Gastroenterologie, Stoffwechselerkrankungen und internistische Intensivmedizin (Medizinische Klinik III) am Universitätsklinikum Aachen, die fachliche Betreuung wurde von Prof. Dr. rer nat Rainer Fischer, Institut für Molekulare Biotechnologie (Biologie VII) der RWTH Aachen, übernommen.

Ich habe keine entgeltliche Hilfe von Vermittlungs- bzw. Beratungsdiensten in Anspruch genommen. Niemand hat von mir unmittelbar oder mittelbar entgeltliche Leistungen für Arbeiten erhalten, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen. Die Synthesen der verwendeten Primer und Antikörper wurden von den jeweils erwähnten Firmen durchgeführt.

Ich habe die Dissertation an folgenden Instituten angefertigt:

Abteilung für Gastroenterologie, Stoffwechselerkrankungen und internistische Intensivmedizin (Medizinische Klinik III) am Universitätsklinikum Aachen.

Die Dissertation wurde bisher nicht für ein Prüfung oder Promotion oder für einen ähnlichen Zweck zur Beurteilung eingereicht.

Ich versichere, dass ich die vorstehenden Angaben nach bestem Wissen vollständig und der Wahrheit entsprechend gemacht habe.

Hiermit bestätige ich, dass die Experimente, die den Ergebnissen dieser Dissertation zu Grunde liegen, ausschließlich von den nachstehend aufgeführten Personen durchgeführt wurden.

Entwicklung und Planung der Versuchsreihen:	Linda Hammerich Prof. Dr. Frank Tacke
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γδTCR; Desmin	Linda Hammerich
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11 Curriculum vitae

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